CYCLINS, HSPs AND E3 LIGASE ACTIVITY IN CELL CYCLE DEREGULATION IN NEURO-MUSCULAR DEGENERATION

THESIS

Submitted By

RENU SHARMA (UGC-SRF)

To The

Delhi Technological University

For the award of the Degree of

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

GUIDE
PROF. PRAVIR KUMAR
DEPARTMENT OF BIOTECHNOLOGY, DTU

DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College Of Engineering)
SHAHBAD DAULATPUR, BAWANA ROAD, DELHI-110042

NOVEMBER 2017

CYCLINS, HSPs AND E3 LIGASE ACTIVITY IN CELL CYCLE DEREGULATION IN NEURO-MUSCULAR DEGENERATION

THESIS

Submitted to the Delhi Technological University

For the award of the degree of

DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY

By

RENU SHARMA (UGC-SRF)

GUIDE: PROF. PRAVIR KUMAR, DTU

DEPARTMENT OF BIOTECHNOLOGY

DELHI TECHNOLOGICAL UNIVERSITY
SHAHBAD DAULATPUR, BAWANA ROAD
DELHI-110042
NOVEMBER 2017

Dedicated to
My
Parents

CERTIFICATE

This is to certify that the thesis entitled **"Cyclins, HSPs and E3 Ligase Activity in Cell Cycle Deregulation in Neuro-Muscular Degeneration"** submitted by **Ms. Renu Sharma** to **Delhi Technological University**, for the award of the degree of "**Doctor of Philosophy**" in Biotechnology is a record of *bona fide* work carried out by her. Ms. Renu Sharma has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to my knowledge has reached the requisite standards.

The results contained in this thesis are original and have not been submitted to any other University or Institute for the award of any degree or diploma.

Prof. Pravir Kumar (Guide)
Professor
Department of Biotechnology
Delhi Technological University
Shahbad Daulatpur, Bawana
Delhi-110042

Prof. D. Kumar
DRC Chairman/HoD
Department of Biotechnology
Delhi Technological University
Shahbad Daulatpur, Bawana
Delhi-110042

ACKNOWLEDGEMENT

It is a great pleasure to express deep gratitude to my thesis supervisor, Prof. Pravir Kumar, Department of Biotechnology, Delhi Technological University, Delhi, for his constant guidance and ever-lasting inspiration in carrying out this work. His knowledge and suggestions were invaluable tools in the completion of my thesis.

I wish to convey my sincere thanks to Dr. Rashmi Ambasta, CSIR Pool Officer, DTU for her constant guidance and motivation in the planning and execution of the experimentations.

I also wish to extend my thanks to my colleagues Niraj Jha, Saurabh Jha and Dhiraj for their support and being an amicable team to work with.

I wish to acknowledge the Senior Management and technical staff of DTU, for providing support to my research work.

Most importantly, credit is due to the amazing group of friends in my life who were always on the stand by to bring me to positivity, hope and smiles when things didn't seem favouring and it seemed a far-fetched journey. Special mention is deserved for my best friend Priya Varma who has always been my guiding light when I didn't believe in myself.

I would also like to thank the University Grants Commission for financially supporting my research work.

Finally, no amount of words can justify the ever-lasting support, encouragement and understanding shown by my family in supporting my research. The constant love, patience, inspiration and mental support by my parents were the guiding force for completing this thesis. Last but not the least, my brothers' love and my nephew Ayush's constant urge to finish the research and be home soon was an internal motivation.

(Renu Sharma)

ABSTRACT

Parkinson's disease (PD) is the most common neurodegenerative disorder after Alzheimer's disease with approximately 7 million patients worldwide which are predicted to get doubled by 2030. The most common form of PD is the sporadic form with no known cause. Amongst many factors responsible for the pathogenesis of sporadic PD, Cell cycle reentry (CCE) with subsequent DNA synthesis in at-risk dopaminergic neurons has been recently identified as the cause of neuronal cell death. Neurons are post-mitotic cells which never divide, but in lieu of their physiological demands certain cell cycle proteins are utilized. However, under the influence of various stressors cell cycle is re-activated in a full blown manner and owing to mitotic incompetence of neurons, drive them to death. Mounting evidence has outlined the causal role of CCE in the pathogenesis of PD and other neurodegenerative disorders. Moreover, certain protective proteins such as ubiquitin E3 ligases and heat shock proteins play crucial role in maintaining protein homeostasis and in alleviating toxic protein burden in various neurodegenerative disorders thereby, promoting neuronal cell survival. The present work aims to study the involvement of cell cycle proteins in neuronal apoptosis and to underline the role of protective proteins especially HSP70 in neuronal cell viability. The study uses widely known toxin rotenone to mimic PD in SH-SY5Y neuroblastoma cell lines. The results show upregulation of cyclin E with subsequent attenuation of ubiquitin E3 ligase parkin and HSP70 in response to rotenone administration. Further, screening of HSP70 inducing biomolecules have clearly outlined their neuroprotective potential in attenuating CCE led neuronal death and in modifying and reversing rotenone induced toxicity. Thus, the present work opens up a new avenue of using HSP70 inducing compounds to target CCE mediated neuronal death in PD which can be extended to other neurodegenerative disorders.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF GRAPHS

CHAPTER V

ABBREVIATIONS

AD	Alzheimer's disease
ADMET	Absorption distribution metabolism excretion toxicity
Akt/GSK3	Akt/Glycogen synthase kinase 3
ALS	Amyotrophic lateral sclerosis
AMD	Age-related macular degeneration
ATM	ataxia-telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
Aβ	Amyloid beta
AβPP	Amyloid beta precursor protein
BAG-1	Bcl2-associated athanogene 1 protein
BBB	Blood brain barrier
BDNF	Brain derived Neurotrophic Factor
BrdU	Bromodeoxyuridine
CCE/CCR	Cell cycle re-entry
CDC20	Cell-division cycle protein-20
CDK	Cyclin dependent kinases
CDKI	Cyclin dependent kinase inhibitors
CFH	Complement factor H
CMA	Chaperone mediated autophagy
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
E2F1	E2F transcription factor 1
EGCG	Epigallocatechin-gallate
FAD	Familial Alzheimer's disease
GSK3β	Glycogen synthase kinase 3 beta
HD	Huntington's disease
Hip	HSP70 interacting protein
HSP	Heat shock protein
IBM	Inclusion body myositis
JNK	c-Jun N-Terminal Kinases
LB	Lewy bodies
LD_{80}	Lethal dose 80
LRRK2	Leucine rich repeat kinase 2
MAO	Monoamine oxidase
MAPK	Mitogen- activated protein kinase
miR	microRNA
mTOR	Mechanistic target of rapamycin
MTT	(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NaB	Sodium butyrate

NDD	Neurodegenerative disorders
NF	Neuro-filaments
NF-κB	Nuclear factor Kb
NGF	Nerve growth factor
NMD	Neuromuscular disorders
NMDA	N-methyl-D-aspartate receptor
OTA	OchratoxinA
p75NTR	p75 neurotrophin receptor
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween-20
PCNA	Proliferating cell nuclear antigen
PCP	Phencyclidine
PD	Parkinson's disease
PDB	Protein Data Bank
PI3K/Akt/GSK3β	Phosphatidylinositol 3-kinase/serine threonine kinase
PM	Polymyositis
PP-1	Protein phosphatase-1
pRb	Phosphorylated retinoblastoma
PS1	Presenilin1
ROS	Reactive oxygen species
SAHA	Suberoylanilidehydroxamic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SNCA	α synuclein
SNpc	Substantia nigrapars compacta
TBI	Traumatic brain injury
TIGAR	TP53-inducible glycolysis and apoptosis regulator
UPS	Ubiquitin proteasome system

CHAPTER I

INTRODUCTION

CHAPTER I: INTRODUCTION

1.1: Introduction

The etiology of neuromuscular degeneration is characterized by the canonical deposition of non-functional/toxic proteins such as amyloid β, tau, parkin, α-synuclein, mutant huntingtin, and chronic inflammations in neuromuscular disorders (NMDs) including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Inclusion body myositis (IBM) and Poly myositis (PM). Though these NMDs have their own distinct pathology, there is a sharing of pathological features between AD, PD and IBM which clearly points towards a common underlying switch to neuro-muscular degeneration marked by alteration of various proteins. For instance, the common morbid feature between IBM and PD includes enrichment of α-synuclein, Parkin and oxidized DJ-1 in muscle fibres. Parkinson's disease is the second most common of all the neurodegenerative disorders (NDD) affecting nearly 1% of population above the age of 65 years. It is a slowly progressive disease represented by two forms; familial and sporadic PD. The cause of familial or genetic PD has been attributed to mutations in six genes namely; α-synuclein also designated as SNCA (PARK1), LRRK2 (PARK8), Parkin (PARK2), PINK1 (PARK6), DJ1 (PARK7) and ATP13A2 (PARK9). However, taken together, mutations in these genes comprise about 30% of familial cases of PD and only 3-5% of sporadic cases. Such observations underlie the fact that sporadic PD is an outcome of a complex interplay of diverse, multi-factors including genes and environmental agents which are mostly unknown. A plethora of studies have demonstrated the involvement of oxidative stress, aging, mitochondrial dysfunction, ubiquitin proteasome system (UPS) shut down and loss of function of several protective proteins in the pathogenesis of PD.

Molecular chaperones such as heat shock proteins regulate protein homeostasis in conjunction with UPS and also regulate cell cycle (Milarski and Morimoto 1986). Ubiquitin E3 ligases are components of UPS involved in warding off misfolded protein burden in cell and thus promoting protein homeostasis along with regulating different phases of cell cycle. While the protective activity of ubiquitin E3 ligases is compromised, expression of HSPs is induced in response to CCE in terminally differentiated neurons and muscles.

The pathology of PD is characterized by dopaminergic neuronal loss which is manifested as a result of α-synuclein (SNCA) positive cytoplasmic inclusions called the Lewy bodies (LB) mostly in the SNPc and cortex region (Hassler 1938; Ehringer and Hornykiewicz 1960). The deposition of pathological proteins or LBs creates a physiological burden on cell that triggers multiple signaling cascades including mitogen- activated protein kinase (MAPK), c-Jun N-Terminal Kinases (JNK), Phosphatidylinositol 3-kinase/serine threonine kinase Akt/Glycogen synthase kinase 3 (PI3K/Akt/GSK3β), Notch and apoptotic signaling pathways. The consequent overexpression of cell cycle markers has been observed with the symptoms of cell cycle re-entry (CCR) (Zheng et al. 2015). Moreover, the co-localization of cell cycle markers with pathological proteins has reinforced the role of cell cycle machinery as a trigger to degeneration (Thakur et al. 2008). Further evidence suggests that re-expression of cell cycle markers occurs at prodromal stages before the appearance of pathological hallmarks in these neuromuscular disorders. Moreover, re-expression of cell cycle markers in terminally differentiated neurons is also reported to fulfill the physiological demands pertaining to synaptic plasticity, neuronal migration and maturation (Lim and Kaldis 2013). Furthermore, prolonged activation or under the influence of acute insults such as DNA damage, oxidative stress, neurotrophic factor

deprivation and altered microRNA (miR) function; cell cycle machinery turns detrimental and drives the cascade of DNA synthesis and consequent neuronal death culminating into neurodegeneration (Yang et al. 2003).

The succeeding sections discuss the normal dynamics of eukaryotic cell cycle along with key role played by UPS and molecular chaperones in cell cycle paradigm and in clearance of toxic protein burden, its post-mitotic physiology and how this machinery turns detrimental and contributes to neurodegeneration in various NDD. Also, emphasis has been laid on the crucial protective role of HSP70 in PD and the significance of designing HSP70 inducing therapeutics to correct PD and other NDD. Moreover, we discuss the factors responsible for triggering cell cycle re-entry in post-mitotic neurons and the altered signalling cascades. The physiology and pathology of Parkinson's disease and the involvement of cell cycle in the etiology of PD has been elaborated. Finally, various therapeutic strategies to target cell cycle along with emerging therapies have been highlighted.

1.2: The Eukaryotic cell cycle

The eukaryotic cell cycle is orchestrated machinery that accomplishes fundamental roles in cell replenishment such as DNA replication, cell growth, repair and birth of new daughter cells from the native mother cell. The coordination of the cell cycle requires complex interplay between the levels of different cyclins and cyclin dependent kinases (CDKs) at different checkpoints. For instance, Cyclin C/Cdk3 complex mark the transition of cells into G1 i.e. preparatory phase. Further, G1/S transition is controlled by the crucial action of cyclin D/Cdk4/6 and cyclin E/Cdk2 complex (Hengstschläger et al. 1999). The DNA replication in S phase is triggered by the cyclin A/Cdk2 and Cyclin A/Cdk1 complex. Cell division in M phase is tightly regulated by the activity of cyclin B/Cdk1complex (Bertoli et al. 2013). The integrity

of the cell cycle is maintained by the timely degradation of cyclins and consequent inactivation of CDKs, which is brought about by a class of cyclin dependent kinase inhibitors (CDKIs). These CDKIs are categorized into Ink family and Cip/Kip family (Ullah et al. 2009). The Ink family is comprised of p15Ink4b, p16Ink4a, p18Ink4c and p19Ink4d that bind with Cdk4/6 and are known to inhibit its association with cyclin D, thereby promoting quiescent stage (Lin et al. 2001). On contrary, members of Cip/Kip family comprised of p21Cip1, p27Kip1 and p57Kip2 that bind and regulate specific Cyclin/Cdk complexes during different stages of cell cycle progression (Starostina and Kipreos 2012). Additionally, activation of different signaling cascades tightly regulates the checkpoints, which are strategically distributed throughout the cell cycle. The checkpoints ensure the correct completion of previous phase before the beginning of new cell cycle phase, and ascertain the correction of any defect and provide protection against the transmittance into daughter cells by halting the cell cycle until the repair has been done or by alternatively triggering cell death pathways. For instance, the G1 phase is regulated by two checkpoints in its early and late phase to assess cell size and growth factors required for promotion to S phase (Foster et al. 2010). While, the S phase is regulated by tumour suppressor gene p53 in response to DNA damage wherein the cell cycle is halted for repair or alternatively triggering apoptosis if the damage cannot be repaired (Velez and Howard 2015). Furthermore, the G2/M transition is tightly controlled by phosphorylation dynamics of Cdk1 where the activatory phosphorylation of Cdk1 is carried out by Cdk-activating kinase complex at Thr160/Thr161 while inhibitory phosphorylation is mediated by Myt1 and Wee1 kinase at Thr14 and Tyr15 respectively. These phosphorylation events can be reversed by the action of cell division cycle 25 (Cdc25) thereby tightly regulating Cdk1 activity (Potapova et al. 2009). In addition to cyclin-cdks, the cell cycle is also

regulated by various other markers for instance, UPS, ubiquitin E3 ligases and molecular chaperones.

1.3: Ubiquitin Proteasome System (UPS) and Molecular Chaperones: An intimate link to cell cycle regulation

The UPS is evident to regulate the cell cycle in two disparate manner via degrading either cyclinD1 or Cdk inhibitors p21/p27 (Kruman 2004). However, oxidative stress is proposed to trigger cell cycle re-entry in response to UPS dysfunction (Fulda et al. 2010). Ubiquitin E3 ligases are components of UPS, which are involved in the degradation of misfolded/ non-functional or harmful proteins in the cell. During normal cycle, the cyclins are degraded by various ubiquitin E3 ligases for progression to the next phase. For instance, while SCF is active throughout the cell cycle (Ang and Harper 2005), APC/cdh regulate mitosis upto G1 phase (Harper et al. 2002). Mounting evidence has suggested the involvement of ubiquitin E3 ligases either in the clearance of toxic aggregates that interfere and deregulate the cell cycle process or in triaging the protein homeostasis with misfolded protein burden, which is important steps between the cell cycle phases and its progression. The Ubiquitin E3 ligase, Parkin protected neurons against cyclin E accumulation by associating with a protein complex, including hSel-10 and Cullin-1 (Duronio and Xiong 2013). Heat shock proteins are molecular chaperones which preserve intracellular protein homeostasis in cooperation with the UPS and are also important regulators of cell cycle. HSPs associate with cdks, Rb protein, in DNA replication (Milarski and Morimoto 1986) and regulate different phases of cell cycle. Likewise, altering the levels of HSPs including HSP27, HSP70 and HSP90, also triggers cell cycle re- entry via controlling different stages of the cell cycle. Therefore, HSPs are also being induced upon the cell cycle trigger in neurons and muscles that regulate the G1 phase. For instance,

blocking of HSP90 action with geldanamycin, halted cell cycle at G0/G1 transition by obstructive action of HSP90- specific client proteins Cdc37 and FKBP52 (Miyata 2005). Another study reported the association between Hsc70 and cyclin D1 to regulate its assembly with Cdk4 and Cip/Kip proteins and formation of a catalytically active complex (Diehl et al. 2003). Moreover, increased expression of HSP70 along with cyclins E/D1 during cell cycle progression has been reported in both IBM and PM muscle and in AD brain. HSP70 is a crucial player in promoting neuronal cell survival and shares an intricate link to PD. The dynamics of HSP70 in reinstating cell viability, promoting toxic protein clearance with special mention in PD and the rationale of designing HSP70 inducing therapeutics is discussed in succeeding section.

1.4: HSP70: The pro-survival switch in NDD

The post-mitotic nature of neurons makes it difficult to clear off toxins, more so in aging brain wherein the protein homeostasis is disturbed or over-burdened. Thus, neurons become very prone to toxic/misfolded proteins aggregate formation owing to compromised UPS, and protein homeostasis machinery such as autophagy and lysosomal degradation pathway as seen in AD, PD, HD, ALS and other NDD (Nah et al. 2015; Cuervo and Wong 2014; Hu et al. 2010; Morimoto et al. 2007; Yu et al. 2005; Nagata et al. 2004; Anglade et al. 1997). Further, accumulating evidence has outlined the direct involvement of Chaperone Mediated Autophagy (CMA) against misfolded protein aggragation in various NDD (Xilouri et al. 2013; Qi et al. 2012; Wang et al. 2009). CMA involves formation of chaperone and co-chaperone complexes such as HSP40, HSP60, HSP70, HSP110, STI1/HOP, Bcl2-associated athanogene 1 protein (BAG-1) and HSP70 interacting protein (Hip) (Agarraberes and Dice 2001). Of these, one of the most widely distributed chaperone is HSP70 which is cytosolic and having homologs BiP/GRP78 in ER and heat shock cognate 70 (Hsc70)

in cytosol respectively. HSP70 has a 40 kDa N-terminal nucleotide binding domain which regulates client protein interaction and the C-terminal substrate binding domain which is 25 kDa and is responsible for recognition of exposed hydrophobic surfaces during early folding stages of client protein (Lackie et al. 2017). Both of these domains are joined by a flexible hydrophobic linker (Bukau et al. 2006; Jiang et al. 2005). Perhaps, the special significance attached to HSP70 can be attributed to the wide array of functions it performs in maintaining protein homeostasis and in NDD. For instance, HSP70 can aid the client protein in sustaining unfolded form until proper fold is maintained (Kastle and Grune 2012). HSP70 can identify proteins in early folding state by recognising leucine rich exposed hydrophobic surfaces (Ciechanover and Kwon 2017; Rudiger et al. 1997). Further, HSP70 can convert misfolded proteins in their native forms by utilizing ATP (Tutar and Tutar 2010; Ranford et al. 2000). Furthermore, in the event of failure in attaining proper folding, HSP70 can act as 'disaggregase' in conjuction with HSP40 and HSP110 to solubilise and exert to unfold protein aggregates into native refolded species by utilising ATP hydrolysis energy (Gao et al. 2015; Nillegoda et al. 2010; DeSantis et al. 2012; Muller et al. 2008; Dickey et al. 2007; Jana et al. 2005; Petrucelli et al. 2004; Meacham et al. 2001). A plethora of evidence has shown direct binding of HSP70 to various misfolded protein aggregates in NDD such as α synuclein in PD, Aβ and tau in AD, mtSOD1 in ALS and mtHTT in poly Q diseases (Luk et al. 2008; Dompierre et al. 2007; Evans et al. 2006; Dedmon et al. 2005; Liu et al. 2005; Choo et al. 2004).

1.4.1: HSP70 and PD: The intricate bond

The evidence of involvement of heat shock proteins in PD was first shown by co-localization of HSP27, HSP40, HSP60, HSP70 and HSP90 with lewy bodies (Leverenz et al. 2007; Uryu et al. 2006; McLean et al. 2002). The human recombinant

HSP70 binded to and decreased chymotrypsin like blocking activity of α synuclein filaments *in vitro* (Lindersson et al. 2004). Further, fibroblasts containing α synuclein generated heat shock response that triggered HSP70 upregulation which in turn, attenuated α synuclein mediated inhibition of proteasome. Furthermore, HSP70 and Hip intercepted α synuclein fibril formation *in vitro*. Moreover, Hip knockdown accelerated α synuclein inclusions formation in HSP70 dependent fashion in *C. elegans* (Roodveldt et al. 2009). Another interesting observation in human neuroglioma cells expressing mutant α synuclein depicted 50% decrease in α synuclein oligomers upon over-expression of HSP70 (Outeiro et al. 2008). Further, HSP70 co-expression revoked dopaminergic neuronal loss in a transgenic model expressing A30P and A53T mutations in α synuclein (Auluck et al. 2002). Furthermore, HSP70 over-expression along with inhibition of HSP90 prevented α synuclein formation and accumulation which in turn led to ablation of α synuclein induced toxicity in human cell lines and in various mice, yeast models (Luk et al. 2008; Flower et al. 2005; Klucken et al. 2004; McLean et al. 2004). Interestingly, HSP70 injection in substantia nigra region abrogated dopaminergic neuronal loss in MPTP treated rats (Dong et al. 2005).

1.4.2: HSP70 mediated therapeutics: Future scope in NDD

The previous section highlighted the crucial role of molecular chaperones particularly, HSP70 in alleviating toxic protein aggregates in PD and other NDD. It is therefore imperative to design therapeutics which can trigger the expression level of these protective proteins and in turn dictate cell survival by clearing misfolded protein accumulation in NDD. Infact, celastrol extracted from the perennial plant of celastraceae family has shown promising results through HSP70 induction in attenuating neuronal loss. The study showed significant decrease in dopaminergic

neuronal loss in substantia nigra upon celastrol treatment in MPTP induced toxicity in mice. Further, celastrol also prevented decline in dopamine level as a result of MPTP toxicity (Cleren et al. 2005).

Given the toxic protein aggregate ablation nature of HSP70, it is logical to design therapeutics which can upregulate the level of HSP70 and thus bring about neuronal survival in PD and other NDD. Thus, the present work is designed with an aim to find and check the efficacy of HSP70 inducing compounds in ameliorating cell cycle re-entry linked neurodegeneration in SH-SY5Y cell line.

In this way, cell cycle markers play a discrete role in the normal physiology of neuronal cells. However, post mitotic expression of these markers is also responsible for meeting physiological demands of neurons and under prolonged activation or as a result of various stressors cell cycle re-entry mediated neuromuscular dysfunction is triggered.

1.5 Post-mitotic cell division: Imperative or Impulsive?

Earlier neurons were believed to be 'truly' post-mitotic and thus being life-long arrested in the quiescent stage G0. Paradoxically, mounting evidence suggested the exit of resting neurons, which actively participated in the untimely cell cycle re-entry upon receiving mitogenic stimuli, synthesized DNA and were subsequently crucified (Bonda et al. 2009). One of the possible explanations to the contradictory premise of 'permanent' post-mitotic neuronal state is to consider that neurons had to constantly suppress the urge of cell cycle reactivation in lieu of their healthy state or face the reverberation of death. For instance, Rb protein is one such cell cycle marker, which serves crucial function of life-long cell cycle suppression in adult neurons (Herrup and Yang 2007). Another candidate ataxia-telangiectasia mutated (ATM). Protein triggered checkpoints during the DNA damage response under physiological

conditions and maintained neurons in quiescent stage in adult CNS (Chou et al. 2015). Moreover, deregulation of ATM signaling is observed in at-risk neurons in AD brains. Likewise, Cdk5, a non-classical Cdk is found to ensure post-mitotic state of neurons by sequestering transcription factor E2F1 and thereby preventing its interaction with the activation subunit. While in response to the encountered neuronal stress, Cdk5 is translocated to the cytoplasm, thus relieving its cell cycle inhibition and inducing cell cycle re-entry. Further ATM, breakdown of Cdk5 also contributed to the pathogenesis of AD (Zhang et al. 2008). Similarly, the ubiquitin E3 ligase APC/Cdh1 also acted as the suppressor of a cell cycle in the adult brain (Li et al. 2009).

However, increasing evidence showed the presence of different cell cycle markers in adult neurons, which was attributed to their requirement in neuronal maturation, migration and synaptic plasticity. For instance, Rb protein served a key post-mitotic role in neuronal migration in conjunction with transcription factor E2F3 (Andrusiak et al. 2011). Further, ATM regulated synaptic functions, including Long-Term Potentiation and was involved in trafficking in adult neurons (Li et al. 2009). Another marker, cell-division cycle protein-20 (cdc20) subunit of ubiquitin E3 ligase APC/C induced NeuroD2 degradation found to promote presynaptic axonal differentiation. Further, cdc20 mediated CaMKIIβ driven retraction and pruning of dendrites. Likewise, Cdh1 modulated growth and patterning in axons, and its inhibition favoured axonal growth. Furthermore, p27 has been proposed to be a microtubule binding protein owing to its binding and disruption of Cofilin-actin complex upon Cdk5 phosphorylation, thereby promoting neuronal migration (Kawauchi et al. 2006). Similarly, p57 mediated cell cycle exit and promoted differentiation and maturation in post-mitotic neurons. Thus, the cell cycle machinery is ‘repurposed’ to serve important demands of the post-mitotic cells. Yet, such re-expression of cell cycle proteins under

various stressors such as oxidative stress, DNA damage and neurotrophic factor deprivation forced mature neurons to re-enter the cell cycle and die at G1/S transition before actual mitosis could begin. Since, during normal development, tetraploid neurons are arrested at G2/M and any attempt to go beyond this checkpoint induces apoptosis (Mosch et al. 2007). Therefore, it is not surprising that the affected neurons in AD completed S phase, arrested the cell cycle at G2/M transition phase and remained in the tetraploid state for some years. These neurons never entered mitotic phase (Currais et al. 2009). The evidence of failed mitosis was further agreed by shunted chromatin condensation and spindle formation. To sum up, erroneous cell cycle re-entry offers two choices to neurons; either they can divide and die in S phase or remain alive with double DNA content at G2/M transition. Since neurons inherently lack mitotic competence, and no evidence of M phase entry has been reported in NDD, cell cycle re-entry in terminally, differentiated neurons drive them to death after successfully synthesizing new DNA. Thus, cell cycle re-entry into adult neurons constituted an early signature of neuromuscular degeneration and CNS injury.

1.6 Aberrant cell cycle re-entry in Neuromuscular Disorders

Scientists are investigating whether the re-expression of various cell cycle markers symbolizes an authentic cell cycle, or it is just a consequence of other pathological events. The growing evidence supports the notion that cyclin's re-expression is a true phenomenon representing actual re-activation of the cell cycle in post-mitotic neurons and muscles. However, cell cycle re-entry in terminally differentiated neurons and muscles is lethal and has been widely implicated in the pathogenesis of degenerative disorders like AD, PD, IBM/PM, HD and ALS (**Figure 1.1**). The co-localization of cell cycle markers with pathological proteins has reinforced the role of cell cycle machinery as a trigger to degeneration (Thakur et al. 2008). Further evidence suggests

that re-expression of cell cycle markers occurs at prodromal stages before the appearance of pathological hallmarks in these neuromuscular disorders. Furthermore, prolonged activation or under the influence of acute insults such as DNA damage, oxidative stress, neurotrophic factor deprivation and altered microRNA (miR) function; cell cycle machinery turns detrimental and drives the cascade of DNA synthesis and consequent neuronal death culminating into neurodegeneration (Yang et al. 2003). Interestingly, numerous studies have established cell cycle re-entry as a true phenomenon in neurodegeneration with actual DNA synthesis and cannot be viewed as an epiphenomenon of other processes.

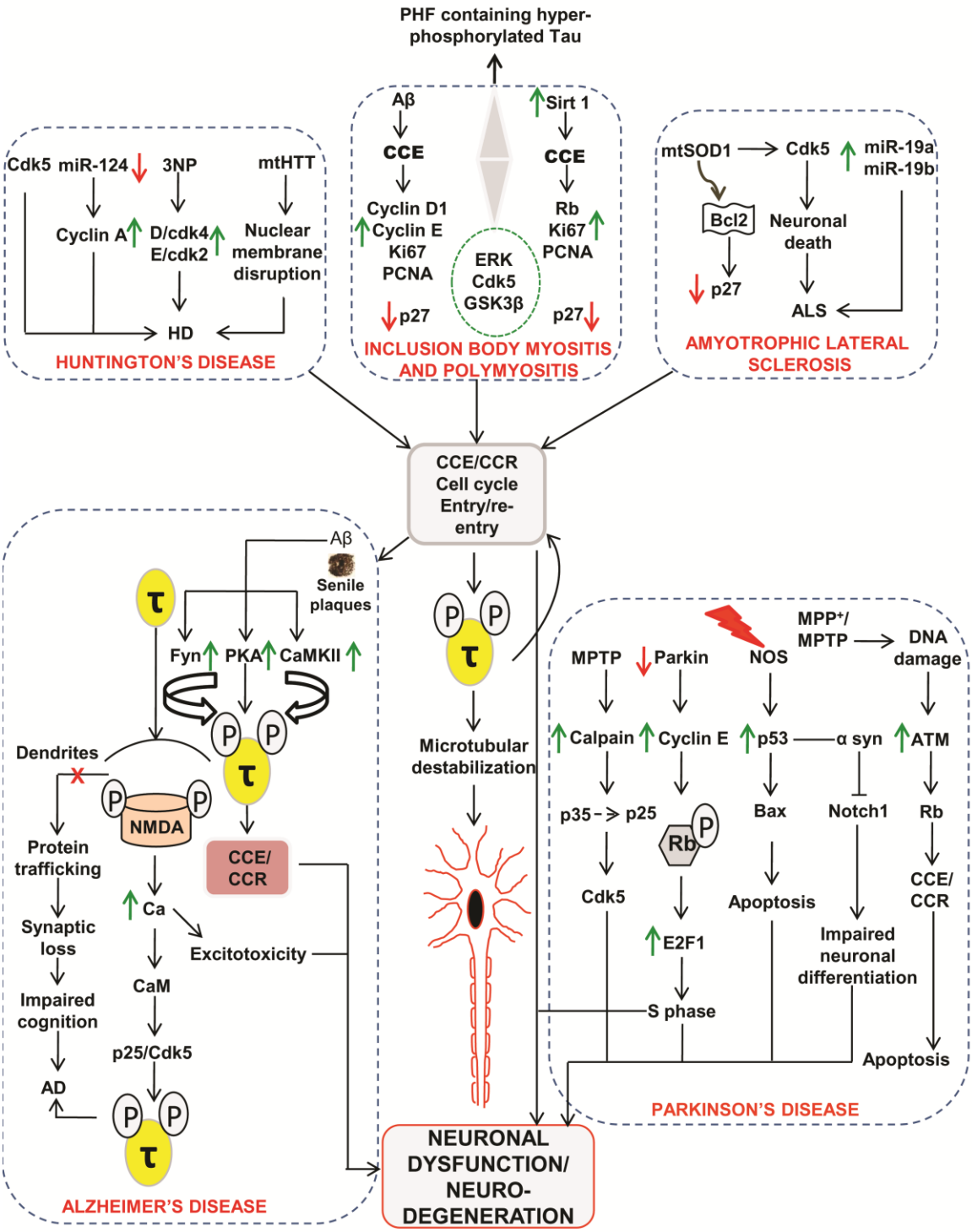


Fig.1.1. Cell cycle re-entry mechanisms in neuromuscular disorders. The pathogenesis of HD is mediated by altered activity of Cdk5, micro RNA-124 and mutant HTT, which triggers the activation of cyclins, disruption of nuclear membrane and thus subsequent CCR. While Neurotoxin (3NP) induced cyclin re-expression is also responsible for CCR in HD. Similarly, Aβ and Sirt-1induced re-

activation of Cyclin D1, Cyclin E, Ki67, PCNA, Rb and reduced p27 level is responsible for CCR in case of IBM/PM. While in case of ALS, mutant SOD1 contributes towards CCR via induction of Cdk5 and reduction of p27. Similarly, microRNA-19 modulates the expression of CCR critical genes and thereby contributes towards CCR in ALS. Likewise, in case of AD, Aβ mediated Tau hyperphosphorylation triggers CCR and causes Cdk5 activation via NMDA induced Calmodulin activation. Further, MPTP activity that induced various factors such as Calpain and ATM, which triggers the cell cycle markers and contributes towards CCR mediated pathogenesis of PD. Additionally, Parkin and NOS also altered the activity of Cyclin E, p53 and α syn that triggers the apoptosis as well as impaired neuronal differentiation in PD progression via CCR. PHF-Paired helical filaments; Cdk-Cyclin dependent Kinase; miR-Micro RNA; 3NP-3-Nitropropionic acid; mtHTT-Mutant huntingtin; Sirt1-Sirtuin 1; PCNA-Proliferating cell nuclear antigen; mtSOD1-Mutant Superoxide dismutase1; Bcl2-B-cell lymphoma 2; PKA-Protein kinase A; CaMKII-Calmodulin-dependent protein kinase II; NMDA-N-methyl-D-aspartate receptor; CaM-Calmodulin; MPTP-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NOS-Nitric oxide synthase; MPP^+-1-methyl-4-phenylpyridinium; ATM-ataxia-telangiectasia mutated; Bax-Bcl-2-associated X protein; Rb–Retinoblastoma

1.6.1 Post-mitotic cell division in the etiology of PD

Recent studies have highlighted the causal role of CCE in Parkinson's disease pathogenesis (Sharma et al. 2017). For instance, the expression of E2F-1, pRb and proliferating cell nuclear antigen (PCNA), a marker of dividing cells has been evident in around 3–6% of substantia nigrapars compacta (SNpc) neurons in PD brains with respect to controls (Levy et al. 2009). Moreover, neurons also showed aneuploidy of selected chromosomes signifying the involvement of DNA replication machinery. Further, the markers of G1/S phase; cyclin D and cyclin E were upregulated upon MPP+ exposure in neuronal culture, suggesting the activation of Cdk4/6 and Cdk2 respectively. Moreover, experimental results suggest that Cdk2/cyclin E complex is more vulnerable than Cdk4/cyclin D or Cdk6/cyclin D complex (Krantic et al. 2005). Another study reported increased level of cyclin D in SNpc subsequent to rotenone administration in rats. Additionally, elevated levels of DNA polymerase β

induced endoreplication were also found to be involved in the pathogenesis of PD (Wang et al. 2014). In addition, MPP induced ROS generation also triggered pRb dependent cell cycle reentry and subsequent ATM mediated DNA damage in animal models as well as in PD patients (Camins et al. 2010). Similarly, Aβ induced aberrant CCR has been shown to be involved in the pathogenesis of IBM and PM. The markers of G1/S transition and proliferation, including cyclin D1, E, and Ki67 were also found to be up-regulated in both IBM and PM samples along with a parallel decrease in the level of cell cycle inhibitor p21 (Kwon et al. 2014). Further, cyclin D1 and E were shown to interact with HSP70 whose level was significantly increased in IBM and PM where heat shock proteins might be playing a role in stabilizing the cyclins. Moreover, the level of various tau- phosphorylating cell cycle kinases including ERK, Cdk5 and GSK3β have been found to co-localize with inclusions containing hyper- phosphorylated tau in degenerating muscle fibers in IBM (Askanas et al. 2015).

1.7 Factors triggering cell cycle re-entry in adult neurons and muscles

There are various factors that are responsible for triggering cell cycle re-entry in neurons and muscles, including environmental toxins, genetic factors, and other cellular stresses. These factors have different mechanistic approaches for eliciting cell cycle abnormalities in neurons and muscles that have been discussed below and elucidated in **Figure 1.2**.

1.7.1 Environmental Factors

Recently, certain environmental factors have been identified, which contributed in the pathogenesis of several neuromuscular disorders. These environmental toxins comprised of heavy metals, pesticides and metal based nanoparticles, which are currently being studied for their role in CCR mediated neuromuscular degeneration. For instance, administration of methyl mercury (MeHg) is found to alter the expression of cell cycle

regulators p16 and p21 in rat embryonic cortical neuronal stem cells. Moreover, compromised cell proliferation and a significant decrease in global DNA methylation were observed subsequent to MeHg treatment in NSCs, supporting the idea that its long-term exposure would lead to neurodevelopmental or neurodegenerative disorders (Bose et al. 2012). Further, exposure to various other toxins likekainic acid, quinolinic acid and heavy metals, including cobalt, lead and iron was shown to be responsible for reactive gliosis. Accordingly, induced reactive gliotic changes led to cell cycle re-entry attempts in neurons (Kerri and Damir 2006). Recently, fluoride and aluminium were found to disrupt neuronal morphology and cell cycle induced progressive cell death by triggering the activation of cell cycle markers and lysosomal proteins (Akinrinade et al. 2015). Additionally, a renal carcinogen OchratoxinA (OTA) induced cell cycle aberrations in renal tubular cells (Taniai et al. 2014). Moreover, environmental factors are found to exacerbate the neuromuscular degeneration in action with other triggering factors. There are numerous reports demonstrating the toxic effect of heavy metals, pesticides and nanoparticles in neurodegeneration, but very few showing the lethal effect of these toxins in the cell cycle re-entry mediated neuromuscular degeneration. Therefore, significant researches need to be carried out to determine the involvement of heavy metals and pesticides in cell cycle induction related neuromuscular toxicity.

1.7.2 Oxidative Stress

Oxidative stress is the most vulnerable and obvious cause for inducing cell cycle irregularities in post mitotic neurons and muscles reported till the date. Reactive oxygen species (ROS) generation is the prime factor responsible for causing oxidative stress, which in turn alters the components of mitogenic signaling cascade by activating various signaling molecules. For instance, mitogen-activated protein kinase (MAPK), nuclear factor κB (NF-κB) and growth factor receptors are activated via ROS and initiate cell

cycle re-entry in postmitotic neurons (Mizukami et al. 2002). Interestingly, ROS also induced DNA damage; chromosomal breaks and base mis-incorporation in cell cycle events (Kruman 2004). Moreover, ROS was also found to abort the activity of DNA repair proteins associated with the DNA replication process in AD. Numerous studies have reported ROS mediated unrepaired, oxidized nitrogenous bases and DNA strand breaks in post-mortem tissues of patients with different neuromuscular disorders (Martin 2008). Hypoxia has emerged as a critical player of cell cycle induction in neurons and muscles. Additionally, various studies have reported DNA replication in post-mitotic neurons upon exposure to hypoxia. Apart from DNA damage, oxidative stress is proposed to trigger cell cycle re-entry in response to ubiquitin proteasome system (UPS) dysfunction (Fulda et al. 2010). Besides, Kwon et al demonstrated the interaction between HSP70 and cyclin D or E1 in IBM and PM biopsy samples depicting their role in cell cycle progression (Kwon et al. 2014). Therefore, oxidative stress in concordance with cell cycle re-entry markers contributed significantly in the etiology of neuromuscular degeneration.

1.7.3 Double stranded DNA breaks (DSBs)

DSB accumulation and its attainment of non-repairable conformation is another crucial factor for initiating cell cycle re-entry in post-mitotic neurons (Leeuwen and Hoozemans 2015). DNA damage activates ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and E2F transcription factor 1 (E2F1), and other proteins, which regulate cell cycle; DNA damages repair, and apoptosis in post-mitotic neurons (Tokarz et al. 2016). Chk1 and chk2 are the checkpoint kinases that are induced in response to DNA damage in the cell cycle. ATM is a well-known checkpoint in the cell cycle which regulates dsDNA break response in a p53 dependent manner and controls cell cycle arrest, apoptosis and mediates protection against oxidative stress (Huang et al. 2008).

Moreover, phosphorylated ATM has been shown to be elevated in cerebellar dentate neurons of AD patients when compared with age-matched controls (Chen et al. 2010). Further, increased ATM expression accompanying down regulation of TP53-inducible glycolysis and apoptosis regulator (TIGAR) protein constituted a key feature of dementia associated with Alzheimer's disease. Furthermore, DSB mediated accumulation of functionally inactive monomers and dimers of TP53 have been reported in AD brain (Katsel et al. 2013). Likewise, E2F complexes are associated with histone-modifying enzymes, histone modifications, including histone acetylation and methylation, and therefore, essential for cell cycle re-entry that may play a regulatory role in DNA repair or apoptosis (Tokarz et al. 2016). Moreover, evidence for DSB had also been reported in s-IBM muscles that resulted in muscle fiber maturation arrest (Rossetto et al. 2010). Importantly, recent studies are going on to explore the relationship between DNA damage and cell cycle re-expression in different systems.

1.7.4 Micro RNAs

Micro RNA (miR) has recently gained attention due to their strategic control of cell cycle regulation in post mitotic neurons. For instance, over-expression of miR26 has been reported to trigger aberrant cell cycle re-entry, DNA replication, and tau hyper phosphorylation in rat primary post mitotic neurons. Moreover, elevated level of miR-26b triggered the re-expression of cell cycle proteins cyclin E1, pRb, PCNA, Ki67 and BrdU in post-mitotic neurons (Absalon et al. 2013). Additionally, miR26b over-activation elicited Cdk5 mediated enhanced tau phosphorylation in vitro and in-vivo studies. It also attenuated the action of cell cycle inhibitors p15 and p27 (Kimura et al. 2014). In another study, increased miR-26b level induced cyclin E1 up-regulation and downregulated p27kip1 expression mediated by Rb1/E2F protein complex, signifying its role in cell cycle regulation (Absalon et al. 2013). Further, genetic and epigenetic

evidence revealed that NF-κB-regulated micro RNAs including miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 are involved in the down regulation of small brain and retinal cell related family of target mRNAs causing complement factor H (CFH) deficiency leading to inflammatory neurodegeneration in AD and age-related macular degeneration (AMD) (Lukiw et al. 2012). Likewise, disruption of miR34a and miR-132 has been linked with AD and other NDDs. For instance, Aβ mediated suppression of miR-34a resulted in unscheduled cell cycle re-entry and apoptosis via MEK-ERK pathway mediated degradation of tumor suppressor TAp73 in AD mouse model. Interestingly, numerous studies are evident for the over-expression of miRNAs in temporal cortex and hippocampus region of AD patients signifying that erroneous miRNA regulation is involved in the etiology of various NDDs (Modi et al. 2015). Recently, altered miRNA expression has been observed in inclusion body myositis, polymyositis and other muscular dystrophies that are involved with myoblast differentiation and muscular regeneration (Luo and Mastaglia 2015). However, few studies reported the role of microRNA in the cell cycle re-entry, but micro RNA research is still at preliminary stage.

1.7.5 Brain derived Neurotrophic Factor

Brain derived Neurotrophic Factor (BDNF) is a member of neurotrophin family of growth factors that plays a crucial role in neuronal cell survival, growth and differentiation. It protects neurons against glutamate and oxidative stress, i.e. NMDA or H_2O_2 mediated toxicity, might be through inhibiting Ras-MAPK pathway. However, altered BDNF has been implicated in the cell cycle re-entry mediated pathogenesis of neuromuscular disorders during oxidative or excite toxic stress responses (Boutahar et al. 2010). For instance, BDNF downregulatedcyclin B and Cdk1 levels (generally localized in NFTs in AD) in differentiating tetraploid neurons in the retina through TrkB

neurotrophic receptor-mediated activity and thereby halted the cell cycle at G2/M checkpoint. The inability of this complex to translocate into the nucleus likely played a role in blocking G2/M transition (Ovejero-Benito and Frade 2013) and abnormal tau phosphorylation, thereby contributing in the pathogenesis of AD (Boutahar et al. 2010). Additionally, BDNF found to trigger inhibition of Cdk1 activity through phosphorylation of the site Tyr15, thereby halting G2/M transition in tetraploid neurons (Ovejero-Benito and Frade 2013). Another study identified that nerve growth factor (NGF) induces cell cycle re-entry through a novel, Cdk4/6-independent pathway, i.e. p38/MAPK-Dependent E2F4 Phosphorylation mediated neurodegeneration (Morillo et al. 2012). The neurotrophin system also exhibits a role in myogenesis and regeneration in skeletal muscle biology. Moreover, differential study of BDNF receptors identified p75 neurotrophin receptor (p75NTR) to be predominantly expressed in human myocytes (Colombo et al. 2013). Presently, BDNF deprivation was well implicated in the cell cycle re-entry mediated pathogenesis in neurons, but its investigation in muscles is still desired.

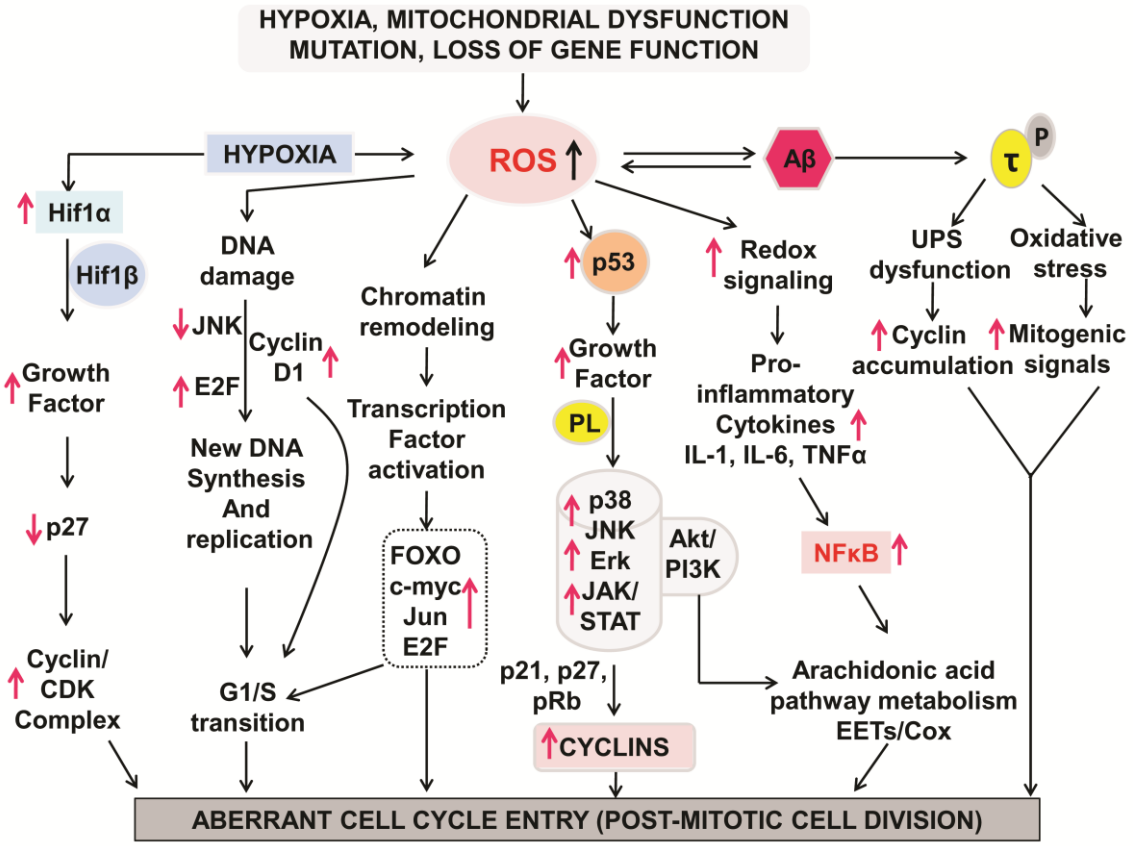


Fig.1.2. Triggering factors associated with the forceful cell cycle re-entries. Hypoxia and oxidative stress are two critical factors responsible for triggering CCR. Hypoxic exposure increases HIF-1α that causes up-regulation of growth factors and subsequent reduction of p27 expression. Reduced p27 expression and increased HIF-1α transactivation induce cyclin/Cdk complex thereby activating cell cycle progression. Further, ROS mediated oxidative stress could lead to aberrant CCR viz. several different pathways. First, ROS accumulation can result in cumulative DNA damage and consequent alteration in new DNA synthesis/replication, chromatin remodeling and transcriptional activation, signaling cascade activation and subsequent activation of cell cycle markers and thus CCR. Further, redox signaling activates pro-inflammatory cytokines (IL-1, IL-6, TNF-α) that triggers NF-κβ mediated arachidonic acid pathway metabolism and consequent CCR. In addition, ROS triggers Aβ mediated Tau phosphorylation and consequent dysfunction of ubiquitin-proteasome system and activation of mitogenic signaling pathways that also induce cell cycle progression. Hif1α-Hypoxia-inducible factor 1-alpha; Hif1β-Hypoxia-inducible factor 1-beta; CDK-Cyclin dependent Kinase; ROS-Reactive oxygen species; JNK- c-Jun N-terminal kinase; JAK/STAT-Janus kinase/signal transducers and activators of transcription; PI3K-Phosphoinositide 3-kinase; FOXO-Forkhead box protein; PL-Phospholipid; pRb- Phosphorylated retinoblastoma; IL-Interleukin; TNFα-Tumor necrosis factor-α; EETs-Epoxyeicosatrienoic acids; Cox-Cyclooxygenase

1.8 Altered signaling cascades in aberrant cell cycle re-entry induced neuromuscular degeneration

Although there are several risks, factors identified to be involved in aberrant CCR but the demonstration of consequent alteration of signaling cascade is still at preliminary stage. However, a growing body of evidences have highlighted the involvement of multiple signaling pathways such as Notch, Wnt/β-catenin, Akt/GSK3β, P38/MAPK and dyotic signaling in the biology of cell cycle re-entry. For instance, treatment with kainic acid is reported to activate notch signaling that augmented the aberrant CCR via activation of CyclinD1-Rb-E2F1 axis (**Fig. 1.3**). Moreover, this abundance of cyclin D1 expression was accomplished by Akt/GSK3β pathway in AD (Marathe et al. 2015). Similarly, elevated levels of β-catenin through wnt/β-catenin signaling in PS1 FAD brain has been found to accelerate CCR simply by upregulating cyclin D1 transcription (Currais et al. 2009). Further, elevated cyclin D1 contributes towards the apoptotic response mediated neuronal cell death. Additionally, disturbed Cdk5 associated neurotrophic signals have also been reported to cause neuronal dysfunction. For instance, Cdk5 mediates phosphorylation of Ape1, MEF2D and GSk-3β and contribute in the pathophysiology of neuromuscular disorders (Zhu et al. 2011). Ape1 plays a crucial role in DNA repair mechanism while MEF2D, a pro-survival transcription factor assists in memory development (Kelley et al. 2012). Moreover, Cdk5 also inhibits protein phosphatase-1 (PP1) and promotes p38/MAPK activity, which in turn leads to tau hyperphosphorylation and consequent microtubule dissociation (Porowska et al. 2014). Further, Cdk5 also involved with Parkin phosphorylation and glutamate or dopamine mediated neurotoxicity in PD and HD respectively (Paoletti et al. 2008). Likewise, Cdk5 is also responsible for altering Drp1 activity thereby increasing mitochondrial fission in HD. Besides, it is also reported to induce tau hyperphosphorylation and consequent increased Perikaryal

inclusions of neuro-filaments (NF) in the SOD1 ALS mice model (Liu et al. 2011). Furthermore, the increased expression of procaspase-3 and cleaved caspase-3 and 9 i.e. apoptotic signaling is observed in IBM/PM sample to be associated with Aβ accumulation. Since, accumulated Aβ in IBM/PM has evidenced to downregulate the Akt expression and subsequent alteration in G1/S phase transition thereby triggering aberrant CCR (Kwon et al. 2014). Recently, dyotic signaling has also been identified to be associated with CCR in post mitotic neuron. Since, this signaling trigger altered AβPP processing and neuronal hypertrophy in M phase of cell cycle, thus governing aberrant CCR in AD (Currais et al. 2009). Moreover, recent studies are going on to identify the cell cycle marker's associated signaling disturbances in post mitotic neurons and muscles to demonstrate the pathophysiology behind the cell cycle re-entry mediated neuromuscular degeneration. Such altered signaling associated with cell cycle markers in different neuromuscular disorders have been summarized in **Table 1**.

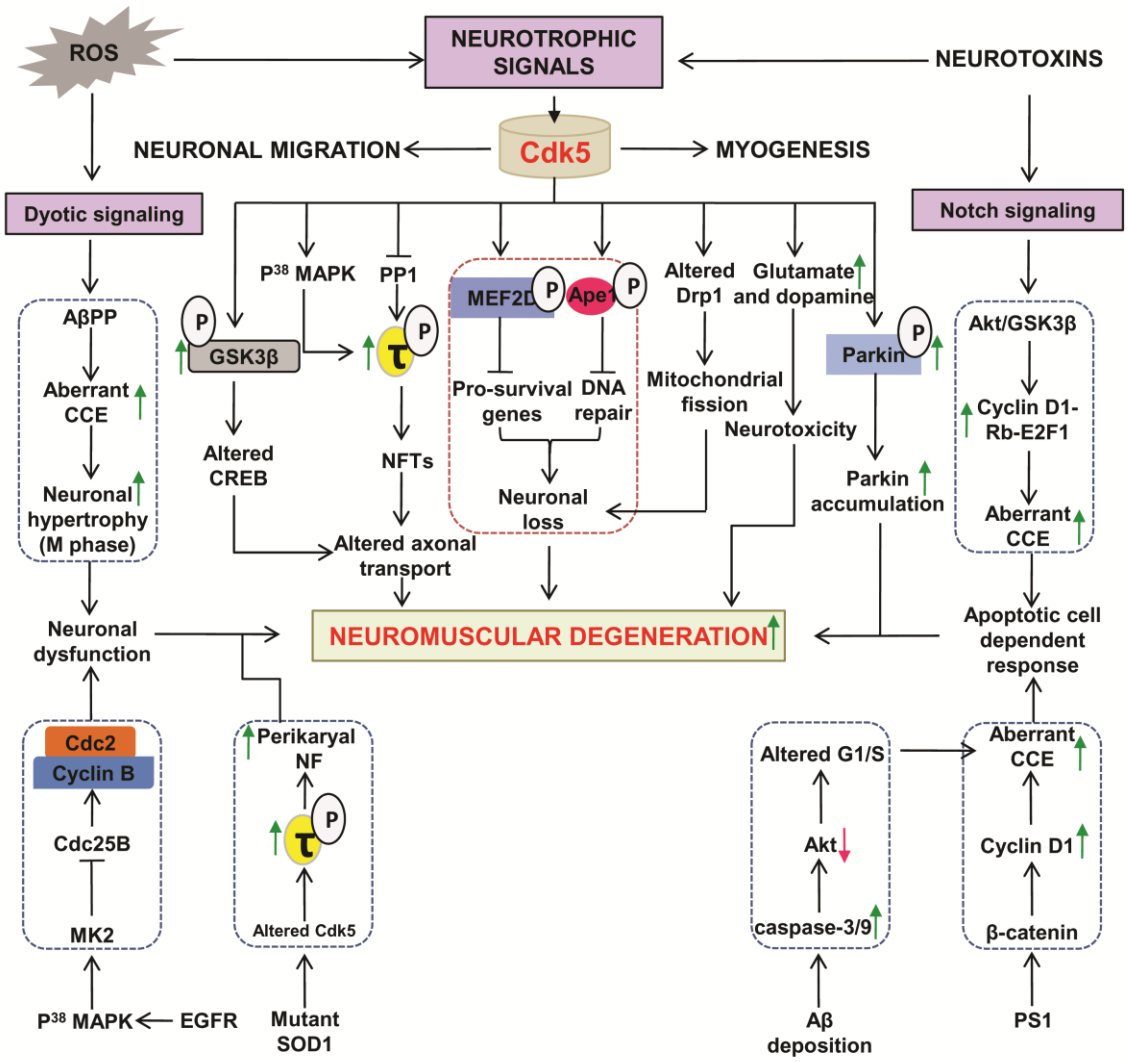


Fig.1.3. Disturbed signaling cascades associated with an aberrant cell cycle re-entry in neuromuscular degeneration. Multiple signaling pathways including Notch, Wnt/β-catenin, Akt/ GSK3β, P^{38}/MAPK and dyotic signaling get affected in response to neurotophic signals, ROS production and neurotoxins attack and contribute towards the pathogenesis of neuromuscular disorders. ROS induced dyotic signaling alters AβPP processing and neuronal hypertrophy in M phase of cell cycle in post-mitotic neurons. Neurotrophic signals mediated Cdk5 activation triggers neuronal dysfunction via phosphorylating Ape1, MEF2D and GSK-3β that govern the onset of NMDs. Further, Cdk5 also inhibits protein phosphatase-1 (PP1) and promotes P^{38}/MAPK activity, which in turn leads to tau hyperphosphorylation and consequent microtubule dissociation. In addition, it also directs Parkin's phosphorylation and glutamate or dopamine mediated neurotoxicity in PD and HD respectively. Similarly, altered Drp1 activity is also mediated via Cdk5 activity that augments mitochondrial fission in HD. Importantly, in case of ALS, mutant SOD1 impairs Cdk5 activity and subsequently

causes tau hyper phosphorylation mediated Perikaryal neurofilaments (NF) accumulation. Further, neurotoxins induced Notch signaling causes aberrant CCR via activation of CyclinD1-Rb-E2F1 axis in AD. Similarly, increased levels of β-catenin viz. wnt/β- catenin pathway accelerate CCR via upregulating cyclin D1 transcription in PS1 FAD brain. Additionally, Aβ mediated increased expression of procaspase-3 and cleaved caspase-3 downregulates Akt expression and subsequent alteration in G1/S phase transition i.e. CCR. Furthermore, EGFR mediated MK2 activation inhibits Cdc25B, which in turn promote Cdc2 and Cyclin B complex and cause neuronal dysfunction. ROS-Reactive oxygen species; Cdk5-Cyclin dependent kinase 5; GSK3β-Glycogen synthase kinase 3β; CREB-cAMP responsive element binding protein; PP1-Protein phosphatases 1; MEF2D-Pro-survival transcription factor; Ape1-Human AP - endonuclease 1; MK2-MAPK-activated protein kinase-2; NF-Neurofilaments; EGFR-Epidermal growth factor receptor; PS1-Presenilin 1

Principle phenotype	Signaling cascades	Check points	Cyclins expression	Cdk/Cdc/Cdki/TFs involved	References
AD	MEK-ERK MAP pathway/p38 MAPK	G0 to G1 and G1/S phase	Cyclin D1 and Cyclin E1	Cdk4 and Ki67 (MKI67)	(Bonda DJ et al. 2010)
	Notch signaling and Akt/GSK3β pathway	G0 to G1 and G1/S phase	CyclinD1	Cdk4/Cdk6 and Rb-E2F1	(Marathe S et al. 2015)
	β-catenin/TCF transcription complex (Wnt signaling)	G1/S phase	Cyclin D1	Cdk4/Cdk6 and Rb-E2F1	(Currais A et al. 2009)
	Not defined	G1/S transition and G2/M phase	Cyclin B, Cyclin C, cyclin D1 and Cyclin E	Cdk1, Cdk2, Cdk4/Cdk6, Cdk5, Cdk7, Cdk11, p15, p16, p18, p19, p21, p27, pRb/p107, Ki67, mpm-2, E2F-1, PCNA, MORF4-related transcription factor and p53	(Arendt T et al.1998 ;Zhou X et al.2010 ;Nagy Z et al. 2007 ;Zhu X et al.2004 ;Jordan-Sciutto KL et al.2002 ;Zhu X et al. 2000,)
PD	Not defined	G1 to S phase	Cyclin D and Cyclin E	Cdk2, Cdk4, Rb-E2F1	(Jordan-Sciutto KL 2003)
	MEK-ERK MAP pathway/p38 MAPK	G0/G1 phase	Cyclin D3	Cdk5, Cdk6/pRb, p21, p25 and p35/p39, Prosurvival transcription factor MEF2D, E2F-1 and p53	(Venderova K et al.2012; Levy OA et al.2009 ; Alquézar C et al.2015 ; Futatsugi A et al. 2012)
HD	Not defined	G0/G1 phase and G1/S phase	Cyclin A2 (CCNA2), Cyclin D1 and Cyclin E	Cdk2, Cdk4, Cdk5 and p25, E2F-1	(Pelegrí C et al.2008; Cherubini M et al.2015 ;Chen J et al. 2010 ;Das E et al. 2013)
ALS	Apoptotic signaling	G1 to S phase	Cyclin D1 and Cyclin F	Cdk4, Cdk5, Cdk6, p25, p35/p39 and pRb/p107,	(Ranganathan S et al. 2003)

				E2F-1	
IBM/PM	Apoptotic signaling and Akt signaling	G1/S phase	Cyclins E and Cyclin D1	Cdk2, Cdk4, Cdk6, Ki-67, PCNA and p21	(Kwon et al. 2014)

Table 1: Consequences of altered cell signaling in the post mitotic cell division

1.9 Parkinson's disease: pathological and clinical manifestations

Parkinson's disease is the second most common of all the neurodegenerative disorders (NDD) affecting nearly 1% of population above the age of 65 years. It is a slowly progressive disease represented by two forms; familial and sporadic PD. PD was first described by a British physician James Parkinson in 1817 who described the major symptoms of the disease and called it 'shaking palsy' in his paper. There are approximately 7 million PD patients worldwide with the highest prevalence in USA followed by Europe. In India, the incidence of PD is relatively low with 70 people out of one lakh. However, the Parsi community in Mumbai has the highest prevalence of PD in the world with 328 per lakh being affected. This observation adds weight to the rationale of studying PD in Indian scenario.

The clinical manifestations of PD are resting tremor, bradykinesia, postural instability and rigor accompanied by impaired cognition (Ebrahimi-Fakhari et al. 2011). Further, secondary symptoms are also present which include freezing or involuntary shaking of gait, involuntary contraction of muscles or dystonia, and progressive constriction in handwriting or micrographia which is attributed to bradykinesia. In addition, various non-motor symptoms are also associated with PD namely, stammering, cognitive decline, difficulty in swallowing, sleep disturbances, hypertension, restlessness, cramps and the typical expression-less face of PD patient.

The chief pathogenesis of PD includes loss of a subset of neurons containing the neurotransmitter dopamine in the substantia nigra (SN) pars compacta (SNPc) region of midbrain. Dopamine is a chemical messenger in the brain which relays signals between

substantia nigra and corpus striatum to effect smooth movements. As a result of loss in Dopamine activity, aberrant nerve firing patterns are triggered which in turn, produces altered movements in PD. The dopaminergic neuronal loss is manifested as a result of α-synuclein (SNCA) positive cytoplasmic inclusions called the Lewy bodies (LB) mostly in the SNPc and cortex region (Hassler 1938; Ehringer and Hornykiewicz 1960). The SNCA is a member of the synuclein family of proteins which are abundantly expressed in the brain. It is a 14.46 kDa protein having 140 amino acids and mostly located in the tips of neurons in pre-synaptic terminals in SN, hippocampus, neocortex, thalamus and cerebellum, though, smaller amounts are also present in heart, muscles and other tissues (Xia et al. 2001; McLean et al. 2000). It is predominantly localized in the nucleus of neurons, also in neuronal mitochondria in striatum, olfactory bulb, hippocampus and thalamus. In its native form, it is unfolded and soluble; present both, in free as well as bound form. It is bound to the phospholipid membrane in α-helix form and upon receiving various stimuli such as phosphorylation by polo like kinase 2, truncation by calpain and on nitration, dissociates from the membrane and attains a monomer form. Further, the monomer form assumes β-sheet like oligomeric conformation which is then changed to insoluble fibrils and finally, LBs are formed with the addition of ubiquitin characteristic of PD pathology (Kim et al. 2007; Spillantini et al. 1997). The Lewy bodies named after their discoverer; Frederic Lewy in 1912, are spherical entities with a dense core surrounded by a halo of fibrils projecting outwards. Occasionally, LBs are also associated with tau protein in addition to ubiquitin in PD (Arima et al. 1999).

The deposition of pathological proteins or LBs creates a physiological burden on cell that triggers multiple signaling cascades including mitogen- activated protein kinase

(MAPK), c-Jun N-Terminal Kinases (JNK), Phosphatidylinositol 3- kinase/serine threonine kinase Akt/Glycogen synthase kinase 3 (PI3K/Akt/GSK3β), Notch and apoptotic signaling pathways.

1.10. Diagnosis

There are no direct tests available for the diagnosis of PD. The various neuro-imaging techniques such as MRI, PET scan and single photo emission computed tomography are employed mainly, to rule out other neurological diseases. Thus, the diagnosis of PD is mainly done by a neuro-surgeon based on a comprehensive neurological examination and presentation of clinical symptoms of the disease. The patient is assessed on various neurological parameters such as mental ability test, movement test, self-awareness test, sensation to pain, touch, light, temperature and knee jerk test. Further, genetic testing for various PD-associated genes such as SNCA and LRRK2, mostly prevalent in familial PD form in Europe, followed by testing for other PD genes like PINK1, DJ1 and parkin may be carried out to aid in PD diagnosis.

1.11 Stages of Parkinson's disease

Clinically, Parkinson's disease is divided into five stages based on the Hoehn and Yahn scale. The Stage I is the mildest form of PD with unilateral manifestations in the body. For instance, some rigidity or tremor may be seen which are usually overlooked in clinical examination owing to age-related similarity. Stage II is marked by bilateral abnormalities related to muscles of trunk, posture and speech, however, balance is not appreciably affected at this stage. In Stage III, imbalance of the body becomes prominent, reflexes are impaired and bradykinesia affects daily activities. Though, the patient is still able to maintain an independent lifestyle. The Stage IV of PD is characterised by severe motor impairment leading to disability, as a result of which the patient is unable to perform simple day-to-day tasks like eating, dressing and thus, personal assistance is

required. Stage V is the most advanced form and associated with aggressive symptoms. The patient is now supported by wheel chair or confined to bed and is very prone to falls. Also, non-motor symptoms become prominent at stage V which includes severe cognitive decline, psychosis, anxiety, hallucinations, depression and sleep disturbances (NINDS homepage).

1.12 Factors responsible for Parkinson's disease

The factors responsible for PD can be divided into two groups (i) Genetic (ii) Environmental.

1.12.1 Genetic factors

The cause of familial or genetic PD has been attributed to mutations in six genes namely; α-synuclein also designated as SNCA (PARK1), LRRK2 (PARK8), Parkin (PARK2), PINK1 (PARK6), DJ1 (PARK7) and ATP13A2 (PARK9).The first two genes represent autosomal dominant mode of inheritance, while, the latter four are responsible for autosomal recessive form of PD (Klein and Westenberger 2012). However, taken together, mutations in these genes comprise about 30% of familial cases of PD and only 3-5% of sporadic cases.

1.12.2 Environmental factors

The environmental factors contributing to PD are mainly constituted by chronic exposure to various pesticides such as rotenone and paraquat. In addition, the herbicides Agent Orange and ziram and organo-chlorines insecticides have been linked to PD. Moreover, studies have shown that exposure to certain heavy metals such as mercury (Hsu et al. 2016; Farina et al. 2013) and manganese (Racette et al. 2017) increased the risk of

developing PD. Also, head injuries are associated with high propensity of PD.

1.13 Available Treatments

Currently, there is no available cure for PD and various drugs provide only symptomatic relief. The course of medications is prescribed based on the stage and symptoms of the disease. During the initial years, symptomatic treatment in the form of levodopa/carbidopa is provided which provides relief from motor dysfunction and rigidity. Levodopa stimulates dopamine synthesis by neuronal cells and addition of carbidopa prevents peripheral carboxylation of levodopa, thereby, increasing its availability in the brain (de Lau and Breteler 2006). Dopamine agonists are another line of treatment in PD. As the name suggests these drugs mimick dopamine activity and thus, increase effective dopamine levels. Dopamine agonists are effective in improving motor functions for a longer period and may be used from mid to late stage of the disease. The examples of dopamine agonists include phencyclidine (PCP), Aripiprazole, Quinpirole which are partial agonists and full efficacy agonists including apomorphine, ciladopa, bromocriptine, ropinirole, epicriptine. The next class of drugs employed in PD are MAO-B inhibitors namely, rasagiline and safinamide. MAO-B inhibitors are known to increase level of dopamine in basal ganglia by inhibiting its metabolism by MAO-B. In addition, anticholinergic drugs which modulate acetylcholine activity may be supplemented to attenuate tremor severity. Finally, in the advanced disease stage where drugs become ineffective, surgery may be adopted to provide relief in severe motor symptoms through deep brain stimulation.

1.14 Emerging therapeutics for CCE induced Parkinson's disease

The ectopic cell cycle re-entry causes abnormal cyclins and CDKs expression and alters the activity of various CDK inhibitors thereby contributing towards cellular

imbalances. In order to overcome such dyshomeostasis, numerous therapeutic strategies are currently being devised that slow down or attenuate the toxicity associated with the abnormal cell cycle re- entries in various neuromuscular disorders. One such strategy involves the implication of various CDK-inhibiting compounds for targeting NMDs. However, these days several compounds/biomolecules that possess anti-apoptotic, anti-proliferative and antioxidant properties have been shown to target various abnormally expressed CDKs and cyclins in NMDs. Most of these biological compounds prevent the neurons and muscles from S phase entry and thereby provide protection against subsequent NMDs. Furthermore, these compounds have defined a beginning point for driving forward screening of potential therapeutic agents with desired pharmacological activity.

Importantly, current studies advocate that aberrant cell cycle re-entry is not a consequence, but rather a cause of neuromuscular degeneration, depicting the importance of targeting cell cycle re-entry as a therapeutic window for treating neuromuscular disorders. Further, the involvement of histone deacetylases inhibitors such as suberoylanilidehydroxamic acid (SAHA) and sodium butyrate (NaB) and rapamycin, an inhibitor of mechanistic target of rapamycin (mTOR) has recently been reported to normalize the proliferation of PD lymphoblasts via preventing the over-expression of the cyclin D3/Cdk6/pRb signaling cascade. These drugs are having neuroprotective roles in both human neuroblastoma SH- SY5Y cells and primary rat mid-brain dopaminergic neuronal cultures toxicity induced by 6-hydroxydopamine (Alquézar et al. 2015). Furthermore, treatment with quercetin has been currently reported to act as a disruptor of the β- catenin/TCF transcription complex and consequently, reduced cyclin D1 levels and reversed the cell cycle/cell

death phenotype in the PS1 Familial Alzheimer's disease (FAD) brain (Currais et al. 2009).

1.15 Methods to study Parkinson's disease

There are three methods through which the etiology of PD can be studied. Each of these can be employed based on their availability and purpose of study. These methods include:

(i) Post-mortem brain sample from PD patients

(ii) Animal models

(iii) Cell culture model

Owing to the limitations of sample availability of PD patients and scarcity of animals, the current research scenario has shifted focus on cellular models of PD which are an excellent source of large drug screening and are easy to maintain.

The present study uses cell culture model to study the toxicity effects of a neurotoxin; rotenone in SH-SY5Y neuroblastoma cell line. Moreover, the neuroprotective effect of biomolecules against rotenone induced toxicity has been studied which will be discussed in *Chapter V*.

1.16 Aims and Objectives of present study

1.16.1 Aim: To investigate the role of protective bio-molecules in stress induced cell cycle deregulation in neuro-muscular degeneration

1.16.2 Objectives

To fulfill the aim of the present work, following objectives were outlined:

1. To investigate the role of cyclins in the progression of cell cycle phases in post-mitotic cells; neurons.
2. To identify the mechanistic approach of ubiquitin E3 ligases and molecular chaperones in the clearance of non-functional proteins.

3. To establish therapeutic action of protective bio-molecules in the correction of aberrant cell cycle entry in neurodegeneration.

1.17 Plan of Work

The present work is divided into different sections to achieve the following objectives

I. Study the repurposing potential of HSP70 inducing compounds using various in silico tools and techniques

a. Assessing the drug-likeliness of given compounds

b. Studying the pharmacokinetic properties and filtering the compounds on given parameters

c. Carrying out molecular docking studies of selected ligands with cell cycle markers to assess their inhibitory potential

d. Studying the shortlisted candidates for interaction with other markers of cell cycle – potential of bimoclomol in attenuating cyclin E level

II. Study the effect of biomolecules in ameliorating toxicity in SH-SY5Y cells

a. Study the dose-dependent and time-dependent effect of toxin; rotenone on neuronal cells

b. Study the effect of rotenone on cell viability

c. Check the dose-dependent and time-dependent efficacy of biomolecules against rotenone toxicity

d. Assess the effect of biomolecules on cell viability

III. Check the expression of cell cycle markers and protective proteins in response to rotenone administration and after treatment with biomolecules

The experimental procedures and methods employed in studying the objectives mentioned above have been discussed in the succeeding chapter.

CHAPTER II

EXPERIMENTAL PROCEDURES AND MATERIALS

CHAPTER II: EXPERIMENTAL PROCEDURES AND MATERIALS

2.1: Introduction

This chapter describes the tools and techniques used in screening of HSP70 inducing compounds and in studying their interaction with cell cycle markers. Also, the experimental procedures employed in sub-culturing SH-SY5Y cell line, administering rotenone and treating cells with biomolecules has been described. All the experiments which have been carried out along with the tools and reagents used are discussed in this chapter.

2.2 *In Silico* Techniques

2.2.1 Data Mining

Data mining was done with the keywords HSP70 inducing compounds in neurodegeneration in the NCBI database. Also, extensive literature survey was carried out. The filter criteria were set to HSP70 inducers in cell cycle and/or neurodegeneration and accordingly, list of 20 potential compounds was prepared.

2.2.2 Retrieval of ligand-protein structure

The sdf file of compounds was retrieved from the PubChem database (http://www.pubchem.ncbi.nlm.nih.gov/). The pubChem database stores physio-chemical and biological information of compounds from three different databases. The pdb file of proteins was retrieved from protein data bank (http://www.rcsb.org/pdb/home/home.do).

2.2.3 Drug-likeliness Analysis

The drug likeness of ligands was tested through Lipinski filter analysis which is used to test compounds for drug ability. The Lipinski's rule of five are: (a) molecular mass <500 Dalton, (b) log P < 5, (c) hydrogen bond donors < 5, (d) hydrogen bond acceptors < 10 and (e) Molar refractivity between 40 -130 (Lipinski et al. 2001). Additionally, two other

parameters; Ghose filter and Veber rules were employed for drug-likeness screening (www.swissadme.ch/index.php). The qualifying parameters of Ghose filter are (a) molecular weight 160-480 (b) number of atoms 20-70 (c) molar refractivity 40-130 (d) molar refractivity -0.4-5.6 (e) polar surface area <140 (Ghose et al. 1999). The Veber rules are (a) rotatable bond count <=10 and (b) polar surface area <=140 (Veber et al. 2002).

2.2.4 ADMET Analysis

The toxicity profiling of compounds was carried out through the online tool Swiss ADME (www.swissadme.ch/index.php). The Swiss ADME tool assessed the ligand on various parameters such as lipophilicity (logP), hydrophilic nature (logS) and BBB permeability.

2.2.5 Pharmacophore based target prediction

Pharmacophore is a spatial arrangement of steric and electronic properties of a compound responsible for its biological response against a particular target. Pharmacophore based target prediction of ligands was done with web server PharmMapper (http://59.78.96.61/pharmmapper/index.php) (Liu et al. 2010).

2.2.6 Protein Homology modelling and Structural validation

The Brookhaven Protein Data Bank (PDB) was searched for suitable templates of cyclin D1 and cyclin C for homology modeling using the BLASTP search with default parameters. Accordingly, PDB ID 2W96.A and 3RGF for cyclin D1 and cyclin C respectively were selected. The homology modeling of given templates was performed using the Swiss Model server (http://swissmodel.expasy.org/) (Soils and Wets 1981). The 3D model so generated was tested for structural and stereo-chemical evaluation using the online server RAMPAGE (http://www.mordred.bioc.cam.ac.uk/~rapper/rampage.php) (Xia et al. 2015). The RAMPAGE tool allowed for residue by residue analysis of cyclin

D1 and cyclin C geometry. Finally, the structural validation and accuracy of the models was checked with Errat (http://nihserver.mbi.ucla.edu/ERRATv2/).

2.2.7 Prediction of physio-chemical properties

The physio-chemical properties of cyclin D1 and cyclin C were predicted using the web based server ProtParam (http://web.expasy.org/protparam/) by using the Uniprot IDP24385 and P24863 respectively.

2.2.8 Active site prediction

The active sites of cyclins C, D1 and E were predicted using the Pock Drug tool (http://pockdrug.rpbs.univ-paris-diderot.fr/cgi-bin/index.py?page=home) (Hussein et al. 2015). The PDB structures of cyclins were uploaded and active sites were predicted using fpocket estimation and setting ligand proximity threshold at 5.5.

2.2.9 Preparation of protein and ligand for docking

The proteins and ligands were prepared for docking using the online Docking Server (http://www.dockingserver.com/web) (Bikadi and Hazai 2009). The protein was cleaned and respective chain was selected for docking. The protein and ligand charge was calculated using Gasteiger method and default solvation parameters were set. The ligand geometry was optimized using MMFF94 method. Further, all non-polar H_2 atoms were merged, rotatable bonds defined and pH set to 7.0.

2.2.10 Molecular Docking

The optimized proteins and ligands were used for molecular docking studies using the online Docking Server (http://www.dockingserver.com/web). The Autodock tool was used for adding Kollman united atom type charges, essential H_2 atoms and solvation parameters. Affinity grid maps were generated with 0.375 Å spacing (Morris and Goodsell 1998). Further, the van der Waals and electrostatic interactions were calculated using Autodock parameter set and distance-dependent dielectric functions respectively.

Furthermore, the Lamarckian genetic algorithm and Soils and Wets local search method was used for docking simulations (Soils and Wets 1981). During docking, all rotatable torsions were dropped. Every docking study was arrived after ten different runs with a cut off energy estimation of 250000. Finally, translational step with 0.2 Å, torsion and quaternion steps of 5 were used with a population size of 150.

3. Cell Culture

3.1 SH-SY5Y Cell line

The SH-SY5Y is a triple cloned cell line of the parent cell line SK-N-SH. It is of human origin and was sub cloned from a four year old female patient suffering from neuroblastoma (Biedler et al. 1978). The cell line is adrenergic and has also been shown to possess moderate levels of dopamine beta hydroxylase activity (Ross and Biedler 1985) and thus has served as an excellent *in vitro* model to study PD associated phenomenon.

3.2 Revival of SH-SY5Y Cell lines

The neuroblastoma cell line SH-SY5Y with an early passage of P4 were grown in Dulbecco's Modified Eagle's Medium/F12 (1:1) growth medium (Gibco; Thermo Fisher Scientific, USA) containing L-Glutamate, 10% heat-inactivated fetal bovine serum, 1mM sodium pyruvate (1:100 dilution) (Sigma-Aldrich; USA), 1X non-essential amino acids (1:100 dilution) (Sigma-Aldrich; USA), sodium bicarbonate (1:50 dilution) (Sigma-Aldrich; USA) and penicillin-streptomycin (Sigma-Aldrich; USA) in T-25 flasks which were pre-coated in a humidified CO_2 incubator with 5% CO_2 and 95% air at 37^0C. The neuroblasts were regularly monitored under microscope for their morphology, growth and confluency.

3.3 Subculturing

The growth medium was changed after two days when neuroblasts reached a confluent stage. Thereafter, cells were regularly monitored for growth and confluency and were prepared for subculturing when they were 60-80% confluent. The growth media was completely removed and cells were washed with 1X dPBS. Next, the cells were incubated with 2 ml Trypsin-EDTA solution (0.05%; Gibco; Thermo Fisher Scientific, USA) for 2 minutes in the CO_2 incubator at 37^0C. The trypsin reaction was stopped by adding growth medium and triturating cells with pipette. Further, the contents of flask were transferred in a 15 ml conical tube and centrifuged at 1000 rpm for 2 minutes. The pellet obtained at the bottom was delicately handled and supernatant removed carefully. The pellet was dissolved in residual medium. Furthermore, cell density was calculated by using 10µL of this solution mixed with 10µL trypan blue solution (0.4%; Sigma-Aldrich; USA) and counting viable cells in 4 different squares of the hemocytometer. Finally, based on calculated cell density, cells were seeded in new T-25 flasks containing fresh medium and incubated in humidified CO_2 incubator with 5% CO_2 and 95% air at 37^0C till they reached 60-80% confluency and were used for further experimentation.

3.4 ROTENONE

The present study uses Rotenone (Sigma-Aldrich; USA) to mimic PD in the SH-SY5Y neuroblastoma cell lines. Rotenone is a pesticide, a broad spectrum insecticide and piscicide which has been linked to the sporadic form of PD (Tanner et al. 2011). It is highly lipophilic, can cross the blood brain barrier and can directly enter cells without the aid of transporters. It impairs mitochondrial respiration by blocking complex I activity and thus augmenting ROS generation. Further, it has also been involved in impairing proteasomal dynamics in the etiology of PD (Cicchetti et al. 2009).

3.5 Rotenone exposure

The cells were subcultured in 24 well plates as described above and labelled each well. Next, stock solutions of rotenone (100% purity) in DMSO (0.5 mg/ml; Sigma-Aldrich; USA) were prepared for 1mM and 1μM concentration respectively. The final concentration of DMSO wherever used in present study was always less than 0.1% to rule out any toxicity linked to DMSO. Rotenone treatment was given to cells with each well containing different concentration in an increasing order ranging from 150nM, 300 nM, 900 nM, 10 μM, 20 μM, 40 μM and 80 μM. The cells were incubated at 37^0C in a CO_2 incubator with 5% CO_2 and 95% air. Next, the cells were observed and photographed under microscope for changes in morphology and confluency at regular intervals of 6 hours, 24 hours and 48 hours and compared with control and sham. The experiment was done to determine dose-dependent and time-dependent effect of rotenone on SH-SY5Y cells and was carried out in triplicates.

3.6 Biomolecules treatment

After determining the LD_{80} concentration of rotenone and the time of incubation required, biomolecules were applied in an increasing dose concentration to control and rotenone treated cells. A stock solution of 1mM sesamol (Sigma-Aldrich; USA) was prepared in DMSO. Similarly, Quercetin (Sigma-Aldrich; USA) was prepared in a stock solution of 1mM in 0.1% DMSO. Sesamol was applied at final concentration of 25μM, 50μM, 75μM, 100μM and 200μM. Further, Quercetin was applied at final concentration of 10μM, 20μM, 50μM, 75μM, and 100μM. The cells were incubated at 37^0C in a CO_2 incubator with 5% CO_2 and 95% air. Further, the cells were observed and photographed under microscope for changes in morphology and confluency at regular intervals of 6 hours, 24 hours and 48 hours and compared with control and sham. The triplicate experimental observations were used to determine the dose-dependent and time-

dependent effect of Sesamol and Quercetin in rotenone induced toxicity in SH-SY5Y cells.

4. Cell viability Assays

4.1 Trypan blue exclusion test

The SH-SY5Y cells were seeded at a density of 2.0 $x10^5$ cells/well in 24 well plates and incubated overnight at 37^0C in a CO_2 incubator with 5% CO_2 and 95% air. Rotenone treatment (20μM) was next given to cells for 24 hours. The biomolecules Sesamol (50 μM) and Quercetin (20 μM) were then added to rotenone treated cells and incubated for 24 hours. Viable cells were counted after rotenone administration and post-biomolecules treatment according to the given protocol. The cell suspension (10μL) and 10μL of trypan blue solution (0.4%; Sigma-Aldrich; USA) was mixed and loaded on the chamber underneath glass cover slip on the hemocytometer. The solution was taken up by capillary action and a clean cover slip was carefully placed. The live cells were counted as unstained and dead as blue stained cells in four different squares of hemocytometer using a cell counter under the inverted microscope. Finally, cell viability for each ml was calculated by using the formula: % Cell viability= (number of live cells/total number of cells) x100. Similarly, cell viability was determined for each concentration of rotenone and biomolecules at defined time periods in the triplicate experimental sets.

4.2 MTT Assay

Cell viability was quantitatively determined using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (HiMedia, India) colorimetric assay which determines the mitochondrial activity of viable cells. SH-SY5Y cells were seeded at the final density of $2x10^5$ cells/ml in 96 well plates and incubated overnight at 37^0C in a CO_2 incubator with 5% CO_2 and 95% air. Rotenone treatment (20μM) was next given to cells for 24 hours. The biomolecules Sesamol (50 μM) and Quercetin (20 μM) were then

added to rotenone treated cells and incubated for 24 hours. MTT assay was done after rotenone administration and post-biomolecules treatment according to the protocol described. Each well was incubated with MTT (0.5mg/ml) reagent for 3 hours in a humidified chamber with 5% CO_2 and 95% air. Next, the yellow coloured formazan crystals were dissolved in dimethyl sulfoxide and absorbance measured at 595nm using a microplate reader. The cell viability was determined as a percentage of control.

5. SDS-PAGE and Western Blotting

The SH-SY5Y cells were lysed using the lysis buffer (50mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 μg/ml PMSF, 0.02% NaN_2, 1 μg/ml aprotinin, 1% Triton X-100) containing the protease inhibitor cocktail (Sigma-Aldrich; USA) for 30 minutes on ice and centrifuged at 12,000g at 4^0C for 30 minutes as described previously (Zhang and Zhao 2003). The protein content was determined by Dot-blot method. Next, each protein sample (50 μg) in Lamelli buffer was heated for 10 minutes at 95^0C, separated on 10% SDS-PAGE gel and finally, electro-blotted (BioRad; USA) onto a PVDF membrane. Next, the membrane was blocked using buffer (5% BSA in 1M PBS, pH 7.4 and 0.1% Tween 20) for 1 hour and incubated with mouse monoclonal primary antibody (1:1000; Santa Cruz; USA) at 4^0C overnight. After PBST washing, the membrane was hybridized with mouse monoclonal HRP-conjugated secondary antibody (1:1000; Santa Cruz; USA) for 1 hour at room temperature. Finally, the Enhanced Chemiluminescence (ECL1 and ECL2) solutions (Sigma-Aldrich; USA) were applied on the membrane, developed and fixed on an X-ray film (Thermo Fisher Scientific; USA) and autoradiography signals detected.

6. Statistical Analysis

All the data are expressed as mean ± SEM. The statistical measurement was done using one way ANOVA followed by Tukey's multiple comparison test. The statistical significant values were considered for $p<0.05$.

Based on the *in silico* tools and techniques detailed in this chapter, we have carried out drug screening of HSP70 inducing compounds to evaluate their repurposing potential in ameliorating CCE led neurodegeneration in the succeeding chapter.

CHAPTER III

REPURPOSING HSP70 INDUCING COMPOUNDS TO TARGET ABERRANT CELL CYCLE IN NDDs

CHAPTER III: REPURPOSING HSP70 INDUCING COMPOUNDS TO TARGET ABERRANT CELL CYCLE IN NDDs

Introduction

This chapter discusses the repurposing potential of HSP70 inducing compounds in targeting ectopic cell cycle related neurodegeneration. Various compounds have been evaluated on drug-likeness and other pharmacokinetic parameters and shortlisted candidates which qualify all the filters are used further in the present study.

3.1 Background

Toxic protein burden has been identified as the common underlying molecular switch to neurodegeneration in several NDD. Despite the knowledge of factors responsible for the onset and progression of these NDD, the quest for new players has been on-going for the simple reason that the known players do not add up to all the outcomes of neurodegeneration.

The ectopic re-entry of cell cycle in post-mitotic cells such as neurons and muscles has been recently identified as a culprit in NMD. Normally, the cell cycle remains suppressed for lifetime and these cells never divide. However, re-expression of cell cycle markers such as cyclin C, cyclin D, cyclin E along with other markers of active cell cycle has been observed in the AD, PD, ALS, PM and IBM. Moreover, the occurrence of cell cycle proteins during early stages of NDD and their co-existence with pathological proteins has placed fresh impetus on cell cycle re-entry (CCE) as a 'causal' phenomenon in NDD (Sharma et al. 2017). Once triggered, the cell cycle ensured DNA synthesis in S phase followed by severe neuronal death and neurodegeneration in various NDD (Herrup 2010). Therefore, the major thrust of present study is to evaluate the role of biomolecules in ameliorating CCE mediated neuronal apoptosis in NDD.

Flavonoids are a class of plant based phenolic compounds with high content in oranges, grapes, lemons, red wine and green tea. The signature properties of flavonoids include

antioxidant, anti-inflammatory, anti-allergic, antiviral, antibacterial, anticancer, anti-hypertensive, insulin-sensitizing and anti-ischemic (Procházková et al. 2011). Moreover, flavonoids are well tolerated in human body and display enhanced bioavailability and negligible toxicity in comparison to their synthetic counter-parts. Furthermore, they have been shown to improve upon disease symptoms by modulating various signal transduction pathways in NDD.

HSPs are molecular chaperones expressed constitutively in the nervous system which are involved in decreasing neurotoxicity and enhancing neuronal cell survival in various NDD (Muchowsk and Walker 2005; Magrane et al. 2004). HSP 70 has been shown to associate with p53and arrest the cell cycle at G1/S. Further, the activity of cell cycle inhibitor p27 was modulated by HSP70 (Liu et al. 2010). Furthermore, the G1/S transition markers cyclin D1 and E were reported to associate with elevated level of HSP70 in IBM and PM thereby speculating their strong co-relation (Kwon et al. 2014). Mounting evidence has outlined HSP70 induction as the major route in mediating pro-survival action of most drugs and biomolecules in PD and other NDD (Bao et al. 2017; Deane and Brown 2016). Moreover, we previously outlined Arimoclomol to be a promising neuroprotectant through HSP70induction in cell cycle driven neurodegeneration (Sharma et al. 2017; Kalmar et al. 2008) (**Table 3.1**). Therefore, it is pertinent to understand that HSP70 inducing compounds could be a new line of neurotherapeutics in CCE mediated neurodegeneration.

S.No.	Compounds	Involved check points	Involved mechanisms	Diseases	References
1	Roscovitine	G1 and G2/M phases of cell cycle	Well-known purine analogue, responsible for suppressing the activation of both Cdk2 and Cdk5. Further, it also inhibits the activity of ERK1 and ERK2 signaling cascade at its higher concentration	AD, PD and ALS	(Wu J et al. 2011)
2	Olomoucine	G1 and G2/M phases of cell cycle	Well-known purine analogue which modulates the activity of Cdk2 and Cdk5 by competitively binding the ATP-binding site. Moreover, neuronal cell death induced by p27	AD, PD and ALS	(Wu J et al. 2011)

			reduction is also inhibited by olomoucine administration		
3	Resveratrol (RSV)	G_0/G_1 phase of cell cycle	It potentiates SIRT1 activity and induces an indirect inhibition of p53 that further inhibit the GSK3β and p53 interaction. Since, GSK3β overactivity leads to increased levels of plaques and tangles and p53 activity induces tau phosphorylation. Further, RSV also protects neuronal cells by toxic effects of mHtt, potentiating SIRT1 activity and inducing an indirect inhibition of p53	AD and HD	(Tellone E et al. 2015)
4	Ibuprofen	S phase of cell cycle	Non-steroidal anti-inflammatory drug Ibuprofen has been shown to abrogate the cell cycle arrest at S phase	AD and PD	(Varvel NH et al. 2009, Elsisi NS et al. 2005)
5	Naproxen	G0/G1 phase of cell cycle	It blocks alterations in brain microglia as well as neuronal cell cycle events (CCEs) in the absence of detectable alterations in AβPP processing and Aβ metabolism	AD	(Varvel NH et al. 2009)
6	Apigenin	G2/M phase of cell cycle	It induces cell cycle arrest preferentially in the G2/M phase	AD and PD	(Elsisi NS et al. 2005)
7	Epigallocatechin-gallate (EGCG)	G0-G1 phase of cell cycle	It is a natural product that has been shown to abrogate the cell cycle at the G0 to G1 phase in various tissues and is known as a cell cycle inhibitor	AD	(Bonda DJ et al. 2010)
8	Tamoxifen	G0-G1 phase of cell cycle	It is a well-tolerated anti-proliferative cancer drug that has been reported to abrogate the cell cycle progression from G0 to G1 in various tissues	AD	(Bonda DJ et al. 2010)
9	Butyrolactone I	G1-S phase of cell cycle	A Cdk5 inhibitor, provides protection against Aβ toxicity in SEK1-AL-expressing cells, advocating that Cdk5 and JNK activation independently contributed to this toxicity	AD	(Liu T et al. 2004)

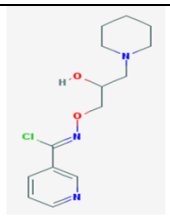
10	Arimoclomol	Not defined	It upregulates the HSP70 expression therefore it might be used as a potential molecule to treat IBM. Since, the interaction between HSP70 and cyclins E/D1 has crucial importance during cell cycle progression in the inflammatory myopathies, probably controlling cyclin maturation or degradation	IBM	(Ahmed M et al. 2016)

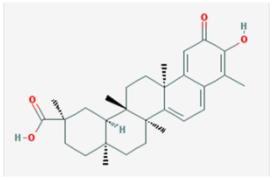
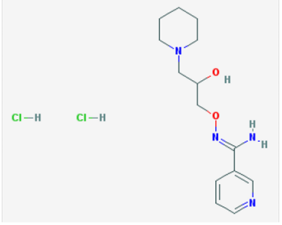
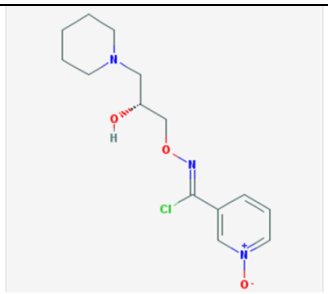
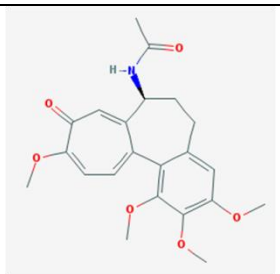
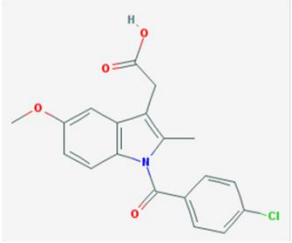
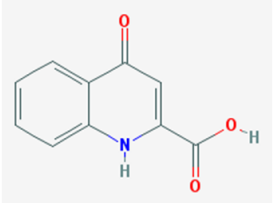
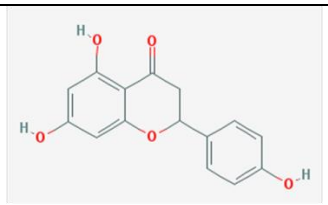
Table 3.1: Different drugs and biomolecules to attenuate the cell cycle progression

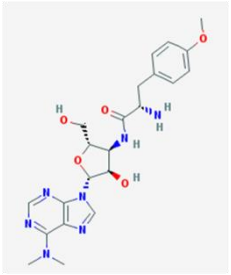
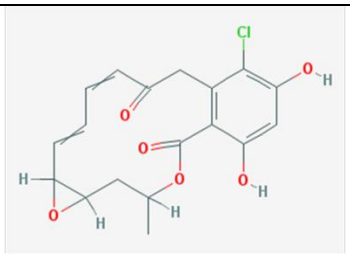
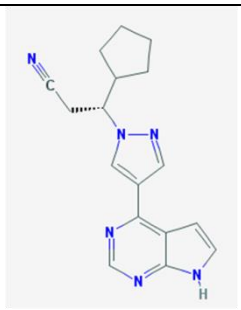
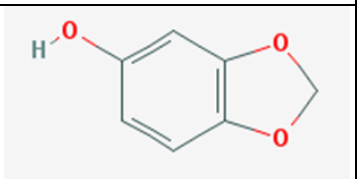
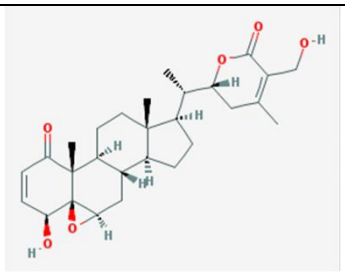
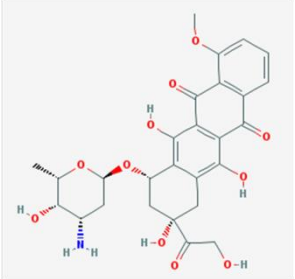
We carried out comprehensive data mining on HSP70 inducers in NDD and carried out the study with twenty compounds. Various virtual screening methods such as, Lipinski filter, Ghose and Veber parameters, pharmacophore generation and ADME analysis were applied to screen drug-like compounds. Further, Homology modelling, 3D structure validation and Ramachandran plots of proteins were performed to establish model accuracy. Finally, ligand-protein interactions were studied with the targets of interests; G0/G1 phase markers i.e. cyclin C and cyclin D1 through molecular docking studies. Our results have outlined strong potential of three HSP70 inducing compounds namely Indomethacin, Bimoclomol and Sesamol in attenuating levels of cyclin D1 and cyclin C. These observations may have promising implications in targeting CCE mediated neurodegeneration in AD, PD and HD.

3.2 Selection of ligands

The compounds along with their structure, physical properties and signalling cascade modulated in NDD and neuro-oncology have been summarised in **Table 3.2**.

S.No.	Compound	Structure	Molecular weight (g/mol)	Molecular formula	Modulated signalling in NDD/Neuro-oncology	References
1	Bimoclomol		297.783	$C_{14}H_{20}ClN_3O_2$	Augmented HSP70 level in ALS	(Benn and Brown 2004)

2	Celastrol		450.619	$C_{29}H_{38}O_4$	Induced HSP70 and acted pro-survival in neurons post TBI damage, anti-inflammatory, Rapid induction of HSF1	(Eroglu et al. 2014)
3	BGP-15		351.272	$C_{14}H_{24}C_{l2}N_4O_2$	Induced HSP70 and acted pro-survival in neurons post TBI damage, anti-inflammatory	(Eroglu et al. 2014)
4	Arimoclomol		313.782	$C_{14}H_{20}ClN_3O_3$	Induced HSP70, delayed progression of ALS	(Benn and Brown 2004)
5	Colchicine		399.443	$C_{22}H_{25}NO_6$	Induced HSPB8 which in turn attenuated accumulation of misfolded TDP-43 and TDP-25 in ALS via HSP70/HSC70-CHIP complex	(Crippa et al. 2016)
6	Indomethacin		357.79	$C_{19}H_{16}ClNO_4$	Induction of HSP70, attenuated Aβ induced damage in AD	(Bernardi et al. 2012)
7	Kyneuric Acid		189.17	$C_{10}H_7NO_3$	Inhibited proliferation, migration and DNA synthesis	(Walczak et al. 2014)
8	Naringenin		272.256	$C_{15}H_{12}O_5$	Rescued against 6-OHDA induced toxicity through Nrf2/ARE signaling	(Lou et al. 2014)

9	puromycin		471.518	$C_{22}H_{29}N_7O_5$	Elicited HSP70 expression in response to ROS	(Moran et al. 2009)
10	Radicicol		364.778	$C_{18}H_{17}ClO_6$	Inhibit huntingtin aggresome, elevated HSP70	(Hay et al. 2004)
11	Ruxolitinib		306.373	$C_{17}H_{18}N_6$	Increased HSP70, Inhibited ERK1/2, Akt, STAT3 and STAT5	(Tavallai et al. 2016)
12	Sesamol		138.122	$C_7H_6O_3$	Protected against amyloidogenesis and cognitive dysfunction through NF-Kß inhibition	(Liu et al. 2016)
13	Withaferin		470.606	$C_{28}H_{38}O_6$	Induction of HSP70,HSP27,MAPK, Inhibition of Akt/mTOR and cell cycle at G2/M	(Grogan et al. 2013)
14	Doxorubicin		543.525	$C_{27}H_{29}NO_{11}$	Induced HSPB8 which in turn attenuated accumulation of misfolded TDP-43 and TDP-25 in ALS via HSP70/HSC70-CHIP complex	(Crippa et al. 2016)

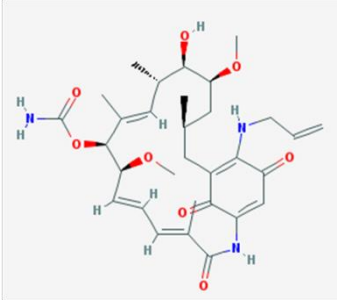
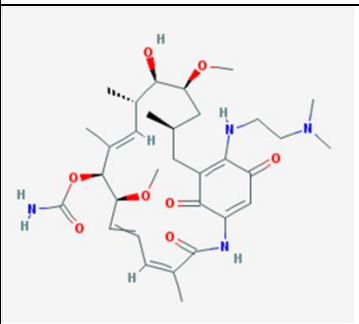
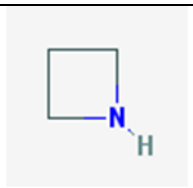
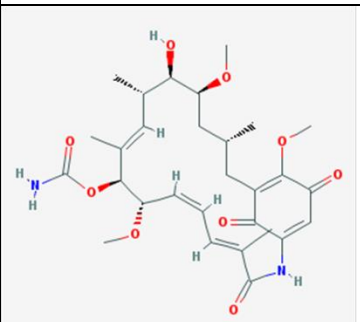
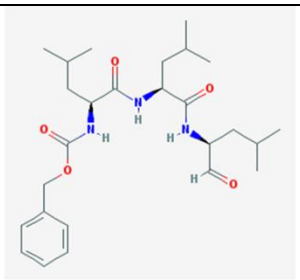
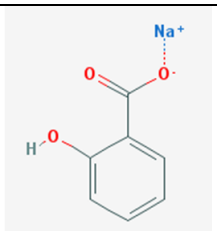
15	17AAG		585.698	$C_{31}H_{43}N_3O_8$	Blocked cell proliferation through Wnt/β catenin pathway attenuation	(Chen et al. 2016)
16	17DMAG		616.756	$C_{32}H_{48}N_4O_8$	HSP70 induction, anti-inflammatory, anti-oxidant	(Wang et al. 2016)
17	Azitidine		57.096	C_3H_7N	Protein synthesis inhibtion, induction of chaperones	(Reina et al. 2012)
18	Geldanamycin		560.644	$C_{29}H_{40}N_2O_9$	Inhibit huntingtin aggresome, elevated molecular chaperones	(Sittler et al. 2001)
19	MG132		475.63	$C_{26}H_{41}N_3O_5$	Induction of HSP70 in response to stress	(Holmberg et al. 2000)
20	Sodium Salicylate		160.104	$C_7H_5NaO_3$	Induction of HSP, imparting neuroprotection in rotenone induced PD	(Thakur and Nehru 2014)

Table 3.2: Physio-chemical properties and modulated signalling pathways of compounds

3.3 Screening for drug-likeness and ADMET Analysis of compounds

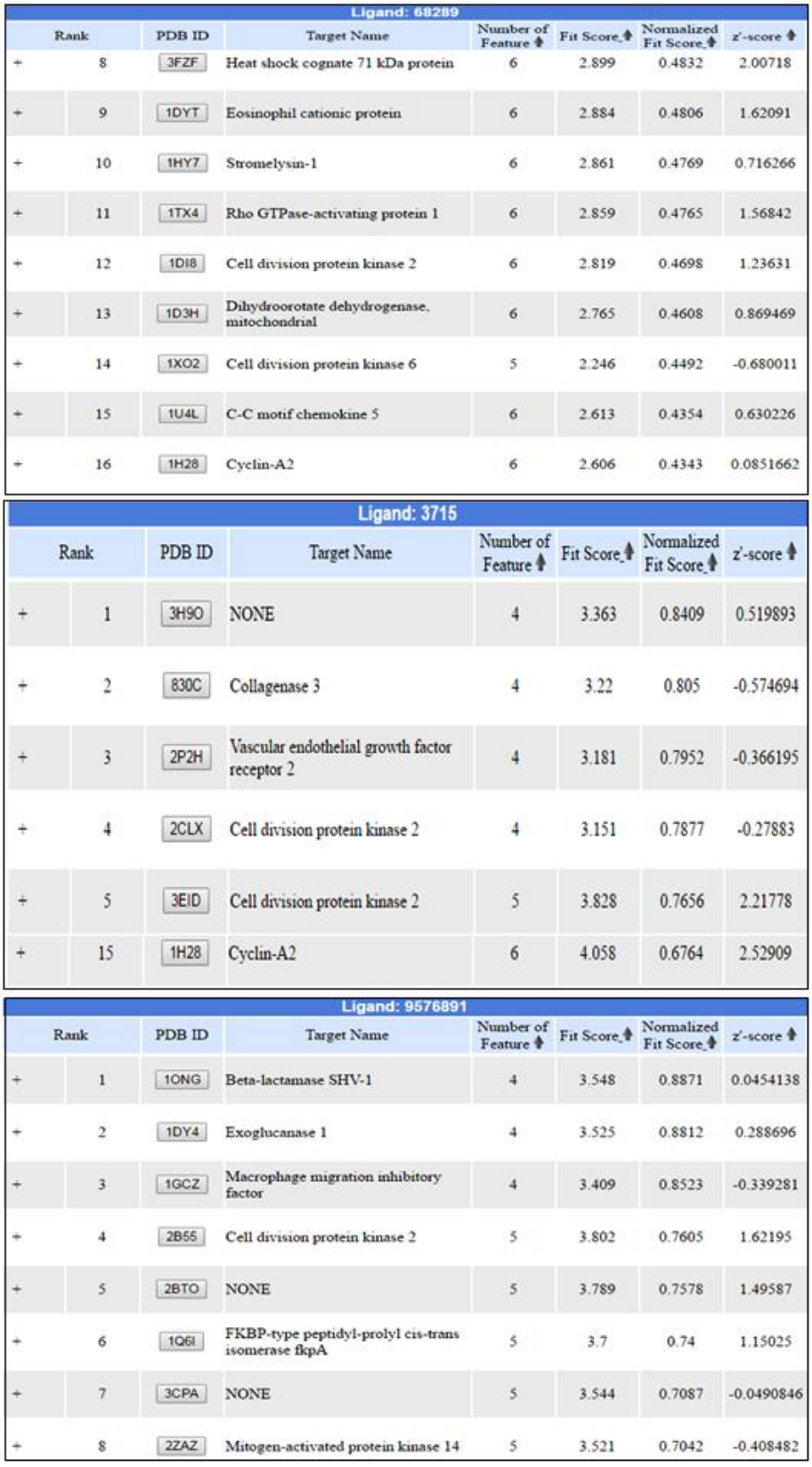
Most of the compounds passed drug-likeness parameters but failed ADMET analysis predictions (**Table 3.3**). Bimoclomol, Indomethacin and Sesamol qualified all the above parameters and were used in further study. While the bioavailability score of Sesamol and Bimoclomol was 0.55, Indomethacin had the highest predicted bioavailability of 0.56.

S. No.	Compound	Drug likeness			Aq. Solubility		Lipophilicity	BBB Permeability	Bioavailability Score
		Lipinski	Ghose	Veber	LogS (ESOL)	GI permeability	XLogP3		
1	**Bimoclomol**	**Y**	**Y**	**Y**	**-2.9**	**High**	**2.21**	**Y**	**0.55**
2	Celastrol	Y	N	Y	-6.31	Low	5.94	N	0.56
3	BGP-15	Y	Y	Y	-3.21	High	2.18	N	0.55
4	Arimoclomol	Y	Y	Y	-2.37	High	1.22	N	0.55
5	Colchicine	Y	Y	Y	-2.9	High	1.03	N	0.55
6	**Indomethacin**	**Y**	**Y**	**Y**	**-4.86**	**High**	**4.27**	**Y**	**0.56**
7	Kyneuric Acid	Y	Y	Y	-2.29	High	1.29	N	0.56
8	Naringenin	Y	Y	Y	-3.49	High	2.52	N	0.55
9	puromycin	Y	N	N	-2.51	Low	0.03	N	0.55
10	Radicicol	Y	Y	Y	-4.4	High	3.36	N	0.55
11	Ruxolitinib	Y	Y	Y	-3.26	High	2.12	N	0.55
12	**Sesamol**	**Y**	**N**	**Y**	**-1.92**	**High**	**1.23**	**Y**	**0.55**
13	Withaferin	Y	N	Y	-4.97	High	3.83	N	0.55
14	Doxorubicin	N	N	N	-3.91	Low	1.27	N	0.17
15	17AAG	Y	N	N	-4.67	Low	2.64	N	0.55
16	17DMAG	Y	N	N	-4.42	Low	2.04	N	0.55
17	Azitidine	Y	N	Y	-0.07	Low	-0.15	N	0.55
18	Geldanamycin	Y	N	N	-4.24	Low	1.99	N	0.11
19	MG132	Y	N	N	-4.77	High	4.83	N	0.55
20	Sodium Salicylate	Y	N	Y	-2.59	High	2.26	N	0.55

Table 3.3: Drug-likeness and ADMET screening analysis

3.4 Pharmacophore based target prediction

The pharmacophore based target prediction of Indomethacin, Bimoclomol and Sesamol outlined various cell cycle proteins such as Cyclin A2, cell division protein kinase 2, VEGFR2 and MAPK18 which further strengthens our premise of their use in targeting cell cycle (**Fig. 3.1**).



Ligand: 68289

Rank		PDB ID	Target Name	Number of Feature	Fit Score	Normalized Fit Score	z'-score
+	8	3FZF	Heat shock cognate 71 kDa protein	6	2.899	0.4832	2.00718
+	9	1DYT	Eosinophil cationic protein	6	2.884	0.4806	1.62091
+	10	1HY7	Stromelysin-1	6	2.861	0.4769	0.716266
+	11	1TX4	Rho GTPase-activating protein 1	6	2.859	0.4765	1.56842
+	12	1DI8	Cell division protein kinase 2	6	2.819	0.4698	1.23631
+	13	1D3H	Dihydroorotate dehydrogenase, mitochondrial	6	2.765	0.4608	0.869469
+	14	1XO2	Cell division protein kinase 6	5	2.246	0.4492	-0.680011
+	15	1U4L	C-C motif chemokine 5	6	2.613	0.4354	0.630226
+	16	1H28	Cyclin-A2	6	2.606	0.4343	0.0851662

Ligand: 3715

Rank		PDB ID	Target Name	Number of Feature	Fit Score	Normalized Fit Score	z'-score
+	1	3H9O	NONE	4	3.363	0.8409	0.519893
+	2	830C	Collagenase 3	4	3.22	0.805	-0.574694
+	3	2P2H	Vascular endothelial growth factor receptor 2	4	3.181	0.7952	-0.366195
+	4	2CLX	Cell division protein kinase 2	4	3.151	0.7877	-0.27883
+	5	3EID	Cell division protein kinase 2	5	3.828	0.7656	2.21778
+	15	1H28	Cyclin-A2	6	4.058	0.6764	2.52909

Ligand: 9576891

Rank		PDB ID	Target Name	Number of Feature	Fit Score	Normalized Fit Score	z'-score
+	1	1ONG	Beta-lactamase SHV-1	4	3.548	0.8871	0.0454138
+	2	1DY4	Exoglucanase 1	4	3.525	0.8812	0.288696
+	3	1GCZ	Macrophage migration inhibitory factor	4	3.409	0.8523	-0.339281
+	4	2B55	Cell division protein kinase 2	5	3.802	0.7605	1.62195
+	5	2BTO	NONE	5	3.789	0.7578	1.49587
+	6	1Q6I	FKBP-type peptidyl-prolyl cis-trans isomerase fkpA	5	3.7	0.74	1.15025
+	7	3CPA	NONE	5	3.544	0.7087	-0.0490846
+	8	2ZAZ	Mitogen-activated protein kinase 14	5	3.521	0.7042	-0.408482

Fig.3.1: Pharmacophore based target prediction of Indomethacin, Sesamol and Bimoclomol (top to bottom in order)

3.5 Homology modeling of proteins

The template shared 100% sequence similarity with cyclin D1 and cyclin C and was used to generate their 3D structures using Swiss Model. The Z QMEAN4 score indicative of overall quality of generated models with respect to non-redundant set of PDB structures was -1.68 and -0.64 for cyclin D1 and cyclin C respectively (**Fig. 3.2**). Thus, the predicted protein structures satisfied good quality models.

3.6 Quality assessment and physio-chemical description of 3D structures

The generated 3D structures were checked for validation in terms of steric and geometric conformations. For this, the Ramachandran plots were generated (**Fig**.**3.2**). The results showed 91.3% residues of cyclin D1 in the most favored region while 5.5% were in the allowed region. Further, 3.1% residues fell in outlier region. Similarly, for cyclin C 98.4% residues were seen in the favored region, 1.4% in the additionally allowed region and only 0.2% residues in the disallowed region. Further, cyclin D1 and cyclin C passed the model accuracy with 85.77% and 90.98% respectively. So overall, the structures of both the proteins were validated with good scores. The predicted physio-chemical properties of the models are summarized in **Table 3.4**.

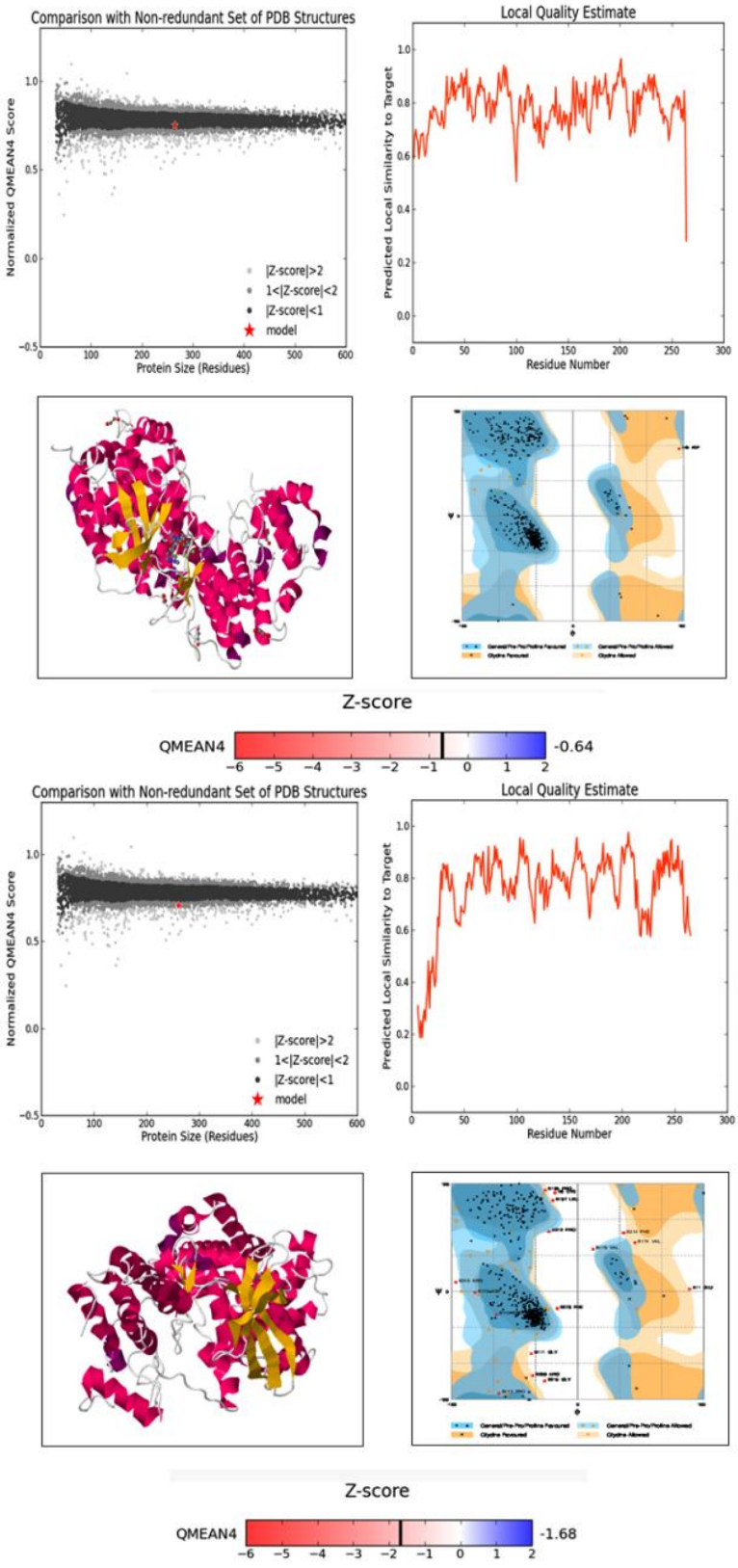


Fig. 3.2: Structural validation of cyclin C and cyclin D1 (top to bottom)

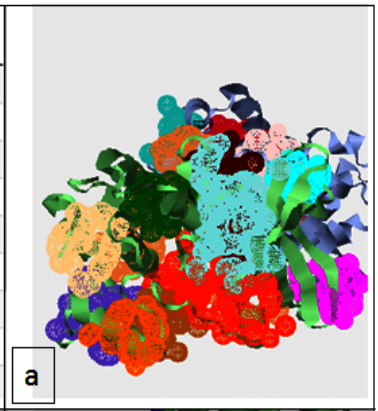
Protein	Mol wt.	Atomic composition	No. of Amino Acids	Theoretical PI	Negatively charged residues Asp+Glu	Positively charged residues Arg+Lys	Instability index	Aliphatic index	GRAVY
Cyclin D1	33729.11	$C_{1480}H_{2386}N_{396}O_{450}S_{25}$	295	4.97	47	34	57.71	92.92	-0.185
Cyclin C	33242.73	$C_{1522}H_{2348}N_{384}O_{417}N_{17}$	283	6.95	32	32	49.97	92.69	-0.158

Table 3.4: Predicted physio-chemical properties of cyclin D1 and cyclin C

3.7 Active site prediction

Based on drugability score, cavity volume and standard deviation, cyclin D1 had best pocket at P5 with a score of 0.95 and 0.01standard deviation (**Fig. 3.3a**). The volume of given pocket was 1079.69 cubic angstroms and 16 residues were involved in interaction. Similarly, P0 was best predicted active site for cyclin C with 0.97 score (**Fig. 3.3b**). The volume of this cavity was found to be 3732.64 cubic angstroms and 38 residues were involved in interaction at this site. These pockets were used for docking the ligands and same residues as predicted were found to be involved during docking.

Pockets	Vol. Hull*	Hydroph. Kyte*	Polar Res.*	Aromatic Res.*	Otyr atom	Nb. Res.*	Drugg Prob*	Standard Deviation
P 0	2609.78	-0.52	0.56	0.13	0.0	32.0	0.59	0.03
P 1	2494.82	-0.36	0.54	0.18	0.0	28.0	0.75	0.08
P 2	1460.86	-0.28	0.45	0.2	0.0	20.0	0.75	0.06
P 3	1097.44	-0.22	0.59	0.12	0.0	17.0	0.68	0.01
P 4	1503.19	-0.54	0.46	0.04	0.0	24.0	0.44	0.04
P 5	1079.69	0.62	0.44	0.13	0.0	16.0	0.95	0.01
P 6	1298.6	-0.34	0.48	0.14	0.02	21.0	0.7	0.03
P 7	988.3	0.28	0.41	0.24	0.0	17.0	0.93	0.04
P 8	598.12	0.79	0.36	0.07	0.03	14.0	0.95	0.02



Pockets	Vol. Hull*	Hydroph. Kyte*	Polar Res.*	Aromatic Res.*	Otyr atom	Nb. Res.*	Drugg Prob*	Standard Deviation
P 0	3732.64	0.58	0.42	0.16	0.01	38.0	0.97	0.0
P 1	2968.13	-1.79	0.77	0.27	0.01	26.0	0.13	0.03
P 11	1218.64	-1.01	0.71	0.12	0.0	17.0	0.25	0.02
P 16	377.41	0.35	0.47	0.07	0.0	15.0	0.84	0.02
P 17	1324.1	-2.19	0.81	0.13	0.0	16.0	0.02	0.0
P 2	2535.02	-0.47	0.48	0.26	0.0	27.0	0.74	0.1
P 28	531.74	0.79	0.27	0.27	0.0	15.0	0.98	0.01
P 3	1432.1	-0.45	0.39	0.17	0.02	18.0	0.67	0.03
P 4	1320.0	-0.47	0.55	0.25	0.02	20.0	0.73	0.03
P 5	868.12	0.14	0.5	0.25	0.02	16.0	0.92	0.01

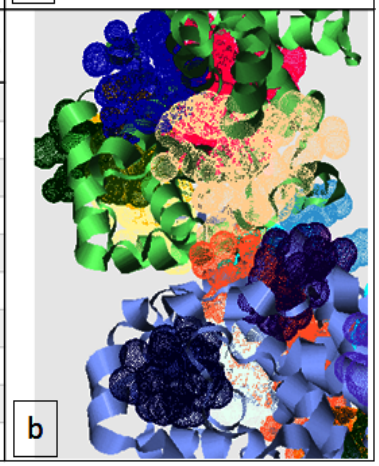


Fig. 3.3: Predicted active sites (top 10) in cyclin D1 (a) and cyclin C (b)

3.8 Molecular docking of ligands with cyclin D1

3.8.1 Bimoclomol and cyclin D1

While the total intermolecular energy of Bimoclomol and cyclin D1 was -5.49kcal/mol, the estimated free energy of binding was found to be -4.77Kcal/mol (**Fig. 3.4a**). Bimoclomol formed hydrogen bonds with LEU148 (-3.9537kcal/mol). Further, ASN151 was involved in polar bond formation with -1.0163kcal/mol and LEU91 formed hydrophobic bond with Bimoclomol (44.6643kcal/mol).

3.8.2 Indomethacin and cyclin D1

The estimated free energy of binding for cyclin D1-Indomethacin interaction was -5.51kcal/mol and total intermolecular energy was -6.68kcal/mol (**Fig. 3.4b**). The H_2bond energy with ALA39 was unfavorable (23.1104kcal/mol). Further, two polar bonds were formed with ARG87 (-6.319kcal/mol) and SER41 (-2.497kcal/mol).

3.8.3 Sesamol and cyclin D1

Sesamol interacted with cyclin C to generate estimated free energy of binding -3.76kcal/mol and total intermolecular energy of -4.06kcal/mol. Polar bond was formed with ASN83 (-0.1798kcal/mol). Four hydrophobic bonds were formed with PRO199 (-0.9286kcal/mol), ALA39 (-0.5013kcal/mol), PRO40 (-0.2763kcal/mol) and PRO200 (-0.195kcal/mol) (**Fig. 3.4c**).

3.9 Molecular docking of ligands with cyclin C

3.9.1 Bimoclomol and cyclin C

The estimated free energy of binding for cyclin C and Bimoclomol was -4.02kcal/mol, while the total intermolecular energy was -6.24kcal/mol. Hydrogen bond with -0.2489kcal/mol energy was formed between THR66. While the polar bond energy of ASP182 was -3.9173kcal/mol, hydrophobic bonds formed with TYR 184 (-

1.8162kcal/mol) and ILE62 (-0.3802kcal/mol). Further, GLN49 formed halogen bond with -7.6881kcal/mol energy (**Fig. 3.4d**).

3.9.2 Indomethacin and cyclin C

Indomethacin interacted with cyclin C and generated high estimated binding energy of -5.68kcal/mol and total intermolecular energy -7.22kcal/mol. Further, five polar bonds were formed with ASN46, ARG185, GLN59, THR66 and GLN49 having energy values of -0.8421, -0.5951, -0.5475, -0.2608 and -0.2379 kcal/mol respectively. Next, two hydrophobic bonds were formed between TRP241 (-0.6165kcal/mol) and ILE62 (-0.4887kcal/mol) (**Fig. 3.4e**).

3.9.3 Sesamol and cyclin C

The estimated free energy of binding for cyclin C-Bimoclomol interaction was -4.31kcal/mol and total intermolecular energy was -4.61kcal/mol. Two polar bonds were formed between TYR37 (-0.686kcal/mol) and ARG25 (-0.4014kcal/mol). Further, three hydrophobic bonds were formed with TYR73 (-0.945kcal/mol), PHE69 (-0.5898kcal/mol) and LEU78 (-0.3605kcal/mol) (**Fig. 3.4f**).

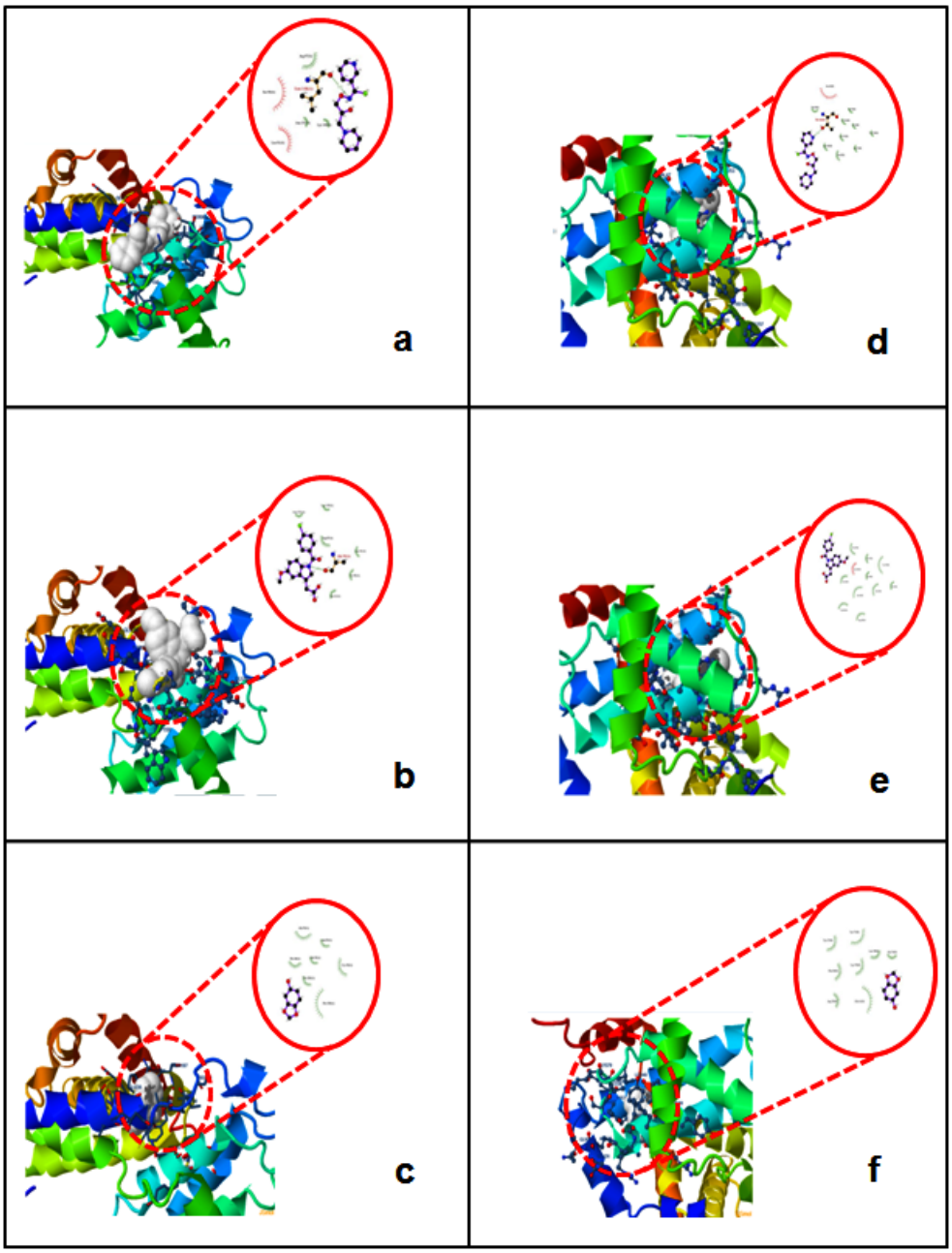


Fig.3.4: Docking of Bimoclomol, Indomethacin and Sesamol with cyclin D1 (a, b, c) and cyclin C (d, e, f) respectively with the interacting residues (inset)

The comparative analysis of docking calculations was done (**Table 3.5**) and Indomethacin was found to be the best compound for targeting and inhibiting cyclin D1 as well as cyclin C thereby, implicating its strong and diverse potential in attenuating G_0/G_1 checkpoints in cell cycle.

Energy Parameters	CYCLIN D1			CYCLIN C		
	Bimoclomol	Indomethacin	Sesamol	Bimoclomol	Indomethacin	Sesamol
Estimated free energy of binding (Kcal/mol)	-4.77	**-5.51**	-3.76	-4.02	**-5.68**	-4.31
Estimated inhibition constant (uM)	317.57	**91.17**	1.74	1.12	**69**	696.84
vdW+Hbond+desolv energy (Kcal/mol)	-5.89	**-6.67**	-4.07	-6.11	**-7.06**	-4.6
Electrostatic energy (Kcal/mol)	0.41	**-0.01**	0.01	-0.13	**-0.16**	0
Total intermolecular energy (Kcal/mol)	-5.49	**-6.68**	-4.06	-6.24	**-7.22**	-4.61
Interacting surface	600.646	**624.148**	366.636	576.194	**645.711**	305.971

Table 3.5: Comparative analysis of ligands-proteins docking calculations

3.10 Discussion

The cell cycle re-entry mediated neurodegeneration contributes heavily in the demise of post-mitotic neurons and muscles. Since cyclins C and D are first respondents of a re-activated cell cycle, thus, targeting these can be 'nib in the bud' strategy in halting/ameliorating the evil cascade of cell cycle led neuronal death. HSPs are molecular chaperones which are upregulated during stress to protect the cell against heat, hypoxia and ROS generation. HSP70 in particular, has been shown to promote neuronal cell survival by inducing autophagy and mediating the activation of pro-survival signaling cascades (Muchowsk and Walker 2005). Moreover, HSP70 is closely associated with cell cycle and interacted with cyclin D1 in IBM and PM (Kwon et al. 2014). It is therefore imperative to search for compounds which can induce the level of HSP70 in NDD as a key neuroprotective strategy. Further, currently available drugs provide only symptomatic relief; therefore, flavonoids are favored by neuroscientists owing to their beneficial effects and negligible toxicity. In the present study, we proposed and tested the efficacy of HSP70 inducing compounds in ameliorating cell cycle led neurodegeneration in various NDD. Since most drugs fail on poor solubility, we screened the compounds for ADMET and pharmacokinetics analysis. It is evident that in vivo bioavailability of an orally administered drug is largely dependent on its

aqueous solubility and dissolution in GI fluids (Khadka et al. 2014). More the water solubility and GI permeability, better the bioavailability. Similarly, lipophilicity of a drug affects various physiological properties such as the rate of metabolism, transport across cell membrane and interaction with binding sites of receptor. Further, drugs intended for CNS should have logP value less than four (Chico et al. 2009; Wager et al. 2010). Indomethacin, Bimoclomol and Sesamol showed logP values of 4.27, 2.21 and 1.23 respectively.

However, the most important property required of a compound to be a neuroprotective agent is the ability to cross Blood brain barrier (BBB). As expected, most compounds failed the BBB permeability parameter. Three biomolecules namely, Bimoclomol, Indomethacin and Sesamol could cross the BBB and combined with their high GI absoption, least violations of drug likeness and good bioavailability score, were the best candidates for targeting NDD in our study. Further, pharmacophore based target prediction of these three compounds listed various cell cycle proteins which further supported our repurposing premise. Finally, molecular docking studies indicated Indomethacin as the best compound for HSP70 mediated targeting of post-mitotic cell cycle based on its high pharmacokinetics and docking calculations.

Further, our results are backed by various *in vitro* and *in vivo* studies wherein these compounds have displayed promising neuroprotective action in various NDD. For instance, Bimoclomol has its derivative Arimoclomol already under Phase II clinical trials in ALS (Hesselink 2016). Indomethacin was shown to ameliorate Aβ1-42 triggered damage in AD mice model as well as in hippocampal cultures (Bernardi et al. 2012). Similarly, Sesamol reversed PD linked symptoms in a rotenone model (Angeline et al. 2013). Hence, our compounds are validated for their neuroprotective action and

yet, add to the hunt for protective biomolecules in alleviating cell cycle led neurodegeneration.

Our study has outlined novel potential of Indomethacin, Bimoclomol and Sesamol in inhibiting/down-regulating the level of cyclin D1 and cyclin C. Out of these, Indomethacin showed best binding with both the cyclins, speculating its strong potential in inhibiting G0/G1 phase reactivation in terminally differentiated neurons in various NDD. Further, the protective action of these compounds in attenuating cell cycle re-entry may be mediated through HSP70. These findings can open up a new window of therapeutics for targeting ectopic cell cycle activation led neurodegeneration and need further validation through *in vitro* and *in vivo* cell cycle studies.

The next chapter discusses the novel potential of one of the above shortlisted compounds; Bimoclomol in targeting another cell cycle marker cyclin E and thus exploring possible outcome in targeting CCE in PD.

CHAPTER IV

NOVEL POTENTIAL OF BIMOCLOMOL IN ATTENUATING POST MITOTIC CELL DIVISION IN PARKINSON'S DISEASE

CHAPTER IV: NOVEL POTENTIAL OF BIMOCLOMOL IN ATTENUATING POST MITOTIC CELL DIVISION IN PARKINSON'S DISEASE

Introduction

This chapter describes the novel potential of Bimoclomol, one of the shortlisted compounds in our study in the previous chapter in targeting post-mitotic cell division in Parkinson's disease. We are targeting another cell cycle marker; cyclin E which holds special significance with PD associated genes. Since the evaluation of Bimoclomol in correction of aberrant cell cycle in PD has not been done before, the study has novelty and the results obtained are interesting.

4.1 Background

The cell cycle proteins share a very intimate relationship with proteins of PD, physiologically as well as pathologically. The PD associated gene; PINK1 was shown to promote cell cycle and PINK1 deletion reversed cell proliferation (O'Flanagan et al. 2015). Further, the ATM gene responsible for DNA damage response and apoptosis has been found to be activated along with retinoblastoma protein leading to neuronal death in MPP^+ induced PD model (Camins et al. 2010). However, the most crucial and strong correlation between cell cycle and PD is provided by the association of cyclin E and PARK2. Cyclin E is G_1/S phase marker of cell cycle and also a substrate of ubiquitin E3 ligase PARK2. Mutations associated with loss of functional PARK2 are linked with cyclin E enrichment led cell cycle and apoptosis through p53 and Bax in PD. Further, mutated PARK2/cyclin E events evoked upregulation of Wnt/β catenin and EGFR/AKT signal transduction pathways (Feng et al. 2015). This intriguing PARK2/cyclin E relation led to speculations that compounds which can bind to and attenuate the level of cyclin E can ameliorate post-mitotic cell division led neurodegeneration in PD.

Numerous studies have highlighted the neuroprotective action of Heat shock proteins (HSP) particularly, HSP70. Further, HSP70 is closely associated with cell cycle regulation and was also found to interact with cyclin E in inclusion body myositis and polymyositis (Kwon et al. 2014). Therefore, we carried out comprehensive data mining for HSP70 inducers in NDD and selected Bimoclomol amongst twenty compounds based on drug-likeness, pharmacokinetics and BBB permeability (Sharma and Kumar 2017b). Bimoclomol is a hydroxylamine derivative with molecular formula $C_{14}H_{20}ClN_3O_2$ and molecular weight of 297.783 g/mol. It has been shown to elicit protective effects through induction of HSP27, HSP70 and HSP90. Moreover, Bimoclomol is currently under Phase II trials in Amyotrophic lateral sclerosis (ALS) disease (Hesselink 2016). Therefore, we investigated the neuroprotective action of Bimoclomol through attenuation of cyclin E in Parkinson's disease. Various virtual screening methods such as, Lipinski filter, Ghose and Veber parameters, pharmacophore modelling based target prediction and ADME analysis were employed to check the efficacy of Bimoclomol as a neuroprotective agent. Further, we studied the cyclin E inhibiting potential of Bimoclomol through molecular docking studies.

4.2 Protein-ligand structure

The 3D structures of Bimoclomol and cyclin E were retrieved from docking server and PDB respectively (**Fig. 4.1**).

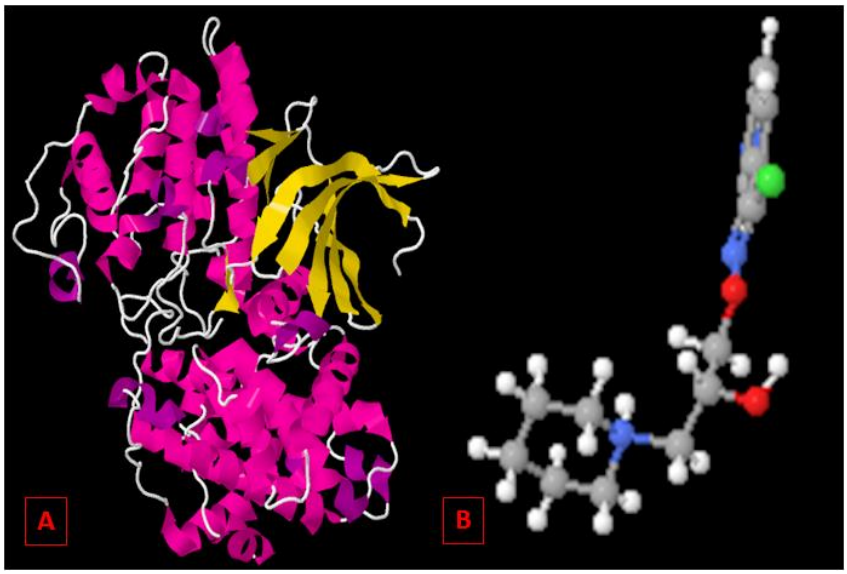


Fig.4.1: 3D structure of cyclin E (A) and Bimoclomol (B)

4.3 Screening for drug-likeness and ADMET Analysis of compounds

Bimoclomol passed all the parameters related to drug-likeness screening namely Lipinski, Ghose and Veber. Most importantly, it can cross the BBB and has high pharmacokinetics values (**Table 4.1**).

GI Permeability	High
LogS (ESOL)	-2.9
XLogP3	2.21
Bioavailability Score	0.55

Table 4.1: ADMET analysis of Bimoclomol

4.4 Pharmacophore based target prediction

The pharmacophore based target prediction of Bimoclomol revealed Mitogen Activated protein kinase 14 as one of the top ten targets with a fit score of 3.521 and Z score value of -0.408482 which supported our premise of its strong potential in inhibiting cell cycle (**Fig. 4.2**).

Ligand: 9576891							
Rank		PDB ID	Target Name	Number of Feature	Fit Score	Normalized Fit Score	z'-score
+	1	1ONG	Beta-lactamase SHV-1	4	3.548	0.8871	0.0454138
+	2	1DY4	Exoglucanase 1	4	3.525	0.8812	0.288696
+	3	1GCZ	Macrophage migration inhibitory factor	4	3.409	0.8523	-0.339281
+	4	2B55	Cell division protein kinase 2	5	3.802	0.7605	1.62195
+	5	2BTO	NONE	5	3.789	0.7578	1.49587
+	6	1Q6I	FKBP-type peptidyl-prolyl cis-trans isomerase fkpA	5	3.7	0.74	1.15025
+	7	3CPA	NONE	5	3.544	0.7087	-0.0490846
+	8	2ZAZ	Mitogen-activated protein kinase 14	5	3.521	0.7042	-0.408482
+	9	2UWL	Coagulation factor X	5	3.394	0.6788	-0.426764
+	10	3MDE	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	6	3.99	0.665	0.078465

Fig. 4.2: Pharmacophore based target prediction of Bimoclomol

4.5 Active site prediction

Out of top ten pockets, cyclin E had best pocket at P20 with a drugability score of 0.94 and 0.01standard deviation (**Fig. 4.3**). The volume of given pocket was 551.26 cubic angstroms and fourteen residues were involved in interaction at this site.

Pockets	Vol. Hull*	Hydroph. Kyte*	Polar Res.*	Aromatic Res.*	Otyr atom	Nb. Res.*	Drugg Prob*	Standard Deviation
P 0	2058.21	-0.36	0.59	0.17	0.0	29.0	0.72	0.06
P 1	2863.51	-1.49	0.71	0.26	0.03	31.0	0.26	0.01
P 15	634.09	0.15	0.53	0.2	0.0	15.0	0.88	0.04
P 17	642.52	0.21	0.4	0.07	0.0	15.0	0.82	0.02
P 2	852.24	0.12	0.41	0.06	0.0	17.0	0.77	0.03
P 20	551.26	0.73	0.29	0.07	0.0	14.0	0.94	0.01
P 3	1146.05	0.04	0.45	0.1	0.0	20.0	0.78	0.01
P 4	1067.3	-0.88	0.67	0.11	0.0	18.0	0.29	0.01
P 5	674.13	-0.63	0.63	0.13	0.0	16.0	0.42	0.02
P 9	1030.55	-0.64	0.57	0.14	0.0	14.0	0.46	0.03

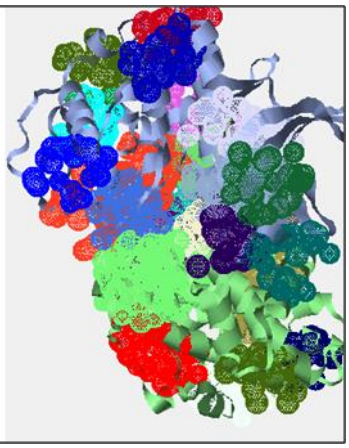


Fig. 4.3: Active sites of cyclin E

4.6 Molecular docking of Bimoclomol with cyclin E

Bimoclomol bound to cyclin E at P20 pocket and same residues as predicted were involved in the interaction (**Fig. 4.4**). The estimated free energy of binding for cyclin E and Bimoclomol was -5.07kcal/mol and total intermolecular energy was -6.48kcal/mol (**Table 4.2**). There were two H_2 bond formations involving GLU188 and LYS186 with bond energies of -0.2603 kcal/mol and -0.2271kcal/mol respectively. Further, a hydrophobic bond was formed with HIS147 (-0.6293 kcal/mol).

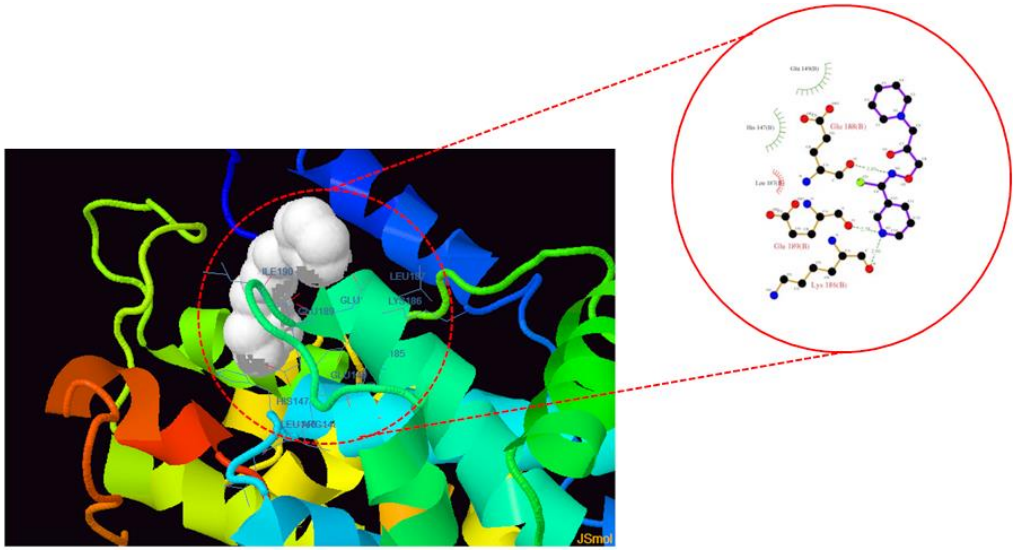


Fig. 4.4: Docking of Bimoclomol with cyclin E and residues involved (inset)

Estimated free energy of binding	-5.07 Kcal/mol
Estimated inhibition constant	191.27 uM
vdW+Hbond+desolv energy	-4.55 Kcal/mol
Electrostatic energy	-1.93 Kcal/mol
Total intermolecular energy	-6.48 Kcal/mol
Interacting surface	541.668

Table 4.2: Energies of binding for cyclin E and Bimoclomol

4.7 DISCUSSION

Parkinson's disease is the second most common age-related neurodegenerative disease affecting those aged above 60 years. Despite the knowledge of several factors which contribute in the occurrence and progression of PD, the exact cause and cure remains elusive. Ectopic activation of cell cycle in terminally differentiated neurons is a recently known phenomenon which has been shown to drive neurodegeneration through actual DNA synthesis followed by apoptosis (Sharma et al. 2017). Moreover, PD associated proteins have shared a very intimate relation with cell cycle markers. The G1/S phase marker cyclin E is a substrate of and ubiquitinated by the E3 ligase activity of PARK2. However, mutations in PARK2 led to loss of function thereby, resulting in cyclin E accumulation which in turn, activated E2F1 and triggered neuronal death in PD (Feng et al. 2015). Thus, it seems imperative to design therapeutic strategies aimed at attenuating the level of cyclin E to inhibit the cascade of neuronal death in PD.

Various biomolecules such as curcumin elicited HSP70 activity and provided protection against neuronal dysfunction, various cancers and in vascular diseases (Xia et al. 2015; Sharma and Kumar 2017; Joshi et al. 2017). Bimoclomol is a hydroxylamine derivative which is non-toxic and elicited its protective effect through HSP induction; including HSP70 (Deane and Brown 2016). HSPs are molecular chaperones which are upregulated in the cell to protect it against heat, ROS and hypoxia. HSP70 has been shown to promote neuronal survival by mediating the activation of pro-survival signalling cascades and through autophagy induction (Sharma et al. 2017). Interestingly, HSP70 has been shown to interact with cyclin E in Aβ induced cell cycle re-entry in inclusion body myositis and polymyositis (Kwon et al. 2014). Taken together, all these data provide convincing

evidence of using HSP70 inducing compound such as Bimoclomol in attenuating the level of cyclin E and in turn, inhibit the cascade of neuronal death in PD.

In the present study, we tested the drug-able efficacy of Bimoclomol for targeting cyclin E in PD. Emphasis was laid on pharmacokinetic analysis as aqueous solubility and dissolution in GI fluids are defining parameters of *in vivo* bioavailability of an orally administered drug (Khadka et al. 2014). Similarly, lipophilicity of a drug directs physiological properties such as rate of metabolism, transport across cell membrane and interaction with binding sites of receptor. Further, CNS drugs should have logP value less than 4 (Chico et al. 2009; Wager et al. 2010). The logP value for Bimoclomol was found to be 2.21.

However, the most important property required of a compound intended to be a neuroprotective agent is Blood brain barrier (BBB) permeability. Bimoclomol qualified all the above mentioned parameters and scored well on pharmacokinetics, bioavailability score and could cross the BBB. Finally, molecular docking studies indicated that Bimoclomol can bind to and attenuate the level of cyclin E and possibly, halt or inhibit cell cycle re-entry mediated neuronal death in PD. These findings can be validated through *in vitro* and *in vivo* cell cycle studies in Parkinson's disease.

The *in silico* results obtained and discussed in the previous two chapters have shown very promising potential of HSP70 inducing compounds in targeting post-mitotic cell division in PD. This encouraged us to undertake *in vitro* studies to confirm the findings of virtual screening. Therefore, in an attempt to study the efficacy of biomolecules in alleviating neurotoxin induced toxicity, we have carried out various *in vitro* experiments in the neuroblastoma cell line in the succeeding chapter.

CHAPTER V

NEUROPROTECTIVE EFFECT OF BIOMOLECULES IN ROTENONE INDUCED TOXICITY IN SH-SY5Y CELL LINES

CHAPTER V: NEUROPROTECTIVE EFFECT OF BIOMOLECULES IN ROTENONE INDUCED TOXICITY IN SH-SY5Y CELL LINES

5.1: Introduction

The previous chapters outlined very promising repurposing potential of HSP70 inducing compounds in alleviating CCE mediated neuronal apoptosis in NDD, especially PD. Thus, in an attempt to verify the virtual screening results, we carried out various studies to check the efficacy of HSP70 inducing biomolecule in ameliorating neuronal cell death in SH-SY5Y cell line. Also, changes in cell viability subsequent to the effect of toxin and upon biomolecule treatment have been assessed. Moreover, the expression level of various cell cycle markers subsequent to rotenone administration and after biomolecules treatment has been described.

5.2: Dose-dependent and time-dependent toxicity assay of Rotenone

A stock of rotenone in DMSO was prepared and from this various gradients of rotenone were prepared in increasing concentration of 150nM, 300nM, 900nM, 10μM, 20μM, 40μM and 80μM. The effect of each of these doses on SH-SY5Y cells was studied in a time period of 6 hours, 12 hours and 24 hours. The results obtained were evaluated through changes in cell morphology, cell viability assay, protein profiling or western blot and finally, statistical calculations and are summarised in succeeding sections.

5.2.1: Rotenone exerts dose-dependent and time-dependent toxicity in SH-SY5Y cells

The results obtained in present study clearly show that rotenone induces toxicity in SH-SY5Y cells in a dose-dependent and time-dependent manner. The change in cell morphology upon rotenone administration in comparison to control cells is clearly evident in **Fig.5.1**.

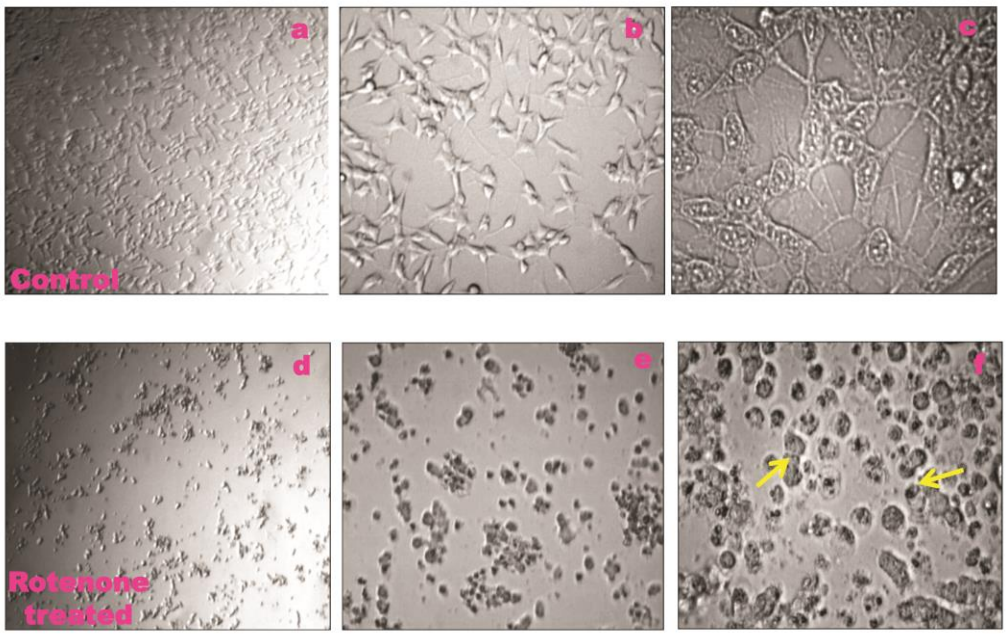


Fig.5.1: Rotenone induced toxicity which is apparent by changed cell morphology (d- 4x; e-10x; f-20x, 24hr) compared to control cells (a-4x; b-10x; c-20x, 24 hr)

5.2.1.1: Rotenone exerts mild toxicity at 150nM-300nM

The SH-SY5Y neuroblastoma cells exhibited nearly normal morphology when compared with control and SHAM post 150nM-300nM rotenone administration after 6-12 hours (**Fig. 5.2**). Most of the cells were alive at these concentrations even after 24 hours. The viability was only slightly decreased at 300nM (**Fig. 5.2 d-f**) in comparison to 150nM concentration (**Fig. 5.2 a-c**).

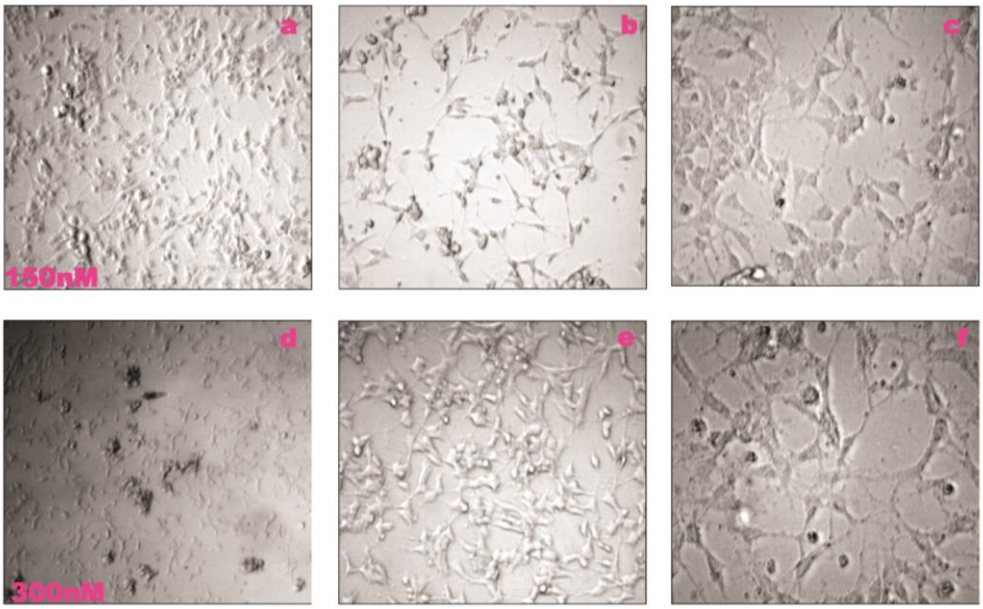


Fig. 5.2: Rotenone exerts mild toxicity at 150nM (a-6hr, 4x; b-12hr, 10x; c-24hr, 20x) and 300nM (d-6hr, 4x; e-12hr, 10x; f-24hr, 20x)

5.2.1.2: Rotenone exerts moderate toxicity at 900nM-10µM

The 900nM concentration of rotenone exerted some toxicity after 6 hours. Further, although cells were mostly alive, toxicity at this concentration was visibly apparent after 12 hours. After 24 hours, the confluency of cells was markedly decreased though; the morphology of cells was somewhat retained (**Fig. 5.3 a-c**). However, the toxicity of 10µM rotenone on neuroblastoma cells was very apparent after 6 hours as cell morphology was visibly distorted. As expected, the morphology degraded after 12 hours and clumps of morphologically abnormal cells were seen after 24 hours of treatment (**Fig. 5.3 d-f**).

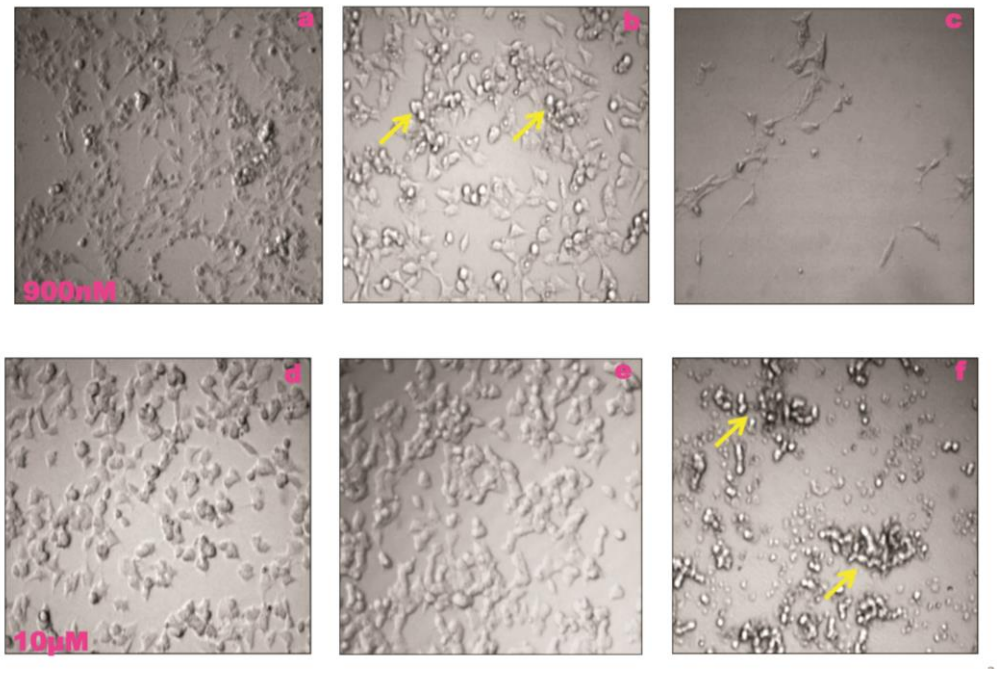


Fig.5.3: Moderate toxicity seen at rotenone doses 900nM (a-6hr, 4x; b-12hr, 10x; c- 24hr, 20x) and 10μM (d-6hr; e-12hr; f-24hr, at 10x magnification)

5.2.1.3: LD_{80} concentration of rotenone is 20μM

The 20μM concentration of rotenone exhibited heavy toxicity on SH-SY5Y cells which was clearly evident after 6 hours of administration in the form of almost complete distortion of neuronal cell morphology. Further, cell morphology was lost after 12 hours and more than 80% cells were dead post 24 hours of 20μM dose administration (**Fig. 5.4 a-c**). Thus, after repeating the experiment in triplicates thrice, 20μM concentration of rotenone was taken as the LD_{80} value in SH-SY5Y cells. Furthermore, the rotenone toxicity was extremely high at 40μM (**Fig. 5.4 d-f**) and 80μM (**Fig. 5.4 g-i**) concentration and immediate deformation of neuronal cells with cell death was visible. Moreover, the morphology and viability was completely lost at 12 hours and cells were floating after 24 hours.

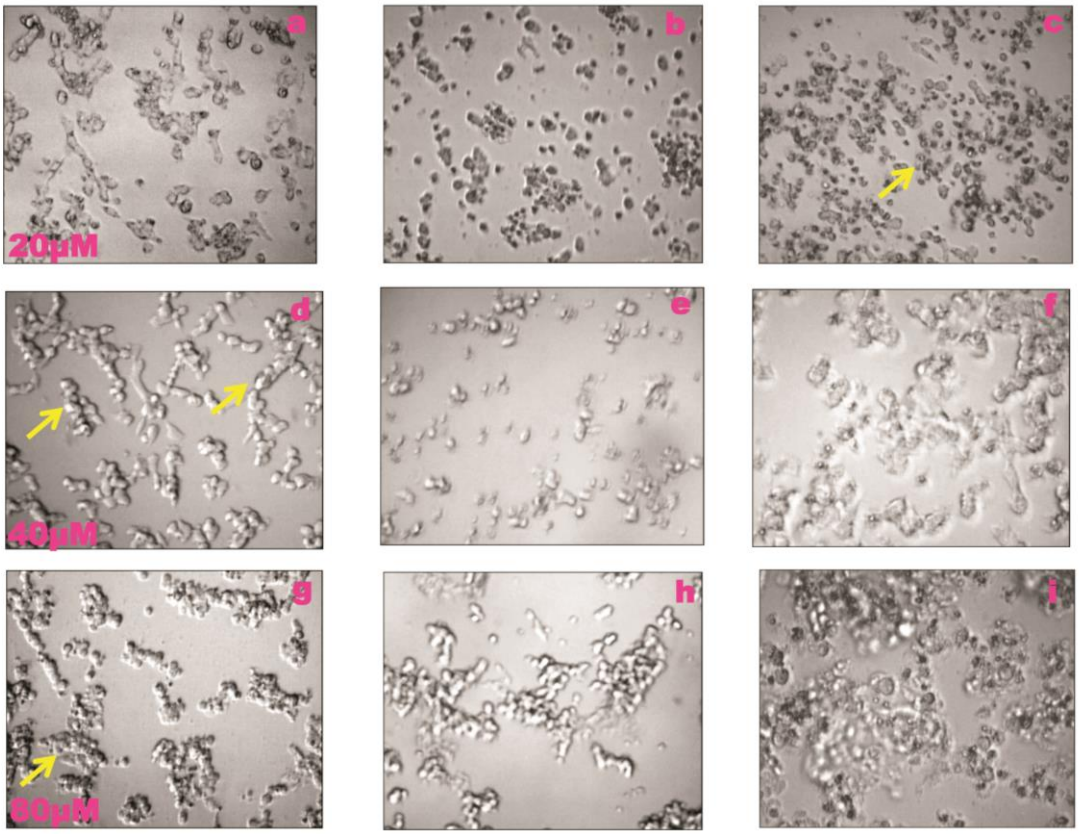
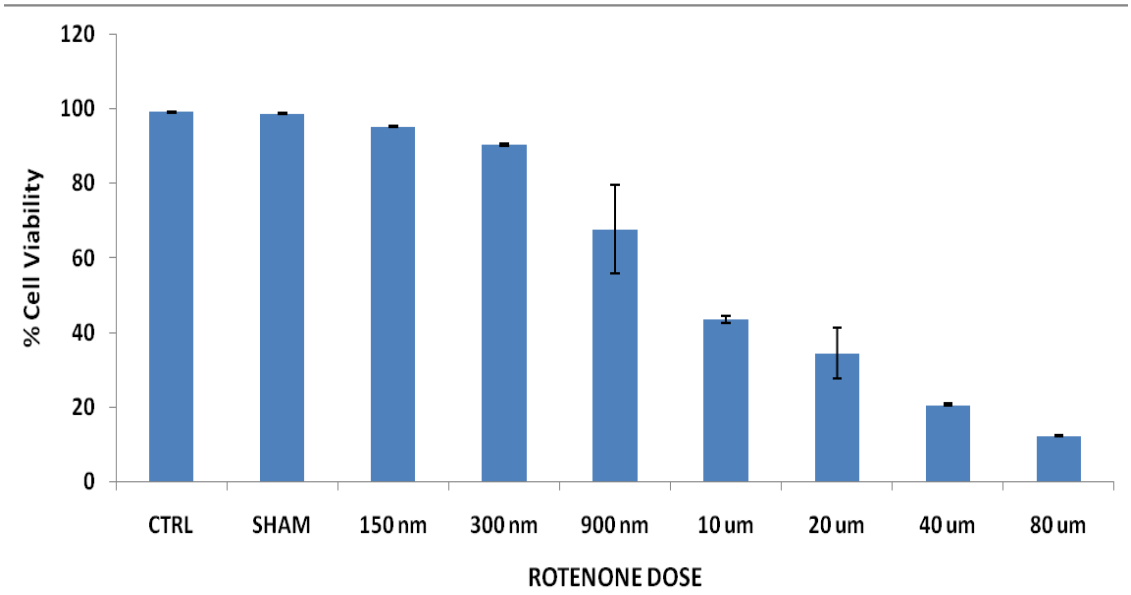


Fig.5.4: Rotenone toxicity is more than 80% at 20µM concentration (a-6hr; b-12hr; c-24hr, 10x). Beyond this dose toxicity is too high and cells are mostly dead within 6h (d) at 40µM (e- 12hr; f-24hr, 10x) and 80µM (g-6hr; h-12hr; i-24hr, 10x) concentration

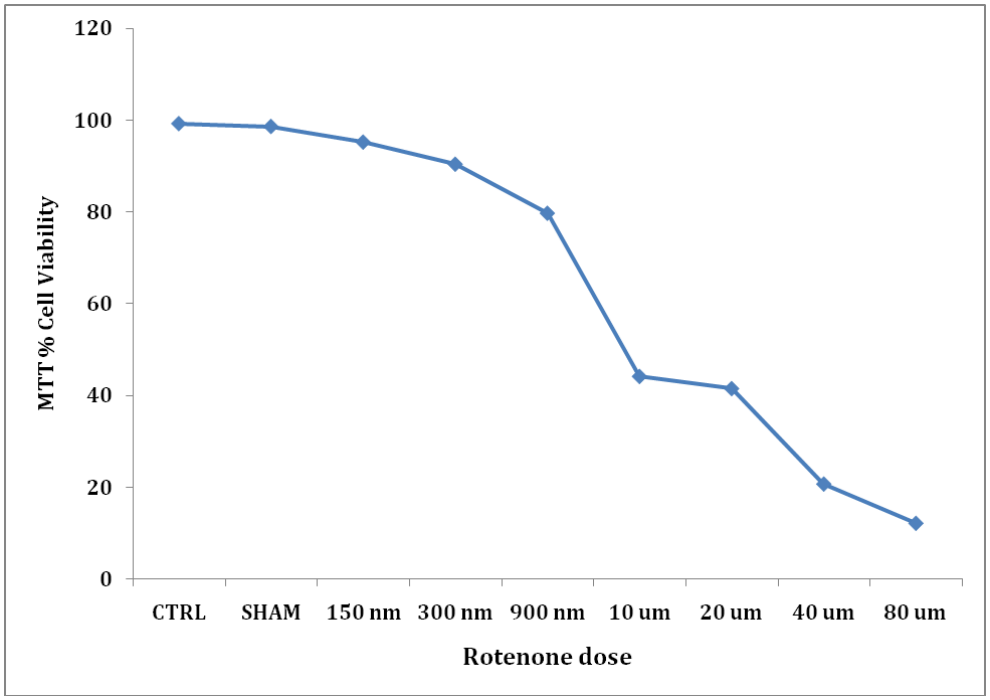
5.2.2 Effect of Rotenone Dose on Cell Viability

To determine the effect of rotenone on cell viability, the trypan blue exclusion test was performed in all the above mentioned sets of experiments. Accordingly, the number of live (unstained) and dead (stained) cells were counted in a hemocytometer and percent cell viability determined in triplicate set of experiments. The results in the form of statistically calculated values were plotted on a graph. The cell viability was found to decrease with increasing concentration of rotenone, further validating the dose-dependent effect of rotenone toxicity on SH-SY5Y cells (**Graph 5.1**). Moreover, the quantification of cell

viability was estimated through the colorimetric MTT Assay which corroborates dose-dependent effect of rotenone in inducing toxicity in SH-SY5Y neuronal cells **(Graph 5.2)**.



Graph 5.1: Dose-dependent effect of rotenone toxicity on SH-SY5Y cells. As the dose of rotenone was increased, cell viability attenuated significantly as deduced from trypan blue exclusion test



Graph 5.2: MTT assay showing percent cell viability as a measure of control against rotenone doses. Cell viability decreased considerably with increasing concentration

Next, we checked the neuroprotective potential of one of the biomolecules outlined in *Chapter III* as HSP70 inducing compound; Sesamol in alleviating rotenone induced toxicity and to check its efficacy on cell viability and on the expression of various cell cycle markers. The results are summarised in the next section.

5.3 Biomolecules reverse rotenone-induced toxicity in SH-SY5Y Cells

5.3.1 Dose-dependent effect of Sesamol in rotenone-induced toxicity in SH-SY5Y Cells

The concentration gradient of Sesamol was prepared in increasing order of 25μM, 50μM, 75μM, 100μM and 125μM respectively. Next, the LD_{80} value of rotenone which was determined as 20μM in preceding experiment was administered to SH-SY5Y neuroblastoma cells for 24 hours. The cell death incurred upon rotenone administration at 20μM was determined through trypan blue cell viability assay and observed morphological deformations. Next, the varying concentrations of Sesamol were used to study its neuroprotective effect on rotenone induced toxicity on SH-SY5Y cells in different time period of 6 hours, 12 hours and 24 hours. The results obtained are summarised below.

5.3.1.1 Sesamol protects against rotenone-induced toxicity at 25μM concentration

Sesamol restored almost 75% cells which were non-viable post-rotenone induced toxicity in SH-SY5Y cells. The rounded morphology of rotenone-treated cells was transformed into well defined neurite-like structures upon 25μM Sesamol treatment (**Fig. 5.5 d-f**). Further, the cell viability was greatly enhanced. Therefore, the present study observed well marked neuroprotective effect of 25μM sesamol against rotenone induced toxicity in SH-SY5Y cells.

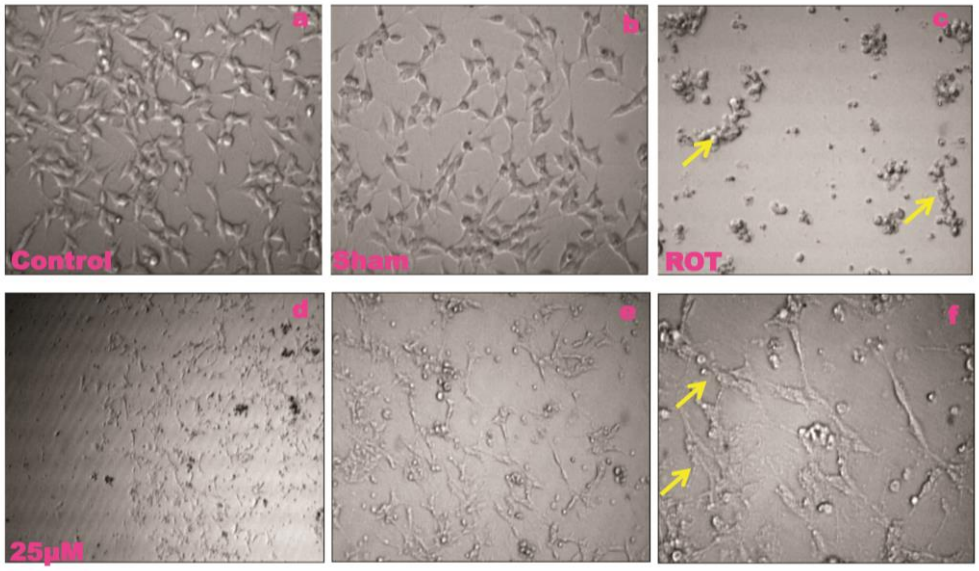


Fig. 5.5: Sesamol is neuroprotective at 25 μM and revived more than 75% cells at this dose (d-6hr, 4x; e-12hr, 10x; f-24hr, 20x) compared to control (a-control; b- Sham; c-rotenone treated; 24hr, 10x)

5.3.1.2 Sesamol reverses rotenone-induced toxicity at 50μM concentration

The concentration of sesamol at which there was almost complete reversal of rotenone-induced toxicity was found to be 50μM. The neuronal morphology was very well defined and long neurites could be seen after treatment with this dose (**Fig. 5.6 d-f**). Moreover, these results are corroborated with cell viability assays. Thus, sesamol rescued neuronal cells from rotenone toxicity at 50μM.

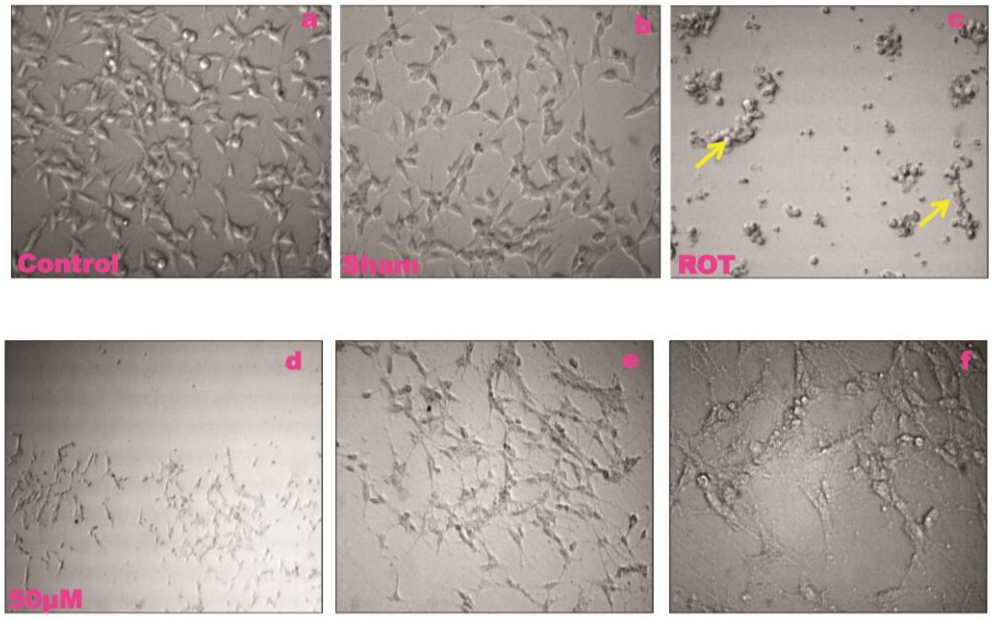


Fig.5.6: Sesamol reverses rotenone toxicity at 50µM concentration (d-4x; e-10x; f-20x, 24hr). At this dose all the cells were rescued and cell viability was greatly enhanced (a-control; b-Sham; c-rotenone treated; 24hr, 10x)

5.3.1.3 Sesamol had varying effect at 75µM-100 µM concentration

In contrast to preceding observations, sesamol did not exhibit much increase in cell viability on further increasing the dose to 75µM (**Fig. 5.7 d-f**) and 100µM (**Fig. 5.7 g-i**) respectively. Infact, the cell viability was found to be attenuated in comparison to the preceding concentration of 50µM. Moreover, the cell morphology and confluency was somewhat diminished compared to 50µM. Though the cell confluency was slightly increased upon 100µM sesamol treatment, the overall cell viability was not appreciably enhanced.

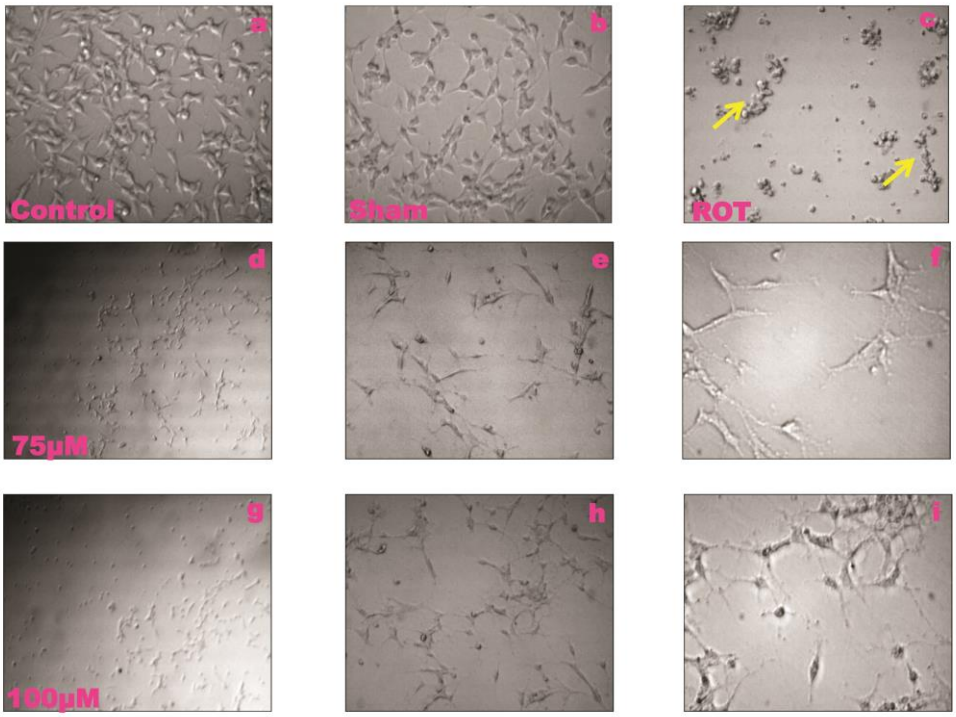


Fig.5.7: Sesamol had varying effects at 75μM-100μM doses. It did not show significant increase in viability on increasing the dose to 75μM concentration (d-4x; e-10x; f-20x, 24hr) and 100 μM concentration (g-4x; h-10x; i-20x, 24hr). Also, there was a slight decrease in cell viability and morphology at these doses

5.3.1.4 Neuroprotective effect of sesamol is resumed at 125μM concentration

The neuroprotective effect of sesamol was found to exert dose-dependent effect on rotenone-induced toxicity in SH-SY5Y neuronal cells. However, cell viability at 75μM and 100μM concentration showed a slight decrease. Interestingly, when sesamol concentration was further increased to 125μM, marked enhancement in cell viability was noted. Further, the confluency and morphology of cells was visibly improved compared to the preceding doses. Therefore, the neuroprotective effect of sesamol was restored at 125μM concentration against rotenone-induced toxicity (**Fig. 5.8 d-f**).

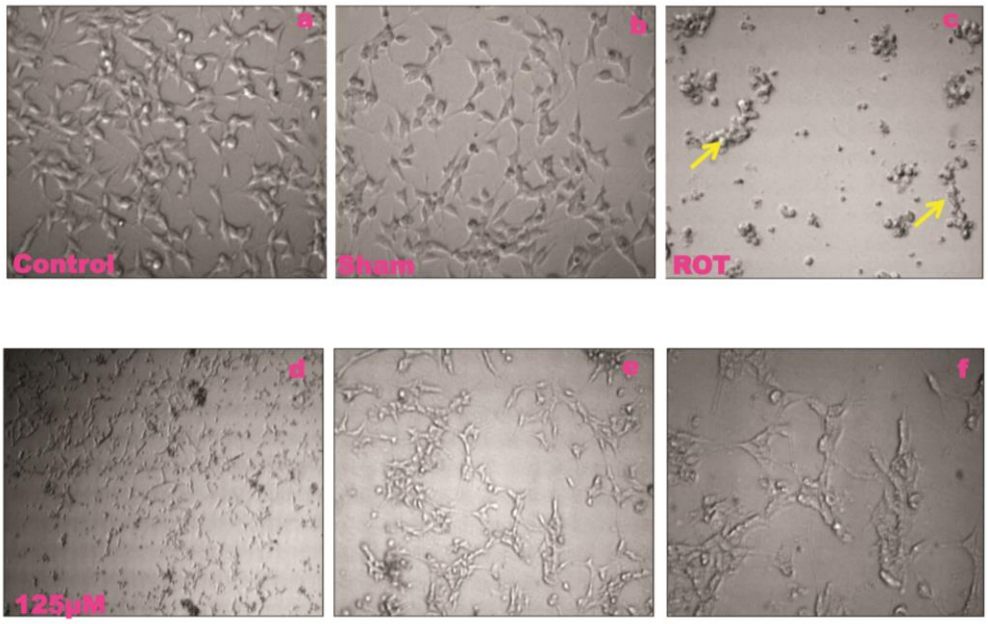
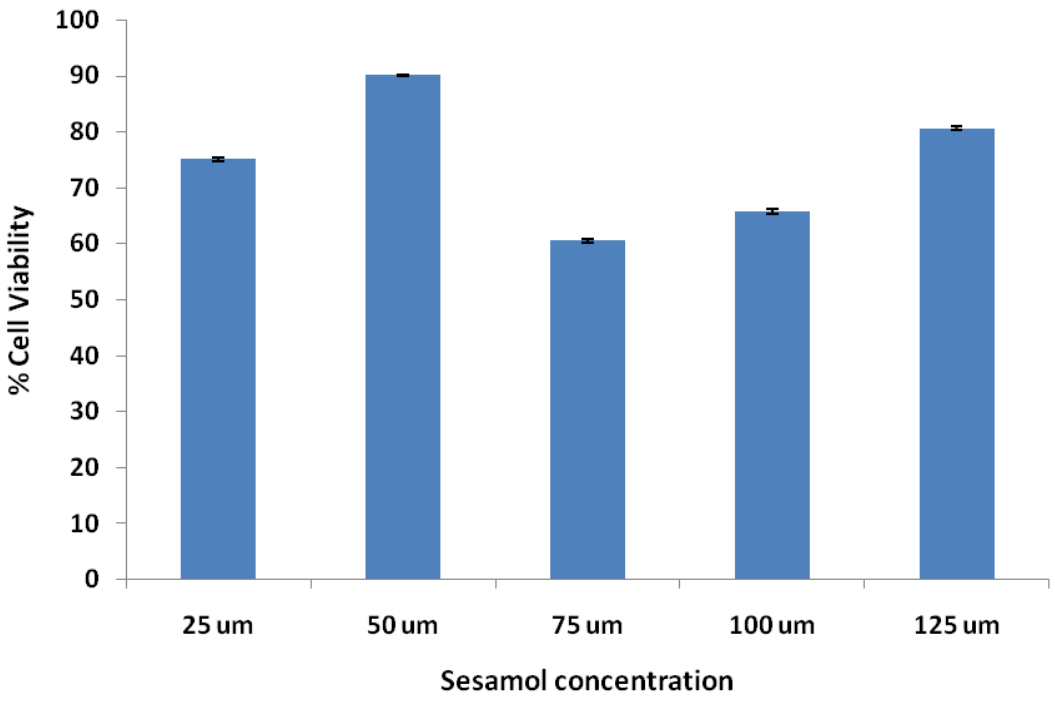


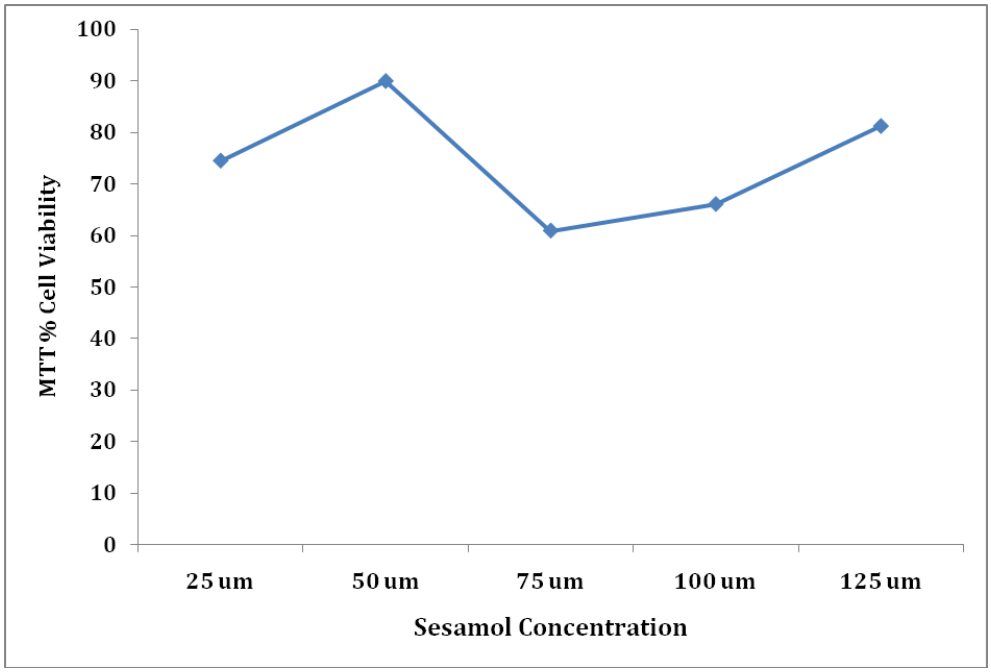
Fig.5.8: Sesamol reinstated neuroprotective action at 125 μM concentration (d-4x; e- 10x; f-20x, 24hr). The cell viability was significantly enhanced along with improved cell morphology

5.3.1.5 Effect of dose of sesamol on cell viability

The neuroprotective effect of sesamol on cell viability in rotenone induced toxicity was determined through trypan blue exclusion test. Post 24 hours of rotenone administration, cells were treated with sesamol for 24 hours. Next, the trypan blue test was performed for each experimental and control group. Accordingly, the number of live (unstained) and dead (stained) cells were counted in a hemocytometer and percent cell viability determined in triplicate set of experiments. The results in the form of statistically calculated values were plotted on a graph **(Graph 5.3)**. Additionally, the quantification of cell viability was estimated through the colorimetric MTT Assay. The values obtained through MTT Assay show dose-dependent effect of sesamol in reviving neuronal cells against rotenone induced toxicity (**Graph 5.4**). To summarise, sesamol treatment increased cell viability in a dose-dependent manner in rotenone induced toxicity in SH-SY5Y cells.



Graph 5.3: Sesamol increased cell viability in a dose-dependent manner against rotenone toxicity. At 50µM sesamol reversed cell toxicity and greatly enhanced cell viability



Graph 5.4: MTT assay showing sesamol exerted neuroprotection in dose-dependent manner against rotenone toxicity. Complete reversal of rotenone toxicity was observed

at 50μM dose and slight decrease observed at 75μM concentration

To further corroborate the previous findings that Sesamol acts through HSP70 induction in reviving neuronal cells against rotenone induced toxicity, we used a HSP70 inhibitor; Quercetin to carry out same set of experiments and the results obtained are summarised in the succeeding section.

5.3.2 Dose-dependent effect of Quercetin in rotenone-induced toxicity

The concentration gradient of quercetin was prepared in increasing order of 10μM, 20μM, 50μM, 75 μM and 100μM respectively. Next, 20μM rotenone was administered to SH-SY5Y neuroblastoma cells for 24 hours. The cell death incurred upon rotenone administration was determined through trypan blue cell viability assay. Next, the effect of varying concentrations of quercetin on rotenone induced toxicity was studied in different time period of 6 hours, 12 hours and 24 hours. The results obtained are summarised below.

5.3.2.1 Quercetin does not attenuate rotenone induced toxicity at 10μM-20μM concentration

Rotenone treated cells were incubated with 10μM and 20μM quercetin respectively for 24 hours. The effect of quercetin was observed after 6 hours, 12 hours and 24 hours. However, quercetin had no effect on rotenone induced toxicity in SH-SY5Y cells at 10μM (**Fig. 5.9 d-f**) and 20μM concentration (**Fig. 5.9 g-i**). The morphology of neuronal cells as well as the cell viability assessed through trypan blue test and MTT assay remained unchanged on treatment with 10μM and 20μM quercetin respectively (**Graph 5.5 and 5.6**).

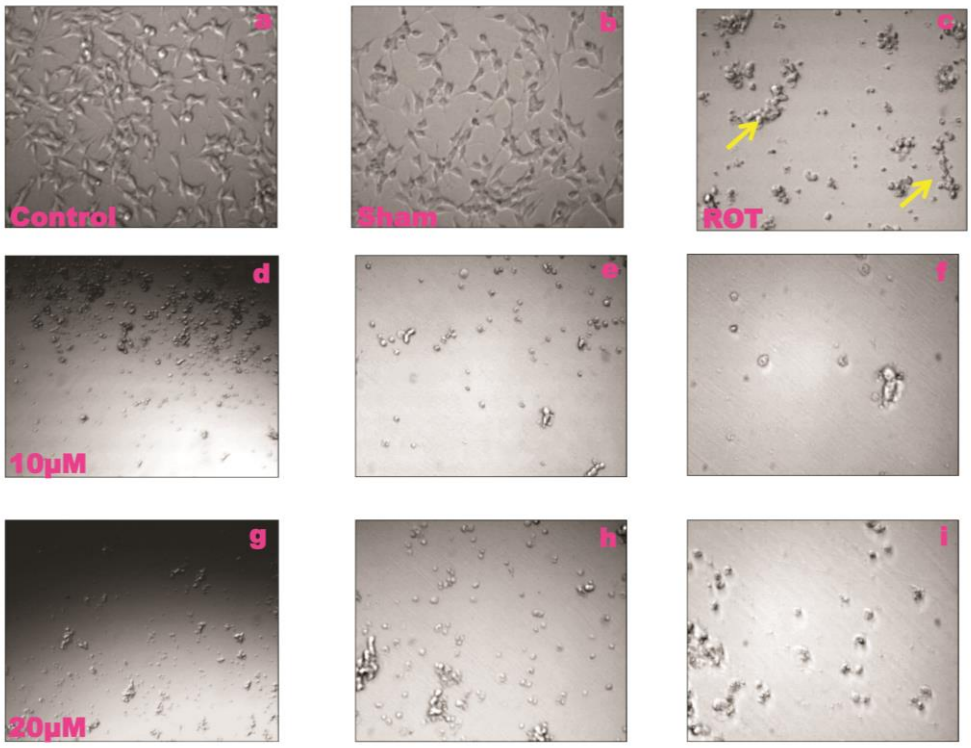


Fig. 5.9: Quercetin had no effect on rotenone toxicity at 10µM (d-4x; e- 10x; f- 20x, 24hr) and 20µM (g- 4x; h- 10x; i- 20x, 24hr) concentration

5.3.2.2 Quercetin shows slight activity at 50µM -75 µM concentration

The dose of quercetin was further increased to study its effect in rotenone induced toxicity. Accordingly, 50µM and 70µM quercetin was used to treat rotenone administered neuronal cells. After 24 hours it was observed that these doses of quercetin had a very slight effect on neuronal cells. Moreover, a very few viable cells were observed in the total population of rotenone induced dead cells. Further, the cell viability was very slightly increased at 50µM quercetin concentration (**Fig. 5.10 d-f**). Interestingly, on further increasing the dose of quercetin to 75µM (**Fig. 5.10 g-i**), attenuation in cell viability as compared to the preceding dose was observed.

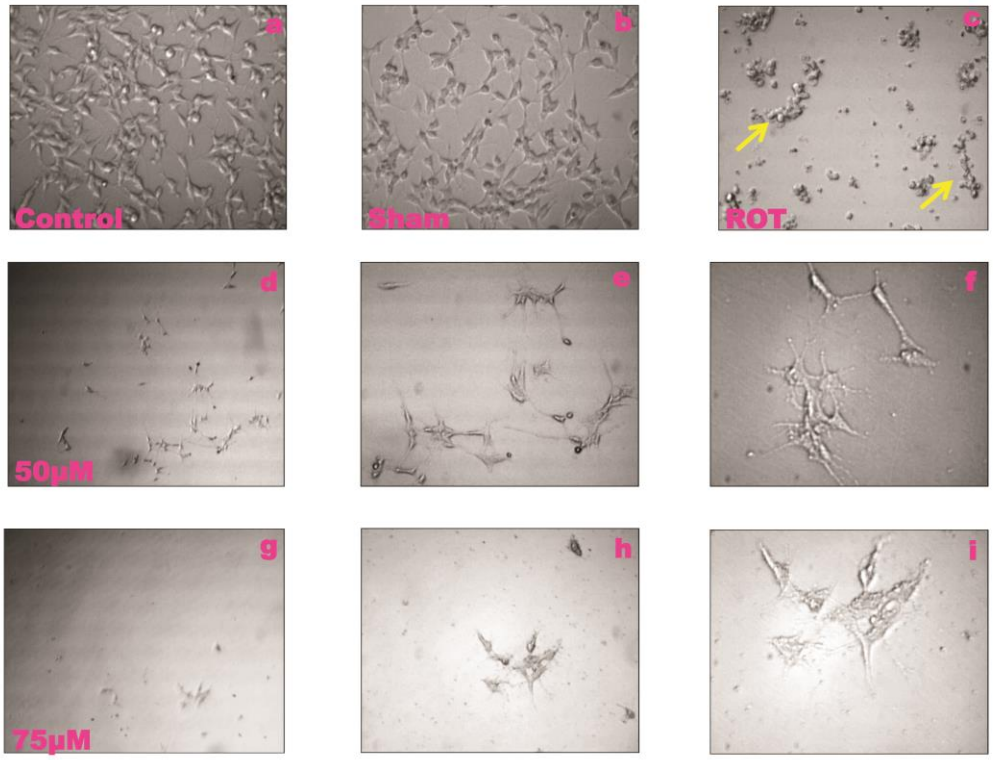


Fig.5.10: Quercetin showed very silght activity at 50µM (d- 4x; e- 10x; f- 20x, 24hr) and 75µM (g- 4x; h- 10x; i- 20x, 24hr) concentration. Although the activity is insignificant in the total population of dead cells

5.3.2.3 Quercetin shows very mild effect at 100 µM concentration

Finally, the dose of quercetin was increased to 100µM concentration. However, even at such high dose not much effect was observed on cells. The neuronal cells did show some response to quercetin treatment at 100 µM concentration (**Fig. 5.11**). A small number of cells were revived at this dose and cell viability slightly enhanced. However, compared to the dose applied the generated response was very mild in the overall population of neuronal cells.

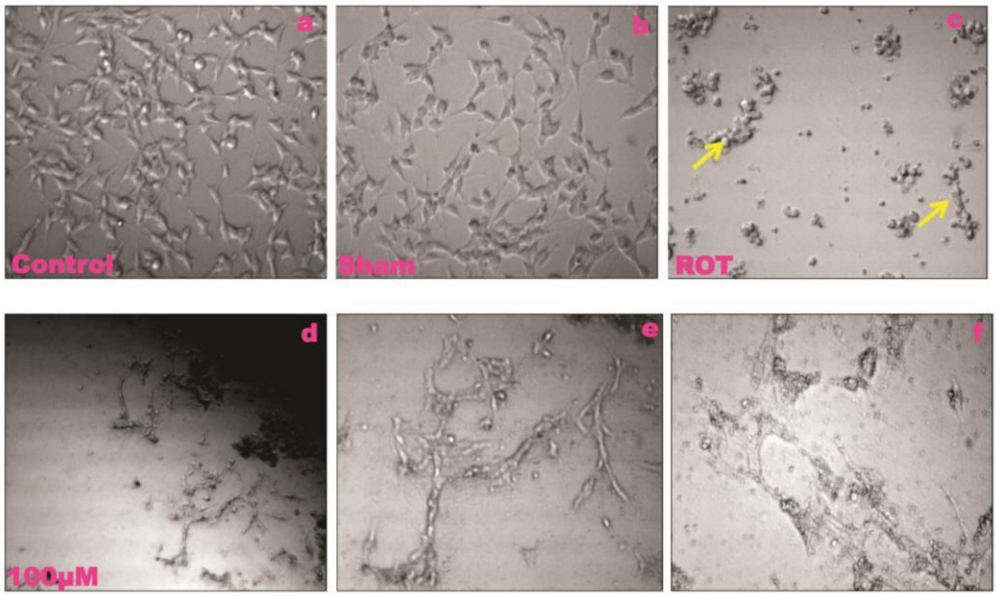
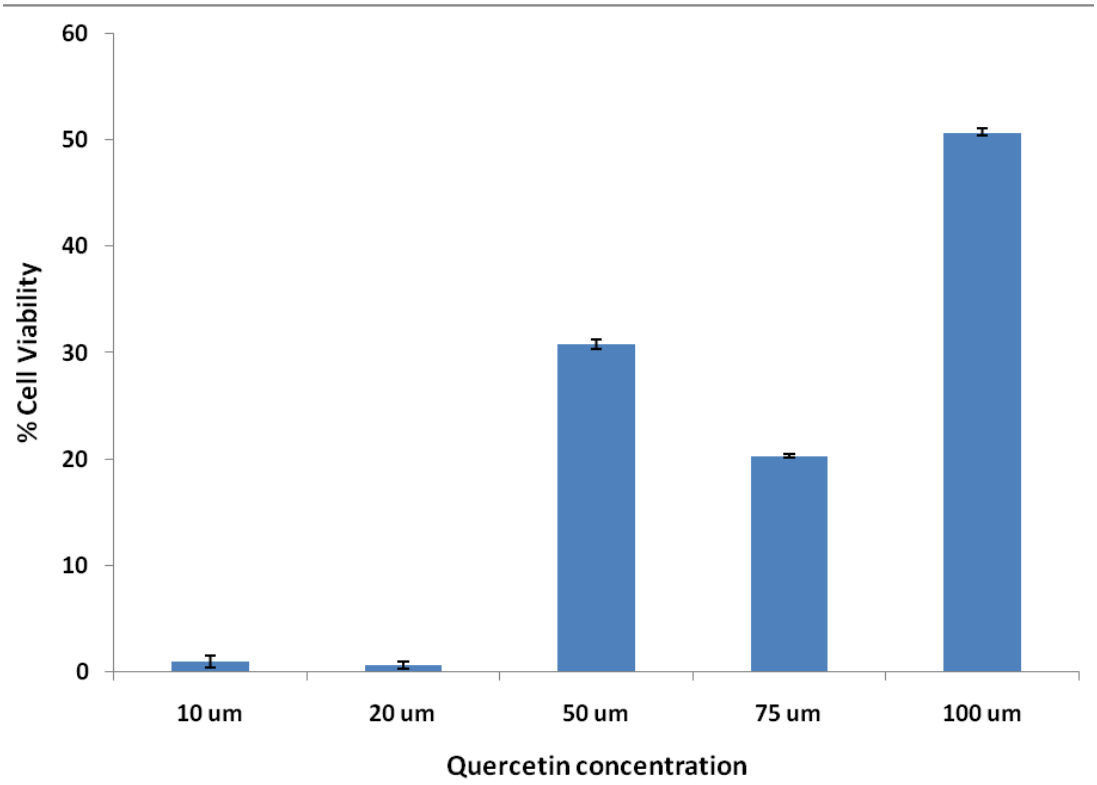


Fig.5.11: Effect of Quercetin on rotenone toxicity at a high dose of 100µM (d-4x; e-10x; f-20x, 24hr) elicited very mild response in cells. Though, overall the observation had not much significance in comparison to dose applied

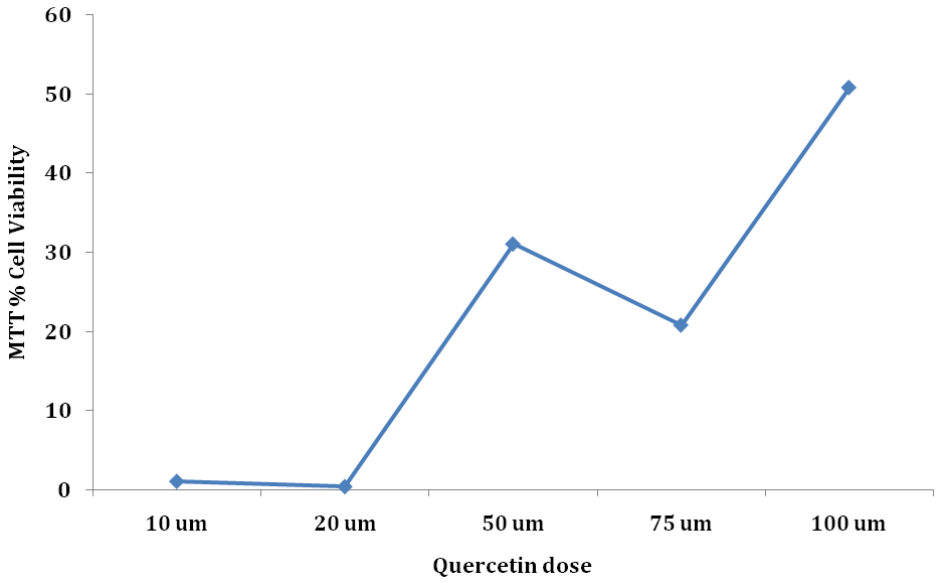
5.3.2.4 Effect of dose of quercetin on cell viability

The neuroprotective effect of quercetin on cell viability in rotenone induced toxicity was determined through trypan blue exclusion test. Rotenone treated cells were incubated with varying concentration of quercetin for 24 hours. Next, the trypan blue test was performed for each experimental and control group. Further, the number of live (unstained) and dead (stained) cells were counted in a hemocytometer and percent cell viability determined in triplicate set of experiments. The results in the form of statistically calculated values were plotted on a graph (**Graph 5.5**). Further, the quantification of cell viability was estimated through the colorimetric MTT Assay. The observations of MTT Assay also show slight effect of quercetin compared to control in rotenone induced toxicity. The cell viability estimation as a measure of control through MTT Assay has been summarised in **Graph 5.6**.

To sum up, quercetin treatment did not exhibit significant increase in cell viability in rotenone induced toxicity in SH-SY5Y cells.



Graph 5.5: Cell viability as a measure of quercetin doses showing no significant results



Graph 5.6: MTT assay showing no significant effect of Quercetin on cell viability. The slight increase at 50µM and 100µM concentration was overall insignificant in total population

5.4 Western blot for various cell cycle and PD related markers

5.4.1 Rotenone induced upregulation of Cyclin E

Cyclin E is a cell cycle marker of G_1/S phase which normally remains suppressed in neurons. Accordingly, level of cyclin E was found to be less in control cells. However, rotenone treatment increased the level of cyclin E, indicative of cell cycle re-entry and progression in G_1/S phase. Rotenone led increase in cyclin E may be attributed to ROS generation.

5.4.2 Rotenone decreased Parkin activity

Parkin is an ubiquitin E3 ligase and a loss in its activity has been linked to increase in various substrates which can have detrimental effect on cells. Rotenone treatment led to attenuation of parkin level when compared to control neuroblastoma cells. Loss in parkin activity can indicate neuronal cell death observed in rotenone treated cells compared to no cell death in control where parkin levels are high. Further, loss of parkin activity may also contribute to increased level of cyclin E as the latter is substrate of parkin.

5.4.3 Rotenone attenuated HSP70 expression

HSP70 is a molecular chaperone which exerts protective function under stress. The administration of rotenone led to attenuated HSP70 in comparison to control cells. Low activity of HSP70 may outline cell death induced by rotenone in SH-SY5Y cells.

5.4.4 Sesamol reduced the level of Cyclin E

Sesamol treatment attenuated the levels of cyclin E which was upregulated in response to rotenone exposure. The decrease in levels of cyclin E may be mediating significant

acceleration in cell viability upon sesamol treatment in neuronal cells. Further, this decrease may signify halt in cell cycle activity.

5.4.5 Sesamol restored Parkin activity

Sesamol restored the activity of parkin which was attenuated in response to rotenone administration. Sesamol mediated increase in parkin level may also explain the decrease in level of parkin substrate cyclin E.

5.4.6 Sesamol treatment enhanced HSP70 expression

The level of HSP70 was enhanced in response to sesamol treatment. Increase in HSP70 may explain augmented cell survival of neuronal cells as HSP70 is a pro-survival marker.

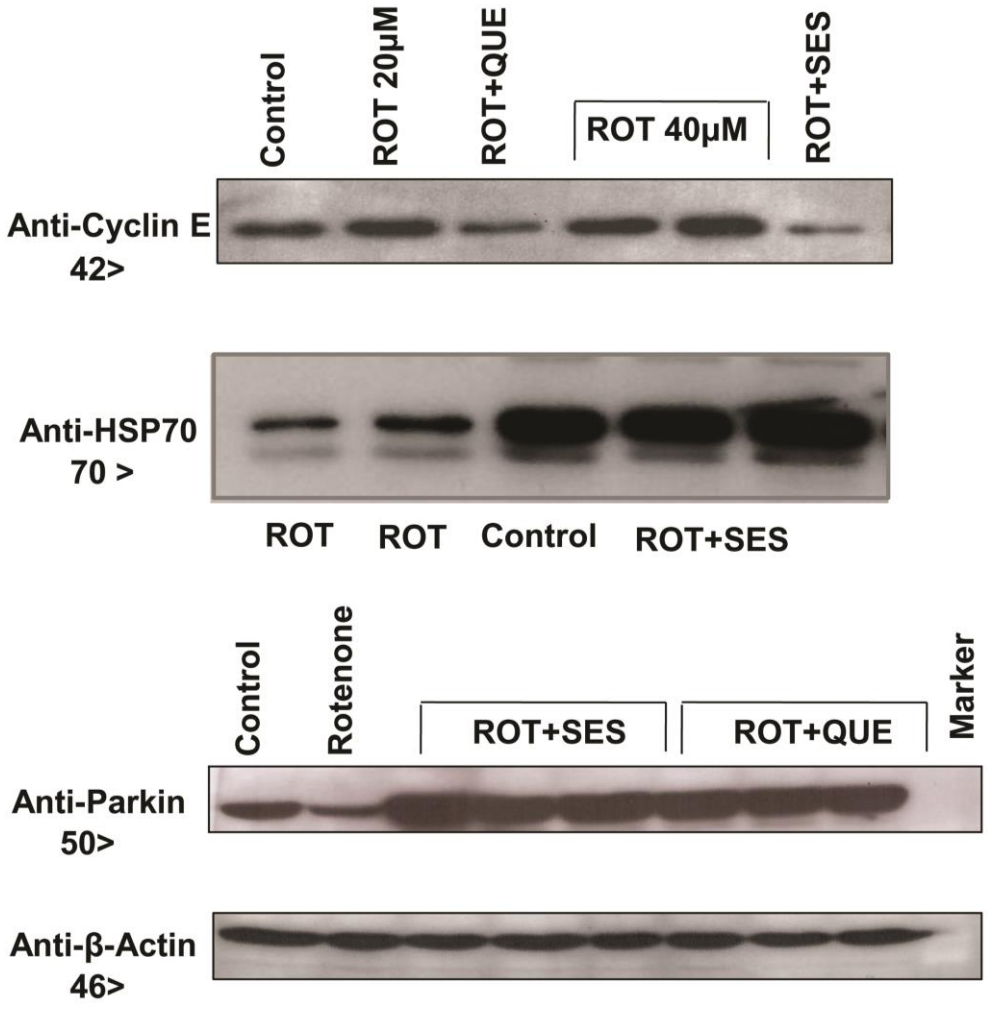


Fig. 5.12: Rotenone increased the level of cyclin E compared to control, with robust expression on administering high dose of 40μM. Sesamol treatment attenuated cyclin E levels (A). Further, the level of HSP70 which was reduced on rotenone treatment was restored upon sesamol treatment (B). Similarly, parkin activity was decreased on rotenone administration and its normal levels were maintained upon treatment with sesamol (C), compared with β-actin levels as loading control (D)

5.4.9 Discussion

The previous chapters described the promising potential of HSP70 inducing compounds in ameliorating CCE mediated neuronal cell death in NDD with an emphasis on Parkinson's disease. Out of the three compounds shortlisted in *Chapter III*, the neuroprotective potential of Sesamol in alleviating neuronal cell death was evaluated in the present chapter. The neurotoxin rotenone which is widely used to mimick PD model was used to instigate toxicity in SH-SY5Y neuroblastoma cell line. The toxicity assay shows dose-dependent and time-dependent effect of rotenone in inducing neuronal cell death and cell viability markedly decreased with increasing dose of rotenone. Moreover, the LD_{80} value of rotenone was determined at 20μM concentration in present study. Further, treatment with sesamol rescued cells against rotenone induced toxicity at 50μM dose and sesamol exhibited dose-dependent and time-dependent neuroprotection in enhancing cell viability.

The protein expression level of various cell cycle markers subsequent to rotenone administration and on sesamol treatment was checked through western blotting. The results show that sesamol attenuated the level of cyclin E which was upregulated on rotenone administration. Also, the activity of parkin was restored upon sesamol treatment which was markedly decreased on rotenone administration. This increase in parkin activity may also explain reduced level of cyclin E which is a substrate of parkin. Further, the level of HSP70

increased upon sesamol treatment which was also attenuated in response to rotenone exposure.

Taken together, results obtained from western blotting experiment corroborate the findings that cell cycle re-entry may be checked by using HSP70 induction strategies. Sesamol restored the activity of parkin and HSP70 further corroborating our theory that induction of HSP70 is a pro-survival pathway in rotenone induced toxicity and HSP70 inducing compounds have a strong potential in attenuating post-mitotic cell division led neuronal death.

Furthermore, to corroborate the finding of HSP70 induction as the possible mechanism of survival in sesamol induced cell viability, the expression of HSP70 was knocked down with the well known compound Querectin. The same sets of experiments were repeated with quercetin against rotenone toxicity. The results obtained show that quercetin has no significant effect on cell viability. Therefore, it can be implied from the given results that sesamol may be rescuing neuronal cells against rotenone induced toxicity through the induction of HSP70. The detailed mechanism of such possible interaction can be checked through further studies.

CHAPTER VI

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVE

CHAPTER VI: SUMMARY, DISCUSSION AND FUTURE PERSPECTIVE

Cell cycle is a crucial phenomenon responsible for generation of new daughter cells, growth, repair and regeneration in the cell. Neurons are post-mitotic cells which retain the cell cycle in a permanently arrested state. However, in lieu of fulfilment of certain physiological demands such as DNA damage repair, synaptic plasticity, neurons may briefly utilize cell cycle markers. Nonetheless, on prolonged activation or under various stressors, the cell cycle machinery turns detrimental and drives the cascade of neurodegeneration by synthesizing DNA in S phase. Thus, re-expression of various cell cycle markers in AD, PD, HD, ALS constitute a sign of cell cycle re-entry led neuronal death (Sharma et al. 2017). It is for this reason that studies related to post-mitotic cell division has gained momentum in various NDD. Moreover, the protective proteins such as molecular chaperones and ubiquitin E3 ligases are responsible for maintaining protein homeostasis in the cell and in the event of failure in attaining native conformation the proteins are tagged for degradation by the UPS machinery. HSPs and E3 ligases are intimately linked to cell cycle regulation. As a result, CCE led neurodegeneration in neurons is exacerbated by loss of activity of HSPs, ubiquitin E3 ligases and impaired UPS system. Further, HSP70 has been shown to act pro-survival in neurodegeneration. Numerous studies have highlighted the crucial role of HSP70 in alleviating misfolded protein burden in PD and other NDD (Gao et al. 2015; Tutar and Tutar 2010). Given the outcome of massive cell death instigated by CCE, it is imperative to design therapeutics targeting post-mitotic cell division through upregulation of HSP70 expression. Such a strategy seems promising and is more justified in the present study given that HSP70 is a key molecule in the regulation of eukaryotic cell cycle.

In the present work, we first attempted to search for HSP70 inducing compounds in NDD. For this, we carried out extensive literature survey with the keywords 'HSP70 inducing compounds' and 'NDD' and prepared a list of twenty compounds. Next, we employed various *in silico* tools and techniques to screen these compounds on drug-likeliness parameters. The compounds were assessed using three parameters namely, Lipinski filter, Ghose and Veber rules and those which passed these filters were then analysed for pharmacokinetic properties. Based on various pharmacokinetic parameters such as aqueous solubility, lipophilicity, GI permeability, bioavailability and BBB permeability scores, the final candidates amongst twenty compounds were obtained. These compounds which passed all the filters and showed good pharmacokinetics included Indomethacin, Bimoclomol and Sesamol. Next, pharmacophore based target prediction of these three compounds listed various cell cycle proteins which added weightage to their repurposing potential.

Indomethacin, Bimoclomol and Sesamol depicted novel potential in attenuating cyclin D1 and cyclin C levels based on interaction energies of molecular docking study (Sharma and Kumar 2017b). These results led to speculations that the given compounds may have strong potential in inhibiting G0/G1 phase reactivation in terminally differentiated neurons in various NDD. Further, the protective action of these compounds in ameliorating cell cycle re-entry led neurodegeneration may be mediated through HSP70 induction.

We took the lead from here and checked for the efficacy of Bimoclomol as a neuroprotectant in attenuating the level of another cyclin, the G1/S phase marker; cyclin E. The rationale behind this study was the intimate link between PD related protein parkin and its substrate cyclin E. Both these proteins share intriguing relationship in cell cycle paradigm and dysregulation of the same leads to enhanced cyclin E, CCE and

accompanying neuronal death in NDD. Bimoclomol is a non-toxic hydroxylamine derivative which elicited protection through HSPs including HSP70 induction (Deane and Brown 2016).

Bimoclomol scored well on pharmacokinetics parameters, bioavailability score and could cross the BBB. Moreover, molecular docking studies suggested that Bimoclomol can bind to and decrease the level of cyclin E which may imply its potential in halting or inhibiting CCE mediated neuronal apoptosis in PD (Sharma and Kumar 2017a).

Finally, we tested the neuroprotective potential of one of these shortlisted biomolecules; sesamol in SH-SY5Y neuroblastoma cell line. The neurotoxin rotenone which is widely used to mimick PD model was used to induce toxicity in the given cell line. Firstly, we tested the dose-dependent and time-dependent effect of rotenone toxicity on cell viability and determined the LD_{80} value of rotenone which was found to be 20μM in a 24 hour time period in the given study. Moreover, we also found that rotenone exerts toxicity in a dose dependent and time-dependent manner and cell viability decreases significantly with increasing dose. Next, we studied the dose-dependent and time-dependent efficacy of sesamol against rotenone induced toxicity in SH-SY5Y cell line. Accordingly, our study outlined neuroprotective potential of sesamol at a dose of 25μM. Further, sesamol reversed rotenone induced toxicity at a dose of 50μM in neuroblastoma cell line. Furthermore, cell viability was significantly upregulated with increasing dose of sesamol. Thus it can be summarised that sesamol exhibited dose dependent efficacy against rotenone toxicity in SH-SY5Y cell lines.

The level of various cell cycle markers was checked subsequent to rotenone administration and on treatment with sesamol using western blotting. Our results showed that sesamol treatment decreased the expression level of G1/S phase marker cyclin E which was increased upon rotenone administration. This decrease in cyclin E may signify

potential of sesamol in attenuating cell cycle re-entry in PD. Further, the level of protective proteins such as parkin and HSP70 were reinstated subsequent to sesamol treatment. The enhanced levels of parkin may further explain decreased activity of its substrate cyclin E. Interestingly, cell viability was markedly increased with decreased cyclin E and increase in level of parkin and HSP70.

To sum up, sesamol attenuated cell cycle re-entry led neuronal death as evident by decreased level of cyclin E. Further, sesamol restored the level of protective proteins parkin and HSP70 and thus, an overall decline in neuronal apoptosis and enhanced cell viability was seen in SH-SY5Y neuroblastoma cell lines. Further, it may be outlined that HSP70 induction mediated a pro-survival pathway which alleviated rotenone induced toxicity in CCE paradigm in SH-SY5Y cell lines. Moreover, the increase in HSP70 expression and concurrent cell viability upon sesamol treatment further support our theory that HSP70 inducing compounds have a strong novel potential in correcting CCE led neuronal apoptosis in PD.

Finally, we tested the HSP70 induction theory by using a compound which is widely employed as HSP70 inhibitor; Quercetin. Similar to sesamol, we carried out the dose-dependent and time-dependent studies of quercetin in rotenone induced toxicity in SH-SY5Y cell line. Interestingly, quercetin treatment neither rescued cells against rotenone toxicity nor improved cell viability. These observations clearly outline that HSP70 induction plays a crucial role in neuronal cell survival in rotenone induced toxicity. Moreover, HSP70 inducing compounds have promising potential in ameliorating CCE led neuronal death in PD. These observations have opened up a new avenue of neuro-therapeutics for further exploration through *in vitro* and *in vivo* cell cycle models in PD and other NDD.

The present study can be extended to check the mRNA levels of the proteins whose activities were found to be altered in the present work. Moreover, the *in silico* results showing efficacy of bimoclomol in attenuating cyclin E can be validated through *in vitro* studies. Additionally, molecular mechanistic involved in sesamol induced HSP70 induction and the accompanied neuronal survival can be further investigated to enable a deeper understanding of mode of action of biomolecules in promoting cell survival in NDD.

REFERENCES

A. Bernardi, R.L. Frozza, A. Meneghetti, J.B. Hoppe, A.M. Battastini, A.R. Pohlmann, S.S. Guterres, C.G. Salbego, Indomethacin-loaded lipid-core nanocapsules reduce the damage triggered by Aβ1-42 in Alzheimer's disease models, Int J Nanomedicine 7 (2012) 4927-42.

A. Camins, J.G. Pizarro, D. Alvira, J. Gutierrez-Cuesta, A.V. de la Torre, J. Folch, F.X. Sureda, E. Verdaguer, F. Junyent, J. Jorda'n, I. Ferrer, M. Palla's, Activation of ataxia telangiectasia muted under experimental models and human Parkinson's disease, Cell Mol. Life Sci. 67 (2010) 3865–3882.

A. Ciechanover, Y.T. Kwon, Protein Quality Control by Molecular Chaperones in Neurodegeneration, Front Neurosci. 11 (2017).

A. Currais, T. Hortobágyi, S. Soriano, The neuronal cell cycle as a mechanism of pathogenesis in Alzheimer's disease, Aging (Albany NY) 1 (2009) 363–371.

A. Futatsugi, E. Utreras, P. Rudrabhatla, H. Jaffe, H.C. Pant, A.B. Kulkarni, Cyclin-dependent kinase 5 regulates E2F transcription factor through phosphorylation of Rb protein in neurons, Cell Cycle 11 (2012) 1603–1610.

A. Mietelska-Porowska, U. Wasik, M. Goras, A. Filipek, G. Niewiadomska, Tau pro- tein modifications and interactions: their role in function and dysfunction, Int. J. Mol. Sci. 15 (2014) 4671–4713.

A. Sittler, R. Lurz, G. Lueder, J. Priller, H. Lehrach, M.K. Hayer-Hartl, F.U. Hartl, E.E. Wanker, Geldanamycin activates a heat shock response and inhibits huntingtin aggregation in a cell culture model of Huntington's disease, Hum Mol Genet. 10 (2001) 1307-15.

A. Thakur, S.L. Siedlak, S.L. James, Retinoblastoma protein phosphorylation at multiple sites is associated with neurofibrillary pathology in Alzheimer disease, Int. J. Clin. Exp. Pathol. 1 (2008) 134–146.

A.K. Ghose, V.N. Viswanadhan, J.J. Wendoloski, A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery, A qualitative and quantitative characterization of known drug databases, J. Com. Chem. 1 (1999) 55-68.

A.M. Abreu Velez, M.S. Howard, Tumor-suppressor genes, cell cycle regulatory checkpoints, and the skin, N. Am. J. Med. Sci. 7 (2015) 176–188.

A.M. Cuervo, E. Wong, Chaperone-mediated autophagy: roles in disease and aging, Cell Res. 24 (2014) 92–104.

B. Bukau, J. Weissman, A. Horwich, Molecular chaperones and protein quality control, Cell 125 (2006) 443–451.

B. Eroglu, D.E. Kimbler, J. Pang, J. Choi, D. Moskophidis, N. Yanasak, K.M. Dhandapani, N.F. Mivechi, Therapeutic inducers of the HSP70/HSP110 protect mice against traumatic brain injury, J Neurochem. 130 (2014) 626-41.

B. Kalmar, S. Novoselov, A. Gray, M.E. Cheetham, B. Margulis, et al, Late stage treatment with arimoclomol delays disease progression and prevents protein aggregation in the SOD1G93A mouse model of ALS, J Neurochem. 107 (2008) 339-350.

B. Kwon, P. Kumar, H.K. Lee, L. Zeng, K. Walsh, Q. Fu, A. Barakat, H.W. Querfurth, Aberrant cell cycle re-entry in human and experimental inclusion body myositis and polymyositis, Hum. Mol. Genet. 23 (2014) 3681–3694.

B. Mosch, M. Morawski, A. Mittag, D. Lenz, A. Tarnok, T. Arendt, Aneuploidy and DNA replication in the normal human brain and Alzheimer's disease, J. Neurosci. 27 (2007) 6859–6867.

B.A. Racette, S. Searles Nielsen, S.R. Criswell, L. Sheppard, N. Seixas, M.N. Warden, H. Checkoway, Dose-dependent progression of parkinsonism in manganese-exposed welders, Neurology 88 (2017) 344-351.

C. Alquézar, E. Barrio, N. Esteras, A. de la Encarnación, F. Bartolomé, J.A. Molina, Á. Martín-Requero, Targeting cyclin D3/CDK6 activity for treatment of Parkinson's disease, J. Neurochem. 133 (2015) 886–897.

C. Bertoli, J.M. Skotheim, R.A. de Bruin, Control of cell cycle transcription during G1 and S phases, Nat. Rev. Mol. Cell Biol. 14 (2013) 518–528.

C. Cleren, N.Y. Calingasan, J. Chen, M.F. Beal, Celastrol protects against MPTP- and 3-nitropropionic acid-induced neurotoxicity, J. Neurochem. 94 (2005) 995–1004.

C. Klein, A. Westenberger, Genetics of Parkinson's disease, Cold Spring Harb Perspect Med. 2 (2012), a008888.

C. Pelegrí, J. Duran-Vilaregut, J. del Valle, N. Crespo-Biel, I. Ferrer, M. Pallàs, A. Camins, J. Vilaplana, Cell cycle activation in striatal neurons from Huntington's disease patients and rats treated with 3-nitropropionic acid, Int. J. Dev. Neurosci. 26 (2008) 665–671.

C. Roodveldt, C.W. Bertoncini, A. Andersson, A.T. van der Goot, S.T. Hsu, R. Fernandez-Montesinos, et al, Chaperone proteostasis in Parkinson’s disease: stabilization of the Hsp70/a-synuclein complex by Hip, EMBO J. 28 (2009) 3758–3770.

C. Xia, Y. Cai, S. Li, J. Yang, G. Xiao, Curcumin increases HSP70 expression in primary rat cortical neuronal apoptosis induced by gp120 V3 loop peptide, Neurochem Res 40 (2015) 1996-2005.

C.A. Deane, I.R. Brown, Induction of heat shock proteins in differentiated human neuronal cells following co-application of celastrol and arimoclomol, Cell Stress Chaperones 21 (2016) 837-48.

C.A. Dickey, A. Kamal, K. Lundgren, N. Klosak, R.M. Bailey, J. Dunmore, et al, The high-affinity HSP90-CHIP complex recognizes and selectively degrades phosphorylated tau client proteins, J. Clin. Invest. 117 (2007) 648–658.

C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv Drug Deliv Rev. 46 (2001) 3-26.

C.H. O'Flanagan, V.A. Morais, W. Wurst, B. De Strooper, C. O'Neill, The Parkinson's gene PINK1 regulates cell cycle progression and promotes cancer-associated phenotypes, Oncogene 34 (2015) 1363–74.

C.I. Holmberg, S.A. Illman, M. Kallio, A. Mikhailov, L. Sistonen, Formation of nuclear HSF1 granules varies depending on stress stimuli, Cell Stress Chaperones 5 (2000) 219-28.

C.M. Tanner, F. Kamel, G.W. Ross, J.A. Hoppin, S.M. Goldman, M. Korell, et al, Rotenone, paraquat, and Parkinson's disease, Environ Health Perspect 119 (2011) 866–72.

C.P. Reina, B.Y. Nabet, P.D. Young, R.N. Pittman, Basal and stress-induced Hsp70 are modulated by ataxin-3, Cell Stress Chaperones 17 (2012) 729-42.

D. Ebrahimi-Fakhari, L. Wahlster, P.J. McLean, Molecular chaperones in Parkinson's disease - Present and future, J Parkinsons Dis 1 (2011) 299-320.

D. Procházková, I. Boušová, N. Wilhelmová, Antioxidant and prooxidant properties of flavonoids, Fitoterapia 82 (2011) 513–523.

D. Rossetto, A.W. Truman, S.J. Kron, J. Côté, Epigenetic modifications in double- strand break DNA damage signaling and repair, Clin. Cancer Res. 16 (2010) 4543–4552.

D. Zheng, G. Zhu, S. Liao, W. Yi, G. Luo, J. He, Z. Pei, G. Li, Y. Zhou, Dysregulation of the PI3K/Akt signaling pathway affects cell cycle and apoptosis of side population cells in nasopharyngeal carcinoma, Oncol. Lett. 10 (2015) 182–188.

D.A. Foster, P. Yellen, L. Xu, M. Saqcena, Regulation of G1 cell cycle progression: distinguishing the restriction point from a nutrient-sensing cell growth check- point(s), Genes Cancer 1 (2010) 1124–1131.

D.D. Feng, W. Cai, X. Chen, The associations between Parkinson's disease and cancer: the plot thickens, Transl Neurodegener. 4 (2015).

D.F. Veber, S.R. Johnson, H.Y. Cheng, B.R. Smith, K.W. Ward, K.D. Kopple, Molecular properties that influence the oral bioavailability of drug candidates, J Med Chem. 45 (2002) 2615-23.

D.G. Hay, K. Sathasivam, S. Tobaben, B. Stahl, M. Marber, R. Mestril, A. Mahal, D.L. Smith, B. Woodman, G.P. Bates, Progressive decrease in chaperone protein levels in a mouse model of Huntington's disease and induction of stress proteins as a therapeutic approach, Hum Mol Genet. 13 (2004) 1389-405.

D.J. Bonda, H.P. Lee, W. Kudo, X. Zhu, M.A. Smith, H.G. Lee, Pathological implications of cell cycle re-entry in Alzheimer disease, Expert Rev. Mol. Med. 12 (2010) e19.

D.J. Bonda, T.A. Evans, C. Santocanale, J.C. Llosá, J. Viña, V. Bajic, R.J. Castellani, S.L. Siedlak, G. Perry, M.A. Smith, H.G. Lee, Evidence for the progression through S- phase in the ectopic cell cycle re-entry of neurons in Alzheimer disease, Aging (Al- bany NY) 1 (2009) 382–388.

D.M. Moran, H. Shen, C.G. Maki, Puromycin-based vectors promote a ROS-dependent recruitment of PML to nuclear inclusions enriched with HSP70 and Proteasomes, BMC Cell Biol 10 (2009).

E. Colombo, F. Bedogni, I. Lorenzetti, N. Landsberger, S.C. Previtali, C. Farina, Auto- crine and immune cell-derived BDNF in human skeletal muscle: implications for myogenesis and tissue regeneration, J. Pathol. 231 (2013) 190–198.

E. Das, N.R. Jana, N.P. Bhattacharyya, MicroRNA-124 targets CCNA2 and regulates cell cycle in STHdh (Q111)/Hdh (Q111) cells, Biochem. Biophys. Res. Commun.437 (2013) 217–224.

E. Lindersson, R. Beedholm, P. Hojrup, T. Moos, W. Gai, K.B. Hendil, et al, Proteasomal inhibition by alpha-synuclein filaments and oligomers, J. Biol. Chem. 279 (2004) 12924–12934.

E. Nagata, A. Sawa, C.A. Ross, S.H. Snyder, Autophagosome like vacuole formation in Huntington's disease lymphoblasts, Neuroreport 15 (2004)1325–1328.

E. Taniai, A. Yafune, M. Nakajima, S.M. Hayashi, F. Nakane, M. Itahashi, M. Shibutani, Ochratoxin A induces karyomegaly and cell cycle aberrations in renal tubular cells without relation to induction of oxidative stress responses in rats, Toxicol. Lett. 224 (2014) 64–72.

E. Tellone, A. Galtieri, A. Russo, B. Giardina, S. Ficarra, Resveratrol: a focus on several neurodegenerative diseases, Oxidative Med. Cell. Longev. 2015 (2015) 392169.

F. Cicchetti, J. Drouin-Ouellet, R.E. Gross, Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol Sci. 30 (2009) 475–83.

F. Hu, T. Padukkavidana, C.B. Vaegter, O.A. Brady, Y. Zheng, I.R. Mackenzie, et al, Sortilin-mediated endocytosis determines levels of the frontotemporal dementia protein, progranulin, Neuron 68 (2010) 654–667.

F.A. Agarraberes, J.F. Dice, A molecular chaperone complex at the lysosomal membrane is required for protein translocation, J. Cell Sci. 114 (2001) 2491–2499.

F.J. Soils, R.J.B. Wets, Minimization by random search techniques, Mathematics of Operations Research 6 (1981) 19-30.

G.C. Meacham, C. Patterson, W. Zhang, J.M. Younger, D.M. Cyr, The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal Degradation, Nat. Cell Biol. 3 (2001) 100–105.

G.M. Morris, D.S. Goodsell, Automated docking using a genetic Lamarckian algorithm and an empirical binding free energy function, Journal of Computational Chemistry 19 (1998) 1639-1662.

H. Ehringer, O. Hornykiewicz, Verteilung von Noradrenalin und Dopamin (3-Hydroxytyramin) im Gehirn des Menschen und ihr Verhalten bei Erkrankungen des extrapyramidalen Systems, Klin. Wochenschr. 38 (1960) 1236– 1239.

H. Joshi, U. Bhandari, B.P. Panda, To assess the potential of curcumin against gut microbiota-induced alteration in choline metabolism in c57bl/6j mice, Int J Pharm Pharm Sci 9 (2017) 215-26.

H. Lou, X. Jing, X. Wei, H. Shi, D. Ren, X. Zhang, Naringenin protects against 6-OHDA-induced neurotoxicity via activation of the Nrf2/ARE signalling pathway, Neuropharmacology 79 (2014) 380-8.

H. Wang, Y. Chen, J. Chen, Z. Zhang, W. Lao, X. Li, J. Huang, T. Wang, Cell cycle regulation of DNA polymerase beta in rotenone-based Parkinson's disease models, PLoS One 9 (2014), e109697.

H.A. Hussein, A. Borrel, C. Geneix, M. Petitjean, L. Regad, A. Camproux, PockDrug-Server: a new web server for predicting pocket druggability on holo and apo proteins, Nucl. Acids Res. (2015).

H.Y. Kim, H. Heise, C.O. Fernandez, M. Baldus, M. Zweckstetter, Correlation of amyloid fibril beta-structure with the unfolded state of alpha-synuclein, Chembiochem. 8 (2007) 1671–4.

I.D. Akinrinade, A.E. Memudu, O.M. Ogundele, Fluoride and aluminium disturb neuronal morphology, transport functions, cholinesterase, lysosomal and cell cycle activities, Pathophysiology 22 (2015) 105–115.

I.I. Kruman, Why do neurons enter the cell cycle, Cell Cycle 3 (2004) 769–773.

J. Magrane, R.C. Smith, K. Walsh, H.W. Querfurth, Heat shock protein 70 participates in the neuroprotective response to intracellularly expressed beta-amyloid in neurons, J Neurosci 24 (2004) 1700-1706.

J. Mizukami, G. Takaesu, H. Akatsuka, H. Sakurai, J. Ninomiya-Tsuji, K. Matsumoto, N. Sakurai, Receptor activator of NF-kappaB ligand (RANKL) activates TAK1 mitogen-activated protein kinase kinase kinase through a signaling complex containing RANK, TAB2, and TRAF6, Mol. Cell Biol. 22 (2002) 992–1000.

J. Chen, M.L. Cohen, A.J. Lerner, Y. Yang, K. Herrup, DNA damage and cell cycle events implicate cerebellar dentate nucleus neurons as targets of Alzheimer's disease, Mol. Neurodegener. 20 (2010).

J. Chen, Z.F. Wang, Roles of cyclin-dependent kinase 5 in central nervous system development and neurodegenerative diseases, Sheng Li Xue Bao. 62 (2010) 295–308.

J. Jiang, K. Prasad, E.M. Lafer, R. Sousa, Structural basis of interdomain communication in the Hsc70 chaperone, Mol. Cell 20 (2005) 513–524.

J. Klucken, Y. Shin, E. Masliah, B.T. Hyman, P.J. McLean, Hsp70 reduces alpha-synuclein aggregation and toxicity, J. Biol. Chem. 279 (2004) 25497–25502.

J. Li, Y.R. Han, M.R. Plummer, K. Herrup, Cytoplasmic ATM in neurons modulates synaptic function, Curr. Biol. 19 (2009) 2091–2096.

J. Lin, S. Jinno, H. Okayama, Cdk6-cyclin D3 complex evades inhibition by inhibitor proteins and uniquely controls cell's proliferation competence, Oncogene 20 (2001) 2000–2009.

J. Liu, D. Zhang, X. Mi, Q. Xia, Y. Yu, Z. Zuo, W. Guo, X. Zhao, J. Cao, Q. Yang, A. Zhu, W. Yang, X. Shi, J. Li, C. Huang, p27 suppresses arsenite-induced Hsp27/Hsp70 expression through inhibiting JNK2/c-Jun- and HSF-1-dependent pathways, J Biol Chem 285 (2010) 26058-65.

J. Nah, J. Yuan, Y.K. Jung, Autophagy in neurodegenerative diseases: from mechanism to therapeutic approach, Mol. Cells 38 (2015) 381–389.

J. Wu, B.A. Stoica, A.I. Faden, Cell cycle activation and spinal cord injury, Neurotherapeutics 8 (2011) 221–228.

J. Zhang, S.A. Cicero, L. Wang, R.R. Romito-Digiacomo, Y. Yang, K. Herrup, Nuclear localization of Cdk5 is a key determinant in the postmitotic state of neurons, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 8772–8777.

J. Zhu, W. Li, Z. Mao, Cdk5: mediator of neuronal development, death and the response to DNA damage, Mech. Ageing Dev. 132 (2011) 389–394.

J.A. Diehl, W. Yang, R.A. Rimerman, H. Xiao, A. Emili, Hsc70 regulates accumulation of cyclin D1 and cyclin D1-dependent protein kinase, Mol. Cell Biol. 23 (2003) 1764–1774.

J.B. Leverenz, I. Umar, Q. Wang, T.J. Montine, P.J. McMillan, D.W. Tsuang, et al, Proteomic identification of novel proteins in cortical lewy bodies, Brain Pathol. 17 (2007) 139–145.

J.C. Ranford, A.R. Coates, B. Henderson, Chaperonins are cell signalling proteins: the unfolding biology of molecular chaperones, Expert Rev. Mol. Med. 2 (2000) 1–17.

J.L. Biedler, S. Roffler-Tarlov, M. Schachner, L.S. Freedman, Multiple Neurotransmitter Synthesis by Human Neuroblastoma Cell Lines and Clones, Cancer Res. 38 (1978) 3751–7.

J.M. Keppel Hesselink, Bimoclomol and Arimoclomol: HSP-co-Inducers for the Treatment of Protein Misfolding Disorders, Neuropathy and Neuropathic Pain, J Pain Relief 6 (2016).

J.W. Harper, J.L. Burton, M.J. Solomon, The anaphase promoting complex: it's not just for mitosis any more, Genes Dev 16 (2002) 2179–2206.

K. Arima, S. Hirai, N. Sunohara, K. Aoto, Y. Izumiyama, K. Uéda, K. Ikeda, M. Kawai, Cellular co-localization of phosphorylated tau- and NACP/alpha-synuclein-epitopes in lewy bodies in sporadic Parkinson's disease and in dementia with Lewy bodies, Brain Research, 843 (1999) 53–61.

K. Herrup, The involvement of cell cycle events in the pathogenesis of Alzheimer's disease, Alzheimers Res Ther. 2 (2010) 13.

K. Herrup, Y. Yang, Cell cycle regulation in the post mitotic neuron: oxymoron or new biology, Nat. Rev. Neurosci. 8 (2007) 368–378.

K. Uryu, C. Richter-Landsberg, W. Welch, E. Sun, O. Goldbaum, E.H. Norris, et al, Convergence of heat shock protein 90 with ubiquitin in filamentous alpha-synuclein inclusions of alpha-synucleinopathies, Am. J. Pathol. 168 (2006) 947–961.

K. Venderova, D.S. Park, Programmed cell death in Parkinson's disease, Cold Spring Harb. Perspect. Med. 2 (2012) a009365.

K. Walczak, S. Deneka-Hannemann, B. Jarosz, W. Zgrajka, F. Stoma, T. Trojanowski, W.A. Turski, W. Rzeski, Kynurenic acid inhibits proliferation and migration of human glioblastoma T98G cells, Pharmacol Rep. 66 (2014) 130-6.

K.C. Luk, I.P. Mills, J.Q. Trojanowski, V.M. Lee, Interactions between Hsp70 and the hydrophobic core of alpha-synuclein inhibit fibril assembly, Biochemistry 47 (2008) 12614–12625.

K.K. Chen, Z.M. He, B.H. Ding, Y. Chen, L.J. Zhang, L. Yu, J. Gao, HSP90 Inhibitor 17-AAG Inhibits Multiple Myeloma Cell Proliferation by Down-regulating Wnt/β-Catenin Signaling Pathway, Zhongguo Shi Yan Xue Ye XueZaZhi 24 (2016) 117-21.

K.L. Jordan-Sciutto, L.M. Malaiyandi, R. Bowser, Altered distribution of cell cycle transcriptional regulators during Alzheimer disease, J. Neuropathol. Exp. Neurol. 61 (2002) 358–367.

K.L. Jordan-Sciutto, R. Dorsey, E.M. Chalovich, R.R. Hammond, C.L. Achim, Expression patterns of retinoblastoma protein in Parkinson disease, J. Neuropathol. Exp. Neurol. 62 (2003) 68–74.

K.L. Milarski, R.I. Morimoto, Expression of human HSP70 during the synthetic phase of the cell cycle, Proc Natl Acad Sci USA 24 (1986) 9517-21.

L. Petrucelli, D. Dickson, K. Kehoe, J. Taylor, H. Snyder, A. Grover, et al, CHIP and Hsp70 regulate tau ubiquitination, degradation and aggregation, Hum. Mol. Genet. 13 (2004) 703–714.

L. Qi, X.D. Zhang, J.C. Wu, F. Lin, J. Wang, M. DiFiglia, et al, The role of chaperone-mediated autophagy in huntingtin degradation, PLoS ONE 7 (2012) :e46834.

L. Tutar, Y. Tutar, Heat shock proteins; an overview, Curr. Pharm. Biotechnol. 11 (2010) 216–222.

L. Xiaofeng, O. Sisheng, Y. Biao, H. Kai, L. Yabo, G. Jiayu, et al, PharmMapper Server: a web server for potential drug target identification via pharmacophore mapping approach, Nucleic Acids Res. 38 (2010) W609-W614.

L.A. van Leeuwen, J.J.M. Hoozemans, Physiological and pathophysiological functions of cell cycle proteins in post-mitotic neurons: implications for Alzheimer's disease, Acta Neuropathol. 129 (2015) 511–525.

L.H. Kerri, J. Damir, Cell Cycle, Neurological Disorders, and Reactive Gliosis, Springer, 2006 163–175 (Book Part III).

L.J. Martin, DNA damage and repair: relevance to mechanisms of neurodegeneration, J. Neuropathol. Exp. Neurol. 67 (2008) 377–387.

L.K. Chico, L.J. Van Eldik, D.M. Watterson, Targeting protein kinases in central nervous system disorders, Nat Rev Drug Discov. 8 (2009) 892-909.

L.M. de Lau, M.M. Breteler, Epidemiology of Parkinson's disease, Lancet Neurol. 5 (2006) 525-35.

M. Ahmed, P.M. Machado, A. Miller, C. Spicer, L. Herbelin, J. He, J. Noel, Y. Wang, A.L. McVey, M. Pasnoor, P. Gallagher, J. Statland, C.H. Lu, B. Kalmar, S. Brady, H. Sethi, G. Samandouras, M. Parton, J.L. Holton, A. Weston, L. Collinson, J.P. Taylor, G. Schiavo, M.G. Hanna, R.J. Barohn, M.M. Dimachkie, L. Greensmith, Targeting protein homeostasis in sporadic inclusion body myositis, Sci. Transl. Med. 8 (2016) 331ra41.

M. Biasini, S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T.G. Cassarino, M. Bertoni, L. Bordoli, T. Schwede, SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information, Nucleic Acids Research 42 (2014) W252-W258.

M. Cherubini, M. Puigdellívol, J. Alberch, S. Ginés, Cdk5-mediated mitochondrial fission: a key player in dopaminergic toxicity in Huntington's disease, Biochim. Biophys. Acta 1852 (2015) 2145–2160.

M. Farina, D.S. Avila, J.B. da Rocha, M. Aschner, Metals, oxidative stress and neurodegeneration: a focus on iron, manganese and mercury, Neurochem Int. 62 (2013) 575–94.

M. Hengstschläger, K. Braun, T. Soucek, A. Miloloza, E. Hengstschläger-Ottnad, Cyclin-dependent kinases at the G1-S transition of the mammalian cell cycle, Mutat. Res. 436 (1999) 1–9.

M. Huang, Z.H. Miao, H. Zhu, Y.J. Cai, W. Lu, J. Ding, Chk1 and Chk2 are differentially involved in homologous recombination repair and cell cycle arrest in response to DNA double-strand breaks induced by camptothecins, Mol. Cancer Ther. 7 (2008) 1440–1449.

M. Kastle, T. Grune, Interactions of the proteasomal system with chaperones: protein triage and protein quality control, Prog. Mol. Biol. Transl. Sci. 109 (2012)113–160.

M. Tavallai, L. Booth, J.L. Roberts, A. Poklepovic, P. Dent, Rationally Repurposing Ruxolitinib (Jakafi (®)) as a Solid Tumor Therapeutic, Front Oncol. 6 (2016).

M. Xilouri, O.R. Brekk, N. Landeck, P.M. Pitychoutis, T. Papasilekas, Z. Papadopoulou-Daifoti, et al, Boosting chaperone-mediated autophagy in vivo mitigates alpha-synuclein-induced neurodegeneration, Brain 136 (2013) 2130–2146.

M.C. Ovejero-Benito, J.M. Frade, Brain-derived neurotrophic factor-dependent cdk1 inhibition prevents G2/M progression in differentiating tetraploid neurons, PLoS One 8 (2013), e64890.

M.E. DeSantis, E.H. Leung, E.A. Sweeny, M.E. Jackrel, M. Cushman-Nick, A. Neuhaus-Follini, et al, Operational plasticity enables hsp104 to disaggregate diverse amyloid and nonamyloid clients, Cell 151 (2012) 778–793.

M.G. Andrusiak, K.A. McClellan, D. Dugal-Tessier, L.M. Julian, S.P. Rodrigues, D.S. Park, T.E. Kennedy, R.S. Slack, Rb/E2F regulates expression of neogenin during neuronal migration, Mol. Cell. Biol. 31 (2011) 238–247.

M.G. Spillantini, M.L. Schmidt, V.M. Lee, J.Q. Trojanowski, R. Jakes, M. Goedert, Alpha-synuclein in Lewy bodies, Nature, 388 (1997) 839–40.

M.R. Kelley, M.M. Georgiadis, M.L. Fishel, APE1/Ref-1 role in redox signaling: translational applications of targeting the redox function of the DNA repair/ redox protein APE1/Ref-1, Curr. Mol. Pharmacol. 5 (2012) 36–53.

N. Boutahar, E. Reynaud, F. Lassabliere, J. Borg, Brain-derived neurotrophic factor inhibits cell cycle re-entry but not endoplasmic reticulum stress in cultured neurons following oxidative or excitotoxic stress, J. Neurosci. Res. 88 (2010) 2263–2271.

N. Morimoto, M. Nagai, Y. Ohta, K. Miyazaki, T. Kurata, M. Morimoto, et al, Increased autophagy in transgenic mice with a G93A mutant SOD1 gene, Brain Res. 1167 (2007) 112–117.

N.B. Nillegoda, M.A. Theodoraki, A.K. Mandal, K.J. Mayo, H.Y. Ren, R. Sultana, et al, Ubr1 and Ubr2 function in a quality control pathway for degradation of unfolded cytosolic proteins, Mol. Biol. Cell 21 (2010) 2102–2116.

N.G. Starostina, E.T. Kipreos, Multiple degradation pathways regulate versatile CIP/ KIP CDK inhibitors, Trends Cell Biol. 22 (2012) 33–41.

N.H. Varvel, K. Bhaskar, M.Z. Kounnas, S.L. Wagner, Y. Yang, B.T. Lamb, K. Herrup, NSAIDs prevent, but do not reverse, neuronal cell cycle re-entry in a mouse model of Alzheimer disease, J. Clin. Invest. 119 (2009) 3692–3702.

N.R. Jana, P. Dikshit, A. Goswami, S. Kotliarova, S. Murata, K. Tanaka, et al, Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes, J. Biol. Chem. 280 (2005) 11635–11640.

N.S. Elsisi, S. Darling-Reed, E.Y. Lee, E.T. Oriaku, K.F. Soliman, Ibuprofen and apigenin induce apoptosis and cell cycle arrest in activated microglia, Neurosci. Lett. 375 (2005) 91–96.

NINDS Parkinson's Disease Information Page, Bethesda (MD): National Institute of Neurological Disorders and Stroke (homepage on the Internet) (http://www.ninds.nih.gov/disorders/parkinsons_disease/parkinsons_disease).

O.A. Levy, C. Malagelada, L.A. Greene, Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps, Apoptosis 14 (2009) 478–500.

P. Anglade, S.Vyas, F. Javoy-Agid, M.T. Herrero, P.P. Michel, J. Marquez, et al, Apoptosis and autophagy in nigral neurons of patients with Parkinson's Disease, Histol. Histopathol. 12 (1997) 25–31.

P. Katsel, W. Tan, P. Fam, D.P. Purohit, V. Haroutunian, Cell cycle checkpoint abnormalities during dementia: a plausible association with the loss of protection against oxidative stress in Alzheimer's disease corrected, PLoS One 8 (2013), e68361.

P. Khadka, R. Jieun, H. Kim, I. Kim, J.T. Kim, H. Kim, et al, Pharmaceutical particle technologies: An approach to improve drug solubility, dissolution and bioavailability, Asian Journal of Pharmaceutical Sciences 9 (2014) 304–316.

P. Muller, R. Hrstka, D. Coomber, D.P. Lane, B. Vojtesek, Chaperone-dependent stabilization and degradation of p53 mutants, Oncogene 27 (2008) 3371–3383.

P. Paoletti, I. Vila, M. Rifé, J.M. Lizcano, J. Alberch, S. Ginés, Dopaminergic and glutamatergic signaling crosstalk in Huntington's disease neurodegeneration: the role of p25/cyclin-dependent kinase 5, J. Neurosci. 28 (2008) 10090–10101.

P. Thakur, B. Nehru, Modulatory effects of sodium salicylate on the factors affecting protein aggregation during rotenone induced Parkinson's disease pathology, Neurochem Int. 75 (2014) 1-10.

P. Tokarz, K. Kaarniranta, J. Blasiak, Role of the cell cycle re-initiation in DNA damage response of post-mitotic cells and its implication in the pathogenesis of neurodegenerative diseases, Rejuvenation Res. 19 (2016) 131–139.

P.J. McLean, H. Kawamata, S. Ribich, B.T. Hyman, Membrane association and protein conformation of alpha-synuclein in intact neurons, Effect of Parkinson's disease-linked mutations, The Journal of Biological Chemistry, 275 (2000) 8812–6.

P.J. McLean, H. Kawamata, S. Shariff, J. Hewett, N. Sharma, K. Ueda, et al, Torsin A and heat shock proteins act as molecular chaperones: suppression of alpha-synuclein aggregation, J. Neurochem. 83 (2002) 846–854.

P.J. McLean, J. Klucken, Y. Shin, B.T. Hyman, Geldanamycin induces Hsp70 and prevents alpha-synuclein aggregation and toxicity in vitro, Biochem. Biophys. Res. Commun. 321 (2004) 665–669.

P.J. Muchowsk, J.L. Walker, Modulation of neurodegeneration by molecular chaperones, Nat. Rev. Neurosci 6 (2005) 11–22.

P.K. Auluck, H.Y. Chan, J.Q. Trojanowski, V.M. Lee, N.M. Bonini, Chaperone suppression of alpha-synuclein toxicity in a Drosophila model for Parkinson's disease, Science 295 (2002) 865–868.

P.K. Modi, S. Jaiswal, P. Sharma, Regulation of neuronal cell cycle and apoptosis by microRNA, Mol. Cell Biol. 36 (2015) 84–94.

P.T. Grogan, K.D. Sleder, A.K. Samadi, H. Zhang, B.N. Timmermann, M.S. Cohen, Cytotoxicity of withaferin A in glioblastomas involves induction of an oxidative stress-mediated heat shock response while altering Akt/mTOR and MAPK signalling pathways, Invest New Drugs 31 (2013) 545-57.

Q. Liu, F. Xie, A. Alvarado-Diaz, M.A. Smith, P.I. Moreira, X. Zhu, G. Perry, Neurofilamentopathy in neurodegenerative diseases, Open Neurol. J. 5 (2011) 58–62.

R. Bose, N. Onishchenko, K. Edoff, A.M. Janson Lang, S. Ceccatelli, Inherited effects of low-dose exposure to methyl mercury in neural stem cells, Toxicol. Sci. 130 (2012) 383–390.

R. Hassler, Zur Pathologic der Paralysis Agitans und des postenzephalitischen Parkinsonismus. J. Psychol. Neurol. 48 (1938) 387– 476.

R. Sharma, D. Kumar, N.K. Jha, S.K. Jha, R.K. Ambasta, P. Kumar, Re-expression of cell cycle markers in aged neurons and muscles: Whether cells should divide or die? Biochim Biophys Acta 1863 (2017) 324-36.

R. Sharma, P. Kumar, Neuroprotective role of bimoclomol in ectopic cell cycle in Parkinson's disease: new insights, Asian J Pharm Clin Res 10 (2017a) 1-4.

R. Sharma, P. Kumar, Repurposing HSP70 inducing compounds for targeting post-mitotic cell division: novel promises as neuroprotectants, J. Chem. Pharm. Res. 9 (2017b) 373-384.

R.A. Ross, J.L. Biedler, Presence and Regulation of Tyrosinase Activity in Human Neuroblastoma Cell Variants in Vitro, Cancer Res, 45 (1985) 1628–32.

R.E. Lackie, A. Maciejewski, V.G. Ostapchenko, J. Marques-Lopes, W.Y. Choy, M.L. Duennwald, V.F. Prado, M.A.M. Prado, The Hsp70/Hsp90 Chaperone Machinery in Neurodegenerative Diseases, Front Neurosci. 11 (2017).

R.J. Duronio, Y. Xiong, Signaling pathways that control cell proliferation, Cold Spring Harb. Perspect. Biol. 5 (2013), a008904.

S. Absalon, D.M. Kochanek, V. Raghavan, A.M. Krichevsky, MiR-26b upregulated in Alzheimer's disease, activates cell cycle entry, tau-phosphorylation, and apoptosis in postmitotic neurons, J. Neurosci. 33 (2013) 14645–14659.

S. Fulda, A.M. Gorman, O. Hori, A. Samali, Cellular stress responses: cell survival and cell death, Int. J. Cell Biol. 214074 (2010).

S. Krantic, N. Mechawar, S. Reix, R. Quirion, Molecular basis of programmed cell death involved in neurodegeneration, Trends Neurosci. 28 (2005) 670–676.

S. Lim, P. Kaldis, Cdks, cyclins and CKIs: roles beyond cell cycle regulation, Development 140 (2013) 3079–3093.

S. Marathe, S. Liu, E. Brai, M. Kaczarowski, L. Alberi, Notch signaling in response to excitotoxicity induces neurodegeneration via erroneous cell cycle re-entry, Cell Death Differ. 22 (2015) 1775–1784.

S. Ranganathan, R. Bowser, Alterations in G (1) to S phase cell-cycle regulators during amyotrophic lateral sclerosis, Am. J. Pathol. 162 (2003) 823–835.

S. Rudiger, L. Germeroth, J. Schneider-Mergener, B. Bukau, Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries, EMBO J. 16 (1997) 1501–1507.

S. Sharma, V. Kumar, In vitro cytotoxicity effect on mcf-7 cell line of co-encapsulated artesunate and curcumin liposome, Int J Pharm Pharm Sci 9 (2017) 123-8.

S.C. Benn, R.H. Brown Jr, Putting the heat on ALS, Nat Med. 10 (2004) 345-7.

S.C. Lovell, I.W. Davis, W.B. Arendall 3rd, P.I. de Bakker, J.M. Word, M.G. Prisant, J.S. Richardson, D.C. Richardson, Structure validation by Calpha geometry: phi, psi and Cbeta deviation, Proteins 50 (2003) 437-50.

S.M. Angeline, A. Sarkar, K. Anand, R.K. Ambasta, P. Kumar, Sesamol and naringenin reverse the effect of rotenone-induced PD rat model, Neuroscience 254 (2013) 379-94.

S.M. Morillo, E.P. Abanto, M.J. Román, J.M. Frade, Nerve growth factor-induced cell cycle re-entry in newborn neurons is triggered by p38MAPK-dependent E2F4 phosphorylation, Mol. Cell. Biol. 32 (2012) 2722–2737.

T. Arendt, M. Holzer, U. Gärtner, Neuronal expression of cycline dependent kinase inhibitors of the INK4 family in Alzheimer's disease, J. Neural Transm. (Vienna) 105 (1998) 949–960.

T. Kawauchi, K. Chihama, Y. Nabeshima, M. Hoshino, Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration, Nat. Cell Biol. 8 (2006) 17–26.

T. Kimura, K. Ishiguro, S. Hisanaga, Physiological and pathological phosphorylation of tau by Cdk5, Front. Mol. Neurosci. 7 (2014).

T. Liu, G. Perry, H.W. Chan, G. Verdile, R.N. Martins, M.A. Smith, C.S. Atwood, Amyloid-beta-induced toxicity of primary neurons is dependent upon differentiation- associated increases in tau and cyclin-dependent kinase 5 expression, J. Neurochem. 88 (2004) 554–563.

T.A. Potapova, J.R. Daum, K.S. Byrd, G.J. Gorbsky, Fine tuning the cell cycle: activation of the Cdk1 inhibitory phosphorylation pathway during mitotic exit, Mol. Biol. Cell. 20 (2009) 1737–1748.

T.F. Outeiro, P. Putcha, J.E. Tetzlaff, R. Spoelgen, M. Koker, F. Carvalho, et al, Formation of toxic oligomeric alpha-synuclein species in living cells, PLoS ONE 3(2008):e1867.

T.R. Flower, L.S. Chesnokova, C.A. Froelich, C. Dixon, S.N. Witt, Heat shock prevents alpha-synuclein-induced apoptosis in a yeast model of Parkinson's disease, J. Mol. Biol. 351 (2005) 1081–1100.

T.T. Wager, X. Hou, P.R. Verhoest, A. Villalobos, Moving beyond rules: the development of a central nervous system multiparameter optimization (CNS MPO) approach to enable alignment of druglike properties, ACS Chem Neurosci. 1 (2010) 435-49.

V. Askanas, W.K. Engel, A. Nogalska, Sporadic inclusion-body myositis: a degenerative muscle disease associated with aging, impaired muscle protein homeostasis and abnormal mitophagy, Biochim. Biophys. Acta 1852 (2015) 633–643.

V. Crippa, V.G. D'Agostino, R. Cristofani, P. Rusmini, M.E. Cicardi, E. Messi, R. Loffredo, M. Pancher, M. Piccolella, M. Galbiati, M. Meroni, C. Cereda, S. Carra, A. Provenzani, A. Poletti, Transcriptional induction of the heat shock protein B8 mediates the clearance of misfolded proteins responsible for motor neuron diseases, Sci Rep. 6 (2016).

W.C. Chou, L.Y. Hu, C.N. Hsiung, C.Y. Shen, Initiation of the ATM-Chk2 DNA damage response through the base excision repair pathway, Carcinogenesis 36 (2015) 832–840.

W.H. Yu, A.M. Cuervo, A. Kumar, C.M. Peterhoff, S.D. Schmidt, J.H. Lee, et al, Macroautophagy–a novel Beta-amyloid peptide generating pathway activated in Alzheimer's disease, J. Cell Biol. 171 (2005) 87–98.

W.J. Lukiw, B. Surjyadipta, P. Dua, P.N. Alexandrov, Common micro RNAs (miRNAs) target complement factor H (CFH) regulation in Alzheimer's disease (AD) and in age-related macular degeneration (AMD), Int. J. Biochem. Mol. Biol. 3 (2012) 105–116.

X. Gao, M. Carroni, C. Nussbaum-Krammer, A. Mogk, N.B. Nillegoda, A. Szlachcic, et al, Human Hsp70 Disaggregase Reverses Parkinson's-Linked a-Synuclein Amyloid Fibrils, Mol. Cell 59 (2015) 781–793.

X. Zhou, J. Jia, P53-mediated G(1)/S checkpoint dysfunction in lymphocytes from Alzheimer's disease patients, Neurosci. Lett. 468 (2010) 320–325.

X. Zhu, A. McShea, P.L. Harris, A.K. Raina, R.J. Castellani, J.O. Funk, S. Shah, C. Bowen, R. Atwood, R. Bowser, L. Morelli, G. Perry, M.A. Smith, Elevated expression of a regulator of the G2/M phase of the cell cycle, neuronal CIP-1-associated regulator of cyclin B, in Alzheimer's disease, J. Neurosci. Res. 75 (2004) 698–703.

X. Zhu, C.A. Rottkamp, A.K. Raina, G.J. Brewer, H.A. Ghanbari, H. Boux, M.A. Smith, Neuronal CDK7 in hippocampus is related to aging and Alzheimer disease, Neurobiol. Aging 21 (2000) 807–813.

X.L. Ang, J. Wade Harper, SCF-mediated protein degradation and cell cycle control, Oncogene 24 (2005) 2860–2870.

X.Q. Bao, X.L. Wang, D. Zhang, FLZ Attenuates α-Synuclein-Induced Neurotoxicity by Activating Heat Shock Protein 70, Mol Neurobiol. 54 (2017) 349-361.

Y. Miyata, Hsp90 inhibitor geldanamycin and its derivatives as novel cancer chemotherapeutic agents, Curr. Pharm. Des. 11 (2005) 1131–1138.

Y. Wang, M. Martinez-Vicente, U. Kruger, S. Kaushik, E. Wong, E.M. Mandelkow, et al, Tau fragmentation, aggregation and clearance: the dual role of lysosomal processing, Hum. Mol. Genet. 18 (2009) 4153–4170.

Y. Xia, T. Saitoh, K. Uéda, S. Tanaka, X. Chen, M. Hashimoto, L. Hsu, C. Conrad, M. Sundsmo, M. Yoshimoto, L. Thal, R. Katzman, E. Masliah, Characterization of the human alpha-synuclein gene: Genomic structure, transcription start site, promoter region and polymorphisms, Journal of Alzheimer's Disease, 3 (2001) 485–494.

Y. Yang, E.J. Mufson, K. Herrup, Neuronal cell death is preceded by cell cycle events at all stages of Alzheimer's disease, J. Neurosci. 23 (2003) 2557–2563.

Y. Zhang, B. Zhao, Green tea polyphenols enhance sodium nitroprusside-induced neurotoxicity in human neuroblastoma SH-SY5Ycells, J. Neurochem. 86 (2003) 1189–1200.

Y.B. Luo, F.L. Mastaglia, Dermatomyositis, polymyositis and immune-mediated necrotising myopathies, Biochim. Biophys. Acta 1852 (2015) 622–632.

Y.C. Hsu, C.W. Chang, H.L. Lee, C.C. Chuang, H.C. Chiu, W.Y. Li, J.T. Horng, E. Fu, Association between History of Dental Amalgam Fillings and Risk of Parkinson's Disease: A Population-Based Retrospective Cohort Study in Taiwan, PLoS One 11 (2016):e0166552.

Y.L. Wang, H.H. Shen, P.Y. Cheng, Y.J. Chu, H.R. Hwang, K.K. Lam, Y.M. Lee, 17-DMAG, an HSP90 Inhibitor, Ameliorates Multiple Organ Dysfunction Syndrome via Induction of HSP70 in Endotoxemic Rats, PLoS One 11 (2016) e0155583.

Z. Bikadi, E. Hazai, Application of the PM6 semi-empirical method to modeling proteins enhances docking accuracy of AutoDock, J. Cheminf. 1 (2009).

Z. Dong, D.P. Wolfer, H.P. Lipp, H. Bueler, Hsp70 gene transfer by adeno-associated virus inhibits MPTP-induced nigrostriatal degeneration in the mouse model of Parkinson disease, Mol. Ther. 11 (2005) 80–88.

Z. Liu, Y. Chen, Q. Qiao, Y. Sun, Q. Liu, B. Ren, X. Liu, Sesamol supplementation prevents systemic inflammation-induced memory impairment and amyloidogenesis via inhibition of nuclear factor kappa B, MolNutr Food Res. 2016.

Z. Nagy, The dysregulation of the cell cycle and the diagnosis of Alzheimer's disease, Biochim. Biophys. Acta 1772 (2007) 402–408.

Z. Ullah, C.Y. Lee, M.L. Depamphilis, Cip/Kip cyclin-dependent protein kinase inhibitors and the road to polyploidy, Cell Div 4 (2009) 10.

APPENDIX- 1

LIST OF INSTRUMENTS USED

1. Laminar Air Flow (vertical)
   The laminar air flow used was the vertical hood with biosafety grade 2 (BS2) level. It was used for keeping the cells contamination-free during media change, toxin and drugs treatment. The manufacturer of the hood is ESCO.

2. CO2 Incubator
   The CO2 incubator was used to provide optimum growing conditions of 5% CO2 and 37°C temperature to the neuroblastoma cells. The NaOH pellet was added to the water tray for maintaining humidity. The make of the incubator is N-Biotek.

3. Inverted Microscope
   Microscopy was done with the help of compound, inverted microscope. The microscope has a mounted camera (Olympus) which is attached to the monitor and images are viewed, clicked and stored with the help of given software.

4. Western Blot Apparatus
   The protein profiling was done using the BioRad western blot apparatus. The instrument used was double-gel casting model. The SDS-PAGE was carried out using 10% polyacrylamide gel.

5. Gel Rocker
   The membrane blocking with BSA and incubation with primary and secondary antibodies during western blotting was carried out on the Tarsons gel rocker.

6. Centrifuge
   The Eppendorf bench top centrifuge was used for separating the required cell constituents and for spinning wherever required during the experiments.

7. Water Bath
   The protein samples were cooked prior to loading onto the gel. The temperature used to denature proteins was 95°C for 15 minutes in the Julabo water bath.

8. Hemocytometer
   The cell density for the purpose of subculturing and cell viability was calculated using the hemocytometer with the cover slip model.

9. Vortex
   The constituents of various solid-liquid formulations were mixed thoroughly with the help of vortex to ensure a uniform composition.

10. Weighing Balance
    The digital electronic weighing balance was used for accurately weighing various compounds and drugs used in the experimentation.

APPENDIX- 2

BUFFERS AND RECEIPES

1. Growth Medium
   DMEM:F12- 1:1
   FBS- 10%
   1mM Sodium Pyruvate- 1:100
   Sodium bicarbonate- 1:50
   1X Non-essential amino acids- 1:100
   PEN-STREP- 1X
   L-Glutamate

2. Resolving Gel (10%) for 5ml
   H_2O - 1.9ml
   30% acrylamide - 1.7ml
   1.5M Tris - 1.3ml
   10% SDS - 0.05ml
   10% APS - 0.05ml
   TEMED - 0.002ml

3. Stacking Gel (5%) for 2ml
   H_2O - 1.4ml
   30% acrylamide - 0.33ml
   1M Tris - 0.25ml
   10% SDS - 0.02ml
   10% APS - 0.02ml
   TEMED - 0.002 ml

4. Running buffer (10X) 1L (pH 8.3)
   Trizma – 30.0g
   Glycine – 144.0g
   SDS – 10.0g
   H_2O – 1000ml

5. Transfer buffer (1X) 1L (pH 8.3)
   Trizma – 25mM
   Glycine – 190mM
   SDS – 0.1%
   Methanol – 20%
   H_2O – make upto 1000ml

6. TBST Buffer (10X) 1L (pH 7.4)
   Trizma – 15g
   NaCl – 44g
   KCl – 1g
   Tween 20 – 10ml

7. PBS Buffer (1X) 1L (pH 7.2)
   NaCl – 8g
   KCl – 0.2g
   Na_2HPO_4 – 1.44g
   KH_2PO_4 – 0.24g

8. Blocking Buffer
   BSA (3%) in TBST

9. Loading Buffer (2X Lammeli) (pH 6.8)
   SDS – 4%
   Betamercaptoethanol – 10%
   Tris-HCl – 0.125M
   Glycerol – 20%
   Bromophenol blue – 0.004%

10. Coomassie blue stain (100ml)
    Coomassie blue – 0.25g
    Methanol – 50ml
    Glacial acetic acid – 10ml
    dH_2O – 40ml

11. Coomassie blue destain (100ml)
    Methanol – 5ml
    Glacial acetic acid – 7ml
    dH_2O – make upto 100ml

12. Ponceau S stain
    Ponceau S – 0.2% (w/v)
    Glacial acetic acid – 5%

LIST OF PUBLICATIONS

Publications from Thesis

1. **Renu Sharma** and Pravir Kumar **(2017)**. *Repurposing HSP70 inducing compounds for targeting post-mitotic cell division: novel promises as neuroprotectants.* ***Journal of Chemical and Pharmaceutical Research 9(3):373-384.***
2. **Renu Sharma** and Pravir Kumar **(2017)**. *Neuroprotective role of bimoclomol in ectopic cell cycle in Parkinson's disease: new insights.* ***Asian Journal of Pharmaceutical and Clinical Research 10(6):1-4.***
3. **Renu Sharma,** Dhiraj Kumar, Niraj Kumar Jha, Saurabh Kumar Jha, Rashmi K Ambasta and Pravir Kumar **(2017)**. *Re-expression of Cell cycle markers in aged Neurons and Muscles: Whether cells should divide or die?* ***Journal of BBA Molecular Basis of Disease****,* **Impact factor: 5.158**

Other Publications

1. Saurabh Kumar Jha, Niraj Kumar Jha, Dhiraj Kumar, **Renu Sharma**, Abhishek Shrivastav, Rashmi K Ambasta, Pravir Kumar*, (**2017**), *Stress-induced synaptic dysfunction and neurotransmitter release in Alzheimer's disease: Can neurotransmitters and neuromodulators be potential therapeutic targets?* **Journal of Alzheimer's Disease**. 57(4):1017-1039. **Impact factor: 3.93**

2. Niraj Kumar Jha, Saurabh Kumar Jha, Dhiraj Kumar, Noopur Kejriwal, **Renu Sharma**, Rashmi K Ambasta and Pravir Kumar. *Impact of IDE and Neprilysin in Alzheimer's Disease Biology: Characterisation of Putative Cognates for Therapeutic Applications*. (Accepted **July 2015**) ***Jounal of Alzheimer's Disease*** **Impact factor 4.18**

3. Rashmi K. Ambasta, Saurabh Kumar Jha, Dhiraj Kumar, **Renu Sharma**, Niraj Kumar Jha, and Pravir Kumar. *Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules*. (Accepted **September 2015**)**,** ***Journal of Translational Medicine,*** **Impact factor: 3.96**

4. Ankit Tripathi*, **Renu Sharma***, Noopur Kejriwal, Rashmi K Ambasta and Pravir Kumar. *Epigenetics Post Transcriptional Mutation In Neuro-Oncology* (**Springer Book Chapter January 2015 *Joint First Author**)

Conference/Workshop Proceedings

1. Participated in the 6th workshop on molecular modeling and drug design (BIF-MMDD) 2016, ACBR 21-23 January, DU, Delhi, INDIA
2. Dhiraj Kumar, Niraj K. Jha, Saurabh K. Jha, **Renu Sharma**, KushiAnand, Rashmi K. Ambasta and Pravir Kumar* (**2014**), Anti cancerous drugs as a neuroprotectant: a therapeutic intervention in neurodegenerative disorders, International Symposium on Translational Neuroscience and XXXII Annual Conference of the Indian Academy of Neurosciences, 01-03 November, NIMHANS Bangalore, INDIA
3. **Renu Sharma**, Rashmi K Ambasta and Pravir Kumar* (**2013**), Cell cycle regulation to programmedcell death: role of cyclins in neurodegeneration, NCRTPSB 2013, JMI 16-18 December, INDIA Journal of Protein and Proteomics, Vol.4, No.2 p63
4. **Renu Sharma**, Ankit Tripathi, SagarVerma, Sakshi Sharma, Pravir Kumar and Rashmi K Ambasta (**2013**), Crosstalk between cancer stem cell markers, chemotherapy and nanoparticles in cancer diagnostics and therapeutics, 09-11 December, Bioworld Conference, IIT Delhi, Delhi, INDIA
5. Niraj kumar Jha, Lakshmi, Binod Koirala, Saurabh kumar Jha, **Renu Sharma**, Rohan Kar, Dhiraj, Jitendra Singh, Rashmi K. Ambasta and Pravir kumar*; (**2013**), Identification and validation of key Ubiquitin E3 ligases in type II diabetes: An in silico work; 09-11 December, Bioworld Conference, IIT Delhi, Delhi, INDIA

BIO-DATA

RENU SHARMA
UGC SRF
sharma.renu2707@gmail.com

OBJECTIVE

To be associated with a progressive organization of a nation that gives me scope to update my knowledge and skills in accordance with the latest trends and technologies. Be a part of the team that leads to the growth of the organization and the country.

EDUCATIONAL QUALIFICATIONS

S.No.	Name of Exam	Year of Passing	University/ Board	Div.	Subjects	%
1	Sec. School	2003	CBSE	I	English,Hindi Science, Maths S.Studies	80.6
2	Sr. Sec. School	2005	CBSE	I	Physics Chemistry Biology, English	74.5
3	B.Sc (H)	2009	Hansraj College, DU	I	Anthropology Chemistry Zoology English	76.2
4	M.Sc.	2011	LNJN NICFS (GGSIPU)	I	Forensic Science	78.92
5	Ph.D	2013-2017	Delhi Technological University	UGC SRF	Human Physiology	-

PROFESSIONAL EXPERIENCE

S. No.	Employer	From	To	Designation	Nature of duties
1	CFSL, CBI	05-7-10	18-7-10	Trainee (Ballistics)	Forensic Analysis

2	FSL, Delhi	05-10-10	20-10-10	Trainee	-do-
3	CFSL,CBI	05-4-11	18-4-11	Trainee (Chemistry)	-do-
4	LNJN NICFS (MHA)	17-1-11	12-5-11	Dessertation on "Detection & identification of date rape drug-KETAMINE"	-do-
5	TRUTH LABS	08-9-11	15-9-12	Scientific Officer	-do-

PEER REVIEWED PUBLICATIONS:

1. **Renu Sharma** and Pravir Kumar (2017). *Repurposing HSP70 inducing compounds for targeting post-mitotic cell division: novel promises as neuroprotectants.* ***Journal of*** **Chemical and Pharmaceutical Research 9(3):373-384.**
2. **Renu Sharma** and Pravir Kumar (2017). *Neuroprotective role of bimoclomol in ectopic cell cycle in Parkinson's disease: new insights.* **Asian Journal of Pharmaceutical and Clinical Research 10(6):1-4.**
3. **Renu Sharma,** Dhiraj Kumar, Niraj Kumar Jha, Saurabh Kumar Jha, Rashmi K Ambasta and Pravir Kumar (2017). *Re-expression of Cell cycle markers in aged Neurons and Muscles: Whether cells should divide or die?* **Journal of BBA Molecular Basis of Disease***, **Impact factor: 5.158**
4. Saurabh Kumar Jha, Niraj Kumar Jha, Dhiraj Kumar, **Renu Sharma**, Abhishek Shrivastav, Rashmi K Ambasta, Pravir Kumar*, (**2017**), *Stress-induced synaptic dysfunction and neurotransmitter release in Alzheimer's disease: Can neurotransmitters and neuromodulators be potential therapeutic targets?* **Journal of Alzheimer's Disease**. 57(4):1017-1039. **Impact factor: 3.93**
5. Niraj Kumar Jha, Saurabh Kumar Jha, Dhiraj Kumar, Noopur Kejriwal, **Renu Sharma**, Rashmi K Ambasta and Pravir Kumar. *Impact of IDE and Neprilysin in Alzheimer's Disease Biology: Characterisation of Putative Cognates for Therapeutic Applications*. (Accepted **July 2015**) **Jounal of Alzheimer's Disease Impact factor 4.18**
6. Rashmi K. Ambasta, Saurabh Kumar Jha, Dhiraj Kumar, **Renu Sharma**, Niraj Kumar Jha, and Pravir Kumar. *Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules.* (Accepted **September 2015**)**, Journal of Translational Medicine, Impact factor: 3.96**

7. Ankit Tripathi*, **Renu Sharma***, Noopur Kejriwal, Rashmi K Ambasta and Pravir Kumar. *Epigenetics Post Transcriptional Mutation In Neuro-Oncology* (**Springer Book Chapter January 2015 *Joint First Author**)

PUBLICATION OF ABSTRACT/POSTER IN JOURNALS/CONFERENCE PROCEEDINGS:

1. Participated in the 6th workshop on molecular modeling and drug design (BIF-MMDD) 2016, ACBR 21-23 January, DU, Delhi INDIA
2. Niraj Kumar Jha, Saurabh Kumar Jha, **Renu Sharma**, Rashmi K Ambasta, Pravir Kumar (2014); Physiological stress in neurodegeneration; Interactomic partners based on Insilico study. IAN 2014, NIMHANS 01-03 November, Bangalore INDIA
3. Dhiraj**, Renu Sharma**, Rashmi K Ambasta and Pravir Kumar (2014); Anticancerous drugs as a neuroprotectant: A therapeutic intervention in neurodegenerative disorders. IAN 2014, NIMHANS 01-03 November, Bangalore INDIA.
4. **Renu Sharma**, Rashmi K Ambasta, Pravir Kumar (2013); Cell cycle regulation to programmed cell death: role of cyclins in neurodegeneration, NCRTPSB 2013, JMI 16-18 December, INDIA Journal of Protein and Proteomics, Vol.4, No.2 p63.
5. **Renu Sharma**, Ankit Tripathi, Sagar Verma, Sakshi Sharma,Pravir Kumar and Rashmi K Ambasta (2013); Crosstalk between cancer stem cell markers, chemotherapy and nanoparticles in cancer diagnostics and therapeutics, 09-11 December, Bioworld Conference, IIT Delhi, Delhi INDIA.
6. Niraj Kumar Jha, Lakshmi, Binod Koirala, Saurabh Kumar Jha, **Renu Sharma**, Rohan Kar, Dhiraj, Jitendra Singh, Rashmi K Ambasta, Pravir Kumar (2013), Identification and validation of key Ubiquitin E3 ligases in type II diabetes: An in silico work; 09-11 December, Bioworld Conference, IIT Delhi, Delhi INDIA

ACHIEVEMENTS

- Qualified UGC NET-JRF in March 2012
- Awarded the highest in merit prize in M.Sc Forensic Science(1^{st} Year) in 2009-10 by GGSIP University

- Won 2nd prize in Hindi debate at the IP University Annual Fest 2010.
- Awarded the highest in merit prize in B.Sc.(H) Anthropology at both Hansraj College and Delhi University in 2008-09
- Highest in merit for AISSC(CBSE) (Medical) during (2005)
- Won special prize at Inter School Hindi debate championship at RIMC, Dehradun in 2003
- Participated in State Level School Volleyball Championships in the year 2000-01
- Highest in merit throughout school years (1992 – 2005)

TECHNIQUES AND INSTRUMENTATION

- Animal Cell Culture
- Western Blotting
- Plasmid DNA Isolation
- Immunofluorescence
- Chorioallantoic Membrane Assay (CAM Assay)
- Electrophoresis
- Microscopy
- UV-Vis Photo spectrophotometer
- Fourier Transform Infra Red Radiation (FTIR)
- Gas Chromatography (GC)
- Thin Layer Chromatography (TLC)
- Molecular Biology Techniques
- Basic Bioinformatics Tools and Techniques

EXTRA CURRICULAR ACTIVITIES & SPORTS

- English/Hindi Debates and Plays
- State Volleyball player
- Singing

KEY SKILLS

- Ambitious and Dynamic
- Capable of facing difficulties as challenges
- Strong team Leader
- Quick grasping of new concepts, good analytical and planning ability
- Committed towards work
- Hardworking, punctual and disciplined

ADDITIONAL SKILLS

- Good Communication skills
- Excellent command over English & Hindi
- Working Knowledge of Computers & Internet

PERSONAL PARTICULARS

- Date of Birth - 27/10/1987
- Gender - Female
- Father's Name - Shri Mahander Singh

Date: 10/11/2017 Renu Sharma

Place: New Delhi

Biochimica et Biophysica Acta 1863 (2017) 324–336

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Re-expression of cell cycle markers in aged neurons and muscles: Whether cells should divide or die?

Renu Sharma, Dhiraj Kumar, Niraj Kumar Jha, Saurabh Kumar Jha, Rashmi K Ambasta, Pravir Kumar *

Molecular Neuroscience and Functional Genomics Laboratory, Delhi Technological University (Formerly DCE), Delhi 110042, India

ARTICLE INFO

Article history:
Received 14 July 2016
Received in revised form 1 September 2016
Accepted 13 September 2016
Available online 14 September 2016

Keywords:
Post-mitotic cell division
Signaling cascade
Neuromuscular degeneration
Cyclins
Therapeutics

ABSTRACT

Emerging evidence revealed that abrogated cell cycle entry into highly differentiated mature neurons and muscles is having detrimental consequences in response to cell cycle checkpoints disruption, altered signaling cascades, pathophysiological and external stimuli, for instance, Aβ, Parkin, p-tau, α-synuclein, impairment in TRK, Akt/GSK3β, MAPK/Hsp90, and oxidative stress. These factors, reinitiate undesired cell division by triggering new DNA synthesis, replication, and thus exquisitely forced mature cell to enter into a disturbed and vulnerable state that often leads to death as reported in many neuro- and myodegenerative disorders. A pertinent question arises how to reverse this unwanted pathophysiological phenomenon is attributed to the usage of cell cycle inhibitors to prevent the degradation of crucial cell cycle arresting proteins, cyclin inhibitors, chaperones and E3 ligases. Herein, we identified the major culprits behind the forceful cell cycle re-entry, elucidated the cyclin re-expression based on disturbed signaling mechanisms in neuromuscular degeneration together with plausible therapeutic strategies.

1. Introduction

The etiology of neuromuscular degeneration is characterized by the canonical deposition of non-functional/toxic proteins such as amyloid β, tau, parkin, α-synuclein, mutant huntingtin, and chronic inflammations in neuromuscular disorders (NMDs) including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Inclusion body myositis (IBM) and Poly myositis (PM). The deposition of pathological proteins or inclusions creates a physiological burden on cell that triggers multiple signaling cascades including mitogen-activated protein kinase (MAPK), c-Jun N-Terminal Kinases (JNK), Phosphatidylinositol 3-kinase/serine threonine kinase Akt/Glycogen synthase kinase 3 (PI3K/Akt/GSK3β), Notch and apoptotic signaling pathways. The consequent overexpression of cell cycle markers has been observed with the symptoms of cell cycle re-entry (CCR) [1]. Moreover, the co-localization of cell cycle markers with pathological proteins has reinforced the role of cell cycle machinery as a trigger to degeneration [2]. Further evidence suggests that re-expression of cell cycle markers occurs at prodromal stages before the appearance of pathological hallmarks in these neuromuscular disorders. A plethora of studies have demonstrated the involvement of oxidative stress, aging, mutation, mitochondrial dysfunction; ubiquitin proteasome system (UPS) shut down and loss of function of several protective proteins in the pathogenesis of these NMDs. For instance, prolonged activation or under the influence of acute insults such as DNA damage, oxidative stress, neurotrophic factor deprivation and altered microRNA (miR) function; cell cycle machinery turns detrimental and drives the cascade of DNA synthesis and consequent neuronal death culminating into neurodegeneration [3]. Interestingly, numerous studies have established cell cycle re-entry as a true phenomenon in neurodegeneration with actual DNA synthesis and cannot be viewed as an epiphenomenon of other processes. Moreover, cell cycle markers' re-expression in terminally differentiated neurons is also reported to fulfill the physiological demands pertaining to synaptic plasticity, neuronal migration and maturation [4]. Importantly, current studies advocate that aberrant cell cycle re-entry is not a consequence, but rather a cause of neuromuscular degeneration, depicting the importance of targeting cell cycle re-entry as a therapeutic window for treating neuromuscular disorders. These days several compounds/biomolecules have been implicated for targeting cell cycle re-entry in neurons and muscles. Moreover, potential role of ubiquitin E3 ligases and heat shock proteins have also been demonstrated to revert the damaging effect caused by cell cycle re-entry in neuromuscular disorders.

2. Relevance of cell cycle markers (cyclins, CDKs, CDKIs) in maintaining cell cycle homeostasis

The eukaryotic cell cycle is orchestrated machinery that accomplishes fundamental roles in cell replenishment such as DNA replication,

* Corresponding author at: Department of Biotechnology, Delhi Technological University (Formerly Delhi College of Engineering), Room # FW4TF3, Mechanical Engineering Building, ShahbadDaulatpur, Bawana Road, Delhi 110042, India.
E-mail addresses: pravirkumar@dce.edu, kpravir@gmail.com (P. Kumar).

http://dx.doi.org/10.1016/j.bbadis.2016.09.010
0925-4439/

cell growth, repair and birth of new daughter cells from the native mother cell. The coordination of the cell cycle requires complex interplay between the levels of different cyclins and cyclin dependent kinases (CDKs) at different checkpoints. For instance, Cyclin C/Cdk3 complex mark the transition of cells into G1 i.e. preparatory phase. Further, G1/S transition is controlled by the crucial action of cyclin D/Cdk4/6 and cyclin E/Cdk2 complex [5]. The DNA replication in S phase is triggered by the cyclin A/Cdk2 and Cyclin A/Cdk1 complex. Cell division in M phase is tightly regulated by the activity of cyclin B/Cdk1complex [6]. The integrity of the cell cycle is maintained by the timely degradation of cyclins and consequent inactivation of CDKs, which is brought about by a class of cyclin dependent kinase inhibitors (CDKIs). These CDKIs are categorized into Ink family and Cip/Kip family [7]. The Ink family comprised $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$ that bind with Cdk4/6 and is known to inhibit its association with cyclin D, thereby promoting quiescent stage [8]. On the contrary, members of Cip/Kip family comprised $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ that bind and regulate specific Cyclin/Cdk complexes during different stages of cell cycle progression [9]. Additionally, activation of different signaling cascades tightly regulates the checkpoints, which are strategically distributed throughout the cell cycle. The checkpoints ensure the correct completion of previous phase before the beginning of new cell cycle phase, and ascertain the correction of any defect and provide protection against the transmittance into daughter cells by halting the cell cycle until the repair has been done or by alternatively triggering cell death pathways. For instance, the G1 phase is regulated by two checkpoints in its early and late phase to assess cell size and growth factors required for promotion to S phase [10]. While, the S phase is regulated by tumor suppressor gene p53 in response to DNA damage wherein the cell cycle is halted for repair or alternatively triggering apoptosis if the damage cannot be repaired [11]. Furthermore, the G2/M transition is tightly controlled by phosphorylation dynamics of Cdk1 where the activatory phosphorylation of Cdk1 is carried out by Cdk-activating kinase complex at Thr160/Thr161 while inhibitory phosphorylation is mediated by Myt1 and Wee1 kinase at Thr14 and Tyr15 respectively. These phosphorylation events can be reversed by the action of cell division cycle 25 (Cdc25) thereby tightly regulating Cdk1 activity [12]. In this way, cell cycle markers play a discrete role in the normal physiology of neuronal cells. However, post mitotic expression of these markers is responsible for the onset of cell cycle re-entry mediated neuromuscular dysfunction.

3. Causative agents that force cell cycle for re-entry into neurons and muscles

There are various factors that are responsible for triggering cell cycle re-entry in neurons and muscles, including environmental toxins, genetic factors, and other cellular stresses. These factors have different mechanistic approaches for eliciting cell cycle abnormalities in neurons and muscles that have been discussed below and elucidated in Fig. 1.

3.1. Potential cell cycle re-entry triggering factors

3.1.1. Environmental factors

Recently, certain environmental factors have been identified, which contributed in the pathogenesis of several neuromuscular disorders. These environmental toxins comprised heavy metals, pesticides and metal based nanoparticles, which are currently being studied for their role in CCR mediated neuromuscular degeneration. For instance, administration of methyl mercury (MeHg) is found to alter the expression of cell cycle regulators p16 and p21 in rat embryonic cortical neuronal stem cells. Moreover, compromised cell proliferation and a significant decrease in global DNA methylation were observed subsequent to MeHg treatment in NSCs, supporting the idea that its long-term exposure would lead to neurodevelopmental or neurodegenerative disorders [13]. Further, exposure to various other toxins like kainic acid, quinolinic acid and heavy metals, including cobalt, lead and iron was shown to be responsible for reactive gliosis. Accordingly, induced reactive gliotic changes led to cell cycle re-entry attempts in neurons [14]. Recently, fluoride and aluminum were found to disrupt neuronal morphology and cell cycle induced progressive cell death by triggering the activation of cell cycle markers and lysosomal proteins [15]. Additionally, a renal carcinogen Ochratoxin A (OTA) induced cell cycle aberrations in renal tubular cells [16]. Moreover, environmental factors are found to exacerbate the neuromuscular degeneration in action with other triggering factors. There are numerous reports demonstrating the toxic effect of heavy metals, pesticides and nanoparticles in neurodegeneration, but very few showing the lethal effect of these toxins in the cell cycle re-entry mediated neuromuscular degeneration. Therefore, significant researches need to be carried out to determine the involvement of heavy metals and pesticides in cell cycle induction related neuromuscular toxicity.

3.1.2. Oxidative stress

Oxidative stress is the most vulnerable and obvious cause for inducing cell cycle irregularities in post-mitotic neurons and muscles reported till the date. Reactive oxygen species (ROS) generation is the prime factor responsible for causing oxidative stress, which in turn alters the components of mitogenic signaling cascade by activating various signaling molecules. For instance, MAPK, nuclear factor κB (NF-κB) and growth factor receptors are activated via ROS and initiate cell cycle re-entry in post-mitotic neurons [17]. Interestingly, ROS also induced DNA damage; chromosomal breaks and base mis-incorporation in cell cycle events [18]. Moreover, ROS was also found to abort the activity of DNA repair proteins associated with the DNA replication process in AD. Numerous studies have reported ROS mediated unrepaired, oxidized nitrogenous bases and DNA strand breaks in post-mortem tissues of patients with different neuromuscular disorders [19]. Hypoxia has emerged as a critical player of cell cycle induction in neurons and muscles. Additionally, various studies have reported DNA replication in post-mitotic neurons upon exposure to hypoxia. Apart from DNA damage, oxidative stress is proposed to trigger cell cycle re-entry in response to UPS dysfunction [20]. However, UPS is evident to regulate the cell cycle in two disparate manner via degrading either cyclin D1 or Cdk inhibitors p21/p27 [18]. Likewise, altering the levels of HSPs including HSP 27, HSP 70 and HSP90, also triggers cell cycle re-entry via controlling different stages of the cell cycle. For instance, blocking of HSP90 action with geldanamycin, halted cell cycle at G0/G1 transition by obstructive action of HSP90-specific client proteins Cdc37 and FKBP52 [21]. Besides Kwon et al. demonstrated the interaction between HSP70 and cyclin D or E1 in IBM and PM biopsy samples depicting their role in cell cycle progression [22]. Another study reported the association between Hsc70 and cyclin D1 to regulate its assembly with Cdk4 and Cip/Kip proteins and formation of a catalytically active complex [23]. Therefore, oxidative stress in concordance with cell cycle re-entry markers contributed significantly in the etiology of neuromuscular degeneration.

3.1.3. Double stranded DNA break (DSB)

DSB accumulation and its attainment of non-repairable conformation is another crucial factor for initiating cell cycle re-entry in post-mitotic neurons [24]. DNA damage activates ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and E2F transcription factor 1 (E2F1), and other proteins, which regulate cell cycle; DNA damages repair, and apoptosis in post-mitotic neurons [25]. Chk1 and chk2 are the checkpoint kinases that are induced in response to DNA damage in the cell cycle. ATM is a well-known checkpoint in the cell cycle which regulates dsDNA break response in a p53 dependent manner and controls cell cycle arrest, apoptosis and mediates protection against oxidative stress [26]. Moreover, phosphorylated ATM has been shown to be elevated in cerebellar dentate neurons of AD patients when compared with age-matched controls [27]. Further, increased

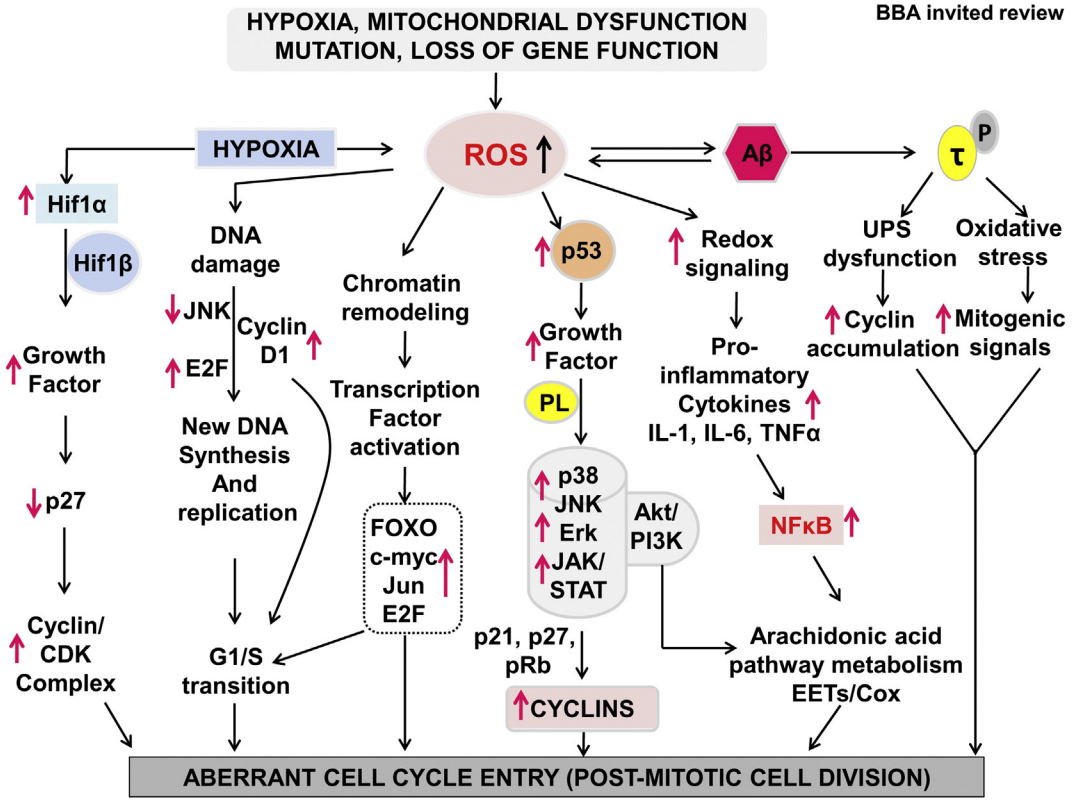


Fig. 1. Triggering factors associated with the forceful cell cycle re-entries. Hypoxia and oxidative stress are two critical factors responsible for triggering CCR. Hypoxic exposure increases HIF-1α that causes up-regulation of growth factors and subsequent reduction of p27 expression. Reduced p27 expression and increased HIF-1α transactivation induce cyclin/Cdk complex thereby activating cell cycle progression. Further, ROS mediated oxidative stress could lead to aberrant CCR viz. several different pathways. First, ROS accumulation can result in cumulative DNA damage and consequent alteration in new DNA synthesis/replication, chromatin remodeling and transcriptional activation, signaling cascade activation and subsequent activation of cell cycle markers and thus CCR. Further, redox signaling activates pro-inflammatory cytokines (IL-1, IL-6, TNF-α) that triggers NF-κβ mediated arachidonic acid pathway metabolism and consequent CCR. In addition, ROS triggers Aβ mediated Tau phosphorylation and consequent dysfunction of ubiquitin-proteasome system and activation of mitogenic signaling pathways that also induce cell cycle progression. Hif1α-Hypoxia-inducible factor 1-alpha; Hif1β-Hypoxia-inducible factor 1-beta; CDK-Cyclin dependent Kinase; ROS-Reactive oxygen species; JNK-c-Jun N-terminal kinase; JAK/STAT-Janus kinase/signal transducers and activators of transcription; PI3K-Phosphoinositide 3-kinase; FOXO-Forkhead box protein; PL-Phospholipid; pRb-Phosphorylated retinoblastoma; IL-Interleukin; TNFα-Tumor necrosis factor-α; EETs-Epoxyeicosatrienoic acids; Cox-Cyclooxygenase.

ATM expression accompanying downregulation of TP53-inducible glycolysis and apoptosis regulator (TIGAR) protein constituted a key feature of dementia associated with Alzheimer's disease. Furthermore, DSB mediated accumulation of functionally inactive monomers and dimers of TP53 have been reported in AD brain [28]. Likewise, E2F complexes are associated with histone-modifying enzymes, histone modifications, including histone acetylation and methylation, and therefore, essential for cell cycle re-entry that may play a regulatory role in DNA repair or apoptosis [25]. Moreover, evidence for DSB had also been reported in s-IBM muscles that resulted in muscle fiber maturation arrest [29]. Importantly, recent studies are going on to explore the relationship between DNA damage and cell cycle re-expression in different systems.

3.1.4. Micro RNAs (miR)

miR has recently gained attention due to their strategic control of cell cycle regulation in post-mitotic neurons. For instance, over-expression of miR26 has been reported to trigger aberrant cell cycle re-entry, DNA replication, and tau hyperphosphorylation in rat primary post-mitotic neurons. Moreover, elevated level of miR-26b triggered the re-expression of cell cycle proteins cyclin E1, pRb, PCNA, Ki67 and BrdU in post-mitotic neurons [30]. Additionally, miR26b over-activation elicited Cdk5 mediated enhanced tau phosphorylation in vitro and in-vivo studies. It also attenuated the action of cell cycle inhibitors p15 and p27 [31]. In another study, increased miR-26b level induced cyclin E1 up-regulation and downregulated $p27^{kip1}$ expression mediated by Rb1/E2F protein complex, signifying its role in cell cycle regulation [30]. Further, genetic and epigenetic evidence revealed that NF-κB-regulated micro RNAs including miRNA-9, miRNA-125b, miRNA-146a and miRNA-155 are involved in the downregulation of small brain and retinal cell related family of target mRNAs causing complement factor H (CFH) deficiency leading to inflammatory neurodegeneration in AD and age-related macular degeneration (AMD) [32]. Likewise, disruption of miR34a and miR-132 has been linked with AD and other NDDs. For instance, Aβ mediated suppression of miR-34a resulted in unscheduled cell cycle re-entry and apoptosis via MEK-ERK pathway mediated degradation of tumor suppressor TAp73 in AD mouse model. Interestingly, numerous studies are evident for the over-expression of miRNAs in temporal cortex and hippocampus region of AD patients signifying that erroneous miRNA regulation is involved in the etiology of various NDDs [33]. Recently, altered miRNA expression has been observed in IBM, PM and other muscular dystrophies that are involved with myoblast differentiation and muscular regeneration [34]. However, few studies reported the role of microRNA in the cell cycle re-entry, but microRNA research is still at preliminary stage.

3.1.5. Brain derived neurotrophic factor (BDNF)

BDNF is a member of neurotrophin family of growth factors that plays a crucial role in neuronal cell survival, growth and differentiation. It protects neurons against glutamate and oxidative stress, i.e. NMDA or H_2O_2 mediated toxicity, might be through inhibiting Ras-MAPK pathway. However, altered BDNF has been implicated in the cell cycle re-entry mediated pathogenesis of neuromuscular disorders during oxidative or excite toxic stress responses [35]. For instance, BDNF down-regulated cyclin B and Cdk1 levels (generally localized in NFTs in AD) in differentiating tetraploid neurons in the retina through TrkB

neurotrophic receptor-mediated activity and thereby halted the cell cycle at G_2/M checkpoint. The inability of this complex to translocate into the nucleus likely played a role in blocking G_2/M transition [36] and abnormal tau phosphorylation, thereby contributing in the pathogenesis of AD [35]. Additionally, BDNF was found to trigger inhibition of Cdk1 activity through phosphorylation of the site Tyr15, thereby halting G2/M transition in tetraploid neurons [36]. Another study identified that nerve growth factor (NGF) induces cell cycle re-entry through a novel, Cdk4/6-independent pathway, i.e. p^{38}/MAPK-Dependent E2F4 Phosphorylation mediated neurodegeneration [37]. The neurotrophin system also exhibits a role in myogenesis and regeneration in skeletal muscle biology. Moreover, differential study of BDNF receptors identified p75 neurotrophin receptor (p75NTR) to be predominantly expressed in human myocytes [38]. Presently, BDNF deprivation was well implicated in the cell cycle re-entry mediated pathogenesis in neurons, but its investigation in muscles is still desired.

3.2. Altered signaling cascades in aberrant cell cycle re-entry induced neuromuscular degeneration

Although there are several risks, factors identified to be involved in aberrant CCR but the demonstration of consequent alteration of signaling cascade is still at preliminary stage. However, a growing body of evidences have highlighted the involvement of multiple signaling pathways such as Notch, Wnt/β-catenin, Akt/GSK3β, P^{38}/MAPK and dyotic signaling in the biology of cell cycle re-entry (Fig. 2). For instance, treatment with kainic acid is reported to activate notch signaling that augmented the aberrant CCR via activation of CyclinD1-Rb-E2F1 axis. Moreover, this abundance of cyclin D1 expression was accomplished by Akt/GSK3β pathway in AD [39]. Similarly, elevated levels of β-catenin through wnt/β-catenin signaling in PS1 FAD brain has been found to accelerate CCR simply by upregulating cyclin D1 transcription [40]. Further, elevated cyclin D1 contributes towards the apoptotic

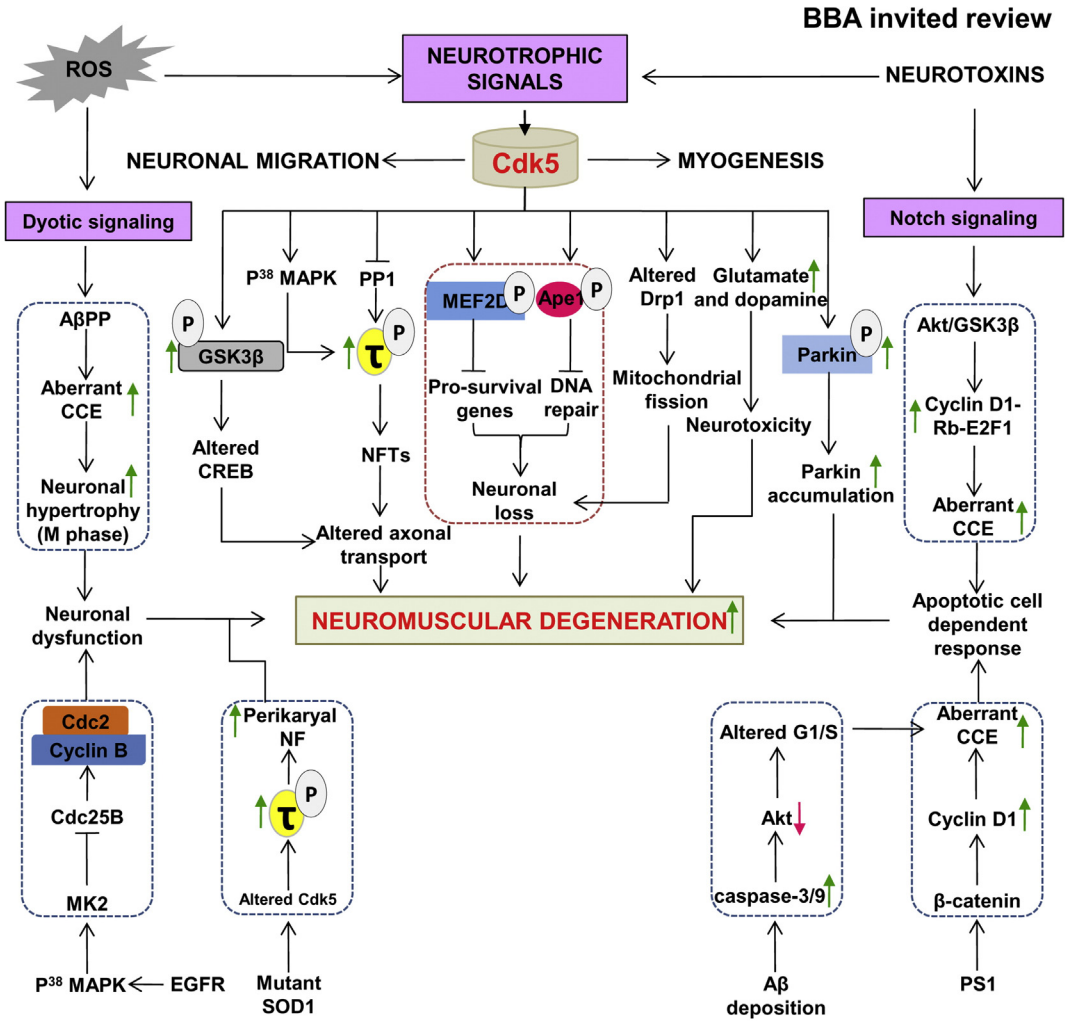


Fig. 2. Disturbed signaling cascades associated with an aberrant cell cycle re-entry in neuromuscular degeneration. Multiple signaling pathways including Notch, Wnt/β-catenin, Akt/GSK3β, P^{38}/MAPK and dyotic signaling get affected in response to neurotophic signals, ROS production and neurotoxins attack and contribute towards the pathogenesis of neuromuscular disorders. ROS induced dyotic signaling alters AβPP processing and neuronal hypertrophy in M phase of cell cycle in post-mitotic neurons. Neurotrophic signals mediated Cdk5 activation triggers neuronal dysfunction via phosphorylating Ape1, MEF2D and GSK-3β that govern the onset of NMDs. Further, Cdk5 also inhibits protein phosphatase-1 (PP1) and promotes P^{38}/MAPK activity, which in turn leads to tau hyperphosphorylation and consequent microtubule dissociation. In addition, it also directs Parkin's phosphorylation and glutamate or dopamine mediated neurotoxicity in PD and HD respectively. Similarly, altered Drp1 activity is also mediated via Cdk5 activity that augments mitochondrial fission in HD. Importantly, in case of ALS, mutant SOD1 impairs Cdk5 activity and subsequently causes tau hyper phosphorylation mediated Perikaryalneurofilaments (NF) accumulation. Further, neurotoxins induced Notch signaling causes aberrant CCR via activation of CyclinD1-Rb-E2F1 axis in AD. Similarly, increased levels of β-catenin viz. wnt/β-catenin pathway accelerate CCR via upregulating cyclin D1 transcription in PS1 FAD brain. Additionally, Aβ mediated increased expression of procaspase-3 and cleaved caspase-3 downregulates Akt expression and subsequent alteration in G1/S phase transition i.e. CCR. Furthermore, EGFR mediated MK2 activation inhibits Cdc25B, which in turn promote Cdc2 and Cyclin B complex and cause neuronal dysfunction. ROS - Reactive oxygen species; Cdk5 - Cyclin dependent kinase 5; GSK3β - Glycogen synthase kinase 3β; CREB - cAMP responsive element binding protein; PP1 - Protein phosphatases 1; MEF2D - Pro-survival transcription factor; Ape1-Human AP - endonuclease 1; MK2 - MAPK-activated protein kinase-2; NF - Neurofilaments; EGFR - Epidermal growth factor receptor; PS1 - Presenilin 1.

response mediated neuronal cell death. Additionally, disturbed Cdk5 associated neurotrophic signals have also been reported to cause neuronal dysfunction. For instance, Cdk5 mediates phosphorylation of Ape1, MEF2D and GSk-3β and contribute in the pathophysiology of neuromuscular disorders [41]. Ape1 plays a crucial role in DNA repair mechanism while MEF2D, a pro-survival transcription factor assists in memory development [42]. Moreover, Cdk5 also inhibits protein phosphatase-1 (PP1) and promotes P^{38}/MAPK activity, which in turn leads to tau hyperphosphorylation and consequent microtubule dissociation [43]. Further, Cdk5 also involved with Parkin phosphorylation and glutamate or dopamine mediated neurotoxicity in PD and HD respectively [44]. Likewise, Cdk5 is also responsible for altering Drp1 activity thereby increasing mitochondrial fission in HD. Besides, it is also reported to induce tau hyperphosphorylation and consequent increased Perikaryal inclusions of neuro-filaments (NF) in the SOD1 amyotrophic lateral sclerosis (ALS) mice model [45]. Furthermore, the increased expression of procaspase-3 and cleaved caspase-3 and 9 i.e. apoptotic signaling is observed in IBM/PM sample to be associated with Aβ accumulation. Since, accumulated Aβ in IBM/PM has evidenced to downregulate the Akt expression and subsequent alteration in G1/S phase transition thereby triggering aberrant CCR [22]. Recently, dyotic signaling has also been identified to be associated with CCR in post-mitotic neuron. Since, this signaling trigger altered AβPP processing and neuronal hypertrophy in M phase of cell cycle, thus governing aberrant CCR in AD [40]. Moreover, recent studies are going on to identify the cell cycle markers associated signaling disturbances in post-mitotic neurons and muscles to demonstrate the pathophysiology behind the cell cycle re-entry mediated neuromuscular degeneration. Such altered signaling associated with cell cycle markers in different neuromuscular disorders have been summarized in Table 1. Nevertheless, numerous signaling pathways are currently being explored in different models to unravel the complexity of cell cycle re-entry mechanisms in various NMDs.

4. Aberrant cell cycle re-entry in neuromuscular disorders

Scientists are investigating whether the re-expression of various cell cycle markers symbolizes an authentic cell cycle, or it is just a consequence of other pathological events. The growing evidence supports the notion that cyclin's re-expression is a true phenomenon representing actual re-activation of the cell cycle in post-mitotic neurons and muscles. However, cell cycle re-entry in terminally differentiated neurons and muscles is lethal and has been widely implicated in the pathogenesis of degenerative disorders like AD, PD, IBM/PM, HD and ALS (Fig. 3).

4.1. Alzheimer's disease

Alzheimer's disease is the most common progressive neurodegenerative disorder, well characterized by the intracellular deposition of hyper-phosphorylated form of microtubule associated protein; Tau and extracellular aggregation of amyloid beta (Aβ) protein manifesting in neurofibrillary tangles (NFT) and senile plaques respectively. However, increasing evidence has defined the role of cell cycle re-entry as one of the keys causal phenomenon in neuronal death associated pathogenesis of AD. Moreover, cell cycle aberrations are directly linked with the increase in pathological accumulation of Aβ and tau hyper-phosphorylation. Furthermore, cell cycle re-entry preceded NFT and senile plaque formation in AD and cell cycle markers were found to co-localize with NFTs, further reinforcing that cell cycle re-entry posits an early laid phenomenon in the pathogenesis of AD [24,63]. The cdc7 dependent phosphorylation of mini-chromosome maintenance protein 2 (Mcm2) at Ser40/41 has been reported in AD neuronal cytoplasm and NFTs, supporting the evidence of cell cycle re-entry in a DNA replication manner. However, it is interesting to note that the Mcm2 expression has not been reported in non-proliferating tissues, rather, its accumulation is observed in the cells re-entering G1 phase. Moreover, phosphorylated Mcm2 gets accumulated in the cytoplasm and is supposed to alter the minichromosome maintenance protein complex (MCM) to prevent cytokinesis in AD neurons [64]. Interestingly, mitotically active neuronal cells also trigger tau phosphorylation with the help of CDKs, which in turn stimulate the NFT formation. In a study of human tau expressing *Drosophila* model, Tau phosphorylation was carried out by Checkpoint kinase 1 & 2 (chk1/2) at Ser 262, which is an AD-related site [65]. In addition, over-expressed chk2 mediated Ser 262 phosphorylation resulted in increased tau-led neurodegeneration [66]. Likewise, APP is also notably upregulated in response to mitogenic stimuli by cell cycle markers. The APP-BP1 adaptor protein, which plays an important role in APP cleavage, is a cell cycle protein involved in S-M phase transition. Augmented APP-BP1 level drives S phase mediated DNA replication in neurons accompanied by expression of cyclin B1 and cdc2 [67]. Similarly, in-vitro studies revealed that Presenilin 1 & 2 (PS1/PS2) overexpression arrested the cycle in G1 phase while its knockdown promoted G1/S transition [40]. Further, transgenic mice studies with human APP mutant model (K670M/N671L), also corroborated the findings of upregulated G1/S cell cycle

Table 1
Consequences of altered cell signaling in the post-mitotic cell division.

Principle phenotype	Signaling cascades	Check points	Cyclins expression	CDK/CDC/CDKI/TFS involved	References
AD	MEK-ERK MAP pathway/p38 MAPK	G0 to G1 and G1/S phase	Cyclin D1 and Cyclin E1	Cdk4 and Ki67 (MKI67)	[46]
	Notch signaling and Akt/GSK3β pathway	G0 to G1 and G1/S phase	CyclinD1	Cdk4/Cdk6 and Rb-E2F1	[39]
	β-catenin/TCF transcription complex (Wnt signaling)	G1/S phase	Cyclin D1	Cdk4/Cdk6 and Rb-E2F1	[40]
	Not defined	G1/S transition and G2/M phase	Cyclin B, Cyclin C, cyclin D1 and Cyclin E	Cdk1, Cdk2, Cdk4/Cdk6, Cdk5, Cdk7, Cdk11, p15, p16, p18, p19, p21, p27, pRb/p107, Ki67, mpm-2, E2F-1, PCNA, MORF4-related transcription factor and p53	[47–52]
PD	Not defined	G1 to S phase	Cyclin D and Cyclin E	Cdk2, Cdk4, Rb-E2F1	[53]
	MEK-ERK MAP pathway/p38 MAPK	G0/G1 phase	Cyclin D3	Cdk5, Cdk6/pRb, p21, p25 and p35/p39, Prosurvival transcription factor MEF2D, E2F-1 and p53	[54–57]
HD	Not defined	G0/G1 phase and G1/S phase	Cyclin A2, Cyclin D1 and Cyclin E	Cdk2, Cdk4, Cdk5 and p25, E2F-1	[58–61]
ALS	Apoptotic signaling	G1 to S phase	Cyclin D1 and Cyclin F	Cdk4, Cdk5, Cdk6, p25, p35/p39 and pRb/p107, E2F-1	[62]
IBM/PM	Apoptotic signaling and Akt signaling	G1/S phase	Cyclins E and Cyclin D1	Cdk2, Cdk4, Cdk6, Ki-67, PCNA and p21	[22]

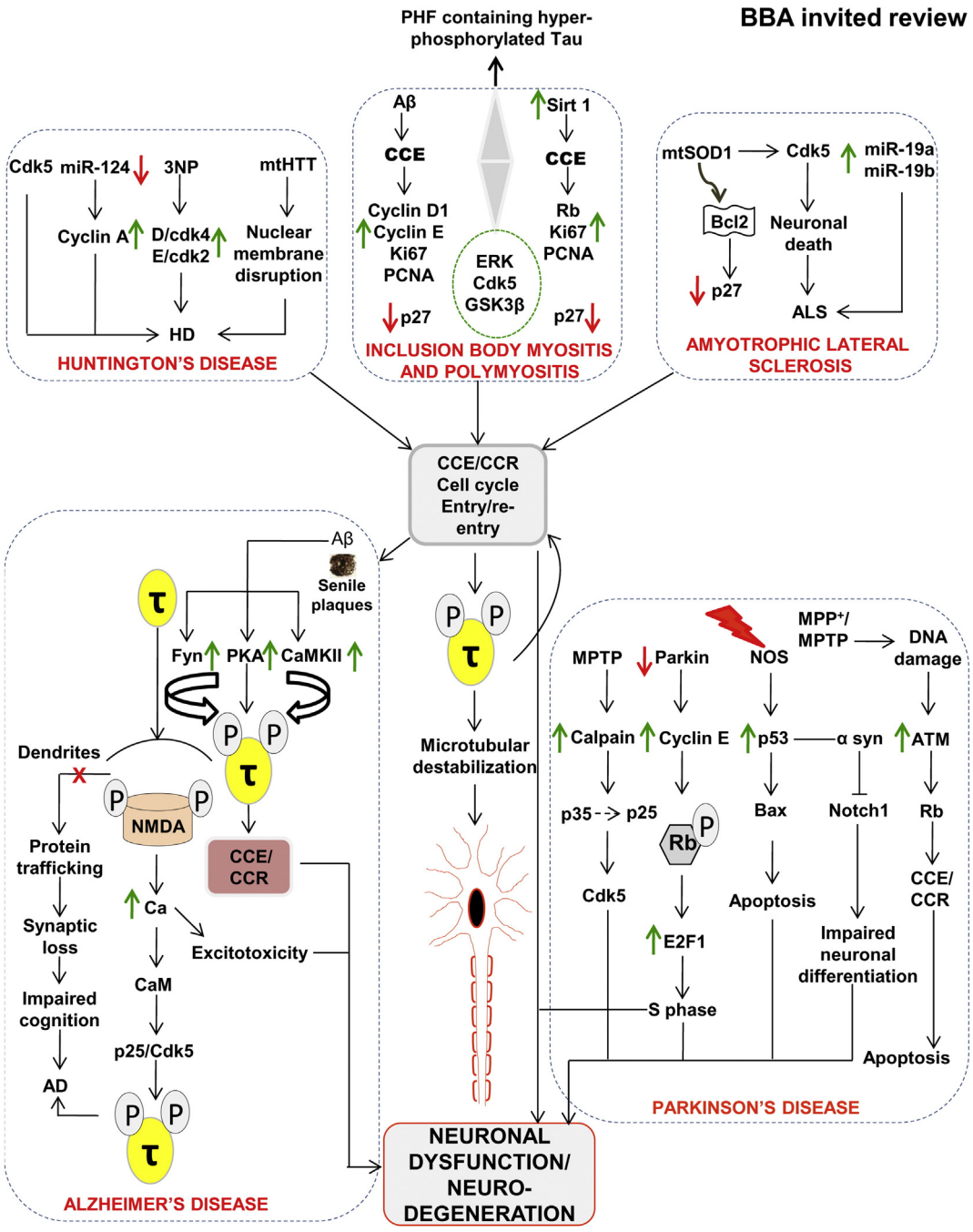


Fig. 3. Cell cycle re-entry mechanisms in neuromuscular disorders. The pathogenesis of HD is mediated by altered activity of Cdk5, micro RNA-124 and mutant HTT, which triggers the activation of cyclins, disruption of nuclear membrane and thus subsequent CCR. While Neurotoxin (3NP) induced cyclin re-expression is also responsible for CCR in HD. Similarly, Aβ and Sirt-1induced re-activation of Cyclin D1, Cyclin E, Ki67, PCNA, Rb and reduced p27 level is responsible for CCR in case of IBM/PM. While in case of ALS, mutant SOD1 contributes towards CCR via induction of Cdk5 and reduction of p27. Similarly, microRNA-19 modulates the expression of CCR critical genes and thereby contributes towards CCR in ALS. Likewise, in case of AD, Aβ mediated Tau hyperphosphorylation triggers CCR and causes Cdk5 activation via NMDA induced Calmodulin activation. Further, MPTP activity that induced various factors such as Calpain and ATM, which triggers the cell cycle markers and contributes towards CCR mediated pathogenesis of PD. Additionally, Parkin and NOS also altered the activity of Cyclin E, p53 and α syn that triggers the apoptosis as well as impaired neuronal differentiation in PD progression via CCR. PHF - Paired helical filaments; Cdk - Cyclin dependent Kinase; miR - Micro RNA; 3NP - 3-Nitropropionic acid; mtHTT - Mutant huntingtin; Sirt1 - Sirtuin 1; PCNA - Proliferating cell nuclear antigen; mtSOD1 - Mutant Superoxide dismutase1; Bcl2 - B-cell lymphoma 2; PKA - Protein kinase A; CaMKII - Calmodulin-dependent protein kinase II; NMDA-N-methyl-D-aspartate receptor; CaM-Calmodulin; MPTP-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NOS - Nitric oxide synthase; MPP^+-1-methyl-4-phenylpyridinium; ATM - ataxia-telangiectasia mutated; Bax - Bcl-2-associated X protein; Rb - Retinoblastoma.

proteins like cyclin D1 and cyclinA in frontal cortical layers [68]. The cyclins re-expression intervened mitogenic signaling is thought to be induced by advanced glycation end products (AGEs) released from activated microglia and astroglia upon Aβ trigger. Further, in an apolipoprotein E-deficient mice model, AGEs deposition triggered cyclin D1 expression and consequent DNA replication through RAGE-ERK1/2 MAP Kinase Signaling Pathway [69]. Further, reports suggest that Aβ induced cell cycle re-entry in neurons can only be driven in the presence of tau phosphorylation carried out by non-receptor tyrosine kinase- fyn, cAMP-regulated protein kinase A and calcium-calmodulin kinase II at Y18, S409 and S416 sites respectively [70]. Moreover, it is also notable that Aβ is itself mitogenic in-vitro, thereby controlling the cell cycle events in AD. The evidence for Aβ induced cell cycle re-entry in post-mitotic neurons was marked by the re-expression of cyclin D1, PCNA

and BrdU incorporation, evidence of new DNA synthesis. Moreover, Aβ exerted a dose dependent effect on the expression of various cyclins. For instance, low Aβ triggered cyclin D1 expression, while high Aβ induced cyclin B1 [63]. Further, cell cycle reactivation is evidence of non-homologous end joining (NHEJ) events in differentiated neurons. Recent studies demonstrated the elevated expression of cyclinD1, phosphorylated retinoblastoma protein (pRb), global cell cycle regulators such as Ki-67 and Mcm2 in relation with NHEJ response [24]. Cell cycle in AD is driven through G1/S phase, which is evident, by the increased level of cyclin E in different regions of the hippocampus in AD brains in comparison with age-matched controls. Moreover, upregulated cyclin E activity matched well with neuritic plaque burden in the neocortex region [46]. The affected neurons in AD also exhibit a crucial role of Cdk5 in cell cycle regulation. It has been demonstrated that nuclear Cdk5 plays a pivotal role in keeping neurons in the post-mitotic state as they mature while loss of nuclear Cdk5 leads to cell cycle entry [71]. Thus, increasing evidences confirm that Aβ and tau have a temporal relationship with the cell cycle re-entry and consequent neurodegeneration.

4.2. Parkinson's disease

The signature, toxic accumulation of alpha-synuclein (α-syn) protein, characterizes the pathology of Parkinson's disease. The phenomenon of cell cycle re-entry has also been observed in PD. For instance, expression of E2F-1, pRb and proliferating cell nuclear antigen (PCNA), a marker of dividing cells has been evident in around 3–6% of substantia nigra pars compacta (SNpc) neurons in PD brains with respect to controls [55]. Moreover, neurons also showed aneuploidy of selected chromosomes signifying the involvement of DNA replication machinery. Further, the markers of G1/S phase; cyclin D and cyclin E were upregulated upon MPP^+ exposure in neuronal culture, suggesting the activation of Cdk4/6 and Cdk2 respectively. Moreover, experimental results suggest that Cdk2/cyclin E complex is more vulnerable than Cdk4/cyclin D or Cdk6/cyclin D complex [72]. Another study reported increased level of cyclin D in SNpc subsequent to rotenone administration in rats. Additionally, elevated levels of DNA polymerase β induced endoreplication were also found to be involved in the pathogenesis of PD [73]. Furthermore, microarray expression analysis revealed the association between cyclin-G-associated kinase (GAK) polymorphism rs1564282 and α-synuclein in post-mortem frontal cortex of PD brain [74]. The non-classical cell cycle protein Cdk5 possibly caused phosphorylation of Parkin, thereby reducing its E3 ligase activity and preventing the ubiquitination of toxic substrates. Moreover, several studies reported that Cdk5 also mediated MPTP induced neuronal cell death. Accordingly, MPTP induced activation of calpains, which triggered cleavage of p35 into p25 thereby inducing over-activation of Cdk5, which, in turn resulted in neuronal apoptosis. Furthermore, Cdk5 mediated higher levels of phosphorylated Peroxiredoxin-2 (Prx2), a neuronal antioxidant is observed in SNpc neurons in PD indicating its involvement in neuronal death [55]. This evidences suggested a potential connection between Cdk5 and cell cycle re-entry in PD. In addition, MPP induced ROS generation also triggered pRb dependent cell cycle reentry and subsequent ATM mediated DNA damage in animal models as well as in PD patients [75]. Thus, experimental studies strongly advocate the causal role of CCR in Parkinson's disease pathogenesis.

4.3. Inclusion body myositis and polymyositis

IBM is the most common, progressive and debilitating, neuro-inflammatory muscle fiber disease among the elderly that is primarily characterized by intra-muscular fiber aggregation of congophilic, misfolded/toxic multi-proteins and vacuolization [76]. Most importantly, IBM shows similar pathogenic manifestations as AD such as Aβ deposits, paired helical filaments containing hyperphosphorylated tau, aggregates of Aβ precursor protein (AβPP) and presenilin1 [77]. Similarly, the common morbid feature between IBM and PD has also been noticed including, enrichment of α-synuclein, Parkin and oxidized DJ-1 in muscle fibers. Such sharing of pathological features between AD, PD and IBM clearly points towards a common underlying switch to neuro-muscular degeneration marked by alteration of these proteins. However, PM has been much debated as to co-exist with IBM than as an independent phenomenon. PM is regarded as a T-cell mediated autoimmune disease typically manifesting as a painless, proximal myopathy [34]. The actual cause of occurrence and series of events leading to IBM/PM has not been fully understood as is the case with NDDs, though multiple factors, including toxic protein burden, UPS failure, aging and oxidative stress remained the common triggering mechanisms. Recently, Aβ induced aberrant CCR has been shown to be involved in the pathogenesis of IBM and PM. The markers of G1/S transition and proliferation, including cyclin D1, E, and Ki67 were also found to be up-regulated in both IBM and PM samples along with a parallel decrease in the level of cell cycle inhibitor p21. Further, cyclin D1 and E were shown to interact with HSP70 whose level was significantly increased in IBM and PM where heat shock proteins might be playing a role in stabilizing the cyclins [22]. Moreover, the level of various tau-phosphorylating cell cycle kinases including ERK, Cdk5 and GSK3β have been found to co-localize with inclusions containing hyper-phosphorylated tau in degenerating muscle fibers in IBM [78]. Further, co-localized ERK in tau containing paired helical filaments (PHFs) strongly suggested its role in directing tau hyper-phosphorylation led pathogenesis in IBM muscle fibers. Additionally, ERK activation was also proposed to contribute in the Activator protein 1 (AP-1) transcription factor mediated phosphorylation of AβPP gene thereby altering Aβ synthesis and processing. Furthermore, post-synaptic activation of ERK at neuromuscular junctions in normal muscles and at non-junctional regions in IBM muscle fibers provided evidence for its role in mediating junctional protein dynamics in health and disease respectively [79]. More evidence of cell cycle activation in IBM/PM has been provided by studies involving the action of NAD^+-dependent histone deacetylase (HDAC), Sirtuin 1 (Sirt1). Sirt1 was shown to promote cell cycle progression in adult skeletal muscle stem cells; muscle precursor cells (MPC) and are involved in the growth, repair and regeneration capability of adult skeletal muscle fibers. The over-expression of Sirt1, induced MPC proliferation through cell cycle activation in G1/S phase as evidenced by Rb phosphorylation and decreased expression of cell cycle inhibitor p21 & p27. Further, Sirt 1 induced cell cycle activation progressively into S phase and directed new DNA synthesis as indicated by the elevated level of PCNA and BrdU [80]. On the contrary, the deacetylase activity of Sirt 1 is neuroprotective and its increased level has been shown to rescue neurons against Aβ induced toxicity through NF-κB inhibition or by inducing α-secretase in various models of tauopathies including AD and ALS [81]. However, the deacetylase activity and protein level of Sirt1 in the nucleus was found to be reduced in human sporadic IBM muscle fibers. Further, Sirt1 proteins are evident for forming cytoplasmic aggregates in response to ER stress. Moreover, increased AβPP and Aβ accumulation in IBM muscle fibers along with NF-κB activation is attributed to decreased Sirt 1 activity. Besides, up-regulation of NF-κB was also reported in PM [82]. Taken together, muscular degeneration in IBM/PM seems to be a manifestation of aberrant CCR mediated cell death through DNA synthesis, as is the case with AD, PD and Poly Q diseases.

4.4. Other neurodegenerative disorders

Numerous studies on post-mortem brain revealed the pathological evidence of cell cycle re-entry in various other neurodegenerative disorders such as epilepsy, ALS, traumatic brain injury (TBI) and cerebral hypoxia-ischemia. The cell cycle re-entry was marked by the formation of cyclin D/Cdk4 complexes (G0/G1 transition) in these NDDs. Recent studies pointed towards oxidative stress as the main mechanism

responsible for CCR. Experimental evidence suggests that oxidative stress mediated DNA damage activate ATM, which in turn activate the expression of E2F1 and consequent neuronal apoptosis. Furthermore, E2F-1 induced apoptosis may be p53-dependent or independent where multiple mitochondrial pathways might be activated including caspase dependent and independent signaling cascades. Similar evidence is observed in the western blot analysis of post-mortem brain samples in Huntington's patients [58]. Further, mHTT-perinuclear inclusions mediated cell cycle re-entry has been observed in primary cortical neurons of transgenic R6/2-J2 HD mice [83]. Another study reported an alteration in the ATF6α/Rheb pathway in HD patients triggering CCR [84]. Another finding identified the role of p25/Cdk5 in dopamine and glutamate neurotoxicity linked with HD [44]. Further, erroneous cell cycle entry and neuronal death have been demonstrated by kainic-acid induction through Notch dependent mechanism in-vivo. Additionally, they identified increased bioavailability of Cyclin D1 through Akt/GSK3β pathway and subsequent CCR mediated neurodegeneration via CyclinD1-Rb-E2F1 axis [39]. Recently, mutant cyclin F, a component of an E3 ubiquitin-protein ligase complex in neuronal cells has been detected through whole exome sequencing of ALS (Amyotrophic lateral sclerosis) and FTD (Frontotemporal dementia) patients. These mutants caused neuronal degeneration by altering ubiquitination process and precipitating ubiquitinated proteins, including TDP-43 [85]. Previous reports on ALS also suggested the involvement of mutant SOD1 in Cdk5 mediated deregulation at G1-S checkpoint in neuronal death [86]. Since there is little evidence of cell cycle re-entry in other neurodegenerative disorders, further researches are required to demonstrate the involvement of cell cycle re-entry in traumatic brain injury (TBI), epilepsy and other neurodegenerative disorders of the CNS.

5. Post-mitotic cell division: imperative or impulsive?

Earlier neurons were believed to be 'truly' post-mitotic and thus being life-long arrested in the quiescent stage G_0. Paradoxically, mounting evidence suggested the exit of resting neurons, which actively participated in the untimely cell cycle re-entry upon receiving mitogenic stimuli, synthesized DNA and were subsequently crucified [64]. One of the possible explanations to the contradictory premise of 'permanent' post-mitotic neuronal state is to consider that neurons had to constantly suppress the urge of cell cycle reactivation in lieu of their healthy state or face the reverberation of death. For instance, Rb protein is one such cell cycle marker, which serves crucial function of life-long cell cycle suppression in adult neurons [87]. Another candidate ataxia-telangiectasia mutated (ATM) protein triggered checkpoints during the DNA damage response under physiological conditions and maintained neurons in quiescent stage in adult CNS [88]. Moreover, deregulation of ATM signaling is observed in at-risk neurons in AD brains.

Likewise, Cdk5, a non-classical Cdk is found to ensure post-mitotic state of neurons by sequestering transcription factor E2F1 and thereby preventing its interaction with the activation subunit. While in response to the encountered neuronal stress, Cdk5 is translocated to the cytoplasm, thus relieving its cell cycle inhibition and inducing cell cycle re-entry. Further ATM, breakdown of Cdk5 also contributed to the pathogenesis of AD [71]. Similarly, the ubiquitin E3 ligase APC/Cdh1 also acted as the suppressor of a cell cycle in the adult brain [89].

However, increasing evidence showed the presence of different cell cycle markers in adult neurons, which was attributed to their requirement in neuronal maturation, migration and synaptic plasticity. Evidences also suggest that cell cycle activation is a crucial event for non-homologous end joining (NHEJ) repair for the double stranded breaks incorporated by excessive ROS under chronic inflammation. It has also been noticed that post-mitotic neurons undergoing DNA repair did not enter S phase, but remain in G1 phase. While unconquerable DNA damage proceed towards G1/S transition evidenced by increased BrdU, CDK2 and cyclin E expression, subsequently leading to apoptosis. For instance, Rb protein served a key post-mitotic role in neuronal migration in conjunction with transcription factor E2F3 [90]. Further, ATM regulated synaptic functions, including Long-Term Potentiation and was involved in trafficking in adult neurons [89]. Another marker, cell-division cycle protein-20 (cdc20) subunit of ubiquitin E3 ligase APC/C induced NeuroD2 degradation found to promote presynaptic axonal differentiation. Further, cdc20 mediated CaMKIIβ driven retraction and pruning of dendrites. Likewise, Cdh1 modulated growth and patterning in axons, and its inhibition favored axonal growth. Furthermore, p27 has been proposed to be a microtubule binding protein owing to its binding and disruption of Cofilin-actin complex upon Cdk5 phosphorylation, thereby promoting neuronal migration [91]. Similarly, p57 mediated cell cycle exit and promoted differentiation and maturation in post-mitotic neurons. Thus, the cell cycle machinery is 'repurposed' to serve important demands of the post-mitotic cells. Yet, such re-expression of cell cycle proteins under various stressors such as oxidative stress, DNA damage and neurotrophic factor deprivation forced mature neurons to re-enter the cell cycle and die at G_1/S transition before actual mitosis could begin. The DNA content and consequent cell death in at-risk neurons in AD have been proposed to corroborate with advancing disease stage. These neurons had diploid DNA content in early AD which shifted to hyperploid DNA in advanced stages of the disease. Additionally, the tetraploid neurons were positive for cyclin B1 which was indicative of re-entry into cell cycle. However, since neurons are mitotically incompetent, only about 2% of these completed DNA synthesis and had double DNA content. Moreover, majority of the tetraploid neurons remained arrested in S phase and at G2/M transition without completing DNA synthesis [92]. Further, these hyperploid neurons represented attenuated viability, breakdown of differentiation control and thus were selectively sacrificed during the progression of the disease [93,94]. Thus, aneuploidy marked an early signature of neuronal death in AD [95]. On the other hand, though a fraction of healthy neurons had more than double DNA content, these lacked the expression of cyclin B1 thus delineating their 'non-cycling' status. The tetraploid neurons in normal brains can be attributed to chromosomal missegregation during mitosis. Further, the constitutive expression of cyclin B1 in normal adult brains can be similarly attributed to extra-cell cycle roles pertaining to microtubule dynamics, neuronal plasticity, differentiation and tau phosphorylation [92]. Since, during normal development, tetraploid neurons are arrested at G_2/M and any attempt to go beyond this checkpoint induces apoptosis [96]. Therefore, it is not surprising that the affected neurons in AD completed S phase, arrested the cell cycle at G_2/M transition phase and remained in the tetraploid state for some years. These neurons never entered mitotic phase [40]. The evidence of failed mitosis was further agreed by shunted chromatin condensation and spindle formation. The role of various cell cycle markers including the evidence of DNA synthesis in CCR mediated NMD is outlined in Table 2. To sum up, erroneous cell cycle re-entry offers two choices to neurons; either they can divide and die in S phase or remain alive with double DNA content at G_2/M transition. Since neurons inherently lack mitotic competence, and no evidence of M phase entry has been reported in NDD, cell cycle re-entry in terminally, differentiated neurons drive them to death after successfully synthesizing new DNA. Thus, cell cycle re-entry into adult neurons constituted an early signature of neuromuscular degeneration and CNS injury.

6. Therapeutic strategies for targeting cell cycle re-entry in neuromuscular degeneration

The ectopic cell cycle re-entry causes abnormal cyclins and CDKs expression and alters the activity of various CDK inhibitors thereby contributing towards cellular imbalances. To target or reverse the chronic effects associated with cell cycle aberrancy in neuromuscular degeneration is a tedious task for neurobiologists. Till date, multiple targets and signaling pathways of the altered cell cycle have been reported that need to be addressed. In order to overcome such dyshomeostatis, numerous therapeutic strategies are currently being

Table 2
A consolidated list of various cell cycle markers that actively participate in post-mitotic neurons and related disorders as listed (shaded in gray); (−) indicates no reported action of that particular cell cycle markers. References: AD: [22,97–100]; PD: [56,73,74,98,101–103]; HD: [58–61]; ALS: [85,104,105]; IBM/PM: [22].

Cell cycle markers	AD	PD	HD	ALS	IBM/PM
Ki67		–	–	–	
BrdU		–	–		
PCNA			–	–	
Mcm2		–	–	–	–
Cyclin A	–				
Cyclin B	–		–	–	–
Cyclin C		–	–		–
Cyclin D					
Cyclin E					
Cyclin G	–		–		–
Cyclin F	–	–	–		–

AD - Alzheimer's disease; PD - Parkinson's disease; HD - Huntington's disease; ALS - Amyotrophic lateral sclerosis; IBM - inclusion body myositis; PM - Polymyositis.

devised that slow down or attenuate the toxicity associated with the abnormal cell cycle re-entries in various neuromuscular disorders. One such strategy involves the implication of various CDK-inhibiting compounds for targeting NMDs. However, these days several compounds/biomolecules that possess anti-apoptotic, anti-proliferative and antioxidant properties have been shown to target various abnormally expressed CDKs and cyclins in NMDs. For instance, the protective role of Flavopiridol has been reported in neurodegenerative disorder like AD, PD and ALS. Flavopiridol is a semi-synthetic flavonoid that found to inhibit all CDKs and reduces cyclin D1 mRNA transcription by arresting G1 and G2/M phase transition of the cell cycle [106]. Similarly, Simvastatin (SIM) has been found to inhibit the serum-mediated enhancement of cell proliferation in AD by blocking the events critical for G1/S transition. Further, it also induces a partial blockade of retinoblastoma (Rb) protein phosphorylation and is also responsible for inhibition of cyclin E/Cdk2 activity associated with increased levels of the CDK inhibitors p21(Cip1) and p27(kip1) [107]. Likewise, the protective role of Interleukin-1β (IL-1β) has also been identified in neurodegeneration through cell cycle arrest mechanism. It induced cell cycle arrest and apoptosis in neural precursor cells (NPCs) through an oxidative stress-dependent mechanism that resulted in p53-mediated induction of the CDK inhibitor p21 and the proapoptotic Bcl-2 family members Puma (p53-upregulated modulator of apoptosis) and Noxa [108]. Moreover, there are numerous other compounds identified so far that have been implicated for targeting aberrant CCR. The mechanism associated with therapeutic action of such compounds at different cell cycle check points is outlined in Table 3. Most of these biological compounds prevent the neurons and muscles from S phase entry and thereby provide protection against subsequent NMDs. Furthermore, these compounds have defined a beginning point for driving forward screening of potential therapeutic agents with desired pharmacological activity.

6.1. Emerging therapies for preventing cell cycle re-entry

Although, the important role of various bio-chemical compounds has been explored extensively to target altered cell cycle re-entry in neuromuscular disorders, yet, several new therapeutic strategies have been recently identified to target such alterations. Various studies have pointed towards the employment of UPS in the correction of CCR induced NMDs. The UPS plays a pivotal role in modulating cell cycle checkpoints, CDKs, transcription factors and other molecules critical for CCR. For instance, when a neuronal cell enters S phase, Cdk5 is transported to the cytoplasm, where it is ubiquitinated and rapidly degraded by the action of ubiquitin E3 ligase APC-Cdh1 complex. Further, APC-Cdh1 is responsible for degrading cyclin B1 where its accumulation was observed in the degenerating brain areas of AD patients [114]. Currently, p10, the N-terminal domain of p35, has also been shown the protective role against Cdk5/p25-induced toxicity in neurons [115]. Since, altered role of Cdk5 has been identified in disease pathology of NDDs. Further, it has been observed that raised expression of Skp1 protein corrects the malfunction of the E3 ligase SCF complex.

Table 3
Different drugs and biomolecules to attenuate the cell cycle progression.

S·no.	Compounds	Involved check points	Involved mechanisms	Diseases	References
1	Roscovitine	G1 and G2/M phases of cell cycle	Well-known purine analog, responsible for suppressing the activation of both Cdk2 and Cdk5. Further, it also inhibits the activity of ERK1 and ERK2 signaling cascade at its higher concentration.	AD, PD and ALS	[106]
2	Olomoucine	G1 and G2/M phases of cell cycle	Well-known purine analog which modulates the activity of Cdk2 and Cdk5 by competitively binding the ATP-binding site. Moreover, neuronal cell death induced by p27 reduction is also inhibited by olomoucine administration.	AD, PD and ALS	[106]
3	Resveratrol (RSV)	G_0/G_1 phase of cell cycle	It potentiates SIRT1 activity and induces an indirect inhibition of p53 that further inhibit the GSK3β and p53 interaction. Since, GSK3β overactivity leads to increased levels of plaques and tangles and p53 activity induces tau phosphorylation. Further, RSV also protects neuronal cells by toxic effects of mHtt, potentiating SIRT1 activity and inducing an indirect inhibition of p53.	AD and HD	[109]
4	Ibuprofen	S phase of cell cycle	Non-steroidal anti-inflammatory drug Ibuprofen has been shown to abrogate the cell cycle arrest at S phase.	AD and PD	[110,111]
5	Naproxen	G0/G1 phase of cell cycle	It blocks alterations in brain microglia as well as neuronal cell cycle events (CCEs) in the absence of detectable alterations in AβPP processing and Aβ metabolism.	AD	[110]
6	Apigenin	G2/M phase of cell cycle	It induces cell cycle arrest preferentially in the G2/M phase.	AD and PD	[111]
7	Epigallocatechin-gallate (EGCG)	G0-G1 phase of cell cycle	It is a natural product that has been shown to abrogate the cell cycle at the G0 to G1 phase in various tissues and is known as a cell cycle inhibitor.	AD	[46]
8	Tamoxifen	G0-G1 phase of cell cycle	It is a well-tolerated anti-proliferative cancer drug that has been reported to abrogate the cell cycle progression from G0 to G1 in various tissues.	AD	[46]
9	Butyrolactone I	G1-S phase of cell cycle	A Cdk5 inhibitor, provides protection against Aβ toxicity in SEK1-AL-expressing cells, advocating that Cdk5 and JNK activation independently contributed to this toxicity.	AD	[112]
10	Arimoclomol	Not defined	It upregulates the HSP70 expression therefore it might be used as a potential molecule to treat IBM. Since, the interaction between HSP70 and cyclins E/D1 has crucial importance during cell cycle progression in the inflammatory myopathies, probably controlling cyclin maturation or degradation	IBM	[113]

Since, malfunction of the E3 ligase SCF complex subsequently results in dysregulation of cell cycle regulators of the G_1/S phase transition like cyclins, CDKs, and cell cycle inhibitors thereby leading to neurodegeneration [116]. Likewise, SCF complex controls the G1/S and G2/M transitions by maintaining the level of β-catenin, p21, p27 and cyclin E in HD. Another Ubiquitin E3 ligase, Parkin protected neurons against cyclin E accumulation by associating with a protein complex, including hSel-10 and Cullin-1 [117]. Importantly, molecular chaperones in general preserve intracellular protein homeostasis in cooperation with the UPS. Therefore, HSPs are also being induced upon the cell cycle trigger in neurons and muscles that regulate the G1 phase. For instance, increased expression of chaperone HSP70 along with cyclins E/D1 during cell cycle progression has been reported in both IBM and PM muscle and in AD brain. Moreover, the interaction between HSP70 and cyclins E/D1 has crucial importance during cell cycle progression in the inflammatory myopathies, probably controlling cyclin maturation or degradation. Similarly, elevated levels of HSP90 in human IBM and PM biopsy specimens have been reported as, presumably in a stress-protective role [22]. Further, targeting cell cycle proteins could be another potential therapeutic approach for treating NMDs. For instance, inhibition of Cdk4 prevents both inductions of Bim expression and thrombin-induced neuronal apoptosis. Since, exposure of neuronal cultures to thrombin causes induction of cell cycle proteins cyclin E and cyclin D1, at both mRNA and protein levels thereby triggering cell cycle re-entry [118].

Interestingly, abnormal activation of the MEK-ERK MAP kinase pathway could be a potential modulator for cell cycle proteins. For instance, aberrant activation of this signaling axis regulates Cyclin D1 expression and forces neurons into the S phase, leading to cell cycle-related neuronal apoptosis (CRNA) [119]. On the contrary, Cyclin D1, in turn, prevents the activation of Cdk5 by its neuronal cyclin-like activator p35 and provides neuroprotection [120]. Similarly, ablating Notch signaling in neurons provides neuroprotection against kainic acid-induced neuronal death in AD. Since, kainic-acid treatment in vivo elicits erroneous CCR and neuronal death through a Notch-dependent mechanism, which further increases the bioavailability of CyclinD1 through Akt/GSK3β pathway, leading to aberrant CCR via activation of CyclinD1-Rb-E2F1 axis [39]. Furthermore, treatment with quercetin has been currently reported to act as a disruptor of the β-catenin/TCF transcription complex and consequently, reduced cyclin D1 levels and reversed the cell cycle/cell death phenotype in the PS1 Familial Alzheimer's disease (FAD) brain [40]. However, elevated expressions of β-catenin in the PS1 FAD brain accelerate CCR simply by increasing cyclin D1 transcription. Further, the induction of numerous cyclin-dependent kinase inhibitors, including, p16, p19, p21 and p27, has been reported as a protective molecule in AD. These inhibitors attenuate the phosphorylation activity of Cdk4 and Cdk6 thereby protecting neurons from apoptotic insults [121]. Currently, the regulatory role of Smad protein has been observed in AD brain via regulating the transcriptional activation of Cdk4. In the normal adult brain, Smad proteins are constitutively phosphorylated and predominantly localized in neuronal nuclei. Under neurodegenerative conditions such as AD, the subcellular localization of phosphorylated forms of Smad protein is heavily disturbed, lead to a loss of neuronal differentiation control and successive cell cycle re-entry [122]. Further, the involvement of histone deacetylases inhibitors such as suberoylanilidehydroxamic acid (SAHA) and sodium butyrate (NaB) and rapamycin, an inhibitor of mechanistic target of rapamycin (mTOR) has recently been reported to normalize the proliferation of PD lymphoblasts via preventing the over-expression of the cyclin D3/Cdk6/pRb signaling cascade. These drugs are having neuroprotective roles in both human neuroblastoma SH-SY5Y cells and primary rat mid-brain dopaminergic neuronal cultures toxicity induced by 6-hidroxydopamine [56]. Furthermore, humungous amount of researchers are still required in the near future to explore the potential and probable therapies against cell cycle aberrancy to alleviate the disease atmosphere of neuromuscular disorders.

7. Conclusion

Re-expression of cell cycle markers in aged neurons and muscles is identified as an intriguing process for aggravating neuromuscular disorders. Earlier its re-expression was confounded by their multiplicity of functions in a bonafide mitotic process, but later it is reported to be showing partial mitotic cell division and subsequent cell death. The mechanism behind the cell cycle re-entry includes the homeostatic imbalances among the levels of different cyclins and Cycin dependent kinases. The cell cycle markers play a pertinent role in marking the cell for mitotic division or for controlling other functions like synapse regulation and damage repair. The recent research has identified the disturbances in multiple signaling cascades controlling the process of cell proliferation and apoptosis. Such CCR mediated disturbed signaling includes Notch, Wnt/β-catenin, Akt/GSK3β, P^{38}/MAPK and dyotic signaling pathways in post-mitotic neurons and muscles. Moreover, numerous triggering factors forcing cell cycle re-entry has been identified, which includes, environmental factors like toxins, pesticides and metal based nanoparticles; oxidative stress, double-stranded DNA break (DSB), UPS dysfunctions, Micro RNAs, and deprivation of the brain derived neurotrophic factor (BDNF). Further, cell cycle aberrations have been widely studied in AD, while its investigation in other neurodegenerative disorders and muscular degenerative disorders like IBM and PM is still at preliminary stage. The upcoming research prospects are to device the potential methods for targeting the cyclin/CDKs dysregulation in neuromuscular degeneration. Currently, in order to target aberrant CCR or reverse the chronic effects of such abnormalities, numerous biomolecules and chaperones mediated therapeutic strategies have been adopted to obviate the disease symptoms. For instance, Flavopiridol, Roscovitine, and Olomoucineas CDKs inhibitor are used as a drug for the treatment of AD, PD, and ALS. Likewise, the potential protective role of numerous flavonoids such as Resveratrol, Apigenin and Epigallocatechin-gallate has also been observed for inducing cell cycle arrest at different checkpoints in AD, PD and HD. Moreover, numerous ubiquitin E3 ligases such as APC-Cdh1 complex has shown the protection against Cdk5/p25-induced toxicity in neurons via its proper ubiquitination and degradation. Similarly, HSP70 along with cyclins E/D1 during cell cycle progression in both IBM and PM muscle, AD brain has shown promising role in controlling cyclin maturation or degradation. Further, it is reasonable to proclaim that the current knowledge on the involvement of aberrant CCR in the pathogenesis of NMDs is still at an elementary stage. However, in light of its substantial progression from both clinical research and therapeutic application perspectives, we look forward to major research efforts being drawn in this field and more approaches being formulated in the near future.

Transparency document

The Transparency document associated with this article can be found, in online version

References

[1] D. Zheng, G. Zhu, S. Liao, W. Yi, G. Luo, J. He, Z. Pei, G. Li, Y. Zhou, Dysregulation of the PI3K/Akt signaling pathway affects cell cycle and apoptosis of side population cells in nasopharyngeal carcinoma, Oncol. Lett. 10 (2015) 182–188.
[2] A. Thakur, S.L. Siedlak, S.L. James, Retinoblastoma protein phosphorylation at multiple sites is associated with neurofibrillary pathology in Alzheimer disease, Int. J. Clin. Exp. Pathol. 1 (2008) 134–146.
[3] Y. Yang, E.J. Mufson, K. Herrup, Neuronal cell death is preceded by cell cycle events at all stages of Alzheimer's disease, J. Neurosci. 23 (2003) 2557–2563.
[4] S. Lim, P. Kaldis, Cdks, cyclins and CKIs: roles beyond cell cycle regulation, Development 140 (2013) 3079–3093.
[5] M. Hengstschläger, K. Braun, T. Soucek, A. Miloloza, E. Hengstschläger-Ottnad, Cyclin-dependent kinases at the G1-S transition of the mammalian cell cycle, Mutat. Res. 436 (1999) 1–9.
[6] C. Bertoli, J.M. Skotheim, R.A. de Bruin, Control of cell cycle transcription during G1 and S phases, Nat. Rev. Mol. Cell Biol. 14 (2013) 518–528.
[7] Z. Ullah, C.Y. Lee, M.L. Depamphilis, Cip/Kip cyclin-dependent protein kinase inhibitors and the road to polyploidy, Cell Div 4 (2009) 10.

[8] J. Lin, S. Jinno, H. Okayama, Cdk6-cyclin D3 complex evades inhibition by inhibitor proteins and uniquely controls cell's proliferation competence, Oncogene 20 (2001) 2000–2009.
[9] N.G. Starostina, E.T. Kipreos, Multiple degradation pathways regulate versatile CIP/KIP CDK inhibitors, Trends Cell Biol. 22 (2012) 33–41.
[10] D.A. Foster, P. Yellen, L. Xu, M. Saqcena, Regulation of G1 cell cycle progression: distinguishing the restriction point from a nutrient-sensing cell growth checkpoint(s), Genes Cancer 1 (2010) 1124–1131.
[11] A.M. Abreu Velez, M.S. Howard, Tumor-suppressor genes, cell cycle regulatory checkpoints, and the skin, N. Am. J. Med. Sci. 7 (2015) 176–188.
[12] T.A. Potapova, J.R. Daum, K.S. Byrd, G.J. Gorbsky, Fine tuning the cell cycle: activation of the Cdk1 inhibitory phosphorylation pathway during mitotic exit, Mol. Biol. Cell. 20 (2009) 1737–1748.
[13] R. Bose, N. Onishchenko, K. Edoff, A.M. Janson Lang, S. Ceccatelli, Inherited effects of low-dose exposure to methyl mercury in neural stem cells, Toxicol. Sci. 130 (2012) 383–390.
[14] L.H. Kerri, J. Damir, Cell Cycle, Neurological Disorders, and Reactive Gliosis, Springer, 2006 163–175 (Book Part III).
[15] I.D. Akinrinade, A.E. Memudu, O.M. Ogundele, Fluoride and aluminium disturb neuronal morphology, transport functions, cholinesterase, lysosomal and cell cycle activities, Pathophysiology 22 (2015) 105–115.
[16] E. Taniai, A. Yafune, M. Nakajima, S.M. Hayashi, F. Nakane, M. Itahashi, M. Shibutani, Ochratoxin A induces karyomegaly and cell cycle aberrations in renal tubular cells without relation to induction of oxidative stress responses in rats, Toxicol. Lett. 224 (2014) 64–72.
[17] J. Mizukami, G. Takaesu, H. Akatsuka, H. Sakurai, J. Ninomiya-Tsuji, K. Matsumoto, N. Sakurai, Receptor activator of NF-kappaB ligand (RANKL) activates TAK1 mitogen-activated protein kinase kinase kinase through a signaling complex containing RANK, TAB2, and TRAF6, Mol. Cell. Biol. 22 (2002) 992–1000.
[18] I.I. Kruman, Why do neurons enter the cell cycle, Cell Cycle 3 (2004) 769–773.
[19] L.J. Martin, DNA damage and repair: relevance to mechanisms of neurodegeneration, J. Neuropathol. Exp. Neurol. 67 (2008) 377–387.
[20] S. Fulda, A.M. Gorman, O. Hori, A. Samali, Cellular stress responses: cell survival and cell death, Int. J. Cell Biol. 214074 (2010).
[21] Y. Miyata, Hsp90 inhibitor geldanamycin and its derivatives as novel cancer chemotherapeutic agents, Curr. Pharm. Des. 11 (2005) 1131–1138.
[22] B. Kwon, P. Kumar, H.K. Lee, L. Zeng, K. Walsh, Q. Fu, A. Barakat, H.W. Querfurth, Aberrant cell cycle re-entry in human and experimental inclusion body myositis and polymyositis, Hum. Mol. Genet. 23 (2014) 3681–3694.
[23] J.A. Diehl, W. Yang, R.A. Rimerman, H. Xiao, A. Emili, Hsc70 regulates accumulation of cyclin D1 and cyclin D1-dependent protein kinase, Mol. Cell Biol. 23 (2003) 1764–1774.
[24] L.A. van Leeuwen, J.J.M. Hoozemans, Physiological and pathophysiological functions of cell cycle proteins in post-mitotic neurons: implications for Alzheimer's disease, Acta Neuropathol. 129 (2015) 511–525.
[25] P. Tokarz, K. Kaarniranta, J. Blasiak, Role of the cell cycle re-initiation in DNA damage response of post-mitotic cells and its implication in the pathogenesis of neurodegenerative diseases, Rejuvenation Res. 19 (2016) 131–139.
[26] M. Huang, Z.H. Miao, H. Zhu, Y.J. Cai, W. Lu, J. Ding, Chk1 and Chk2 are differentially involved in homologous recombination repair and cell cycle arrest in response to DNA double-strand breaks induced by camptothecins, Mol. Cancer Ther. 7 (2008) 1440–1449.
[27] J. Chen, M.L. Cohen, A.J. Lerner, Y. Yang, K. Herrup, DNA damage and cell cycle events implicate cerebellar dentate nucleus neurons as targets of Alzheimer's disease, Mol. Neurodegener. 20 (5) (2010) 60.
[28] P. Katsel, W. Tan, P. Fam, D.P. Purohit, V. Haroutunian, Cell cycle checkpoint abnormalities during dementia: a plausible association with the loss of protection against oxidative stress in Alzheimer's disease corrected, PLoS One 8 (2013), e68361.
[29] D. Rossetto, A.W. Truman, S.J. Kron, J. Côté, Epigenetic modifications in double-strand break DNA damage signaling and repair, Clin. Cancer Res. 16 (2010) 4543–4552.
[30] S. Absalon, D.M. Kochanek, V. Raghavan, A.M. Krichevsky, MiR-26b upregulated in Alzheimer's disease, activates cell cycle entry, tau-phosphorylation, and apoptosis in postmitotic neurons, J. Neurosci. 33 (2013) 14645–14659.
[31] T. Kimura, K. Ishiguro, S. Hisanaga, Physiological and pathological phosphorylation of tau by Cdk5, Front. Mol. Neurosci. 7 (2014) 65.
[32] W.J. Lukiw, B. Surjyadipta, P. Dua, P.N. Alexandrov, Common micro RNAs (miRNAs) target complement factor H (CFH) regulation in Alzheimer's disease (AD) and in age-related macular degeneration (AMD), Int. J. Biochem. Mol. Biol. 3 (2012) 105–116.
[33] P.K. Modi, S. Jaiswal, P. Sharma, Regulation of neuronal cell cycle and apoptosis by microRNA, Mol. Cell Biol. 36 (2015) 84–94.
[34] Y.B. Luo, F.L. Mastaglia, Dermatomyositis, polymyositis and immune-mediated necrotising myopathies, Biochim. Biophys. Acta 1852 (2015) 622–632.
[35] N. Boutahar, E. Reynaud, F. Lassabliere, J. Borg, Brain-derived neurotrophic factor inhibits cell cycle re-entry but not endoplasmic reticulum stress in cultured neurons following oxidative or excitotoxic stress, J. Neurosci. Res. 88 (2010) 2263–2271.
[36] M.C. Ovejero-Benito, J.M. Frade, Brain-derived neurotrophic factor-dependent cdk1 inhibition prevents G2/M progression in differentiating tetraploid neurons, PLoS One 8 (2013), e64890.
[37] S.M. Morillo, E.P. Abanto, M.J. Román, J.M. Frade, Nerve growth factor-induced cell cycle re-entry in newborn neurons is triggered by p38MAPK-dependent E2F4 phosphorylation, Mol. Cell. Biol. 32 (2012) 2722–2737.
[38] E. Colombo, F. Bedogni, I. Lorenzetti, N. Landsberger, S.C. Previtali, C. Farina, Autocrine and immune cell-derived BDNF in human skeletal muscle: implications for myogenesis and tissue regeneration, J. Pathol. 231 (2013) 190–198.
[39] S. Marathe, S. Liu, E. Brai, M. Kaczarowski, L. Alberi, Notch signaling in response to excitotoxicity induces neurodegeneration via erroneous cell cycle re-entry, Cell Death Differ. 22 (2015) 1775–1784.
[40] A. Currais, T. Hortobágyi, S. Soriano, The neuronal cell cycle as a mechanism of pathogenesis in Alzheimer's disease, Aging (Albany NY) 1 (2009) 363–371.
[41] J. Zhu, W. Li, Z. Mao, Cdk5: mediator of neuronal development, death and the response to DNA damage, Mech. Ageing Dev. 132 (2011) 389–394.
[42] M.R. Kelley, M.M. Georgiadis, M.L. Fishel, APE1/Ref-1 role in redox signaling: translational applications of targeting the redox function of the DNA repair/redox protein APE1/Ref-1, Curr. Mol. Pharmacol. 5 (2012) 36–53.
[43] A. Mietelska-Porowska, U. Wasik, M. Goras, A. Filipek, G. Niewiadomska, Tau protein modifications and interactions: their role in function and dysfunction, Int. J. Mol. Sci. 15 (2014) 4671–4713.
[44] P. Paoletti, I. Vila, M. Rifé, J.M. Lizcano, J. Alberch, S. Ginés, Dopaminergic and glutamatergic signaling crosstalk in Huntington's disease neurodegeneration: the role of p25/cyclin-dependent kinase 5, J. Neurosci. 28 (2008) 10090–10101.
[45] Q. Liu, F. Xie, A. Alvarado-Diaz, M.A. Smith, P.I. Moreira, X. Zhu, G. Perry, Neurofilamentopathy in neurodegenerative diseases, Open Neurol. J. 5 (2011) 58–62.
[46] D.J. Bonda, H.P. Lee, W. Kudo, X. Zhu, M.A. Smith, H.G. Lee, Pathological implications of cell cycle re-entry in Alzheimer disease, Expert Rev. Mol. Med. 12 (2010), e19.
[47] T. Arendt, M. Holzer, U. Gärtner, Neuronal expression of cycline dependent kinase inhibitors of the INK4 family in Alzheimer's disease, J. Neural Transm. (Vienna) 105 (1998) 949–960.
[48] X. Zhou, J. Jia, P53-mediated G(1)/S checkpoint dysfunction in lymphocytes from Alzheimer's disease patients, Neurosci. Lett. 468 (2010) 320–325.
[49] Z. Nagy, The dysregulation of the cell cycle and the diagnosis of Alzheimer's disease, Biochim. Biophys. Acta 1772 (2007) 402–408.
[50] X. Zhu, A. McShea, P.L. Harris, A.K. Raina, R.J. Castellani, J.O. Funk, S. Shah, C. Bowen, R. Atwood, R. Bowser, L. Morelli, G. Perry, M.A. Smith, Elevated expression of a regulator of the G2/M phase of the cell cycle, neuronal CIP-1-associated regulator of cyclin B, in Alzheimer's disease, J. Neurosci. Res. 75 (2004) 698–703.
[51] K.L. Jordan-Sciutto, L.M. Malaiyandi, R. Bowser, Altered distribution of cell cycle transcriptional regulators during Alzheimer disease, J. Neuropathol. Exp. Neurol. 61 (2002) 358–367.
[52] X. Zhu, C.A. Rottkamp, A.K. Raina, G.J. Brewer, H.A. Ghanbari, H. Boux, M.A. Smith, Neuronal CDK7 in hippocampus is related to aging and Alzheimer disease, Neurobiol. Aging 21 (2000) 807–813.
[53] K.L. Jordan-Sciutto, R. Dorsey, E.M. Chalovich, R.R. Hammond, C.L. Achim, Expression patterns of retinoblastoma protein in Parkinson disease, J. Neuropathol. Exp. Neurol. 62 (2003) 68–74.
[54] K. Venderova, D.S. Park, Programmed cell death in Parkinson's disease, Cold Spring Harb. Perspect. Med. 2 (2012) a009365.
[55] O.A. Levy, C. Malagelada, L.A. Greene, Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps, Apoptosis 14 (2009) 478–500.
[56] C. Alquézar, E. Barrio, N. Esteras, A. de la Encarnación, F. Bartolomé, J.A. Molina, Á. Martín-Requero, Targeting cyclin D3/CDK6 activity for treatment of Parkinson's disease, J. Neurochem. 133 (2015) 886–897.
[57] A. Futatsugi, E. Utreras, P. Rudrabhatla, H. Jaffe, H.C. Pant, A.B. Kulkarni, Cyclin-dependent kinase 5 regulates E2F transcription factor through phosphorylation of Rb protein in neurons, Cell Cycle 11 (2012) 1603–1610.
[58] C. Pelegrí, J. Duran-Vilaregut, J. del Valle, N. Crespo-Biel, I. Ferrer, M. Pallàs, A. Camins, J. Vilaplana, Cell cycle activation in striatal neurons from Huntington's disease patients and rats treated with 3-nitropropionic acid, Int. J. Dev. Neurosci. 26 (2008) 665–671.
[59] M. Cherubini, M. Puigdellívol, J. Alberch, S. Ginés, Cdk5-mediated mitochondrial fission: a key player in dopaminergic toxicity in Huntington's disease, Biochim. Biophys. Acta 1852 (2015) 2145–2160.
[60] J. Chen, Z.F. Wang, Roles of cyclin-dependent kinase 5 in central nervous system development and neurodegenerative diseases, Sheng Li Xue Bao. 62 (2010) 295–308.
[61] E. Das, N.R. Jana, N.P. Bhattacharyya, MicroRNA-124 targets CCNA2 and regulates cell cycle in STHdh (Q111)/Hdh (Q111) cells, Biochem. Biophys. Res. Commun. 437 (2013) 217–224.
[62] S. Ranganathan, R. Bowser, Alterations in G (1) to S phase cell-cycle regulators during amyotrophic lateral sclerosis, Am. J. Pathol. 162 (2003) 823–835.
[63] S. Majd, A. Zarifkar, K. Rastegar, M.A. Takhshid, Different fibrillar Abeta 1-42 concentrations induce adult hippocampal neurons to reenter various phases of the cell cycle, Brain Res. 1218 (2008) 224–229.
[64] D.J. Bonda, T.A. Evans, C. Santocanale, J.C. Llosá, J. Viña, V. Bajic, R.J. Castellani, S.L. Siedlak, G. Perry, M.A. Smith, H.G. Lee, Evidence for the progression through S-phase in the ectopic cell cycle re-entry of neurons in Alzheimer disease, Aging (Albany NY) 1 (2009) 382–388.
[65] J. Mendoza, M. Sekiya, T. Taniguchi, K.M. Iijima, R. Wang, K. Ando, Global analysis of phosphorylation of tau by the checkpoint kinases Chk1 and Chk2 in vitro, J. Proteome Res. 12 (2013) 2654–2665.
[66] Z. Liu, T. Li, P. Li, N. Wei, Z. Zhao, H. Liang, X. Ji, W. Chen, M. Xue, J. Wei, The ambiguous relationship of oxidative stress, tau hyperphosphorylation, and autophagy dysfunction in Alzheimer's disease, Oxidative Med. Cell. Longev. 2015 (2015) 352723.
[67] Z. Liu, Y. Chen, R. Neve, H. Zheng, W. Griffin, S. Barger, R. Mrak, Cycle on wheels: is APP key to the AppBp1 pathway, Austin Alzheimers Park. Dis. 1 (2014) (pii: id1008).

[68] W. Ouyang, Q. Ma, J. Li, D. Zhang, Z.G. Liu, A.K. Rustgi, C. Huang, Cyclin D1 induction through IkappaB kinase beta/nuclear factor-kappaB pathway is responsible for arsenite-induced increased cell cycle G1-S phase transition in human keratinocytes, Cancer Res. 65 (2005) 9287–9293.
[69] K. Bhaskar, N. Maphis, G. Xu, N.H. Varvel, O.N. Kokiko-Cochran, J.P. Weick, S.M. Staugaitis, A. Cardona, R.M. Ransohoff, K. Herrup, B.T. Lamb, Microglial derived tumor necrosis factor-α drives Alzheimer's disease-related neuronal cell cycle events, Neurobiol. Dis. 62 (2014) 273–285.
[70] M.E. Seward, E. Swanson, A. Norambuena, A. Reimann, J.N. Cochran, R. Li, E.D. Roberson, G.S. Bloom, Amyloid-β signals through tau to drive ectopic neuronal cell cycle re-entry in Alzheimer's disease, J. Cell Sci. 126 (2013) 1278–1286.
[71] J. Zhang, S.A. Cicero, L. Wang, R.R. Romito-Digiacomo, Y. Yang, K. Herrup, Nuclear localization of Cdk5 is a key determinant in the postmitotic state of neurons, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 8772–8777.
[72] S. Krantic, N. Mechawar, S. Reix, R. Quirion, Molecular basis of programmed cell death involved in neurodegeneration, Trends Neurosci. 28 (2005) 670–676.
[73] H. Wang, Y. Chen, J. Chen, Z. Zhang, W. Lao, X. Li, J. Huang, T. Wang, Cell cycle regulation of DNA polymerase beta in rotenone-based Parkinson's disease models, PLoS One 9 (2014), e109697.
[74] A. Dumitriu, C.D. Pacheco, J.B. Wilk, K.E. Strathearn, J.C. Latourelle, S. Goldwurm, G. Pezzoli, J.C. Rochet, S. Lindquist, R.H. Myers, Cyclin-G-associated kinase modifies α-synuclein expression levels and toxicity in Parkinson's disease: results from the gene PD study, Hum. Mol. Genet. 20 (2011) 1478–1487.
[75] A. Camins, J.G. Pizarro, D. Alvira, J. Gutierrez-Cuesta, A.V. de la Torre, J. Folch, F.X. Sureda, E. Verdaguer, F. Junyent, J. Jorda'n, I. Ferrer, M. Palla's, Activation of ataxia telangiectasia muted under experimental models and human Parkinson's disease, Cell Mol. Life Sci. 67 (2010) 3865–3882.
[76] V. Askanas, W.K. Engel, A. Nogalska, Inclusion body myositis: a degenerative muscle disease associated with intra-muscle fiber multi-protein aggregates, proteasome inhibition, endoplasmic reticulum stress and decreased lysosomal degradation, Brain Pathol. 19 (2009) 493–506.
[77] V. Askanas, W.K. Engel, Does overexpression of beta APP in aging muscle have a pathogenic role and a relevance to Alzheimer's disease? Clues from inclusion body myositis, cultured human muscle, and transgenic mice, Am. J. Pathol. 153 (1998) 1673–1677.
[78] V. Askanas, W.K. Engel, A. Nogalska, Sporadic inclusion-body myositis: a degenerative muscle disease associated with aging, impaired muscle protein homeostasis and abnormal mitophagy, Biochim. Biophys. Acta 1852 (2015) 633–643.
[79] S. Pedrini, T.L. Carter, G. Prendergast, S. Petanceska, M.E. Ehrlich, S. Gandy, Modulation of statin-activated shedding of Alzheimer APP ectodomain by ROCK, PLoS Med. 2 (2005), e18.
[80] C.R. Rathbone, F.W. Booth, S.J. Lees, Sirt1 increases skeletal muscle precursor cell proliferation, Eur. J. Cell Biol. 88 (2009) 35–44.
[81] A.Z. Herskovits, L. Guarente, SIRT1 in neurodevelopment and brain senescence, Neuron 81 (2014) 471–483.
[82] A. Nogalska, C. D'Agostino, W.K. Engel, K.J. Davies, V. Askanas, Decreased SIRT1 deacetylase activity in sporadic inclusion-body myositis muscle fibers, Neurobiol. Aging 31 (2010) 1637–1648.
[83] K.Y. Liu, Y.C. Shyu, B.A. Barbaro, Y.T. Lin, Y. Chern, L.M. Thompson, C.K. James Shen, J.L. Marsh, Disruption of the nuclear membrane by perinuclear inclusions of mutant huntingtin causes cell-cycle re-entry and striatal cell death in mouse and cell models of Huntington's disease, Hum. Mol. Genet. 24 (2015) 1602–1616.
[84] M.R. Fernandez-Fernandez, I. Ferrer, J.J. Lucas, Impaired ATF6α processing, decreased Rheb and neuronal cell cycle re-entry in Huntington's disease, Neurobiol. Dis. 41 (2011) 23–32.
[85] K.L. Williams, S. Topp, S. Yang, B. Smith, J.A. Fifita, S.T. Warraich, K.Y. Zhang, N. Farrawell, C. Vance, X. Hu, A. Chesi, C.S. Leblond, A. Lee, S.L. Rayner, V. Sundaramoorthy, C. Dobson-Stone, M.P. Molloy, M. van Blitterswijk, D.W. Dickson, R.C. Petersen, N.R. Graff-Radford, B.F. Boeve, M.E. Murray, C. Pottier, E. Don, C. Winnick, E.P. McCann, A. Hogan, H. Daoud, A. Levert, P.A. Dion, J. Mitsui, H. Ishiura, Y. Takahashi, J. Goto, J. Kost, C. Gellera, A.S. Gkazi, J. Miller, J. Stockton, W.S. Brooks, K. Boundy, M. Polak, J.L. Muñoz-Blanco, J. Esteban-Pérez, A. Rábano, O. Hardiman, K.E. Morrison, N. Ticozzi, V. Silani, J. de Belleroche, J.D. Glass, J.B. Kwok, G.J. Guillemin, R.S. Chung, S. Tsuji, R.H. Brown Jr., A. García-Redondo, R. Rademakers, J.E. Landers, A.D. Gitler, G.A. Rouleau, N.J. Cole, J.J. Yerbury, J.D. Atkin, C.E. Shaw, G.A. Nicholson, I.P. Blair, CCNF mutations in amyotrophic lateral sclerosis and frontotemporal dementia, Nat. Commun. 7 (2016) 11253.
[86] M.D. Nguyen, M. Boudreau, J. Kriz, S. Couillard-Després, D.R. Kaplan, J.P. Julien, Cell cycle regulators in the neuronal death pathway of amyotrophic lateral sclerosis caused by mutant superoxide dismutase 1, J. Neurosci. 23 (2003) 2131–2140.
[87] K. Herrup, Y. Yang, Cell cycle regulation in the post mitotic neuron: oxymoron or new biology, Nat. Rev. Neurosci. 8 (2007) 368–378.
[88] W.C. Chou, L.Y. Hu, C.N. Hsiung, C.Y. Shen, Initiation of the ATM-Chk2 DNA damage response through the base excision repair pathway, Carcinogenesis 36 (2015) 832–840.
[89] J. Li, Y.R. Han, M.R. Plummer, K. Herrup, Cytoplasmic ATM in neurons modulates synaptic function, Curr. Biol. 19 (2009) 2091–2096.
[90] M.G. Andrusiak, K.A. McClellan, D. Dugal-Tessier, L.M. Julian, S.P. Rodrigues, D.S. Park, T.E. Kennedy, R.S. Slack, Rb/E2F regulates expression of neogenin during neuronal migration, Mol. Cell. Biol. 31 (2011) 238–247.
[91] T. Kawauchi, K. Chihama, Y. Nabeshima, M. Hoshino, Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration, Nat. Cell Biol. 8 (2006) 17–26.
[92] B. Mosch, M. Morawski, A. Mittag, D. Lenz, A. Tarnok, T. Arendt, Aneuploidy and DNA replication in the normal human brain and Alzheimer's disease, J. Neurosci. 27 (2007) 6859–6867.
[93] T. Arendt, M.K. Brückner, A. Lösch, Regional mosaic genomic heterogeneity in the elderly and in Alzheimer's disease as a correlate of neuronal vulnerability, Acta Neuropathol. 130 (2015) 501–510.
[94] T. Arendt, M.K. Brückner, B. Mosch, A. Lösche, Selective cell death of hyperploids neurons in Alzheimer's disease, Am. J. Pathol. 177 (2010) 15–20.
[95] T. Arendt, Cell cycle activation and aneuploid neurons in Alzheimer's disease, Mol. Neurobiol. 46 (2012) 125–135.
[96] J.M. Frade, M.C. Ovejero-Benito, Neuronal cell cycle: the neuron itself and its circumstances, Cell Cycle 14 (2015) 712–720.
[97] U. Ueberham, A. Hessel, T. Arendt, Cyclin C expression is involved in the pathogenesis of Alzheimer's disease, Neurobiol. Aging 24 (2003) 427–435.
[98] S.B. Wharton, G.H. Williams, K. Stoeber, C.H. Gelsthorpe, L. Baxter, A.L. Johnson, P.G. Ince, MRC-CFAS. Expression of Ki67, PCNA and the chromosome replication licensing protein Mcm2 in glial cells of the ageing human hippocampus increases with the burden of Alzheimer-type pathology, Neurosci. Lett. 383 (2005) 33–38.
[99] L.C. Chuang, L.K. Teixeira, J.A. Wohlschlegel, M. Henze, J.R. Yates, J. Méndez, S.I. Reed, Phosphorylation of Mcm2 by Cdc7 promotes pre-replication complex assembly during cell-cycle re-entry, Mol. Cell 35 (2009) 206–216.
[100] H.G. Lee, G. Casadesus, X. Zhu, R.J. Castellani, A. McShea, G. Perry, R.B. Petersen, V. Bajic, M.A. Smith, Cell cycle re-entry mediated neurodegeneration and its treatment role in the pathogenesis of Alzheimer's disease, Neurochem. Int. 54 (2009) 84–88.
[101] J.G. Pizarro, F. Junyent, E. Verdaguer, J. Jordan, C. Beas-Zarate, M. Pallàs, A. Camins, J. Folch, Effects of MPP+ on the molecular pathways involved in cell cycle control in B65 neuroblastoma cells, Pharmacol. Res. 61 (2010) 391–399.
[102] X.F. Fei, Z.H. Qin, B. Xiang, L.Y. Li, F. Han, K. Fukunaga, Z.Q. Liang, Olomoucine inhibits cathepsin L nuclear translocation, activates autophagy and attenuates toxicity of 6-hydroxydopamine, Brain Res. 1264 (2009) 85–97.
[103] S.S. Lee, Y.M. Kim, E. Junn, G. Lee, K.H. Park, M. Tanaka, R.D. Ronchetti, M.M. Quezado, M.M. Mouradian, Cell cycle aberrations by alpha-synuclein overexpression and cyclin B immunoreactivity in Lewy bodies, Neurobiol. Aging 24 (2003) 687–696.
[104] A. Appert-Collin, B. Hugel, R. Levy, N. Niederhoffer, G. Coupin, Y. Lombard, P. André, P. Poindron, J.P. Gies, Cyclin dependent kinase inhibitors prevent apoptosis of postmitotic mouse motoneurons, Life Sci. 79 (2006) 484–490.
[105] Y.M. Jiang, M. Yamamoto, Y. Kobayashi, T. Yoshihara, Y. Liang, S. Terao, H. Takeuchi, S. Ishigaki, M. Katsuno, H. Adachi, J. Niwa, F. Tanaka, M. Doyu, M. Yoshida, Y. Hashizume, G. Sobue, Gene expression profile of spinal motor neurons in sporadic amyotrophic lateral sclerosis, Ann. Neurol. 57 (2005) 236–251.
[106] J. Wu, B.A. Stoica, A.I. Faden, Cell cycle activation and spinal cord injury, Neurotherapeutics 8 (2011) 221–228.
[107] S.G. Sala, U. Muñoz, F. Bartolomé, F. Bermejo, A. Martín-Requero, HMG-CoA reductase inhibitor simvastatin inhibits cell cycle progression at the G1/S checkpoint in immortalized lymphocytes from Alzheimer's disease patients independently of cholesterol-lowering effects, J. Pharmacol. Exp. Ther. 324 (2008) 352–359.
[108] J. Guadagno, P. Swan, R. Shaikh, S.P. Cregan, Microglia-derived IL-1β triggers p53-mediated cell cycle arrest and apoptosis in neural precursor cells, Cell Death Dis. 6 (2015), e1779.
[109] E. Tellone, A. Galtieri, A. Russo, B. Giardina, S. Ficarra, Resveratrol: a focus on several neurodegenerative diseases, Oxidative Med. Cell. Longev. 2015 (2015) 392169.
[110] N.H. Varvel, K. Bhaskar, M.Z. Kounnas, S.L. Wagner, Y. Yang, B.T. Lamb, K. Herrup, NSAIDs prevent, but do not reverse, neuronal cell cycle re-entry in a mouse model of Alzheimer disease, J. Clin. Invest. 119 (2009) 3692–3702.
[111] N.S. Elsisi, S. Darling-Reed, E.Y. Lee, E.T. Oriaku, K.F. Soliman, Ibuprofen and apigenin induce apoptosis and cell cycle arrest in activated microglia, Neurosci. Lett. 375 (2005) 91–96.
[112] T. Liu, G. Perry, H.W. Chan, G. Verdile, R.N. Martins, M.A. Smith, C.S. Atwood, Amyloid-beta-induced toxicity of primary neurons is dependent upon differentiation-associated increases in tau and cyclin-dependent kinase 5 expression, J. Neurochem. 88 (2004) 554–563.
[113] M. Ahmed, P.M. Machado, A. Miller, C. Spicer, L. Herbelin, J. He, J. Noel, Y. Wang, A.L. McVey, M. Pasnoor, P. Gallagher, J. Statland, C.H. Lu, B. Kalmar, S. Brady, H. Sethi, G. Samandouras, M. Parton, J.L. Holton, A. Weston, L. Collinson, J.P. Taylor, G. Schiavo, M.G. Hanna, R.J. Barohn, M.M. Dimachkie, L. Greensmith, Targeting protein homeostasis in sporadic inclusion body myositis, Sci. Transl. Med. 8 (2016) 331ra41.
[114] M. Veas-Pérez de Tudela, C. Maestre, M. Delgado-Esteban, J.P. Bolaños, A. Almeida, Cdk5-mediated inhibition of APC/C-Cdh1 switches on the cyclin D1-Cdk4-pRb pathway causing aberrant S-phase entry of postmitotic neurons, Sci. Rep. 5 (2015) 18180.
[115] L. Zhang, W. Liu, K.K. Szumlinski, J. Lew, p10 the N-terminal domain of p35, protects against CDK5/p25-induced neurotoxicity, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 20041–20046.
[116] T. Fishman-Jacob, L. Reznichenko, M.B. Youdim, S.A. Mandel, A sporadic Parkinson disease model via silencing of the ubiquitin-proteasome/E3 ligase component SKP1A, J. Biol. Chem. 284 (2009) 32835–32845.
[117] R.J. Duronio, Y. Xiong, Signaling pathways that control cell proliferation, Cold Spring Harb. Perspect. Biol. 5 (2013) a008904.
[118] H.V. Rao, L. Thirumangalakudi, P. Desmond, P. Grammas, Cyclin D1, cdk4, and Bim are involved in thrombin-induced apoptosis in cultured cortical neurons, J. Neurochem. 101 (2007) 498–505.
[119] T. Boutros, E. Chevet, P. Metrakos, Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer, Pharmacol. Rev. 60 (2008) 261–310.

[120] K. Shah, D.K. Lahiri, Cdk5 activity in the brain - multiple paths of regulation, J. Cell Sci. 127 (2014) 2391–2400.
[121] B.B. McConnell, F.J. Gregory, F.J. Stott, E. Hara, G. Peters, Induced expression of p16(INK4a) inhibits both CDK4- and CDK2-associated kinase activity by reassortment of cyclin-CDK-inhibitor complexes, Mol. Cell. Biol. 19 (1999) 1981–1989.
[122] R. Von Bernhardi, F. Cornejo, G.E. Parada, J. Eugenín, Role of TGFβ signaling in the pathogenesis of Alzheimer's disease, Front. Cell. Neurosci. 9 (2015) 426.

Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2017, 9(3):373-384

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Repurposing HSP70 Inducing Compounds for Targeting Post-Mitotic Cell Division: Novel Promises as Neuroprotectants

Renu Sharma and Pravir Kumar*

Molecular Neuroscience and Functional Genomics Laboratory, Delhi Technological University (Formerly DCE), Delhi, India

---

ABSTRACT

The recent findings related to cell cycle re-entry mediated neurodegeneration in post mitotic neurons have triggered rampant research in this area. Cell cycle has been identified as a true, causative phenomenon occurring during prodromal stages of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Heat shock proteins are internal 'stress absorbing' machinery which comes into force to protect the cell against heat, oxidative stress. Owing to its chaperonic activity, HSP70 has been shown to mediate pro-survival pathways in several diseases including neurodegenerative diseases. We therefore set out to check whether HSP70 inducing compounds can be repurposed to target post-mitotic cell division. Various in silico methods such as homology modelling, Ramachandran plots, Lipinski filter, ADMET analysis and molecular docking studies were performed. We report novel potential of some HSP70 inducing compounds in ameliorating post-mitotic cell division led neurodegeneration which has wide implications in Alzheimer's disease and Parkinson's disease.

Keywords: Cell cycle; HSP70; Therapeutics; Neurodegeneration

INTRODUCTION

Toxic protein burden has been identified as the common underlying molecular switch to neurodegeneration in several neurodegenerative diseases (NDD) such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), Inclusion body myositis (IBM) and Poly myositis (PM). While a host of stressors which induce neuro-muscular degeneration (NMD) namely aging, oxidative stress, impaired ubiquitin proteasome system (UPS), mitochondrial breakdown, loss of function of protective proteins and mutations have been identified, the quest for new players has been on-going for the simple reason that the known players do not add up to all the outcomes of neurodegeneration. The ectopic re-entry of cell cycle in post-mitotic cells such as neurons and muscles has been recently identified as a culprit in NMD. Normally, the cell cycle remains suppressed for lifetime and these cells never divide. However, re-expression of cell cycle markers such as cyclin C, cyclin D, cyclin E along with other markers of active cell cycle has been observed in the AD, PD, ALS, PM and IBM. Moreover, the occurrence of cell cycle proteins during early stages of NDD and their co-existence with pathological proteins has placed fresh impetus on cell cycle re-entry (CCE) as a 'causal' phenomenon in NDD [1]. Once triggered, the cell cycle ensured DNA synthesis in S phase followed by severe neuronal death and neurodegeneration in various NDD [2]. The major thrust of present study is flavonoids which are a class of plant based phenolic compounds with high content in oranges, grapes, lemons, red wine and green tea. The signature properties of flavonoids include antioxidant, anti-inflammatory, anti-allergic, antiviral, antibacterial, anticancer, anti-hypertensive, insulin-sensitizing and anti-ischemic [3]. Moreover, flavonoids are well tolerated in human body and display enhanced bioavailability and negligible toxicity in comparison to their synthetic counter-parts. Furthermore, they have been shown to improve upon disease symptoms by modulating various signal transduction pathways. Molecular chaperones are a class of intracellular proteins which assist the misfolded/toxic protein in regaining its native conformation or alternatively mediating its degradation via UPS thus triaging protein homeostasis inside the cell. Heat shock proteins (HSPs) are molecular chaperones

expressed constitutively in the nervous system which are involved in decreasing neurotoxicity and enhancing neuronal cell survival in various NDD [4,5]. HSP 70 has been shown to associate with p53and arrest the cell cycle at G_1/S. Further, the activity of cell cycle inhibitor p27 was modulated by HSP70 [6]. Furthermore, the G1/S transition markers cyclin D1 and E were reported to associate with elevated level of HSP70 in IBM and PM thereby speculating their strong co-relation [7]. Mounting evidence has outlined HSP70 induction as the major route in mediating pro-survival action of most drugs and biomolecules in PD and other NDD [8,9]. Moreover, we previously outlined Arimoclomol to be a promising neuroprotectant through HSP70 induction in cell cycle driven neurodegeneration [1,10]. Therefore, it is pertinent to understand that HSP70 inducing compounds could be a new line of neurotherapeutics in CCE mediated neurodegeneration. We carried out comprehensive data mining on HSP70 inducers in NDD and carried out the study with twenty compounds. Various virtual screening methods such as, Lipinski filter, Ghose and Veber parameters, pharmacophore generation and ADME analysis were applied to screen drug-like compounds. Further, Homology modelling, 3D structure validation and Ramachandran plots of proteins were performed to establish model accuracy. Finally, ligand-protein interactions were studied with the targets of interests; G_0/G_1 phase markers i.e. cyclin C and cyclin D1 through molecular docking studies. Our results have outlined strong potential of three HSP70 inducing compounds namely Indomethacin, Bimoclomol and Sesamol in attenuating levels of cyclin D1 and cyclin C. These observations may have promising implications in targeting CCE mediated neurodegeneration in AD, PD and HD. Our results have reinforced the promising potential of HSP70 inducers as novel neuroprotectants in ameliorating CCE mediated neurodegeneration.

MATERIALS AND METHODS

Data mining

Data mining was done with the keywords HSP70 inducing compounds in neurodegeneration in the NCBI database. Also, extensive literature survey was carried out. The filter criteria were set to HSP70 inducers in cell cycle and/or neurodegeneration and accordingly, list of 20 potential compounds was prepared.

Retrieval of ligand structure

The sdf files of all the 20 compounds were retrieved from the PubChem database (http://www.pubchem.ncbi.nlm.nih.gov/). The pubChem database stores physio-chemical and biological information of compounds from three different databases. Additionally, their structures, physical and chemical properties were also obtained.

Drug-likeliness analysis

The drug ability of all the 20 potential candidates was tested through Lipinski filter analysis via the online tool. As the name suggests, Lipinski's rule of five is used to distinguish between compounds which may be converted into drugs from the negative candidates of drug-likeliness. The five rules of Lipinski are: (a) molecular mass <500 Dalton, (b) logP < 5, (c) hydrogen bond donors< 5, (d) hydrogen bond acceptors< 10 and (e) Molar refractivity between 40 -130 [11]. The other two markers used for drug-likeness screening were Ghose filter and Veber rules (www.swissadme.ch/index.php). The qualifying parameters of Ghose filter are (a) molecular weight 160-480 (b) number of atoms 20-70 (c) molar refractivity 40-130 (d) molar refractivity -0.4-5.6 (e) polar surface area <140 [12]. Finally, the Veber rules of (a) rotatable bond count <=10 and (b) polar surface area <=140 were applied to the compounds [13].

ADMET analysis

The toxicity profiling of ligands was carried out through the online tool SwissADME (www.swissadme.ch/index.php). The Swiss ADME tool assessed the ligands on various parameters such as lipophilicity (logP), hydrophilic nature (logS) and Blood Brain Barrier (BBB) permeability.

Pharmacophore based target prediction

The pharmacophore is a spatial arrangement of electronic and steric properties of a ligand which are responsible for its biological response against a particular target. Pharmacophore based target prediction was done with web server PharmMapper(http://59.78.96.61/pharmmapper/index.php) [14].

Protein Homology modelling and Structural validation

The Brookhaven Protein Data Bank (PDB) was searched for suitable templates of cyclin D1 and cyclin C for homology modeling using the BLASTP search with default parameters. Accordingly, PDB ID 2W96.A and 3RGF for cyclin D1 and cyclin C respectively were selected. The homology modeling of given templates was performed using the Swiss Model server (http://swissmodel.expasy.org/) [15].

The 3D model so generated was tested for structural and stereo-chemical evaluation using the online server RAMPAGE (http://www.mordred.bioc.cam.ac.uk/~rapper/rampage.php) [16]. The RAMPAGE tool allowed for residue by residue analysis of cyclin D1 and cyclin C geometry. Finally, the structural validation and accuracy of the models was checked with Errat(http://nihserver.mbi.ucla.edu/ERRATv2/).

Prediction of physio-chemical properties

The physio-chemical properties of cyclin D1 and cyclin C were predicted using the web based server ProtParam (http://web.expasy.org/protparam/) by using the Uniprot IDP24385 and P24863 respectively.

Active site prediction

The active sites of cyclin D1 and cyclin C were predicted using the Pock Drug tool(http://pockdrug.rpbs.univ-paris-diderot.fr/cgi-bin/index.py?page=home) [17]. The PDB structures of cyclin D1 and cyclin C were uploaded and binding pockets were predicted using the fpocket estimation and setting ligand proximity threshold at 5.5.

Preparation of proteins and ligands for docking

The proteins and ligands were prepared for docking using the online Docking Server (http://www.dockingserver.com/web) [18]. The proteins were cleaned and appropriate chain; A and B for cyclin D1 and cyclin C respectively selected for docking. Next, charge onprotein and ligands was added using Gasteiger method and solvation parameters set to default. The ligand geometry was optimized using MMFF94 method. Further, all non-polar H_2 atoms were merged, rotatable bonds defined and pH set to 7.0.

Molecular docking

The optimized proteins and ligands were used for molecular docking studies using the online Docking Server (http://www.dockingserver.com/web).The Autodock tool was used for adding Kollman united atom type charges, essential H_2 atoms and solvation parameters. Affinity grid maps were generated with 0.375 Å spacing [19]. Further, the van der Waals and electrostatic interactions were calculated using Autodock parameter set and distance-dependent dielectric functions respectively. Furthermore, the Lamarckian genetic algorithm and Soils and Wets local search method was used for docking simulations [20]. During docking, all rotatable torsions were dropped. Every docking study was arrived after ten different runs with a cut off energy estimation of 250000. Finally, translational step with 0.2Å, torsion and quaternion steps of 5 were used with a population size of 150.

RESULTS

Selection of ligands

The compounds along with their structure, physical properties and signalling cascade modulated in NDD and neuro-oncology have been summarised in Table 1.

Screening for drug-likeness and ADMET Analysis of compounds

Most of the compounds passed drug-likeness parameters but failed ADMET analysis predictions (Table 2). Bimoclomol, Indomethacin and Sesamol qualified all the above parameters and were used in further study. While the bioavailability score of Sesamol and Bimoclomol was 0.55, Indomethacin had the highest predicted bioavailability of 0.56.

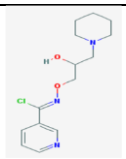
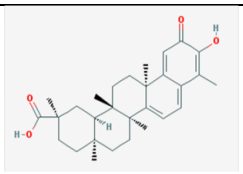
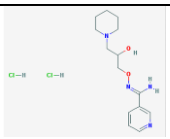
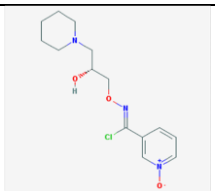
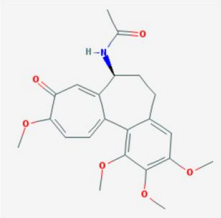
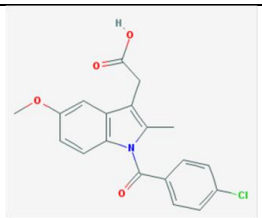
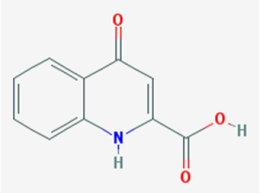
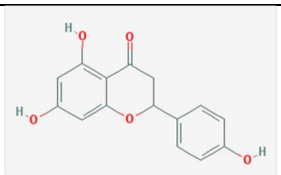
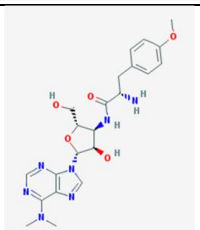
Pharmacophore based target prediction

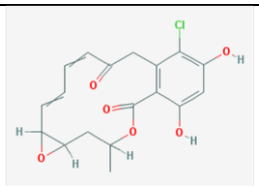
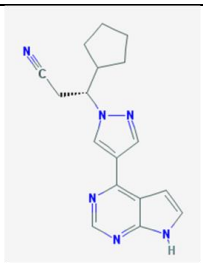
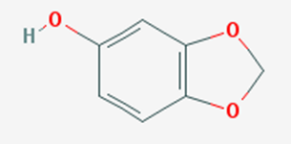
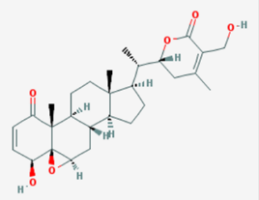
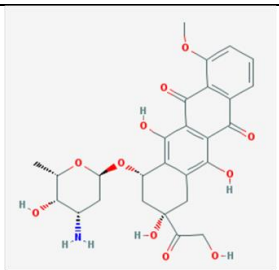
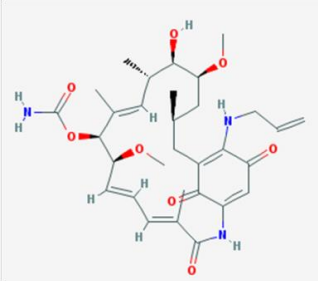
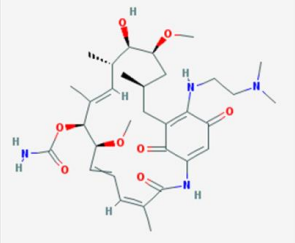
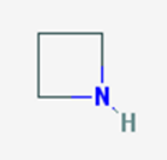
The pharmacophore based target prediction of Indomethacin, Bimoclomol and Sesamol outlined various cell cycle proteins such as Cyclin A2, cell division protein kinase 2, VEGFR2 and MAPK18 which further strengthens our premise of their use in targeting cell cycle (Figure 1).

Homology modeling of proteins

The template shared 100% sequence similarity with cyclin D1 and cyclin C and was used to generate their 3D structures using Swiss Model. The Z QMEAN4 score indicative of overall quality of generated models with respect to non-redundant set of PDB structures was -1.68 and -0.64 for cyclin D1 and cyclin C respectively (Figure 2). Thus, the predicted protein structures satisfied good quality models.

Table 1: Physio-chemical properties and modulated signalling pathways of compounds

S.No.	Compound	Structure	Molecular weight (g/mol)	Molecular formula	Modulated signalling in NDD/Neuro-oncology	References
1	Bimoclomol		297.783	$C_{14}H_{20}ClN_3O_2$	Augmented HSP70 level in ALS	[21]
2	Celastrol		450.619	$C_{29}H_{38}O_4$	Induced HSP70 and acted pro-survival in neurons post TBI damage, anti-inflammatory, Rapid induction of HSF1	[22]
3	BGP-15		351.272	$C_{14}H_{24}Cl_2N_4O_2$	Induced HSP70 and acted pro-survival in neurons post TBI damage, anti-inflammatory	[22]
4	Arimoclomol		313.782	$C_{14}H_{20}ClN_3O_3$	Induced HSP70, delayed progression of ALS	[21]
5	Colchicine		399.443	$C_{22}H_{25}NO_6$	Induced HSPB8 which in turn attenuated accumulation of misfolded TDP-43 and TDP-25 in ALS via HSP70/HSC70-CHIP complex	[23]
6	Indomethacin		357.79	$C_{19}H_{16}ClNO_4$	Induction of HSP70, attenuated Aβ induced damage in AD	[24]
7	Kyneuric Acid		189.17	$C_{10}H_7NO_3$	Inhibited proliferation, migration and DNA synthesis	[25]
8	Naringenin		272.256	$C_{15}H_{12}O_5$	Rescued against 6-OHDA induced toxicity through Nrf2/ARE signaling	[26]
9	puromycin		471.518	$C_{22}H_{29}N_7O_5$	Elicited HSP70 expression in response to ROS	[27]

10	Radicicol		364.778	$C_{18}H_{17}ClO_6$	Inhibit huntingtin aggresome, elevated HSP70	[28]
11	Ruxolitinib		306.373	$C_{17}H_{18}N_6$	Increased HSP70, Inhibited ERK1/2, Akt, STAT3 and STAT5	[29]
12	Sesamol		138.122	$C_7H_6O_3$	Protected against amyloidogenesis and cognitive dysfunction through NF-Kß inhibition	[30]
13	Withaferin		470.606	$C_{28}H_{38}O_6$	Induction of HSP70,HSP27,MAPK, Inhibition of Akt/Mtor and cell cycle at G2/M	[31]
14	Doxorubicin		543.525	$C_{27}H_{29}NO_{11}$	Induced HSPB8 which in turn attenuated accumulation of misfolded TDP-43 and TDP-25 in ALS via HSP70/HSC70-CHIP complex	[23]
15	17AAG		585.698	$C_{31}H_{43}N_3O_8$	Blocked cell proliferation through Wnt/β catenin pathway attenuation	[32]
16	17DMAG		616.756	$C_{32}H_{48}N_4O_8$	HSP70 induction, anti-inflammatory, anti-oxidant	[33]
17	Azitidine		57.096	C_3H_7N	protein synthesis inhibition, induction of chaperones	[34]

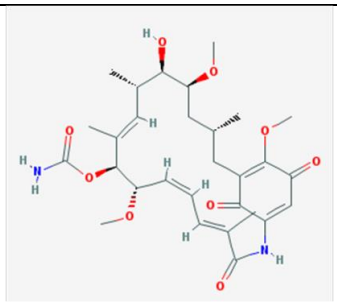
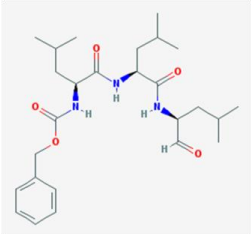
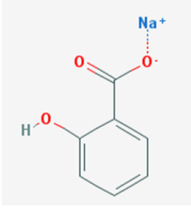
18	Geldanamycin		560.644	$C_{29}H_{40}N_2O_9$	Inhibit huntingtin aggresome, elevated molecular chaperones	[35]
19	MG132		475.63	$C_{26}H_{41}N_3O_5$	Induction of HSP70 in response to stress	[36]
20	Sodium Salicylate		160.104	$C_7H_5NaO_3$	Induction of HSP, imparting neuroprotection in rotenone induced PD	[37]

Table 2: Drug-likeness and ADMET screening analysis

S.No.	Compound	Drug likeness			Aq. Solubility		Lipophilicity	BBB Permeability	Bioavailability Score
		Lipinski	Ghose	Veber	LogS(ESOL)	GI permeability	XLogP3		
1	Bimoclomol	Y	Y	Y	-2.9	High	2.21	Y	0.55
2	Celastrol	Y	N	Y	-6.31	Low	5.94	N	0.56
3	BGP-15	Y	Y	Y	-3.21	High	2.18	N	0.55
4	Arimoclomol	Y	Y	Y	-2.37	High	1.22	N	0.55
5	Colchicine	Y	Y	Y	-2.9	High	1.03	N	0.55
6	Indomethacin	Y	Y	Y	-4.86	High	4.27	Y	0.56
7	Kyneuric Acid	Y	Y	Y	-2.29	High	1.29	N	0.56
8	Naringenin	Y	Y	Y	-3.49	High	2.52	N	0.55
9	puromycin	Y	N	N	-2.51	Low	0.03	N	0.55
10	Radicicol	Y	Y	Y	-4.4	High	3.36	N	0.55
11	Ruxolitinib	Y	Y	Y	-3.26	High	2.12	N	0.55
12	Sesamol	Y	N	Y	-1.92	High	1.23	Y	0.55
13	Withaferin	Y	N	Y	-4.97	High	3.83	N	0.55
14	Doxorubicin	N	N	N	-3.91	Low	1.27	N	0.17
15	17AAG	Y	N	N	-4.67	Low	2.64	N	0.55
16	17DMAG	Y	N	N	-4.42	Low	2.04	N	0.55
17	Azitidine	Y	N	Y	-0.07	Low	-0.15	N	0.55
18	Geldanamycin	Y	N	N	-4.24	Low	1.99	N	0.11
19	MG132	Y	N	N	-4.77	High	4.83	N	0.55
20	Sodium Salicylate	Y	N	Y	-2.59	High	2.26	N	0.55

Quality assessment and physio-chemical description of 3D structures

The generated 3D structures were checked for validation in terms of steric and geometric conformations. For this, the Ramachandran plots were generated (Figure 2). The results showed 91.3% residues of cyclin D1 in the most favored region while 5.5% were in the allowed region. Further, 3.1% residues fell in outlier region. Similarly, for cyclin C 98.4% residues were seen in the favored region, 1.4% in the additionally allowed region and only 0.2% residues in the disallowed region. Further, cyclin D1 and cyclin C passed the model accuracy with 85.77% and 90.98% respectively. So overall, the structures of both the proteins were validated with good scores. The predicted physio-chemical properties of the models are summarized in Table 3.

Ligand: 68289

	Rank	PDB ID	Target Name	Number of Feature	Fit Score	Normalized Fit Score	z'-score
+	8	3FZF	Heat shock cognate 71 kDa protein	6	2.899	0.4832	2.00718
+	9	1DYT	Eosinophil cationic protein	6	2.884	0.4806	1.62091
+	10	1HY7	Stromelysin-1	6	2.861	0.4769	0.716266
+	11	1TX4	Rho GTPase-activating protein 1	6	2.859	0.4765	1.56842
+	12	1DI8	Cell division protein kinase 2	6	2.819	0.4698	1.23631
+	13	1D3H	Dihydroorotate dehydrogenase, mitochondrial	6	2.765	0.4608	0.869469
+	14	1XO2	Cell division protein kinase 6	5	2.246	0.4492	-0.680011
+	15	1U4L	C-C motif chemokine 5	6	2.613	0.4354	0.630226
+	16	1H28	Cyclin-A2	6	2.606	0.4343	0.0851662

Ligand: 3715

	Rank	PDB ID	Target Name	Number of Feature	Fit Score	Normalized Fit Score	z'-score
+	1	3H9O	NONE	4	3.363	0.8409	0.519893
+	2	830C	Collagenase 3	4	3.22	0.805	-0.574694
+	3	2P2H	Vascular endothelial growth factor receptor 2	4	3.181	0.7952	-0.366195
+	4	2CLX	Cell division protein kinase 2	4	3.151	0.7877	-0.27883
+	5	3EID	Cell division protein kinase 2	5	3.828	0.7656	2.21778
+	15	1H28	Cyclin-A2	6	4.058	0.6764	2.52909

Ligand: 9576891

	Rank	PDB ID	Target Name	Number of Feature	Fit Score	Normalized Fit Score	z'-score
+	1	1ONG	Beta-lactamase SHV-1	4	3.548	0.8871	0.0454138
+	2	1DY4	Exoglucanase 1	4	3.525	0.8812	0.288696
+	3	1GCZ	Macrophage migration inhibitory factor	4	3.409	0.8523	-0.339281
+	4	2B55	Cell division protein kinase 2	5	3.802	0.7605	1.62195
+	5	2BTO	NONE	5	3.789	0.7578	1.49587
+	6	1Q6I	FKBP-type peptidyl-prolyl cis-trans isomerase fkpA	5	3.7	0.74	1.15025
+	7	3CPA	NONE	5	3.544	0.7087	-0.0490846
+	8	2ZAZ	Mitogen-activated protein kinase 14	5	3.521	0.7042	-0.408482

Figure 1: Pharmacophore based target prediction of Indomethacin, Sesamol and Bimoclomol (top to bottom in order)

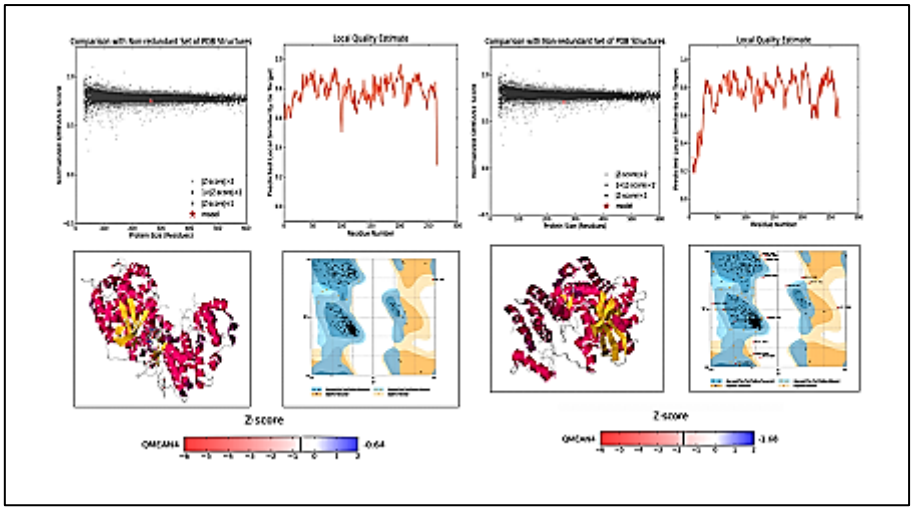


Figure 2: Structural validation of cyclin C and cyclin D1 (left to right)

Table 3: Predicted physio-chemical properties of cyclin D1 and cyclin C

Protein	Mol wt.	Atomic composition	No. of Amino Acids	Theoretical PI	Negatively charged residues Asp+Glu	Positively charged residues Arg+Lys	Instability index	Aliphatic index	GRAVY
Cyclin D1	33729.11	$C_{1480}H_{2386}N_{396}O_{450}S_{25}$	295	4.97	47	34	57.71	92.92	-0.185
Cyclin C	33242.73	$C_{1522}H_{2348}N_{384}O_{417}N_{17}$	283	6.95	32	32	49.97	92.69	-0.158

Active site prediction

Based on drugability score, cavity volume and standard deviation, cyclin D1 had best pocket at P5 with a score of 0.95 and0.01standard deviation (Figure 3a). The volume of given pocket was 1079.69 cubic angstroms and 16 residues were involved in interaction. Similarly, P0 was best predicted active site for cyclin C with 0.97 score (Figure 3b). The volume of this cavity was found to be 3732.64 cubic angstroms and 38 residues were involved in interaction at this site. These pockets were used for docking the ligands and same residues as predicted were found to be involved during docking.

Pockets	Vol. Hull*	Hydroph. Kyte*	Polar Res.*	Aromatic Res.*	Otyr atom	Nb. Res.*	Drugg Prob*	Standard Deviation
P 0	2609.78	-0.52	0.56	0.13	0.0	32.0	0.89	0.03
P 1	2494.82	-0.36	0.54	0.18	0.0	28.0	0.75	0.08
P 2	1460.86	-0.28	0.45	0.2	0.0	20.0	0.75	0.06
P 3	1097.44	-0.22	0.59	0.12	0.0	17.0	0.68	0.01
P 4	1503.19	-0.54	0.46	0.04	0.0	24.0	0.44	0.04
P 5	1079.69	0.62	0.44	0.13	0.0	16.0	0.95	0.01
P 6	1298.6	-0.34	0.48	0.14	0.02	21.0	0.7	0.03
P 7	988.3	0.28	0.41	0.24	0.0	17.0	0.93	0.04
P 8	598.12	0.79	0.36	0.07	0.03	14.0	0.95	0.02

a

Pockets	Vol. Hull*	Hydroph. Kyte*	Polar Res.*	Aromatic Res.*	Otyr atom	Nb. Res.*	Drugg Prob*	Standard Deviation
P 0	3732.64	0.58	0.42	0.16	0.01	38.0	0.97	0.0
P 1	2968.13	-1.79	0.77	0.27	0.01	26.0	0.13	0.03
P 11	1218.64	-1.01	0.71	0.12	0.0	17.0	0.25	0.02
P 16	377.41	0.35	0.47	0.07	0.0	15.0	0.84	0.02
P 17	1324.1	-2.19	0.81	0.13	0.0	16.0	0.02	0.0
P 2	2535.02	-0.47	0.48	0.26	0.0	27.0	0.74	0.1
P 28	531.74	0.79	0.27	0.27	0.0	15.0	0.98	0.01
P 3	1432.1	-0.45	0.39	0.17	0.02	18.0	0.87	0.03
P 4	1320.0	-0.47	0.55	0.25	0.02	20.0	0.73	0.03
P 5	868.12	0.14	0.5	0.25	0.02	16.0	0.92	0.01

b

Figure 3: Predicted active sites (top 10) in cyclin D1 (a) and cyclin C (b)

Molecular docking of ligands with cyclin D1

Bimoclomol and cyclin D1: While the total intermolecular energy of Bimoclomol and cyclin D1 was -5.49kcal/mol, the estimated free energy of binding was found to be -4.77Kcal/mol (Figure 4a). Bimoclomol formed hydrogen bonds with LEU148 (-3.9537kcal/mol). Further, ASN151 was involved in polar bond formation with -1.0163kcal/mol and LEU91formed hydrophobic bond with Bimoclomol (44.6643kcal/mol).

Indomethacin and cyclin D1: The estimated free energy of binding for cyclin D1-Indomethacin interaction was -5.51kcal/mol and total intermolecular energy was -6.68kcal/mol (Figure 4b). The H_2bond energy with ALA39 was unfavorable (23.1104kcal/mol). Further, two polar bonds were formed with ARG87 (-6.319kcal/mol) and SER41 (-2.497kcal/mol).

Sesamol and cyclin D1: Sesamol interacted with cyclin C to generate estimated free energy of binding -3.76kcal/mol and total intermolecular energy of -4.06kcal/mol. Polar bond was formed with ASN83 (-0.1798kcal/mol). Four hydrophobic bonds were formed with PRO199 (-0.9286kcal/mol), ALA39 (-0.5013kcal/mol), PRO40 (-0.2763kcal/mol) and PRO200 (-0.195kcal/mol) (Figure 4c).

Molecular docking of ligands with cyclin C

Bimoclomol and cyclin C: The estimated free energy of binding for cyclin C and Bimoclomol was -4.02kcal/mol, while the total intermolecular energy was -6.24kcal/mol. Hydrogen bond with -0.2489kcal/mol energy was formed between THR66. While the polar bond energy of ASP182 was -3.9173kcal/mol, hydrophobic bonds formed with TYR 184(-1.8162kcal/mol) and ILE62 (-0.3802kcal/mol). Further, GLN49 formed halogen bond with -7.6881kcal/mol energy (Figure 4d).

Indomethacin and cyclin C: Indomethacin interacted with cyclin C and generated high estimated binding energy of -5.68kcal/mol and total intermolecular energy -7.22kcal/mol. Further, five polar bonds were formed with ASN46, ARG185, GLN59, THR66 and GLN49 having energy values of -0.8421, -0.5951, -0.5475, -0.2608 and -0.2379 kcal/mol respectively. Next, two hydrophobic bonds were formed between TRP241 (-0.6165kcal/mol) and ILE62 (-0.4887kcal/mol) (Figure 4e).

Sesamol and cyclin C: The estimated free energy of binding for cyclin C-Bimoclomol interaction was -4.31kcal/mol and total intermolecular energy was -4.61kcal/mol. Two polar bonds were formed between TYR37 (-0.686kcal/mol) and ARG25 (-0.4014kcal/mol). Further, three hydrophobic bonds were formed with TYR73(-0.945kcal/mol), PHE69(-0.5898kcal/mol) and LEU78(-0.3605kcal/mol) (Figure 4f).

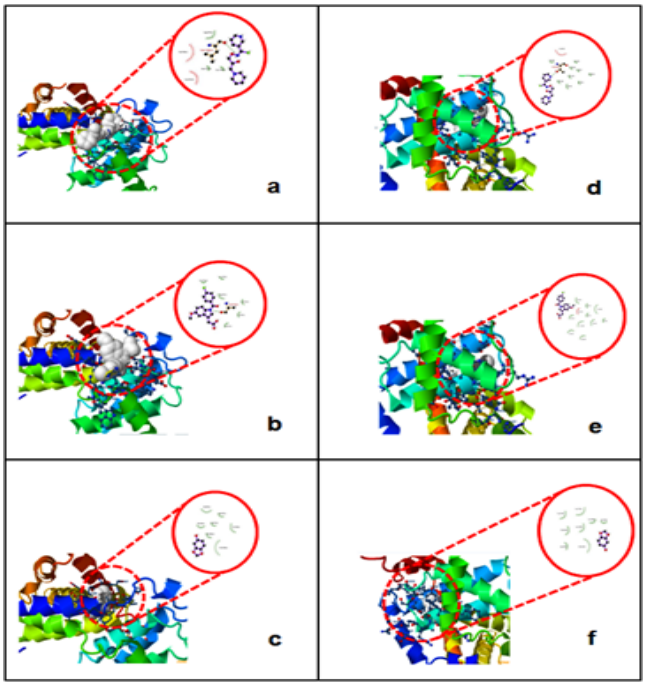


Figure 4: Docking of cyclin D1 with Bimoclomol, Indomethacin and Sesamol (a,b,c) and cyclin C (d,e,f) respectively with the interacting residues (inset)

The comparative analysis of docking calculations was done (Table 4) and Indomethacin was found to be the best compound for targeting and inhibiting cyclin D1 as well as cyclin C thereby, implicating its strong and diverse potential in attenuating G_0/G_1 checkpoints in cell cycle.

Table 4: Comparative analysis of ligands-proteins docking calculations

Energy Parameters	CYCLIN D1			CYCLIN C		
	Bimoclomol	**Indomethacin**	**Sesamol**	**Bimoclomol**	**Indomethacin**	**Sesamol**
Estimated free energy of binding (Kcal/mol)	-4.77	-5.51	-3.76	-4.02	-5.68	-4.31
Estimated inhibition constant (uM)	317.57	91.17	1.74	1.12	69	696.84
vdW+Hbond+desolv energy (Kcal/mol)	-5.89	-6.67	-4.07	-6.11	-7.06	-4.6
Electrostatic energy (Kcal/mol)	0.41	-0.01	0.01	-0.13	-0.16	0
Total intermolecular energy (Kcal/mol)	-5.49	-6.68	-4.06	-6.24	-7.22	-4.61
Interacting surface	600.646	624.148	366.636	576.194	645.711	305.971

DISCUSSION

The cell cycle re-entry mediated neurodegeneration contributes heavily in the demise of post-mitotic neurons and muscles. Since cyclins C and D are first respondents of a re-activated cell cycle,thus, targeting these can be 'nib in the bud' strategy in halting/ameliorating the evil cascade of cell cycle led neuronal death. HSPs are molecular chaperones which are upregulated during stress to protect the cell against heat, hypoxia and ROS generation. HSP70 in particular, has been shown to promote neuronal cell survival by inducing autophagy and mediating the activation of pro-survival signaling cascades [4]. Moreover, HSP70 is closely associated with cell cycle and interacted with cyclin D1 in IBM and PM [7]. It is therefore imperative to search for compounds which can induce the level of HSP70 in NDD as a key neuroprotective strategy. Further, currently available drugs provide only symptomatic relief; therefore, flavonoids are favored by neuroscientists owing to their beneficial effects and negligible toxicity. In the present study, we proposed and tested the efficacy of HSP70 inducing compounds in ameliorating cell cycle led neurodegeneration in various NDD. Since most drugs fail on poor solubility, we screened the compounds for ADMET and pharmacokinetics analysis. It is evident that *in vivo* bioavailability of an orally administered drug is largely dependent on its aqueous solubility and dissolution in GI fluids [38]. More the water solubility and GI permeability, better the bioavailability. Similarly, lipophilicity of a drug affects various physiological properties such as the rate of metabolism, transport across cell membrane and interaction with binding sites of receptor. Further, drugs intended for CNS should have logP value less than four [39,40]. Indomethacin, Bimoclomol and Sesamol showed logP values of 4.27, 2.21 and 1.23 respectively.However, the most important property required of a compound to be a neuroprotective agent is the ability to cross Blood brain barrier (BBB). As expected, most compounds failed the BBB permeability parameter. Three biomolecules namely, Bimoclomol, Indomethacin and Sesamol could cross the BBB and combined with their high GI absoption, least violations of drug likeness and good bioavailability score, were the best candidates for targeting NDD in our study. Further, pharmacophore based target prediction of these three compounds listed various cell cycle proteins which further supported our repurposing premise. Finally, molecular docking studies indicated Indomethacin as the best compound for HSP70 mediated targeting of post-mitotic cell cycle based on its high pharmacokinetics and docking calculations. Further, our results are backed by various *in vitro* and *in vivo* studies wherein these compounds have displayed promising neuroprotective action in various NDD. For instance, Bimoclomol has its derivative Arimoclomol already under Phase II clinical trials in ALS [41]. Indomethacin was shown to ameliorate Aβ1-42 triggered damage in AD mice model as well as in hippocampal cultures [24]. Similarly, Sesamol reversed PD linked symptoms in a rotenone model [42]. Hence, our compounds are validated for their neuroprotective action and yet, add to the hunt for protective biomolecules in alleviating cell cycle led neurodegeneration.Our study has outlined novel potential of Indomethacin, Bimoclomol and Sesamol in inhibiting/down-regulating the level of cyclin D1 and cyclin C. Out of these, Indomethacin showed best binding with both the cyclins, speculating its strong potential in inhibiting G_0/G_1 phase reactivation in terminally differentiated neurons in various NDD. Further, the protective action of these compounds in attenuating cell cycle re-entry may be mediated through HSP70. These findings can open up a new window of therapeutics for targeting ectopic cell cycle activation led neurodegeneration and need further validation through *in vitro* and *in vivo* cell cycle studies.

CONCLUSION

The study evaluated the potential of HSP70 inducing compounds for targeting post-mitotic cell cycle in neurodegenerative disorders. Based on BBB permeability, pharmacokinetic properties and ADMET analysis, we have shortlisted Indomethacin, Bimoclomol and Sesamol amongst twenty compounds for targeting cell cycle proteins; cyclin D1 and cyclin C. Further, our study demonstrated that Indomethacin has the highest potential in stalling or inhibiting cell cycle, based on high free energy of binding with both the markers of G_0/G_1 phase.

Moreover, the cell cycle inhibiting effect of these compounds may be elicited through HSP70 induction. To the best of our knowledge, these compounds are novel for their use in targeting post mitotic cell division in neurodegenerative disorders

ACKNOWLEDGEMENT

The authors would like to thank management of Delhi Technological University for their encouragement and support. The authors would also like to thank University Grants Commission for providing Senior Research Fellowship (SRF) to R.S.

REFERENCES

[1] R Sharma; D Kumar; NK Jha; SK Jha; RK Ambasta; P Kumar. *Biochim Biophys Acta*, **2017**, 1863(1), 324-336.
[2] K Herrup. *Alzheimers Res Ther*, **2010**, 2(3), 13.
[3] D Procházková; I Boušová; N Wilhelmová. *Fitoterapia*, **2011**, 82, 513–523.
[4] PJ Muchowsk; JL Walker. *Nat Rev Neurosci* , **2005**, 6, 11–22.
[5] J Magrane; RC Smith; K Walsh; HW Querfurth. *J Neurosci*, **2004**, 24, 1700-1706.
[6] J Liu; D Zhang; X Mi; Q Xia; Y Yu; Z Zuo; W Guo; X Zhao; J Cao; Q Yang; A Zhu; W Yang; X Shi; J Li; C Huang. *J Biol Chem*, **2010**, 285(34), 26058-26065.
[7] B Kwon; P Kumar; HK Lee; L Zeng; K Walsh; Q Fu. *Hum Mol Genet*, **2014**, 23, 3681–3694.
[8] XQ Bao; XL Wang; D Zhang. *Mol Neurobiol*, **2017**, 54 (1), 349-361.
[9] CA Deane; IR Brown. *Cell Stress Chaperon*, **2016**, 21(5), 837-48.
[10] DA Parfitt; M Aguila; CH McCulley; D Bevilacqua; HF Mendes; D Athanasiou; SS Novoselov; N Kanuga; PM Munro; PJ Coffey; B Kalmar. *J Neurochem*, **2008**, 107, 339-350.
[11] CA Lipinski; F Lombardo; BW Dominy; PJ Feeney. *Adv Drug Deliv Rev*, **2001**, 46 (1-3), 3-26.
[12] AK Ghose; VN Viswanadhan; JJ Wendoloski. *J Com Chem*, **1999**, 1, 55-68.
[13] DF Veber; SR Johnson; HY Cheng; BR Smith; KW Ward; KD Kopple. *J Med Chem*, **2002**, 45 (12), 2615-2623.
[14] L Xiaofeng; O Sisheng; Y Biao; H Kai; L Yabo; G Jiayu; Z Sisuan; L Zhihua; L Honglin; J Hualiang. *Nucleic Acid Res*, **2010**, 38, W609-W614.
[15] M Biasini; S Bienert; A Waterhouse; K Arnold; G Studer; T Schmidt; F Kiefer; TG Cassarino; M Bertoni; L Bordoli; T Schwede. *Nucleic Acids Res*, **2014**, 42(W1), W252-W258
[16] SC Lovell; IW Davis; WB 3rd Arendall; PI de Bakker; JM Word; MG Prisant; JS Richardson; DC Richardson. *Proteins*, **2003**, 50(3), 437-50.
[17] HA Hussein; A Borrel; C Geneix; M Petitjean; L Regad; A Camproux. *Nucl Acids Res*, **2015**.
[18] Z Bikadi; E Hazai. *J Cheminf*, **2009**, 1, 15.
[19] GM Morris; DS Goodsell. *J Computational Chem*, **1998**, 19(14), 1639-1662.
[20] FJ Soils; RJB Wets. *Mathematics of Operations Research*, **1981**, 6(1), 19-30.
[21] SC Benn; RH Jr Brown. *Nat Med*, **2004**, 10(4), 345-7.
[22] B Eroglu; DE Kimbler; J Pang; J Choi; D Moskophidis; N Yanasak; KM Dhandapani; NF Mivechi. *J Neurochem*, **2014**, 130(5), 626-41.
[23] V Crippa; VG D'Agostino; R Cristofani; P Rusmini; ME Cicardi; E Messi; R Loffredo; M Pancher; M Piccolella; M Galbiati; M Meroni; C Cereda; S Carra; A Provenzani; A Poletti. *Sci Rep*, **2016**, 6, 22827.
[24] A Bernardi; RL Frozza; A Meneghetti; JB Hoppe; AM Battastini; AR Pohlmann; SS Guterres; CG Salbego. *Int J Nanomedicine*, **2012**, 7, 4927-4942.
[25] K Walczak; S Deneka-Hannemann; B Jarosz; W Zgrajka; F Stoma; T Trojanowski; WA Turski; W Rzeski. *Pharmacol Rep*, **2014**, 66(1), 130-136.
[26] H Lou; X Jing; X Wei; H Shi; D Ren; X Zhang. *Neuropharmacol*, **2014**, 79, 380-388.
[27] DM Moran; H Shen; CG Maki. *BMC Cell Biol*, **2009**, 10, 32.
[28] DG Hay; K Sathasivam; S Tobaben; B Stahl; M Marber; R Mestril; A Mahal; DL Smith; B Woodman; GP Bates. *Hum Mol Genet*, **2004**, 13(13), 1389-1405.
[29] M Tavallai; L Booth; JL Roberts; A Poklepovic P Dent. *Front Oncol*, **2016**, 6,142.
[30] Z Liu; Y Chen; Q Qiao; Y Sun; Q Liu; B Ren; X Liu. *MolNutr Food Res*, **2016**.
[31] PT Grogan; KD Sleder; AK Samadi; H Zhang; BN Timmermann; MS Cohen. *Invest New Drugs*, **2013**, 31(3), 545-557.
[32] KK Chen; ZM He; BH Ding; Y Chen; LJ Zhang; L Yu; J Gao. *Zhongguo Shi Yan Xue Ye XueZaZhi*, **2016**, 24(1), 117-121.

[33] YL Wang; HH Shen; PY Cheng; YJ Chu; HR Hwang; KK Lam; YM Lee. *PLoS One*, **2016**, 11(5), e0155583.
[34] CP Reina; BY Nabet; PD Young; RN Pittman. *Cell Stress Chaperon*, **2012**, 17(6), 729-742.
[35] A Sittler; R Lurz; G Lueder; J Priller; H Lehrach; MK Hayer-Hartl; FU Hartl; EE Wanker. *Hum Mol Genet*, **2001**, 10(12), 1307-1315.
[36] CI Holmberg; SA Illman; M Kallio; A Mikhailov; L Sistonen. *Cell Stress Chaperon*, **2000**, 5(3), 219-228.
[37] P Thakur; B Nehru. *Neurochem Int*, **2014**, 75, 1-10.
[38] P Khadka; R Jieun; H Kim; I Kim; JT Kim; H Kim; G Yun; J Lee. *Asian J Pharm Sci*, **2014**, 9(6), 304-316.
[39] LK Chico; LJ Van Eldik; DM Watterson. *Nat Rev Drug Discov*, **2009**, 8(11), 892-909.
[40] TT Wager; X Hou; PR Verhoest; A Villalobos. *ACS ChemNeurosci*, **2010**, 1(6), 435-449.
[41] JM Keppel Hesselink. *J Pain Relief*, **2016**, 6, 279.
[42] SM Angeline; A Sarkar; K Anand; RK Ambasta; P Kumar. *Neurosci*, **2013**, 254, 379-394.

ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 6, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

NEUROPROTECTIVE ROLE OF BIMOCLOMOL IN ECTOPIC CELL CYCLE IN PARKINSON'S DISEASE: NEW INSIGHTS

RENU SHARMA[1], PRAVIR KUMAR[2]*

[1]Department of Biotechnology, Molecular Neuroscience and Functional Genomics Laboratory, Delhi Technological University (Formerly DCE), New Delhi, India. [2]Department of Biotechnology, Molecular Neuroscience and Functional Genomics Laboratory, Delhi Technological University (Formerly DCE), New Delhi, India. Email: pravirkumar@dce.edu/kpravir@gmail.com

Received: 17 February 2017, Revised and Accepted: 11 March 2017

ABSTRACT

Objective: Parkinson's disease (PD) is a debilitating age-related neurodegenerative disease characterized by the canonical formation of intracellular Lewy bodies comprising α-synuclein protein. Despite the knowledge of factors causing PD, it remains irreversible and incurable. Recent studies have highlighted the physiological and pathological involvement of cell cycle proteins in PD. The intriguing relationship between PARK2 and cyclin E which leads to upregulation of cyclin E in the absence of functional PARK2 contributes heavily in the onset and progression of PD. The objective of this study is to explore neuroprotective action of bimoclomol in attenuating the level of cyclin E and inhibiting post-mitotic cell division led neurodegeneration in PD.

Methods: We employed various *in silico* methods such as drug-likeness parameters, namely, Lipinski filter analysis, Ghose parameters, Veber rules, absorption, distribution, metabolism, and excretion - toxicity analysis, pharmacophore based target prediction, active site prediction, and molecular docking studies.

Results: The binding of bimoclomol inhibited cyclin E, thereby, attenuating post-mitotic cell division led neurodegeneration in PD.

Conclusion: This study outlines the novel potential of bimoclomol in attenuating cyclin E led neuronal death in PD which may be mediated by heat shock proteins (HSP70).

Keywords: Parkinson's disease, Bimoclomol, Cell cycle, Heat shock proteins 70, Therapeutics.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i6.17971

INTRODUCTION

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder affecting 2% of population aged above 65 years in industrialized nations. It is characterized by pathogenic protein burden and intracellular inclusion body formation, namely, Lewy bodies and Lewy neurites constituted by α-synuclein protein. The clinical cardinal features of PD include resting tremor, bradykinesia, postural instability and rigor, often accompanied by impaired cognition [1]. These symptoms are an outcome of dopaminergic neuronal loss in the substantia nigra pars compacta region of the brain.

Genetic and animal studies have outlined various causative phenomenon in PD including mutations in genes predominantly; Parkin, α-synuclein, PINK1 and DJ-1, oxidative stress, aging, impaired ubiquitin proteasome system, and dysfunctional mitochondrial system. Despite the availability of this knowledge, the etiology of PD remains incurable and irreversible. Moreover, recent studies have highlighted the involvement of aberrant cell cycle in PD [2]. Interestingly; cell cycle proteins share a very intimate relationship with proteins of PD, physiologically as well as pathologically. The PD-associated gene; PINK1 was shown to promote cell cycle, and PINK1 deletion reversed cell proliferation [3]. Further, the ATM gene responsible for DNA damage response and apoptosis has been found to be activated along with retinoblastoma protein leading to neuronal death in MPP^+ induced PD model [4]. However, the most crucial and strong correlation between cell cycle and PD is provided by the association of cyclin E and PARK2. Cyclin E is G_1/S phase marker of the cell cycle and also a substrate of ubiquitin E3 ligase PARK2. Mutations associated with loss of functional PARK2 are linked with cyclin E enrichment led cell cycle and apoptosis through p53 and Bax in PD. Further, mutated PARK2/cyclin E events evoked upregulation of Wnt/β catenin and EGFR/AKT signal transduction pathways [5]. This intriguing PARK2/cyclin E relation led to speculations that compounds which can bind to and attenuate the level of cyclin E can ameliorate post-mitotic cell division led neurodegeneration in PD.

Numerous studies have highlighted the neuroprotective action of heat shock proteins (HSP) particularly, HSP70. Further, HSP70 is closely associated with cell cycle regulation and was also found to interact with cyclin E in inclusion body myositis and polymyositis [6]. Therefore, we carried out comprehensive data mining for HSP70 inducers in neurodegenerative diseases and selected bimoclomol among 20 compounds based on drug-likeness, pharmacokinetics, and blood–brain barrier (BBB) permeability (unpublished results). Bimoclomol is a hydroxylamine derivative with molecular formula $C_{14}H_{20}ClN_3O_2$ and molecular weight of 297.783 g/mol. It has been shown to elicit protective effects through induction of HSP27, HSP70, and HSP90. Moreover, bimoclomol is currently under Phase II trials in amyotrophic lateral sclerosis disease [7]. Therefore, we investigated the neuroprotective action of bimoclomol through attenuation of cyclin E in PD. Various virtual screening methods such as Lipinski filter, Ghose and Veber parameters, pharmacophore modeling based target prediction, and ADME analysis were employed to check the efficacy of bimoclomol as a neuroprotective agent. Further, we studied the cyclin E inhibiting potential of bimoclomol through molecular docking studies.

METHODS

Retrieval of ligand-protein structure

The SDF file of bimoclomol was retrieved from the PubChem database (http://www.pubchem.ncbi.nlm.nih.gov/). The pubChem database stores physiochemical and biological information of compounds from

three different databases. The protein data bank (PDB) file of cyclin E was retrieved from PDB (http://www.rcsb.org/pdb/home/home.do).

Drug-likeliness analysis

The drug-likeness of bimoclomol was tested through Lipinski filter analysis which is used to test compounds for drug ability. The Lipinski's rule of five is: (a) Molecular mass <500 Dalton, (b) lipophilicity (logP) <5, (c) hydrogen bond donors <5, (d) hydrogen bond acceptors <10, and (e) molar refractivity between 40 and 130 [8]. In addition, two other parameters; Ghose filter and Veber rules were employed for drug-likeness screening (www.swissadme.ch/index.php). The qualifying parameters of Ghose filter are (a) molecular weight 160-480, (b) number of atoms 20-70, (c) molar refractivity 40-130, (d) molar refractivity - 0.4-5.6, and (e) polar surface area <140 [9]. The Veber rules are (a) rotatable bond count ≤10 and (b) polar surface area ≤140 [10].

Absorption, distribution, metabolism, and excretion - toxicity (ADMET) analysis

The toxicity profiling of bimoclomol was carried out through the online tool Swiss ADME (www.swissadme.ch/index.php). The Swiss ADME tool assessed the ligand on various parameters such as logP, hydrophilic nature (logS), and BBB permeability.

Pharmacophore based target prediction

A pharmacophore is a spatial arrangement of steric and electronic properties of a compound responsible for its biological response against a particular target. Pharmacophore-based target prediction of bimoclomol was done with web server PharmMapper (http://59.78.96.61/pharmmapper/index.php) [11].

Active site prediction

The active sites of cyclin E were predicted using the pockdrug tool (http://pockdrug.rpbs.univ-paris-diderot.fr/cgi-bin/index.py?page=home) [12]. The PDB structure of cyclin E was uploaded, and active sites were predicted using fpocket estimation and setting ligand proximity threshold at 5.5.

Preparation of protein and ligand for docking

Cyclin E and bimoclomol were prepared for docking using the online docking server (http://www.dockingserver.com/web) [13]. The protein was cleaned, and chain A of cyclin E was selected for docking. The protein and ligand charge was calculated using Gasteiger method, and default solvation parameters were set. The ligand geometry was optimized using the MMFF94 method. Further, all non-polar H_2 atoms were merged, rotatable bonds defined and pH set to 7.0.

Molecular docking

The optimized proteins and ligands were used for molecular docking studies using the online docking server (http://www.dockingserver.com/web). The Autodock tool was used for adding Kollman united atom type charges, essential H_2 atoms, and solvation parameters. Affinity grid maps were generated with 0.375 Å spacing [14]. Further, the van der Waals and electrostatic interactions were calculated using Autodock parameter set and distance-dependent dielectric functions, respectively. Furthermore, the Lamarckian genetic algorithm and Soils and Wets local search method was used for docking simulations [15]. During docking, all rotatable torsions were dropped. Every docking study was arrived after 10 different runs with a cut off energy estimation of 250000. Finally, translational step with 0.2 Å, torsion and quaternion steps of five were used with a population size of 150.

RESULTS

Protein-ligand structure

The 3D structures of bimoclomol and cyclin E were retrieved from docking server and PDB, respectively (Fig. 1).

Screening for drug-likeness and ADMET analysis of compounds

Bimoclomol passed all the parameters related to drug-likeness screening, namely, Lipinski, Ghose and Veber. Most importantly, it can cross the BBB and has high pharmacokinetics values (Table 1).

Pharmacophore based target prediction

The pharmacophore based target prediction of bimoclomol revealed mitogen-activated protein kinase 14 as one of the top 10 targets with a fit score of 3.521 and Z-score value of −0.408482 which supported our premise of its strong potential in inhibiting cell cycle (Fig. 2).

Active site prediction

Out of top 10 pockets, cyclin E had best pocket at P20 with a drug ability score of 0.94 and 0.01 standard deviation (Fig. 3). The volume of given pocket was 551.26 cubic angstroms and 14 residues were involved in interaction at this site.

Molecular docking of bimoclomol with cyclin E

Bimoclomol bound to cyclin E at P20 pocket and same residues as predicted were involved in the interaction (Fig. 4). The estimated free energy of binding for cyclin E and bimoclomol was −5.07 kcal/mol, and total intermolecular energy was −6.48 kcal/mol (Table 2). There were two H_2 bond formations involving GLU188 and LYS186 with bond energies of −0.2603 kcal/mol and −0.2271 kcal/mol, respectively. Further, a hydrophobic bond was formed with HIS147 (−0.6293 kcal/mol).

DISCUSSION

PD is the second most common age-related neurodegenerative disease affecting those aged above 60 years. Despite the knowledge of several factors which contribute in the occurrence and progression of PD, the exact cause and cure remain elusive. Ectopic activation of the cell cycle in terminally differentiated neurons is a recently known phenomenon which has been shown to drive neurodegeneration through actual DNA

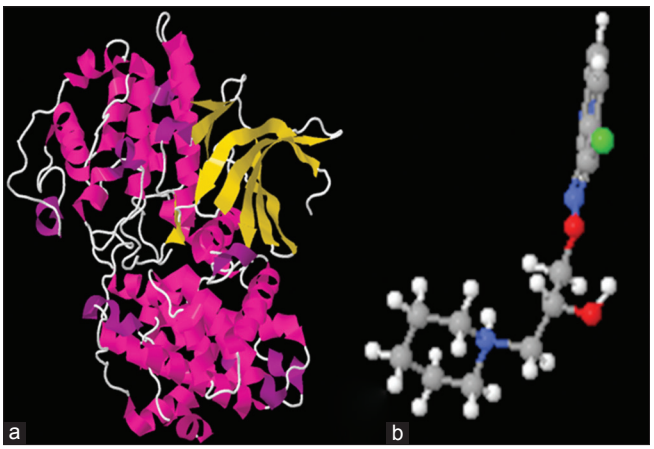


Fig. 1: Three-dimensional structure of cyclin E (a) and bimoclomol (b)

Ligand: 9576891

	Rank	PDB ID	Target Name	Number of Feature	Fit Score	Normalized Fit Score	z'-score
+	1	1ONG	Beta-lactamase SHV-1	4	3.548	0.8871	0.0454138
+	2	1DY4	Exoglucanase 1	4	3.525	0.8812	0.288696
+	3	1GCZ	Macrophage migration inhibitory factor	4	3.409	0.8523	-0.339281
+	4	2B55	Cell division protein kinase 2	5	3.802	0.7605	1.62195
+	5	2BTO	NONE	5	3.789	0.7578	1.49587
+	6	1Q6I	FKBP-type peptidyl-prolyl cis-trans isomerase fkpA	5	3.7	0.74	1.15025
+	7	3CPA	NONE	5	3.544	0.7087	-0.0490846
+	8	2ZAZ	Mitogen-activated protein kinase 14	5	3.521	0.7042	-0.408482
+	9	2UWL	Coagulation factor X	5	3.394	0.6788	-0.426764
+	10	3MDE	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	6	3.99	0.665	0.078465

Fig. 2: Pharmacophore based target prediction of bimoclomol

Pockets	Vol. Hull*	Hydroph. Kyte*	Polar Res.*	Aromatic Res.*	Otyr atom	Nb. Res.*	Drugg Prob*	Standard Deviation
P 0	2058.21	-0.36	0.59	0.17	0.0	29.0	0.72	0.06
P 1	2863.51	-1.49	0.71	0.26	0.03	31.0	0.26	0.01
P 15	634.09	0.15	0.53	0.2	0.0	15.0	0.88	0.04
P 17	642.52	0.21	0.4	0.07	0.0	15.0	0.82	0.02
P 2	852.24	0.12	0.41	0.06	0.0	17.0	0.77	0.03
P 20	551.26	0.73	0.29	0.07	0.0	14.0	0.94	0.01
P 3	1146.05	0.04	0.45	0.1	0.0	20.0	0.78	0.01
P 4	1067.3	-0.88	0.67	0.11	0.0	18.0	0.29	0.01
P 5	674.13	-0.63	0.63	0.13	0.0	16.0	0.42	0.02
P 9	1030.55	-0.64	0.57	0.14	0.0	14.0	0.46	0.03

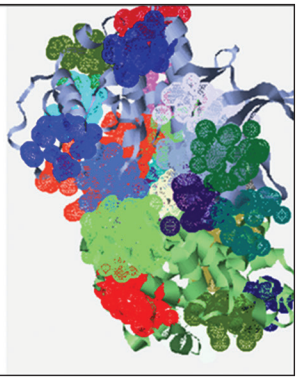


Fig. 3: Active sites of cyclin E

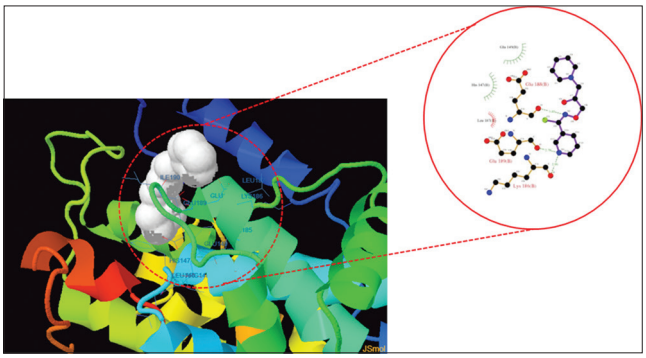


Fig. 4: Docking of bimoclomol with cyclin E and residues involved (inset)

Table 1: ADMET analysis of Bimoclomol

GI permeability	High
LogS (ESOL)	−2.9
XLogP3	2.21
Bioavailability score	0.55

ADMET: Absorption, distribution, metabolism, and excretion - toxicity, GI: Gastrointestinal, ESOL: Estimated aqueous solubility

Table 2: Energies of binding for cyclin E and bimoclomol

Estimated free energy of binding	−5.07 Kcal/mol
Estimated inhibition constant	191.27 uM
vdW+Hbond+desolv energy	−4.55 Kcal/mol
Electrostatic energy	−1.93 Kcal/mol
Total intermolecular energy	−6.48 Kcal/mol
Interacting surface	541.668

synthesis followed by apoptosis [2]. Moreover, PD-associated proteins have shared a very intimate relation with cell cycle markers. The G1/S phase marker cyclin E is a substrate for PARK2 and participates in ubiquitination process. However, mutations in PARK2 led to the loss of function thereby, resulting in cyclin E accumulation which in turn, activated E2F1 and triggered neuronal death in PD [5]. Thus, it seems imperative to design therapeutic strategies aimed at attenuating the level of cyclin E to inhibit the cascade of neuronal death in PD.

Various biomolecules such as curcumin elicited HSP70 activity and provided protection against neuronal dysfunction, various cancers, and in vascular diseases [16-18]. Bimoclomol is a hydroxylamine derivative which is nontoxic and elicited its protective effect through HSP induction; including HSP70 [19]. HSPs are molecular chaperones which are upregulated in the cell to protect it against heat, ROS and hypoxia. HSP70 has been shown to promote neuronal survival by mediating the activation of pro-survival signaling cascades and through autophagy induction [2]. Interestingly, HSP70 has been shown to interact with cyclin E in Aβ induced cell cycle re-entry in inclusion body myositis and polymyositis [6]. Taken together, all these data provide convincing evidence of using HSP70 inducing compound such as bimoclomol in attenuating the level of cyclin E and in turn, inhibit the cascade of neuronal death in PD.

In this study, we tested the drug-able efficacy of bimoclomol for targeting cyclin E in PD. Emphasis was laid on pharmacokinetic analysis as aqueous solubility, and dissolution in gastrointestinal fluids are defining parameters of *in vivo* bioavailability of an orally administered drug [20]. Similarly, the lipophilicity of a drug directs physiological properties such as rate of metabolism, transport across cell membrane and interaction with binding sites of the receptor. Further, CNS drugs should have logP <4 [21,22]. The logP value for bimoclomol was found to be 2.21.

However, the most important property required of a compound intended to be a neuroprotective agent is BBB permeability. Bimoclomol qualified all the above-mentioned parameters and scored well on pharmacokinetics, bioavailability score and could cross the BBB. Finally, molecular docking studies indicated that bimoclomol can bind to and attenuate the level of cyclin E and possibly, halt or inhibit cell cycle re-entry mediated neuronal death in PD. These findings can be validated through *in vitro* and *in vivo* cell cycle studies in PD.

CONCLUSION

The results of our study provide the novel potential of bimoclomol in attenuating the level of cyclin E which has wider implications in inhibiting cell cycle re-entry mediated neurodegeneration in PD.

ACKNOWLEDGMENT

The authors would like to thank senior management of Delhi Technological University for their encouragement and support. The authors would also like to thank University Grants Commission for providing Senior Research Fellowship to R.S.

REFERENCES

1. Ebrahimi-Fakhari D, Wahlster L, McLean PJ. Molecular chaperones in Parkinson's disease - Present and future. J Parkinsons Dis 2011;1(4):299-320.
2. Sharma R, Kumar D, Jha NK, Jha SK, Ambasta RK, Kumar P. Re-expression of cell cycle markers in aged neurons and muscles: Whether cells should divide or die? Biochim Biophys Acta 2017;1863(1):324-36.
3. O'Flanagan CH, Morais VA, Wurst W, De Strooper B, O'Neill C. The Parkinson's gene PINK1 regulates cell cycle progression and promotes cancer-associated phenotypes. Oncogene 2015;34(11):1363-74.
4. Camins A, Pizarro JG, Alvira D, Gutierrez-Cuesta J, de la Torre AV, Folch J, *et al.* Activation of ataxia telangiectasia muted under experimental models and human Parkinson's disease. Cell Mol Life Sci

2010;67(22):3865-82.
5. Feng DD, Cai W, Chen X. The associations between Parkinson's disease and cancer: The plot thickens. Transl Neurodegener 2015;4:20.
6. Kwon B, Kumar P, Lee HK, Zeng L, Walsh K, Fu Q, *et al.* Aberrant cell cycle reentry in human and experimental inclusion body myositis and polymyositis. Hum Mol Genet 2014;23(14):3681-94.
7. Keppel Hesselink JM. Bimoclomol and arimoclomol: HSP-co-inducers for the treatment of protein misfolding disorders, neuropathy and neuropathic pain. J Pain Relief 2016;6:279.
8. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 2001;46(1-3):3-26.
9. Ghose AK, Viswanadhan VN, Wendoloski JJ. A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery 1. A qualitative and quantitative characterization of known drug databases. J Comb Chem 1999;1(1):55-68.
10. Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW, Kopple KD. Molecular properties that influence the oral bioavailability of drug candidates. J Med Chem 2002;45(12):2615-23.
11. Liu X, Ouyang S, Yu B, Liu Y, Huang K, Gong J, *et al.* PharmMapper server: A web server for potential drug target identification using pharmacophore mapping approach. Nucleic Acids Res 2010;38:W609-14.
12. Hussein HA, Borrel A, Geneix C, Petitjean M, Regad L, Camproux AC. PockDrug-server: A new web server for predicting pocket druggability on holo and apo proteins. Nucleic Acids Res 2015;43:W436-42.
13. Bikadi Z, Hazai E. Application of the PM6 semi-empirical method to modeling proteins enhances docking accuracy of AutoDock. J Cheminform 2009;1:15.
14. Morris GM, Goodsell DS. Automated docking using a genetic lamarckian algorithm and an empirical binding free energy function. J Comput Chem 1998;19(14):1639-62.
15. Soils FJ, Wets RJ. Minimization by random search techniques. Math Oper Res 1981;6(1):19-30.
16. Xia C, Cai Y, Li S, Yang J, Xiao G. Curcumin increases HSP70 expression in primary rat cortical neuronal apoptosis induced by gp120 V3 loop peptide. Neurochem Res 2015;40:1996-2005.
17. Sharma S, Kumar V. *In vitro* cytotoxicity effect on mcf-7 cell line of co-encapsulated artesunate and curcumin liposome. Int J Pharm Pharm Sci 2017;9(3):123-8.
18. Joshi H, Bhandari U, Panda BP. To assess the potential of curcumin against gut microbiota-induced alteration in choline metabolism in c57bl/6j mice. Int J Pharm Pharm Sci 2017;9(3):215-26.
19. Deane CA, Brown IR. Induction of heat shock proteins in differentiated human neuronal cells following co-application of celastrol and arimoclomol. Cell Stress Chaperones 2016;21(5):837-48.
20. Khadka P, Jieun R, Kim H, Kim I, Kim JT, Kim H, *et al*. Pharmaceutical particle technologies: An approach to improve drug solubility, dissolution and bioavailability. Asian J Pharm Sci 2014;9(6):304-16.
21. Chico LK, Van Eldik LJ, Watterson DM. Targeting protein kinases in central nervous system disorders. Nat Rev Drug Discov 2009;8(11):892-909.
22. Wager TT, Hou X, Verhoest PR, Villalobos A. Moving beyond rules: The development of a central nervous system multiparameter optimization (CNS MPO) approach to enable alignment of druglike properties. ACS Chem Neurosci 2010;1(6):435-49.

Journal of Alzheimer's Disease 57 (2017) 1017–1039
DOI 10.3233/JAD-160623
IOS Press

Review

Stress-Induced Synaptic Dysfunction and Neurotransmitter Release in Alzheimer's Disease: Can Neurotransmitters and Neuromodulators be Potential Therapeutic Targets?

Saurabh Kumar Jha, Niraj Kumar Jha, Dhiraj Kumar, Renu Sharma, Abhishek Shrivastava, Rashmi K. Ambasta and Pravir Kumar*
Department of Biotechnology, Molecular Neuroscience and Functional Genomics Laboratory, Delhi Technological University (Formerly DCE), Delhi, India

Accepted 10 August 2016

Abstract. The communication between neurons at synaptic junctions is an intriguing process that monitors the transmission of various electro-chemical signals in the central nervous system. Albeit any aberration in the mechanisms associated with transmission of these signals leads to loss of synaptic contacts in both the neocortex and hippocampus thereby causing insidious cognitive decline and memory dysfunction. Compelling evidence suggests that soluble amyloid-β (Aβ) and hyper-phosphorylated tau serve as toxins in the dysfunction of synaptic plasticity and aberrant neurotransmitter (NT) release at synapses consequently causing a cognitive decline in Alzheimer's disease (AD). Further, an imbalance between excitatory and inhibitory neurotransmission systems induced by impaired redox signaling and altered mitochondrial integrity is also amenable for such abnormalities. Defective NT release at the synaptic junction causes several detrimental effects associated with altered activity of synaptic proteins, transcription factors, Ca^{2+} homeostasis, and other molecules critical for neuronal plasticity. These detrimental effects further disrupt the normal homeostasis of neuronal cells and thereby causing synaptic loss. Moreover, the precise mechanistic role played by impaired NTs and neuromodulators (NMs) and altered redox signaling in synaptic dysfunction remains mysterious, and their possible interlink still needs to be investigated. Therefore, this review elucidates the intricate role played by both defective NTs/NMs and altered redox signaling in synaptopathy. Further, the involvement of numerous pharmacological approaches to compensate neurotransmission imbalance has also been discussed, which may be considered as a potential therapeutic approach in synaptopathy associated with AD.

Keywords: Amyloid-β, neurotransmitters/neuromodulators, redox signaling, synaptic dysfunction, tau, therapeutics

*Correspondence to: Dr. Pravir Kumar, PhD, Department of Biotechnology, Delhi Technological University (Formerly Delhi college of engineering), Room # FW4TF3, Mechanical Engineering Building, Shahbad Daulatpur, Bawana Road, Delhi 110042, India. Tel.: +91 9818898622; E-mails: pravirkumar@dce.edu; kpravir@gmail.com.

INTRODUCTION

To maintain brain homeostasis, synapses and their associated neurotransmitters (NTs) play the role where synapses are specialized structures that form a network to transmit electrochemical signals

or information from one neuron to another. The signal transmission involves a complex process of NT release and uptake at synaptic junctions [1] where dysregulation of the synaptic junction in response to numerous insults and aberrant NT releases or its receptors lead to synaptopathy associated with Alzheimer's disease (AD) [2]. It has been demonstrated that soluble amyloid plaques and hyperphosphorylated tau served as toxins in disrupting synaptic plasticity and NT release at synapses, thereby causing a cognitive decline in AD [3, 4]. In addition, free radicals, oxidative stress, and mitochondrial dysfunctions have also contributed significantly toward synaptic loss [5]. Redox signaling has also been shown to alter the signaling cascades associated with the pathophysiology of synaptic loss and thus cause vulnerability to neuronal cells in AD [6]. Since the exact mechanisms associated with synaptic dysfunction induced by impaired NTs and altered redox signaling remain enigmatic, their plausible association is under investigation. In this regard, the present review underlines the involvement of both defective NTs and altered redox signaling in the etiology of synaptic loss associated with AD, and also demonstrates the involvement of numerous biological compounds and recent therapeutic strategies for targeting synaptic loss induced by defective NTs and neuromodulators (NMs).

SYNAPTOPATHY IN ALZHEIMER'S DISEASE: CORRELATION BETWEEN SYMPTOMS AND SYNAPTIC FAILURE

Synapses are an alliance of specialized structures that allow a neuron to pass a chemical or an electrical signal to another neuron. This electrochemical transmission is a complex interplay among NT release at presynaptic terminals and its detection at receptors of postsynaptic terminals of a neuron [7, 8]. While any dysregulation in synaptic transmission leads to a number of chronic brain disorders, including addiction, depression, anxiety, and dementia like AD and Parkinson's disease [9]. There is numerous evidence depicting the promising role of synaptic plasticity in memory formation and its stabilization. Recent methodological advancements have uncovered the mystery behind synaptopathy and its consequent dysregulation in neural circuitry [10]. For instance, amyloid-β (Aβ) and tau proteins were reported to function normally at synaptic junctions while their overburden caused neuronal toxicity and thus synaptic loss in the case of AD [11]. Hyperphosphorylated and aggregated forms of tau are leading agents for synaptic dysfunction, behavioral impairment, and neuronal death in neurodegenerative disorders (NDDs) [12]. It is reported to directly interact with postsynaptic signaling complexes to regulate synaptic transmission [13]. On the other hand, major kinases such as glycogen synthase-3β (GSK3β), cyclic adenosine monophosphate response element-binding protein (CREB), extracellular receptor kinase (ERK), and mitogen-activated protein kinase (MAPK) are found to induce synaptic dysfunction through their dynamic association with stress-mediated abnormal hyperphosphorylated or accumulated forms of tau in the AD brain [5]. Recently, abnormal acetylation at K281 and K274 of tau protein has been reported to promote synaptic loss in the AD brain viz. reduction of AMPA receptors trafficking, damaged actin dynamics, and diminished postsynaptic KIdney/BRAin (KIBRA) signaling pathways [14]. Additionally, several groups demonstrated the pathogenic role of soluble Aβ in dendritic spine injury in cultured neurons, while its monomeric and fibrillar forms remained inert to synaptic loss [15]. The available evidence suggests that abnormally acetylated and phosphorylated forms of tau, aggregated forms of Aβ, and impaired synaptic plasticity are the key components involved in the synaptopathy of AD [16]. Unlike Aβ and tau, various other factors have also been identified to cause synaptic dysfunction in AD (Table 1). These factors significantly affect neurotransmission and correlate with the disease symptoms including cognitive decline and dementia in AD.

THE PERTINENT ROLE OF AMYLOID-β IN SYNAPTIC BIOLOGY OF ALZHEIMER'S DISEASE

Aβ is a prevalent toxic protein deposited as senile plaques and is likely to be involved in the impairment of synaptic plasticity in both sporadic and familiar forms of AD [35]. One and foremost among the numerous proposed mechanisms associated with Aβ-mediated synaptic dysfunction is toxicity due to its self-aggregation and interaction with various other membranous proteins at synaptic junctions [36]. Importantly, Aβ is found to moderately terminate mGluR-dependent synaptic long-term depression (LTD), thereby signifying its role in modulating synaptic plasticity [37]. Growing

Table 1
Causative factors and associated mechanisms of synaptic dysfunction in AD

SN	Causative Factors	Associated Mechanisms (Synaptic Dysfunctions)	Disease Involved	References
1	Factors associated with oxidative stress and mitochondrial dysfunction	Causes apoptotic cell death and elevation of caspase-3 activity associated with enhanced level of long-term depression (LTD), which further leads to synaptic loss	Alzheimer's disease	[17]
2	Ca^{2+} dyshomeostasis	Affects calcineurin (CaN) activity that causes alteration in N-methyl-D-aspartate (NMDA) receptor activity and impairment of long-term potentiation (LTP), leading to synaptic loss		[18]
3	Cholesterol and lipid depletion	Induces formation of protein aggregates that leads to the impairment of both NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which further affects glutamate transmission, alters the LTP and synaptic loss		[19]
4	Factors associated with inhibition of protein phosphatases	Increases cytoskeletal protein phosphorylation which further induces Ca^{2+} dyshomeostasis and glutamate excitotoxicity, thereby leading to loss of synaptic activity in the brain		[20]
5	$STEP_{61}$	Increased activity of $STEP_{61}$ causes inactivation of ERK1/2 signaling and alteration in NMDA receptor		[21]
6	MAPK phosphatases	Inactivate Mitogen-activated protein kinases (MAPK) and cause neuronal excitability and synaptic dysfunction		[22]
7	Dyshomeostasis of Fe^{2+}, Cu^{2+}, Zn^{2+} and other metal ions	Causes alteration in glutamate activity and modulates various ionotropic, metabotropic, and postsynaptic receptors such as NMDA and AMPA receptors		[23]
8	Factors associated with microglial dysfunction	Induces the release of proinflammatory cytokines such as IL-1β, TNFα, and TNFR1, thus causes apoptosis. Moreover, it also causes alteration in the AMPA receptors at the synapse		[24]
9	GABAergic and Glutamatergic excitotoxicity	Causes alteration in Ca^{2+} homeostasis, involved in the modulation of various receptors such as NMDA and AMPA, and also responsible for the production of free radicals thereby leading to apoptosis of neuronal cells		[4, 25]
10	Glutamate transporter 1 (GLT1)	Dysfunction of GLT1 causes excessive glutamate release and impairment of NMDA and AMPA receptors		[26]
11	Mutations in AβPP/PS1	Causes alteration in stathmin signaling, 14-3-3-mediated signaling, CREB signaling, and protein kinase A (PKA) mediated signaling pathways. Further, it also alters the activity of both NMDA receptor and glutamate release		[27]
12	Aβ	Alters acetylcholine, NMDA, and AMPA receptor activity. It also affects Wnt signaling pathway and causes alteration in glutamate, norepinephrine, and GABA release that further leads to the accumulation of tau at postsynaptic terminals		[28–30]
13	Tau	Mislocalization of hyperphosphorylated tau in dendritic spines causes both the disruption of glutamate receptor and Aβ-related synaptic excitotoxicity		[31]
14	GirK Channels	Aβ interferes with GirK channels, curtailing LTP and increasing LTD, thereby mediating synaptic dysfunction		[32]
15	IGF-1 depletion	Causes defects in both LTP and LTD and augments incorporation of glutamate receptors through the activation of PI3K/Akt signaling		[33]
16	ApoE4	Affects PKA, calcineurin, BDNF, and CREB mediated signaling pathways thereby leading to synaptic loss		[34]

evidence suggests that Aβ oligomers also interact with various NTs/NMs to inhibit synaptic transmission by dysregulation of these receptors. For instance, glutamatergic, GABAergic, and serotoninergic receptors were observed with compromised expression at neuronal synaptic junctions [38, 39]. Similarly, Aβ oligomers interacted with a dozen receptors to trigger the distribution of critical synaptic proteins and induce hyperactivity in ionotropic and metabotropic glutamate receptors [40]. Likewise, there is evidence indicating the interaction of Aβ oligomers with glutamatergic receptors to either facilitate or inhibit the uptake of glutamate and thus cause Aβ-mediated synaptic loss [26]. Moreover, glutamatergic receptors (AMPA and NMDA) are found to regulate Aβ-mediated synaptic dysfunction

via aberrant redox signaling and cytoplasmic Ca^{2+} overload, which triggers downstream pathways including protein phosphatase 2A (PP2A) and Ca^{2+} dependent protein phosphatase calcineurin/PP2B [39]. Additionally, NMDA receptors insults are also found to promote the amyloidogenic processing of amyloid-β protein precursor (AβPP) to induce oligomeric Aβ production and trigger synaptic failure and memory loss [41]. Multiple studies also revealed that Aβ causes neuroinflammation via activation of microglial cells and alters the level of ERK, CaMII, and pCREB to impair long-term potentiation (LTP) and LTD. Such alterations trigger a negative feedback mechanism to deplete the regulation of GSK3β and consequent abnormal Aβ and oligomeric tau production at synapses leading to synaptic dysfunction and memory impairment [42–44]. Besides, numerous studies on hippocampal neurons explored the effects of Aβ and human amylin on LTP with the expression of amylin receptors, while its blockade led to LTP enhancement in transgenic mice to trigger Aβ burden in brain [45]. In this way, multi-disciplinary research has been carried out extensively that identified potential receptors involved in synaptic loss, which could be potential targets for therapeutic intervention.

TAU PROTEIN AS A CULPRIT OF SYNAPTOPATHY IN ALZHEIMER'S DISEASE

Tau protein is another significant pathological hallmark of AD in its hyperphosphorylated form as neurofibrillary tangles (NFTs) and is associated with cognitive decline, memory impairment, synaptic dysfunction, and neuronal loss [46]. Nevertheless, tau phosphorylation is also known for synaptic plasticity during the early stages of neuronal development, but it declines with the aging brain [47]. However, NFTs are reported in varying degrees in the brain before the onset and throughout the progression of AD, but it was not proportionate with the neuronal death. Even the neuronal death exceeded the amount of NFTs indicating that it is not a prominent cause for neuronal death [48]. Furthermore, researchers revealed that it is not the number of NFTs that are responsible for dementia, but it is the aggregated form of tau at synaptic junctions that causes synaptic dysfunction and is vulnerable to neurons [46]. Recent evidence showed that tau-mediated memory impairment is partly associated with decreased RNA translation, due to very close association of ribosomes with tau proteins in AD with respect to control brains [49]. Another study identified that impaired synthesis of postsynaptic density protein-95 (PSD-95) contributes toward the decline of synaptic plasticity that is crucial for learning and memory [50]. Similarly, earlier studies on human fetal cerebral cortical neurons reported the association of an aberrant rise in Ca^{2+} levels with tau hyperphosphorylation leading to microtubular degeneration in AD [51]. Further, chronic exposure of inorganic Arsenic compounds (iAs) and its metabolites facilitated tau hyperphosphorylation and increased AβPP expression. Besides, it also causes altered NT synthesis, increased glutamate receptors activation, and reduced glutamate transporters expression, thereby affecting synaptic transmission [52]. A study demonstrated the increase in accumulation of phosphorylated tau that triggered synaptic loss, neurite retraction, Ca^{2+} dyshomeostasis, and altered NT release (reduced acetylcholine (ACh) levels) in tau oligomer treated neurons [53]. Tau phosphorylation is also modulated by Bcl2 Associated Athanogene-2 (BAG2) expression, since it controls a functional intracellular switch between the p38-dependent functions of nicotine on tau phosphorylation levels via the α7 nicotinic receptor [54]. Furthermore, glucocorticoid (GC, stress hormones) mediated synaptic loss has been evident in AD models via tau hyperphosphorylation, missorting, and mislocation [55, 56]. Recently, abnormal acetylation of K274 and K281 sites on tau has been reported to stimulate disruption of synaptic plasticity and memory by reducing postsynaptic KIBRA (a memory-associated protein) in transgenic mice [14]. Interestingly, another variant of human tau A152T (hTau-A152T) is found to increase the risk for synaptic loss by increasing hyperphosphorylated forms of tau protein and by promoting network hyperexcitability that triggered age dependent neuronal loss at synapses in AD [57]. Apart from Aβ and tau, oxidative stress and mitochondrial dysfunction also play a key role in synaptic dysfunction.

HOW OXIDATIVE STRESS LINKED MITOCHONDRIAL DYSFUNCTION IS A CAUSE FOR SYNAPTIC LOSS

Mitochondria possess an extensive role in ATP production, reactive oxygen species (ROS) generation, Ca^{2+} homeostasis, and apoptotic signaling; while being a great source of intracellular ROS, they are mainly vulnerable to oxidative stress [17].

Nowadays, oxidative stress and subsequent damage to mitochondrial integrity has been widely implicated in various NDDs including AD, Parkinson's disease, Huntington's disease, multiple sclerosis, and amyotrophic lateral sclerosis [58]. Since neurons are energy dependent on mitochondrial activities for its critical functioning, including axonal/dendritic transport, synaptic transmission, and ion pumps and channels, oxidative stress-mediated mitochondrial damage is the predetermining factor for causing synaptic loss in neurons [59, 60]. Moreover, it has been observed that under extreme conditions of oxidative stress, mitochondrial damage takes place primarily at complex IV (cytochrome oxidase) of the electron transport system [61]. This damage ultimately leads to synaptic loss in association with oxidative alteration of the mitochondrial membrane phospholipid; cardiolipin [62]. Synapse maintenance is a prerequisite for proper communication and therefore, synapses are densely packed with mitochondria in order to fulfill their high-energy demands and Ca^{2+} buffering requirements for synaptic transmission [63]. Moreover, synaptic mitochondria are responsible for clearing Ca^{2+} either directly or by providing ATP to Na^{+}/Ca^{2+} exchangers to maintain Ca^{2+} homeostasis in order to govern normal synaptic function [64]. Defective mitochondrial buffering mediated Ca^{2+} overburden causes severe brain tissue injury in response to glutamate excitotoxicity [65]. Likewise, elevated ROS accumulation causes vulnerability to cells in response to compromised shock regulatory proteins and leads to the formation of the mitochondrial permeability transition pore (mPTP). The prolonged opening of mPTP can cause both necrosis and apoptosis via cytochrome C release and consequent activation of caspases [66]. Furthermore, oxidative stress was shown to also contribute significantly toward neuronal damage in the substantia nigra via dopamine-mediated quinone formation [67]. Nevertheless, deficits in axonal transport of mitochondria from soma to distal synapses are prevalent in NDDs. Moreover, some genetic factors have been identified that regulate mitochondrial transport; for instance, PTEN-induced putative kinase 1 (PINK1) is found to interact with Miro and Milton's protein to govern mitochondrial trafficking and distribution [68]. In another study, perturbed mitochondrial fusion/fission proteins are found to affect dendritic mitochondrial populations thereby affecting synaptic plasticity [69]. Likewise, many compounds and elements have been identified so far, which are responsible for oxidative stress/mitochondrial dysfunction mediated synaptic loss in AD (Table 2). The investigations suggest that synaptic dysfunction is presumably one of the initial events in the majority of NDDs associated with mitochondrial abnormalities or irregular mitochondrial distribution in neurons causing clinical symptoms such as motor dysfunction, cognitive decline, and memory loss.

FREE RADICALS, REACTIVE OXYGEN SPECIES, AND CELL-SIGNALING IN SYNAPTIC DYSFUNCTION

Free radicals are highly reactive chemical species having one or more unpaired electrons, and are generated in the complex I and complex III of mitochondria. The iron-sulfide centers and semiquinone or cytochrome b are believed to be likely candidates for its generation in mitochondrial complex I and III, respectively [81, 82]. The altered ROS homeostasis activates various signaling pathways underlying cell inflammation; for instance, ROS and other reactive species regulate the expression of numerous inflammatory mediators including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), lipoxygenase (LOX), cycloxygenase-2 (COX-2), and cell adhesion molecules (VCAM-1, ICAM-1, P & E-selectin) [83–85]. Additionally, ROS has been identified as key modulators of signal transduction cascades pertaining to synaptic plasticity and memory functions without the help of GTPases, phosphatases, protein kinases, Ca^{2+}- dependent enzymes, and other transcription/translation factors [86, 87]. For instance, ROS sensitive calcineurin (PP2B) is believed to suppress LTP by inhibiting LTP-inducing kinases CaMKII and PKC. Moreover, ROS exhibits two-fold roles in LTP; one is stimulation and other is inhibition. For example, in rodent hippocampus, superoxide scavenging blocked high frequency stimulated LTP (HFS-LTP), while superoxide dismutase (SOD) mediated H_2O_2 burden caused LTP inhibition [88]. NMDA receptor is another candidate, which is directly attacked by ROS to affect synaptic plasticity and long-term memory formation. Another reactive species, nitric oxide (NO) plays a dual role in neurobiology by provoking both neuroprotection and neurodegeneration. For instance, NO boosts synaptic plasticity by evoking dendritic Ca^{2+} release via ryanodine receptor (RyR) while its aberrant production triggers synaptic loss via enhanced activation of soluble guanylyl cyclase (sGC)/protein kinase G

Table 2
Key compounds and elements involved in oxidative stress/mitochondrial dysfunction mediated synaptopathy

SN	Compounds and Elements	Mechanisms associated with Synaptic Loss	Disease Involved	References
1	Methotrexate (MTX)	MTX treatment increases the levels of phosphorylated tau, AβPP, and β-secretase thereby causing oxidative stress mediated synaptic loss	Alzheimer's disease	[70]
2	Okadaic acid (OKA)	It induces neurodegeneration by provoking tau hyperphosphorylation, GSK3β overstimulation, oxidative stress, neuroinflammation, altered neurotransmission, and neurotoxicity. Moreover, it also acts as a selective inhibitor of protein phosphatase, PP1 and PP2A		[71]
3	Acrolein	Acrolein is a peroxidation product of arachidonic acid (AA) that augments tau phosphorylation at the site recognized by PHF-1 thereby causing oxidative stress in primary neuronal cultures		[72]
4	Cocaine	Peritoneal injection of cocaine promotes Alzheimer-like hyperphosphorylation of tau and neurofilaments thereby causing oxidative stress in rat brain. Further, it also increases the levels of dopamine, as does nicotine		[73]
5	Lipopolysaccharide	Lipopolysaccharide treatment causes both synaptic alteration and learning-memory deficit by inducing paired immunoglobulin-like receptor B (PirB) expression		[74]
6	Rotenone	Rotenone treatment causes inhibition of complex I of the electron transport chain (ETC), thus induces cell death of cholinergic neurons		[75]
7	Paraquat	Paraquat exposure causes increased oxidative damage specifically in mitochondria of cerebral cortex and manifests mitochondrial dysfunction, thus leads to synaptic loss		[76]
8	1-methyl-4-phenylpyridinium (MPP(+))	Treatment with MPP (+) causes generation of ROS from inhibition of complex I of the mitochondrial respiratory chain and inactivation of aconitase. Further, it also causes ROS-mediated oxidative damage and apoptosis, thereafter lead to synaptic loss		[77]
9	Copper (Cu^{2+})	Chronic exposure of Cu^{2+} causes altered spatial memory with selective loss of synaptic proteins pre-synaptic protein synapsin 1 and post PSD-95 through the mechanisms including activation of PKR/eIF2α signaling pathway		[78]
10	Iron (Fe^{2+})	Fe^{2+} is responsible for triggering synaptic loss and apoptotic cell death by affecting mitochondrial dynamics		[79]
11	Aluminum (Al^{3+})	Al^{3+} exposure decreased the levels of NTs and acetylcholinesterase activity in the brain. Further, it promotes cross-linking of toxic Aβ thereby causing both oxidative stress and synaptic dysfunction		[80]

(PKG) pathway and RhoA/Rho kinase (ROCK) signaling pathway [89, 90]. Furthermore, ROS also directly modulates voltage-dependent Ca^{2+} channels and thus altering synaptic transmission. Additionally, the protein RanBP9 is found to elicit ROS production, mitochondrial dysfunction, and Ca^{2+} dysregulation in AD models [91]. Accumulating evidence suggests that synaptic loss is also caused by altered insulin signaling pathway. This signaling is triggered via an insulin receptor substrate (IRS) that further interacts with numerous other receptor tyrosine kinases including IGF1/2, tropomyosin-related kinase receptor B (TrkB), and ErbB. The phosphorylation of IRS1 on tyrosine residues thereafter leads to the activation of downstream signaling including, Akt, mTOR, and GSK3. Furthermore, the phosphorylation of IRS1 on multiple serine (Ser) residues inhibits IRS1 activity leading to insulin resistance (IR), which further contributes to both Aβ accumulation and tau phosphorylation associated with synaptic loss (Fig. 1). Most importantly, IR is also accountable for altered insulin degrading enzyme (IDE) and neprilysin (NEP) activity, which is induced by accumulated Aβ in AD [92, 93]. Similarly, ROS-mediated microglial activation induced by toxic Aβ is another cause for both neuroinflammation and synaptic dysfunction in AD. Because, Aβ activated microglia is responsible for synaptic loss by releasing numerous neurotoxic mediators including cytokines, interleukin, and TNF-α that propagate an inflammatory

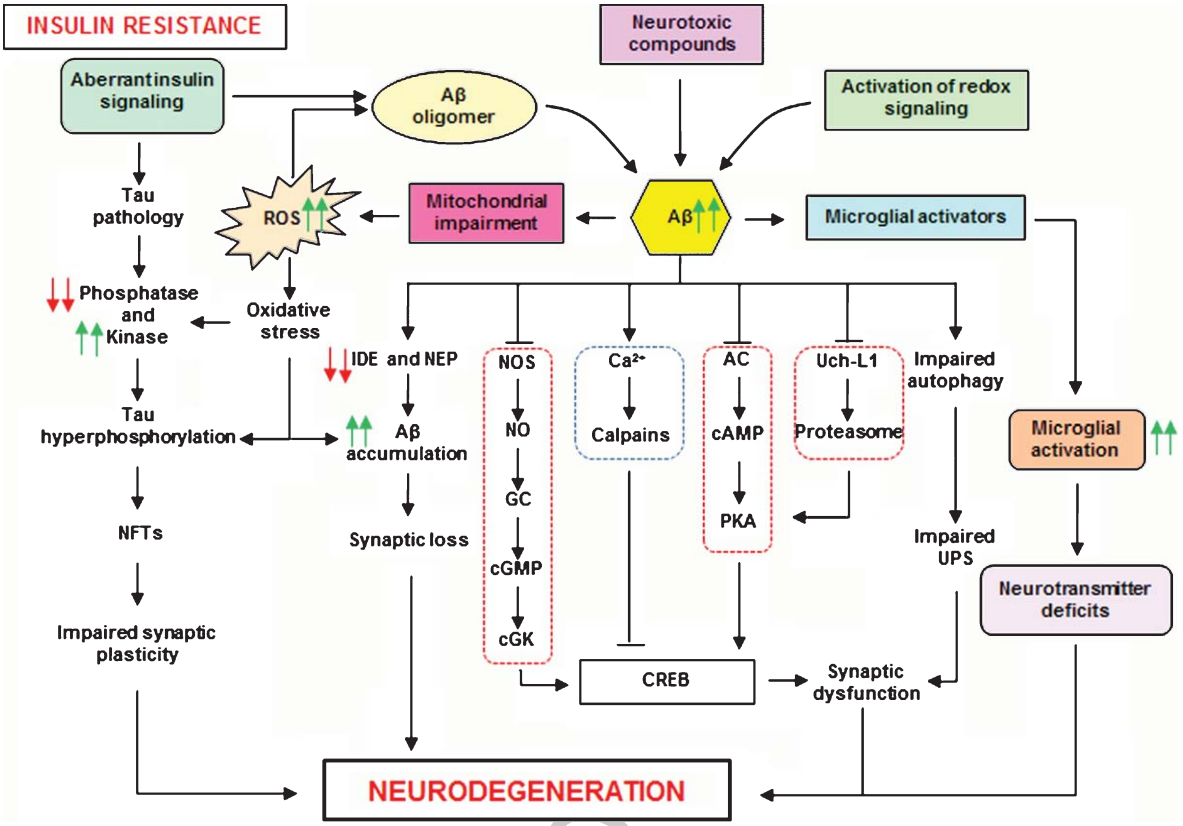


Fig. 1. Schematic illustration showing the different signaling axis that is involved in Aβ-induced synaptic dysfunction and its associated factors. ROS, reactive oxygen species; NFTs, neurofibrillary tangles; IDE, insulin-degrading enzyme; NEP, neprilysin; NOS, nitric oxide synthase; NO, nitric oxide; GC, guanylyl cyclase; cGMP, cyclic guanosine monophosphate; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CREB, cyclic adenosine monophosphate response element-binding protein; UPS, ubiquitin proteasome system.

cycle [94]. Furthermore, CREB acts as a central converging point of diverse signaling cascades that are involved in synaptic strengthening and memory formation and is also reported to be altered by Aβ accumulation [95]. Further research is required to determine other important targets of ROS signaling to investigate their significance in synaptic transmission and neuronal homeostasis.

NEUROTRANSMITTERS/ NEUROMODULATORS: A KEY MEDIATOR OF CELLULAR HOMEOSTASIS OR SYNAPTOPATHY?

NTs are a diverse group of endogenous compounds that act as chemical messengers to transmit the electrical/chemical information throughout the body. Nerve impulses rely on synapses for the release of NTs from presynaptic axons and its detection at postsynaptic terminals [8]. These NTs normally maintain cellular homeostasis via regulation of interdependent elements/chemicals through a number of physiological processes, while its depletion may affect different processes like concentration, mood, sleep, weight, and various others leading to altered cellular homeostasis [96]. For instance, NTs like glutamate, aspartate, GABA, and ACh have been implicated in synaptic dysfunction associated with the progression of AD [4]. The mechanisms underlying synaptic dysfunction are linked to the alteration of their receptors that lead to the pathogenic events in the progression of NDDs. The mechanisms associated with NT/NM-mediated synaptic dysfunction in AD are discussed here.

Acetylcholine

ACh release is responsible for the regulation of memory storage and cognition in cortex and subcortical circuits [97]. ACh interacts with two types

of receptors; G protein coupled muscarinic acetyl cholinergic receptors (mAChR) and ionotropic nicotinic acetyl cholinergic receptors (nAChR) and triggers distinct responses to different modulators [98]. The α7 nAChR receptors are found to regulate intracellular Ca^{2+} and NT release at synaptic junctions. nAChR are also evident for synaptic plasticity by stimulating the upregulation of LTP in the hippocampus [99]. In fact, the progressive loss of cholinergic signaling is among the major breakdown mechanisms associated with the etiology of AD. For instance, dysfunctional nAChR/mAChR in the cortex resulted in cognitive decline in AD. However, this failure of cholinergic transmission is not merely a loss of ACh containing neurons but also marked by attenuated acetylcholine esterase and choline acetyl transferase activity [100]. Further, SNPs associated with nAChR genes (α3, α4, α7, and β2) have been reported to cause the pathogenesis of AD [101]. Besides, nAChRs are found to trigger synaptopathy due to its up- and downregulation at different sites in AD brain. For instance, chronic Aβ exposure enhanced the levels of α7-nAChR in the hippocampus, cortex, and amygdala both *in vivo* and *in vitro* while marked reduction in α4β2-nAChR is observed in the cortical regions of AD patients [102, 103]. Additionally, the interaction between Aβ and α7-nAChR has also been documented in postmortem AD brains [104].

Dopamine (DA)

DA is a major NT in the central nervous system (CNS) and is characterized as an important modulator of synaptic plasticity. Failure of the DA transmission system results in apathy, a negative prognostic indicator of normal aging as well as AD. The occurrences of apathy and motor dysfunctions were predictive of rapid cognitive loss and shorter lifespan in AD patients [105]. Various studies have identified a reduced level of DA at the site of Aβ plaques and NFTs in nigrostriatal regions of AD brain, depicting its prominent role in pathogenesis and impaired cognition [106]. In a study of the 3xTg AD model, Aβ-induced impaired dopaminergic neurotransmission resulted in conversion of LTP into LTD, which led to poor memory and neuroplastic insults in the basolateral amygdaloid nucleus-insular cortex pathway [107]. Moreover, Aβ favored LTD upon low frequency stimulation while restricted LTP after high frequency stimulation. It has also been observed that expression of dopaminergic receptors D1 and D2 is significantly reduced in the prefrontal cortex and hippocampus region of AD patients, while stimulation of dopaminergic transmission improved cognitive function in various animal models of AD. Recently, DA has been shown to possess anti-amyloidogenic and antioxidant effects in mice [108]. Interestingly, the administration of dopaminergic drugs exhibited better cortical plasticity and memory functions in AD patients.

Gamma-amino butyric acid (GABA)

GABA is the principal inhibitory NT in the CNS, which is synthesized by decarboxylation of glutamate with the help of glutamic acid decarboxylase enzyme. Synthesized GABA is transported into vesicles by vesicular GABA transporter at presynaptic terminals of neurons [109]. There are three distinct receptor subfamilies of GABA namely $GABA_A$, $GABA_B$, and $GABA_C$ receptors that contribute toward their inhibitory effects, where $GABA_A$ and $GABA_C$ receptors are ligand-gated chloride (Cl^-) channels, while $GABA_B$ receptors are G-protein coupled receptors [110]. The alteration in the balance between inhibitory GABA and excitatory glutamate NTs were found to be one of the pathological factors contributing toward synaptic dysfunction. Aβ fibrils were found to cause perforations in the cell membrane leading to enhanced Ca^{2+} influx mediated over-excitation and consequent epileptic seizures in the hippocampus and cortex. The increased seizures in turn cause alterations in GABAergic sprouting and synaptic inhibition as a protective mechanism to overcome the hyper-excitation of neurons [38]. Immunohistochemical study of GABAergic receptors revealed that α2, β1, and γ1 subunits of $GABA_A$ receptors get upregulated whereas the levels of α1 and γ2 subunits get downregulated in AD brains, indicating a functional remodeling of GABAergic neurotransmission in the cortex of AD patients [111]. Furthermore, elevated inhibitory function of GABAergic synapses induced by glutamate mediated NMDA receptor activation affects the processes required for LTP in dentate gyrus. Therefore, crosstalk between $GABA_A$ receptors and postsynaptic glutamate NMDA receptors are evident in AD pathology [112].

Glutamate

Glutamate is one of the important excitatory NTs that play a crucial role in neural activation with the help of its receptors localized on neuronal mem-

brane. A wide variety of glutamatergic receptors, namely NMDA and AMPA receptors, have been implicated in synaptopathy while normally they are known to regulate synaptic plasticity, neurotransmission, learning, and memory [113]. Numerous studies reported that glutamatergic neurons get lost in response to Aβ accumulation selectively at some synapses in the pathogenesis of AD. These Aβ oligomers also upregulate the extracellular concentration of glutamate in hippocampus of AD brain [39] and directly impact AMPA and NMDA receptors through various subunits including GluR2 and GluN2B. Moreover, Aβ is found to bind with GluR2 subunit via clathrin mediated activation of calcineurin and dynamin [114] and further downregulate AMPA-mediated signal transmission and synaptic plasticity via nuclear translocation of Jacob protein and induction of accompanying CREB shutoff signaling [115]. Furthermore, oligomeric Aβ exposure upregulates GluN2B containing NMDA and extrasynaptic NMDA receptors and disturbs signal transmission [116]. Another study reported an increase in STriatal-Enriched protein tyrosine Phosphatase (STEP) activity upon Aβ and tau exposure leading to GluN2B containing NMDA receptor endocytosis via dephosphorylation of Src kinases Fyn and GluN2B at tyrosine (Y1472) [117]. The increase in STEP activity further disrupts synaptic plasticity and affects cognitive functions in AD. It has also been revealed that $A\beta_{1\text{-}42}$ oligomers form clusters at synaptic junctions and trigger subsequent decreases in the mGlu5 receptor's mobility and distribution leading to intracellular Ca^{2+} release. The disrupted Ca^{2+} homeostasis triggers mitochondrial dysfunction mediated ATP loss and ROS generation, ultimately causing LTD via GSK3β and calcineurin over-stimulation [5]. Furthermore, Aβ-induced reduction in LTP specifically involves caspase 3 activation and subsequent Akt cleavage in AD patients [118].

Histamine (HA)

HA is a NT that directs crucial physiological functions such as sleep cycle, synaptic plasticity, cognition, and movement. The hypothalamic tuberomammillary nucleus (TMN) is the site in the adult mammalian brain where somas of HA producing neurons are located and extends throughout the CNS [119]. The action of HA is mediated by the activation of four G coupled protein receptors, namely H1R, H2R, H3R, and H4R, which are widespread in the brain. Specifically, H1 receptors were reported to be reduced in the frontal and temporal regions of AD brain. The key association between HA and AD can be ascertained from the fact that the level of HA is markedly elevated in different regions of CNS in AD patients [120]. HA is also found to regulate neuroinflammation along with TNF-α and IL-1β in hippocampal neurons, which is responsible for poor cognition and impaired cerebrovascular functions in AD. Moreover, an association between microglial activation and APOE has been reported in AD patients, where HA levels were found to correlate with APOE; for instance, patients carrying the APOE-4 alleles had lowest HA levels in the brain [121]. Furthermore, it has been identified that HA-containing neurons in the TMN get reduced in association with accumulated NFTs while its level was found to be upregulated in cerebrospinal fluid and serum of AD patients [122].

Norepinephrine

Norepinephrine is a catecholamine, synthesized through a cascade of enzymatic reactions where dopamine is converted into norepinephrine through the action of dopamine β-hydroxylase. Norepinephrine either can act on target receptors (α1, α2, and β) or can be re-uptaken into presynaptic neurons via Na/K-dependent norepinephrine transporters. The primary function of noradrenergic transmission includes regulation of spatial working memory, neuroinflammation, and cellular metabolism [123]. Moreover, norepinephrine also regulates neuroinflammation through adrenergic receptors present in astrocytes and glial cells where any aberration in adrenergic signaling leads to the progression of AD [124]. A recent study evidenced the administration of a selective neurotoxin DSP-4 against noradrenergic neurons that caused enrichment of Aβ deposition, altered spatial memory, and impaired receptor binding sites of α1, α2, and β and upregulation of hyperphosphorylated tau in a transgenic mice model of AD. Furthermore, several studies have also reported impaired LTP and cognition in norepinephrine-compromised hybrid AD mice models [125]. A few reports have linked polymorphisms in the dopamine β-hydroxylase gene leading to reduced norepinephrine production in selective Caucasian populations with AD [126]. In another experiment, the endogenous α2A receptors are shown to contribute in the cascade for AD progression [127]. Likewise, Aβ activates β2 receptors to trigger the

hyperphosphorylation of tau via protein kinase-A and c-Jun N-terminal kinase (PKA-JNK) signaling in AβPP/PS1 mice model [128].

Serotonin

Serotonin is a biogenic monoamine, which regulates important physiological functions in CNS such as mood, pain, anger, aggression, sleep, and appetite [129], and it serves as both NT as well as NM [130]. In general, serotonin regulates crucial mechanisms like learning and memory both in healthy as well as in aged individuals. This is the reason that neurological disorders such as AD are marked by aberrant serotonergic signaling and altered 5-hydroxytryptamine (5-HT) metabolism in the CNS [131]. A specific class of receptors called the 5-HT receptors orchestrates the activity of serotonin. Though a number of 5HTRs (5HT2AR, 5HT2CR, 5HT4R etc.) are involved in AβPP processing, 5HT4R has gained attention by reinstating a neuroprotective environment by inducing non-amyloidogenic AβPP cleavage mediated release of soluble AβPPα [132, 133]. The investigations reported reduced levels of serotonergic neurons and 5HT metabolites in the raphe nuclei of AD postmortem brains [134, 135]. Likewise, Aβ plaques in the projection site of serotonergic neurons triggered 5HT neuronal apoptosis accompanied by loss of neuronal cell bodies in an AβPP transgenic mice model [136]. Additionally, a link between tau phosphorylation and 5HTRs have further strengthened the notion that 5HTRs are closely associated with AD via Fyn mediated ERK1/2 activation [137]. Moreover, tau hyperphosphorylation in the raphe nuclei is also evident to induce 5HT-mediated neuronal cell death in AD brain [138]. The altered NTs/NMs and their associated factors in the etiology of AD have been depicted in Fig. 2.

OTHER NEUROTRANSMITTERS/NEUROMODULATORS

Additionally, various other NTs/NMs such as Reelin, Interleukin-33 (IL-33), Purinergic receptors, and TRP channels are identified that contribute significantly in the synaptopathy of AD. For instance, disruption in the activity of Reelin causes aberrant hyperphosphorylation of tau by altering the signaling cascades associated with GSK3β activity [139]. Another NT, interleukin-33 (IL-33), is found to regulate inflammation in neuronal cell since IL-33 depletion released IL-1β and TNF-α that contributed well in neuroinflammatory synaptic loss in AD brain [140]. Likewise, dysfunction of purinergic receptors also causes neuroinflammation, ATP release, and alteration in Ca^{2+} influx and thus induces LTD at synaptic junctions [141]. There are several TRP channels (TRPC, TRPV, TRPM, TRPP, TRPML, and TRPA) that are involved in the alteration of Ca^{2+} influx, modulation of the PSD95 pathway, and GSK3β phosphorylation mediated synaptic plasticity [142, 143]. Herein, the potential mechanisms of synaptopathy associated with NTs/NMs have been described in (Table 3).

CORRECTION MECHANISMS TO TARGET PERTURBED NEUROTRANSMITTERS/NEUROMODULATORS IN SYNAPTOPATHY

Presently, treatment against defective NT/NM-mediated synaptopathy in AD has become a demanding task for neurobiologists since there is not a single factor responsible for such abnormalities but a massive numbers of factors associated with widely dispersed signaling cascades. Nevertheless, defective NT release at the synaptic junction causes several detrimental effects, which are associated with the altered activity of synaptic proteins, transcription factors, Ca^{2+} homeostasis, and other molecules critical for neuronal plasticity [168]. These detrimental effects further disrupt the neuronal homeostasis and thereby cause the synaptic insults. In order to overcome such complications, numerous therapeutic strategies are currently being devised that alleviate the toxicity associated with defective NTs/NMs. For instance, numerous biological compound-based therapies have been designed to overcome the problems associated with these defective NTs/NMs in AD. Moreover, current therapeutic approaches have also been discussed here that can slow down the pathophysiology behind defective NT/NM-mediated synaptic dysfunction.

Biological compound-mediated therapy for altered neurotransmitters/neuromodulators in synaptopathy

The reduction in cholinergic neurotransmission in AD has led to the development of numerous compounds as the first-line of treatment for the pathological phenomenon of this disease. The clinical

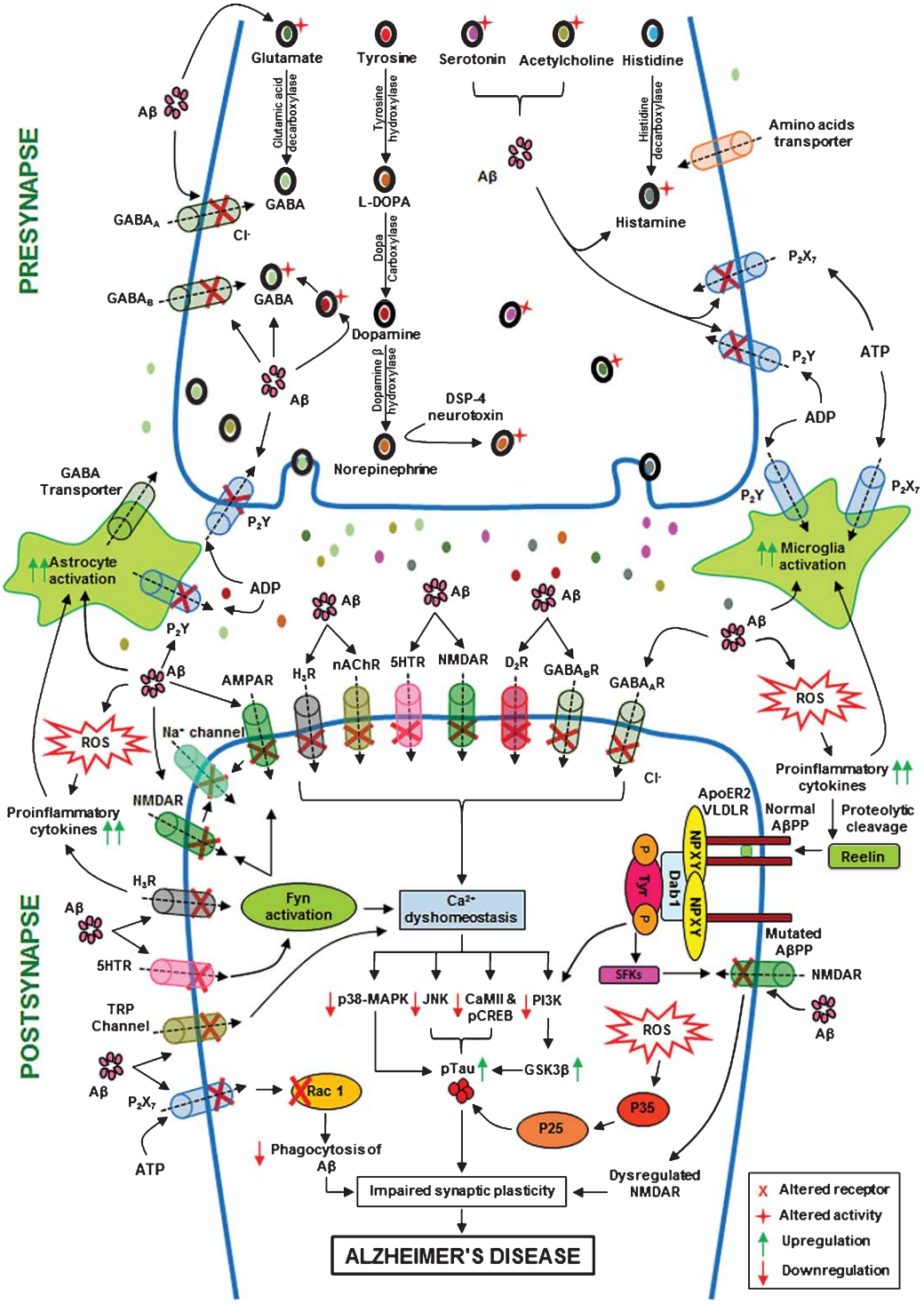


Fig. 2. Molecular mechanisms associated with defective neurotransmitters/neuromodulators (NTs/NMs) in Alzheimer's disease and their associated factors. GABA, γ-Aminobutyric acid; L-DOPA, L-3,4-dihydroxyphenylalanine P_2X_7, purinoceptor 7, P_2Y, purinoceptor; DSP-4, neurotoxin; NMDAR, N-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; H_3R, histamine receptor, nAChR, nicotinic acetylcholine receptor; 5HTR, 5-hydroxytryptamine (serotonin receptor); D_2R, dopamine receptor D2; AβPP, amyloid-β protein precursor; ATP, adenosine triphosphate; ADP, adenosine diphosphate; ROS, reactive oxygen species; VLDLR, very-low-density-lipoprotein receptor; TRP, transient receptor potential.

Table 3
List of neurotransmitters/neuromodulators and their mechanism associated with synaptopathy in AD

Neurotransmitters/ Neuromodulators	Involved causative factors/receptors	Involved mechanisms in synaptopathy	Principle phenotype	References
Acetylcholine (ACh)	Aβ, tau, cholesterol depletion, metal ion dyshomeostasis, and oxidative stress	Abnormalities in AChR causes downregulation of both MAPK-CREB and PI3K-Akt signaling cascade and also causes alteration in the level of glutamate that affects synaptic plasticity		[144, 145]
Glutamate	Factors associated with inhibition of protein phosphatases, Aβ, tau, cholesterol depletion, metal ion dyshomeostasis, IGF-1 depletion, altered level of dopamine, and elevated level of glucose	Glutamate toxicity causes alteration in the level of intracellular Na^+, K^+, and Ca^{2+}. It also causes impairment in NMDA, AMPA, and mGlu receptor and ultimately impairs LTP and LTD thus induces synaptic loss associated cognitive decline		[39, 114, 146–149]
Gamma Amino Butyric Acid (GABA)	KNCQ Channels, AβPP, Aβ, mitochondrial dysfunction, lipid depletion, and altered level of dopamine	GABAergic dysfunction causes alteration in Ca^{2+} channels and block μ-opioid receptors thus impairs synaptic plasticity		[38, 150–153]
Noradrenaline/ Norepinephrine	Microglia dysfunction, involvement of complement system, usage of DSP-4 and reserpine, and knockout of DβH enzyme	Norepinephrine dysfunction causes alteration in PKA-JNK pathway, microglia activation, and impairs LTP and LTD, thereby leading to synaptic loss associated with cognitive decline		[123, 154, 155]
Serotonin	Aβ and tau	Dysfunction of serotonin impairs cholinergic receptors, diminishes arachidonic acid (AA) uptake and causes axonal dysfunction thereby leading to the degeneration of raphe monoamine neurons and triggers synaptic loss associated with memory loss	Alzheimer's Disease	[138, 156, 157]
Histamine (HA)	Impact of GABAergic and glutamatergic receptors and dysregulation of mast cells	HA dysfunction impairs cholinergic and NMDA receptors, leading to synaptic dysfunction		[158]
Reelin	APOER2, VLDLR, DAB1 dysfunction, and Aβ	Depletion of Reelin causes alteration of signaling pathways that regulates GSK3β activity and enhances the level of hyperphosphorylated tau thereby leading to dysfunction of synapses		[159–161]
Purinoceptors	Loss of $P2X_7$ and $P2Y_2$, microglia activation, and Aβ	Loss of $P2X_7$ and $P2Y_2$ receptors causes neuroinflammation via activation of microglia and dysfunction of Rac1 protein which thereafter causes dysregulation of Aβ phagocytosis that ultimately leads to synaptic loss		[162–164]
Interleukin-33 (IL-33)	Microglia activation, Aβ, increased level of IL-1β and TNF-α	IL-33 is proinflammatory in CNS and activates inflammatory cells thereby leading to the disruption of synapses		[140, 165]
TRP Channels	Factors associated with oxidative stress and mitochondrial dysfunction, Aβ, mutated PSEN1 and AβPP	TRP channels disrupt Ca^{2+} homeostasis and alters various signaling cascade leading to synaptic loss		[166]
Dopamine (DA)	Aβ, Ca^{2+}, degeneration of cholinergic neurons, and inactivation of p38-MAPK pathways	DA dysfunction causes alteration in glutamatergic and cholinergic transmission, impairs physiological cell metabolism, activates various enzymatic pathways, leading to apoptosis and synaptic loss		[106, 167]

Table 4
List of potent biological compounds involved in alleviating the neurotoxic effect of altered neurotransmitters/neuromodulators in synaptopathy

SN	Biological compounds	Associated protective mechanisms against synaptic loss	Principle phenotype	References
1	Anthocyanine (ANT)	It regulates ion pump activity and cholinergic neurotransmission. Further, it also enhances memory and act as an anxiolytic compound	Alzheimer's disease	[174]
2	Oridonin (Ori)	Augments the expression of PSD-95 and synaptophysin and also promotes mitochondrial dynamics in the synaptosomes of AD mice thereby leading to attenuate synaptic loss and cognitive deficits. Moreover, it also activates the BDNF/TrkB/CREB signaling pathway in the hippocampus of AD mice		[175]
3	Asiaticoside	It prevents spatial learning and memory decline by scavenging free radicals, decreasing the level of Aβ, upregulating the activity of antioxidant enzymes, ameliorating dysfunction in synaptic plasticity and reversing abnormal changes in ACh level and AChE activity		[176]
4	Cotinine	The beneficial effects of cotinine are accompanied by an increase in the expression of the active form of PKB and the PSD-95 in the hippocampi and frontal cortices of Tg6799 mice. This PSD95 plays a crucial role in promoting brain plasticity by modulating NMDA receptor signaling. Additionally, it also increases 5-HT levels in the rat brains thereby stimulating the serotonergic activity in AD brain		[177]
5	Piracetam	It is a derivative of the neurotransmitter GABA and modulates neurotransmission in a range of transmitter systems (including cholinergic and glutamatergic), thus improves neuroplasticity. It also significantly restores impaired synaptic function by attenuating numerous mitochondrial dysfunctions such as, mitochondrial membrane potential and ATP production, induced by Aβ in PC12 cells		[178]
6	Naringin	It improves long-term learning and memory ability by both enhancing the autophosphorylation of CaMKII and increasing the phosphorylation of the AMPA receptor at a CaMKII-dependent site		[179]
7	7, 8-dihydroxyflavone (7,8-DHF)	Inhibits the loss of hippocampal synapses, restores synapse number and synaptic plasticity, and prevents memory deficits induced by impaired BDNF/TrkB signaling pathway in the hippocampus of AD mice		[180]
8	Memantine	A non-competitive NMDA receptor antagonist attenuates cognitive impairment and synaptic dysfunction by restoring protein phosphatase-2A activity via decreasing its demethylation at Leu309 selectively in adeno-associated virus vector-1-I1PP2A rats. Further, it also alleviates glutamate mediated cell excitotoxicity by excessive stimulation of NMDA receptors in astrocytes and neurons		[181]
9	Propofol	Acts as a $GABA_A$ receptor agonist which reduces Aβ generation and accelerates Aβ degradation. Further, it ameliorates cognitive function and attenuates caspase-3, caspase-9 activation in AD mice model		[112]
10	Metformin	It alleviates the increase of total tau, phospho-tau and activated JNK. Further, it also attenuates the reduction of synaptophysin, a synaptic protein in the db/db mouse hippocampus		[182]
11	Cannabidiol (CBD)	It shows neuroprotection by normalizing the caspase 3 activity, the main integral transmembrane protein of synaptic vesicles (synaptophysin) and mitochondrial fission protein DNM1L expression levels in rats with brain iron overload. CBD treatment has also been reported to improve dopamine neurotransmission in in a mouse model of tauopathy		[79, 183]

(*Continued*)

Table 4
(*Continued*)

SN	Biological compounds	Associated protective mechanisms against synaptic loss	Principle phenotype	References
12	Dizocilpine (MK801)	MK801 alleviates glutamate mediated cell excitotoxicity by excessive stimulation of NMDA receptors in astrocytes and neurons		[184]
13	UBP141	UBP141, an astroglial NMDA receptors antagonist plays a potential therapeutic role against synapses loss		[185]
14	Levetiracetam	Chronic levetiracetam administration reduces glutamate excitotoxicity and enhances the levels of inhibitory neurotransmission thereby attenuating cognitive abnormalities in AD		[186]
15	Muscimol	Selective $GABA_A$ receptor agonist provides protection against Aβ-induced neurotoxicity in hippocampal, retinal and cortical neurons in rodents		[187]
16	Etazolate (EHT-0202)	The $GABA_A$ receptor modulator protects neurons against Aβ-induced toxicity thereby displaying anti-inflammation effect after traumatic brain injury and improves cognition in mice models		[188]
17	Pentobarbital	Stimulation of GABA receptors by pentobarbital restores neuronal maturation and neurogenesis in apolipoprotein E4 (APOE4) knocking mice. APOE4 is known as a genetic risk factor for early onset AD perhaps by accelerating Aβ plaque formation, or by impairing neuron repair		[112]
18	Flumazenil or picrotoxin	Chronic systemic treatment with $GABA_A$ antagonists restores memory loss in AD		[189]
19	EVP-6124	EVP-6124, a selective agonist of the α7 nAChR, exerts an excitatory effect on the postsynaptic neuron thereby leading to LTP and memory formation		[190]
20	D-serine acid oxidase	Treatment with D-serine acid oxidase attenuates NMDA-induced excitotoxicity		[191]
21	5,7-dicholorokynurenic acid	The physiological glycine site antagonist kynurenic acid protects against NMDA-induced excitotoxicity		[191]
22	Methyl-4-carboxyphenylglycine (MCPG)	MCPG, a non-selective group I/II mGluR antagonist, prevents the reduction induced by Aβ in miniature excitatory post-synaptic cells		[192]
23	2-methyl-6-(phenylethynyl)-pyridine	An mGluR5 antagonist rescues LTP facilitation in murine hippocampal slices induced by oligomers of Aβ extracted from AD brains		[192]
24	SIB1757	An mGluR5 antagonist prevents LTD facilitation in rat hippocampal slices induced by oligomers of Aβ extracted from AD brains		[193]
25	Melatonin	It shows neuroprotection against Aβ induced neurotoxicity, mediated by activation of GABA receptors and the resulting hyper-polarization of the neurons		[194]
26	6,7-dinitroquinoxaline-2,3-dione	An AMPA receptor and kainate receptor antagonist attenuates excitotoxic cell death induced by Aβ retinal cell cultures		[192]

advantages of these compounds include significant improvements in NT release, improvement in altered synaptic plasticity, and attenuation of memory loss and cognitive failure. Several compounds have been identified so far to target altered NT/NM activity in AD. Curcumin, a major active component of turmeric, has been found to regulate the levels of dopamine, norepinephrine, serotonin, and glutamate in the brain and thus significantly reduce behavioral symptoms of AD. Moreover, it also acts as an inhibitor of mono-amine oxidase (MAO)-A and MAO-B enzyme, which is also crucial for depleting dopamine and serotonin [169]. Similarly, galantamine, an acetylcholinesterase inhibitor and an allosteric regulator of nAChR, has been reported in the treatment of severe dementia associated with AD. Additionally, it also influences diverse other NT systems, possibly modulating the activity of dopamine, serotonin, glutamate, and GABA in certain nerve tracts [170]. Further, numerous other chemical compounds, including donepezil (E2020), rivastigmine, and tacrine have been introduced for

the inhibition of acetylcholinesterase activity in AD [171]. Currently, nicotine has also been reported to reduce Aβ toxicity through the activation of α7 nicotinic acetylcholine receptor/phosphatidylinositol 3-kinase (α7nAChR/PI3K) signaling pathway and its cross-talk with the Wnt signaling pathway [172]. In a study, the effects of natural cannabinoids (Sativex®) have been reported to improve dopamine neurotransmission [173]. Similarly, many other biological compounds have been identified and implicated so far to target altered NT activity in the AD brain, which have been outlined in Table 4. These compounds bind to NTs and their specific receptors thereby reducing the severity of disease atmosphere.

Recent therapeutic strategies for targeting perturbed neurotransmitters/neuromodulators in synaptopathy

Although the significant role of distinct chemical compounds has been explored extensively to target altered NTs/NMs in synaptopathy, there are numerous other new therapeutic strategies that have been identified so far to target such alterations. For instance, the neuroprotective effect of Nanodiamond against memory deficits has currently been reported, where it showed a protective role by modulating NF-kB and STAT3 signaling cascade, the effects mediated by the regulation of NMDARs [195]. Further, treatment using vitamin D3 has shown a

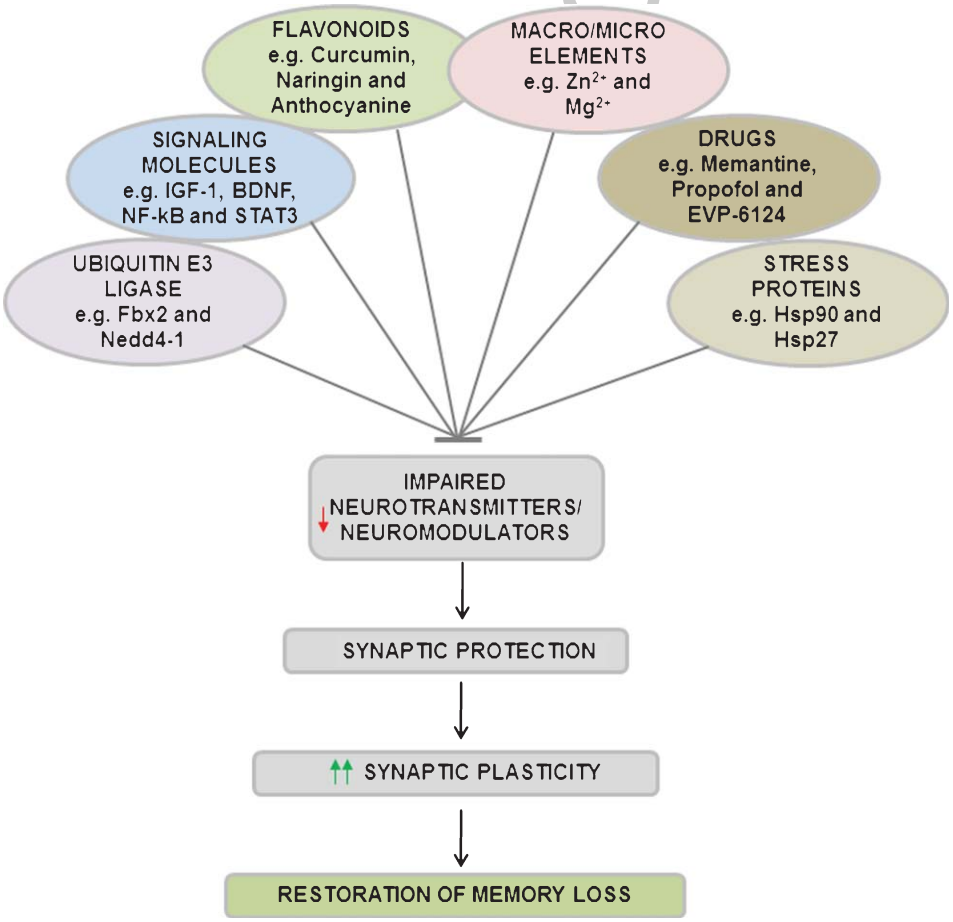


Fig. 3. Pictorial representation showing the involvement of different therapeutic approaches against synaptic loss associated with impaired neurotransmitters/neuromodulators (NTs/NMs). Fbx2, F-Box Protein 2; IGF-1, insulin-like growth factor 1; brain-derived neurotrophic factor; NF-kB, Nuclear factor-κB; STAT3, signal transducer and activator of transcription 3; Hsp90, heat shock protein 90; Hsp27, heat shock protein 27.

protective role against synaptic loss via significantly increasing the levels of ACh in neurons [196]. Treatment with zinc ion (Zn^{2+}) showed enhanced levels of NT release in aluminum (Al^{3+})-treated animals, thereby showing their protective role against synaptic injury, since Al^{3+} exposure decreases the level of NTs and acetylcholinesterase activity in brain and leads to the neuronal dysfunction [197]. Similarly, elevated levels of magnesium ion (Mg^{2+}) also exert substantial synaptoprotective effects in AD brains where it controls the synaptic density/plasticity bt preventing the onset of impaired NMDA receptor signaling pathway [198]. Interestingly, insulin is being implicated nowadays to attenuate the cognitive dysfunctions through its regulatory effect on the expression of NMDA receptors and on the associated insulin signaling cascade in AD [199]. Another therapeutics strategy to ameliorate Aβ-mediated synaptic loss is using Substance P, which is a member of the tachykinin family, distributed widely in the CNS and acts as a NT/NM as well as neurotrophic factor. Further, Substance P is able to provoke non-amyloidogenic AβPP processing, thereby curtailing the feasibility of Aβ peptides production in the brain [200]. Additionally, several studies have highlighted the role of the ubiquitin-proteasome system as a therapeutic approach to target synaptic loss induced by altered release of NTs at synapses. The ubiquitin-proteasome system is found to modulate NTs/NMs, synaptic proteins, transcription factors, and other molecules critical for neuronal plasticity. For instance, altered NMDA receptors are retro-translocated and degraded by a F-box protein called Fbx2, advocating that SCF-type ligases targets NMDA receptors for ubiquitination [201]. Another protein Nedd4-1, known as a HECT E3 ubiquitin ligase, has also found to target Aβ-induced reductions in surface AMPARs, dendritic spine density, and synaptic strength [202]. Likewise, heat shock protein (HSP)-based therapeutic approach has also been currently addressed in numerous studies. For example, HSP90 is being used nowadays to modulate NT release at the presynaptic terminals independently by mediating the continuous cycling of synaptic AMPA receptors [203]. Moreover, the hypothetical representation of numerous pharmacological approaches to compensate neurotransmission imbalance has been depicted in Fig. 3. Nevertheless, several other suitable approaches still need to be devised in the near future for effective treatment against synaptic dysfunctions mediated by altered NTs/NMs.

CONCLUSION

In this review, we have discussed the pertinent role of synaptic plasticity in memory formation, its stabilization, and associated abnormalities due to Aβ accumulation and tau phosphorylation. For instance, increased levels of toxic Aβ and tau oligomers at synaptic junctions are responsible for neuronal toxicity, which is associated with synaptic loss in AD. Similarly, involvement of oxidative stress, activation of redox signaling, and subsequent damage to mitochondrial integrity in synaptic alteration has also been elucidated. Additionally, the altered activity of various NTs/NMs including glutamatergic, GABAergic, and acetylcholinergic receptors with respect to Aβ accumulation and tau phosphorylation has also been extensively reviewed, since NTs/NMs in their normal form play a crucial role in maintaining neuronal homeostasis in the brain. However, any alterations in their proper functioning cause several neurotoxic effects associated with altered activity of synaptic proteins, transcription factors, Ca^{2+} homeostasis, and other molecules critical for neuronal plasticity. These factors under the diseased state disrupt normal homeostasis of neurons, thereby causing synaptic loss. Furthermore, in order to target the malfunctioning NTs/NMs or reverse their associated chronic effects, numerous biological compound-mediated therapeutic strategies have been discussed to obviate the disease symptoms of AD. Additionally, recent therapeutic strategies for targeting synaptic loss induced by defective NTs/NMs have been addressed. Finally, this review accentuates the savvy of altered redox signaling and impaired neurotransmission in synaptic dysfunction during synaptopathy that could unveil mechanism-based therapeutics and ameliorated inferential strategies.

ACKNOWLEDGMENTS

The authors would like to thank the DTU senior management for encouragement and facility.

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/16-0623r1).

REFERENCES

[1] Parikh V, Bernard CS, Naughton SX, Yegla B (2014) Interactions between Aβ oligomers and presynaptic cholinergic signaling: Age-dependent effects on attentional capacities. *Behav Brain Res* **274**, 30-42.

[2] Zarate CA Jr, Manji HK (2008) The role of AMPA receptor modulation in the treatment of neuropsychiatric diseases. *Exp Neurol* **211**, 7-10.
[3] Guerrero-Muñoz MJ, Gerson J, Castillo-Carranza DL (2015) Tau oligomers: The toxic player at synapses in Alzheimer's disease. *Front Cell Neurosci* **9**, 464.
[4] Nava-Mesa MO, Jiménez-Díaz L, Yajeya J, Navarro-Lopez JD (2014) GABAergic neurotransmission and new strategies of neuromodulation to compensate synaptic dysfunction in early stages of Alzheimer's disease. *Front Cell Neurosci* **8**, 167.
[5] Kamat PK, Kalani A, Rai S, Swarnkar S, Tota S, Nath C, Tyagi N (2016) Mechanism of oxidative stress and synapse dysfunction in the pathogenesis of Alzheimer's disease: Understanding the therapeutics strategies. *Mol Neurobiol* **53**, 48-61.
[6] Butterfield DA, Perluigi M, Reed T, Muharib T, Hughes CP, Robinson RA, Sultana R (2012) Redox proteomics in selected neurodegenerative disorders: From its infancy to future applications. *Antioxid Redox Signal* **17**, 1610-1655.
[7] Missler M, Südhof TC, Biederer T (2012) Synaptic cell adhesion. *Cold Spring Harb Perspect Biol* **4**, a005694.
[8] Henstridge CM, Pickett E, Spires-Jones TL (2016) Synaptic pathology: A shared mechanism in neurological disease. *Ageing Res Rev* **28**, 72-84.
[9] Duric V, Clayton S, Leong ML, Yuan LL (2016) Comorbidity factors and brain mechanisms linking chronic stress and systemic illness. *Neural Plast* **2016**, 5460732.
[10] Takeuchi T, Duszkiewicz AJ, Morris RG (2013) The synaptic plasticity and memory hypothesis: Encoding, storage and persistence. *Philos Trans R Soc Lond B Biol Sci* **369**, 20130288.
[11] Hamley IW (2012) The amyloid beta peptide: A chemist's perspective. Role in Alzheimer's and fibrillization. *Chem Rev* **112**, 5147-5192.
[12] Ballatore C, Lee VM, Trojanowski JQ (2007) Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat Rev Neurosci* **8**, 663-672.
[13] Mondragón-Rodríguez S, Trillaud-Doppia E, Dudilot A, Bourgeois C, Lauzon M, Leclerc N, Boehm J (2012) Interaction of endogenous tau protein with synaptic proteins is regulated by N-Methyl-d-aspartate receptor-dependent tau phosphorylation. *J Biol Chem* **287**, 32040-32053.
[14] Tracy TE, Sohn PD, Minami SS, Wang C, Min SW, Li Y, Zhou Y, Le D, Lo I, Ponnusamy R, Cong X, Schilling B, Ellerby LM, Huganir RL, Gan L (2016) Acetylated tau obstructs KIBRA-mediated signaling in synaptic plasticity and promotes tauopathy-related memory loss. *Neuron* **90**, 245-260.
[15] Shankar GM, Walsh DM (2009) Alzheimer's disease: Synaptic dysfunction and Abeta. *Mol Neurodegener* **4**, 48.
[16] Overk CR, Masliah E (2014) Pathogenesis of synaptic degeneration in Alzheimer's disease and Lewy body disease. *Biochem Pharmacol* **88**, 508-516.
[17] Moreira PI, Carvalho C, Zhu X, Smith MA, Perry G (2010) Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology. *Biochim Biophys Acta* **1802**, 2-10.
[18] Woods NK, Padmanabhan J (2012) Neuronal calcium signaling and Alzheimer's disease. *Adv Exp Med Biol* **740**, 1193-1217.
[19] Rushworth JV, Hooper NM (2010) Lipid rafts: Linking Alzheimer's amyloid-β production, aggregation, and toxicity at neuronal membranes. *Int J Alzheimers Dis* **2011**, 603052.
[20] Malchiodi-Albedi F, Petrucci TC, Picconi B, Iosi F, Falchi M (1997) Protein phosphatase inhibitors induce modification of synapse structure and tau hyperphosphorylation in cultured rat hippocampal neurons. *J Neurosci Res* **48**, 425-438.
[21] Jang SS, Royston SE, Lee G, Wang S, Chung HJ (2016) Seizure-induced regulations of amyloid-β, STEP61, and STEP61 substrates involved in hippocampal synaptic plasticity. *Neural Plast* **2016**, 2123748.
[22] Abdul Rahman NZ, Greenwood SM, Brett RR, Tossell K, Ungless MA, Plevin R, Bushell TJ (2016) Mitogen-activated protein kinase phosphatase-2 deletion impairs synaptic plasticity and hippocampal-dependent memory. *J Neurosci* **36**, 2348-2354.
[23] Maynard CJ, Bush AI, Masters CL, Cappai R, Li QX (2005) Metals and amyloid-beta in Alzheimer's disease. *Int J ExpPathol* **86**, 147-159.
[24] Wang WY, Tan MS, Yu JT, Tan L (2015) Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Ann Transl Med* **3**, 136.
[25] Rudy CC, Hunsberger HC, Weitzner DS, Reed MN (2015) The role of the tripartite glutamatergic synapse in the pathophysiology of Alzheimer's disease. *Aging Dis* **6**, 131-148.
[26] Mookherjee P, Green PS, Watson GS, Marques MA, Tanaka K, Meeker KD, Meabon JS, Li N, Zhu P, Olson VG, Cook DG (2011) GLT-1 loss accelerates cognitive deficit onset in an Alzheimer's disease animal model. *J Alzheimers Dis* **26**, 447-455.
[27] Kempf SJ, Metaxas A, Ibáñez-Vea M, Darvesh S, Finsen B, Larsen MR (2016) An integrated proteomics approach shows synaptic plasticity changes in an APP/PS1 Alzheimer's mouse model. *Oncotarget*. doi: 10.18632/oncotarget.9092
[28] Tu S, Okamoto S, Lipton SA, Xu H (2014) Oligomeric Aβ-induced synaptic dysfunction in Alzheimer's disease. *Mol Neurodegener* **9**, 48.
[29] Marttinen M, Kurkinen KM, Soininen H, Haapasalo A, Hiltunen M (2015) Synaptic dysfunction and septin protein family members in neurodegenerative diseases. *Mol Neurodegener* **10**, 16.
[30] Ross JA, McGonigle P, Van Bockstaele EJ (2015) Locus coeruleus, norepinephrine and Aβ peptides in Alzheimer's disease. *Neurobiol Stress* **2**, 73-84.
[31] Sheng M, Sabatini BL, Südhof TC (2012) Synapses and Alzheimer's disease. *Cold Spring Harb Perspect Biol* **4**, a005777.
[32] Nava-Mesa MO, Jiménez-Díaz L, Yajeya J, Navarro-Lopez JD (2013) Amyloid-β induces synaptic dysfunction through G protein-gated inwardly rectifying potassium channels in the fimbria-CA3 hippocampal synapse. *Front Cell Neurosci* **7**, 117.
[33] Deak F, Sonntag WE (2012) Aging, synaptic dysfunction, and insulin-like growth factor (IGF)-1. *J Gerontol A Biol Sci Med Sci* **67**, 611-625.
[34] Liu DS, Pan XD, Zhang J, Shen H, Collins NC, Cole AM, Koster KP, Ben Aissa M, Dai XM, Zhou M, Tai LM, Zhu YG, LaDu M, Chen XC (2015) APOE4 enhances age-dependentdecline in cognitive function by down-regulating an NMDA receptor pathway inEFAD-Tg mice. *Mol Neurodegener* **10**, 7.
[35] Ardiles AO, Tapia-Rojas CC, Mandal M, Alexandre F, Kirkwood A, Inestrosa NC, Palacios AG (2012) Postsynaptic dysfunction is associated with spatial and object recognition memory loss in a natural model of

Alzheimer's disease. *Proc Natl Acad Sci U S A* **109**, 13835-13840.

[36] Mucke L, Selkoe DJ (2012) Neurotoxicity of amyloid β-protein: Synaptic and network dysfunction. *Cold Spring Harb Perspect Med* **2**, a006338.

[37] Parihar MS, Brewer GJ (2010) Amyloid-β as a modulator of synaptic plasticity. *J Alzheimers Dis* **22**, 741-763.

[38] Palop JJ, Mucke L (2010) Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: From synapses toward neural networks. *Nat Neurosci* **13**, 812-818.

[39] Danysz W, Parsons CG (2012) Alzheimer's disease, β-amyloid, glutamate, NMDA receptors and memantine–searching for the connections. *Br J Pharmacol* **167**, 324-352.

[40] Viola KL, Klein WL (2015) Amyloid β oligomers in Alzheimer's disease pathogenesis, treatment, and diagnosis. *Acta Neuropathol* **129**, 183-206.

[41] Kamenetz F, Tomita T, Hsieh H, Seabrook G, Borchelt D, Iwatsubo T, Sisodia S, Malinow R (2003) APP processing and synaptic function. *Neuron* **37**, 925-937.

[42] Malm TM, Jay TR, Landreth GE (2015) The evolving biology of microglia in Alzheimer's disease. *Neurotherapeutics* **12**, 81-93.

[43] Mandrekar-Colucci S, Landreth GE (2010) Microglia and Inflammation in Alzheimer's disease. *CNS Neurol Disord Drug Targets* **9**, 156-167.

[44] Mosher KI, Wyss-Coray T (2014) Microglial dysfunction in brain aging and Alzheimer's disease. *Biochem Pharmacol* **88**, 594-604.

[45] Kimura R, MacTavish D, Yang J, Westaway D, Jhamandas JH (2012) Beta amyloid-induced depression of hippocampal long-term potentiation is mediated through the amylin receptor. *J Neurosci* **32**, 17401-17406.

[46] Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT (2011) Neuropathological alterations in Alzheimer disease. *Cold Spring Harb Perspect Med* **1**, a006189.

[47] Khan SS, Bloom GS (2016) Tau: The center of a signaling nexus in Alzheimer's disease. *Front Neurosci* **10**, 31.

[48] Eckert A, Schmitt K, Götz J (2011) Mitochondrial dysfunction – the beginning of the end in Alzheimer's disease? Separate and synergistic modes of tau and amyloid-β toxicity. *Alzheimers Res Ther* **3**, 15.

[49] Meier S, Bell M, Lyons DN, Rodriguez-Rivera J, Ingram A, Fontaine SN, Mechas E, Chen J, Wolozin B, LeVine H, 3rd, Zhu H, Abisambra JF (2016) Pathological tau promotes neuronal damage by impairing ribosomal function and decreasing protein synthesis. *J Neurosci* **36**, 1001-1007.

[50] Nagura H, Ishikawa Y, Kobayashi K, Takao K, Tanaka T, Nishikawa K, Tamura H, Shiosaka S, Suzuki H, Miyakawa T, Fujiyoshi Y, Doi T (2012) Impaired synaptic clustering of postsynaptic density proteins and altered signal transmission in hippocampal neurons, and disrupted learning behavior in PDZ1 and PDZ2 ligand binding-deficient PSD-95 knockin mice. *Mol Brain* **5**, 43.

[51] Mietelska-Porowska A, Wasik U, Goras M, Filipek A, Niewiadomska G (2014) Tau protein modifications and interactions: Their role in function and dysfunction. *Int J Mol Sci* **15**, 4671-4713.

[52] Usenovic M, Niroomand S, Drolet RE, Yao L, Gaspar RC, Hatcher NG, Schachter J, Renger JJ, Parmentier-Batteur S (2015) Internalized tau oligomers cause neurodegeneration by inducing accumulation of pathogenic tau in human neurons derived from induced pluripotent stem cells. *J Neurosci* **35**, 14234-14250.

[53] Zhou Y, Zhao Y, Xie H, Wang Y, Liu L, Yan X (2015) Alteration in amyloid β42, phosphorylated tau protein, interleukin 6, and acetylcholine during diabetes-accelerated memory dysfunction in diabetic rats: Correlation of amyloid β42 with changes in glucose metabolism. *Behav Brain Funct* **11**, 24.

[54] De Oliveira AS, Santiago FE, Balioni LF, Ferrari Mde F, Almeida MC, Carrettiero DC (2016) BAG2 expression dictates a functional intracellular switch between the p38-dependent effects of nicotine on tau phosphorylation levels via the α7 nicotinic receptor. *Exp Neurol* **275**, 69-77.

[55] Sotiropoulos I, Sousa N (2016) Tau as the converging protein between chronic stress and alzheimer's disease synaptic pathology. *Neurodegener Dis* **16**, 22-25.

[56] Lopes S, Vaz-Silva J, Pinto V, Dalla C, Kokras N, Bedenk B, Mack N, Czisch M, Almeida OF, Sousa N, Sotiropoulos I (2016) Tau protein is essential for stress-induced brain pathology. *Proc Natl Acad Sci U S A* **113**, E3755-E3763.

[57] Maeda S, Djukic B, Taneja P, Yu GQ, Lo I, Davis A, Craft R, Guo W, Wang X, Kim D, Ponnusamy R, Gill TM, Masliah E, Mucke L (2016) Expression of A152T human tau cause age-dependent neuronal dysfunction and loss in transgenic mice. *EMBO Rep* **17**, 530-551.

[58] Kovacic P, Somanathan R (2012) Redox processes in neurodegenerative disease involving reactive oxygen species. *Curr Neuropharmacol* **10**, 289-302.

[59] Arun S, Liu L, Donmez G (2016) Mitochondrial biology and neurological diseases. *Curr Neuropharmacol* **14**, 143-154.

[60] Gandhi S, Abramov AY (2012) Mechanism of oxidative stress in neurodegeneration. *Oxid Med Cell Longev* **2012**, 428010.

[61] Srinivasan S, Avadhani NG (2012) Cytochrome c oxidase dysfunction in oxidative stress. *Free Radic Biol Med* **53**, 1252-1263.

[62] Pope S, Land JM, Heales SJ (2008) Oxidative stress and mitochondrial dysfunction in neurodegeneration; cardiolipin a critical target? *Biochim Biophys Acta* **1777**, 794-799.

[63] Vos M, Lauwers E, Verstreken P (2010) Synaptic mitochondria in synaptic transmission and organization of vesicle pools in health and disease. *Front Synaptic Neurosci* **2**, 139.

[64] Zenisek D, Matthews G (2000) The role of mitochondria in presynaptic calcium handling at a ribbon synapse. *Neuron* **25**, 229-237.

[65] Walker KR, Tesco G (2013) Molecular mechanisms of cognitive dysfunction following traumatic brain injury. *Front Aging Neurosci* **5**, 29.

[66] Rao VK, Carlson EA, Yan SS (2014) Mitochondrial permeability transition pore is a potential drug target for neurodegeneration. *Biochim Biophys Acta* **1842**, 1267-1272.

[67] Santiago-López D, Bautista-Martínez JA, Reyes-Hernandez CI, Aguilar-Martínez M, Rivas-Arancibia S (2010) Oxidative stress, progressive damage in the substantia nigra and plasma dopamine oxidation, in rats chronically exposed to ozone. *Toxicol Lett* **197**, 193-200.

[68] Sheng ZH (2014) Mitochondrial trafficking and anchoring in neurons: New insight and implications. *J Cell Biol* **204**, 1087-1098.

[69] Chen H, Chan DC (2009) Mitochondrial dynamics-fusion, fission, movement, and mitophagy-in neurodegenerative diseases. *Hum Mol Genet* **18**, 69-76.

[70] Yoon SY, Choi HI, Choi JE, Sul CA, Choi JM, Kim DH (2007) Methotrexate decreases PP2A methylation and increases tau phosphorylation in neuron. *Biochem Biophys Res Commun* **363**, 811-816.

[71] Kamat PK, Nath C (2015) Okadaic acid: A tool to study regulatory mechanisms for neurodegeneration and regeneration in Alzheimer's disease. *Neural Regen Res* **10**, 365-367.

[72] Gómez-Ramos A, Díaz-Nido J, Smith MA, Perry G, Avila J (2003) Effect of the lipid peroxidation product acrolein on tau phosphorylation in neural cells. *J Neurosci Res* **71**, 863-870.

[73] Liu SJ, Fang ZY, Yang Y, Deng HM, Wang JZ (2003) Alzheimer-like phosphorylation of tau and neurofilament induced by cocaine *in vivo*. *Acta Pharmacol* **24**, 512-518.

[74] Deng XH, Ai WM, Lei DL, Luo XG, Yan XX, Li Z (2012) Lipopolysaccharide induces paired immunoglobulin-like receptor B (PirB) expression, synaptic alteration, and learning-memory deficit in rats. *Neuroscience* **209**, 161-170.

[75] Ullrich C, Humpel C (2009) Rotenone induces cell death of cholinergic neurons in an organotypic co-culture brain slice model. *Neurochem Res* **34**, 2147-2153.

[76] Chen L, Yoo SE, Na R, Liu Y, Ran Q (2012) Cognitive impairment and increased Aβ levels induced by paraquat exposure are attenuated by enhanced removal of mitochondrial H (2)O(2), *Neurobiol Aging* **33**, 432.e15-26.

[77] Shang T, Kotamraju S, Kalivendi SV, Hillard CJ, Kalyanaraman B (2004) 1-Methyl-4-phenylpyridinium-induced apoptosis in cerebellar granule neurons is mediated by transferrin receptor iron-dependent depletion of tetrahydrobiopterin and neuronal nitric-oxide synthase-derived superoxide. *J Biol Chem* **279**, 19099-19112.

[78] Ma Q, Ying M, Sui X, Zhang H, Huang H, Yang L, Huang X, Zhuang Z, Liu J, Yang X (2015) Chronic copper exposure causes spatial memory impairment, selective loss of hippocampal synaptic proteins, and activation of PKR/eIF2α pathway in mice. *J Alzheimers Dis* **43**, 1413-1427.

[79] Da Silva VK, de Freitas BS, da Silva Dornelles A, Nery LR, Falavigna L, Ferreira RD, Bogo MR, Hallak JE, Zuardi AW, Crippa JA, Schröder N (2014) Cannabidiol normalizes caspase 3, synaptophysin, and mitochondrial fission protein DNM1L expression levels in rats with brain iron overload: Implications for neuroprotection. *Mol Neurobiol* **49**, 222-233.

[80] Kawahara M, Kato-Negishi M (2011) Link between aluminum and the pathogenesis of Alzheimer's disease: The integration of the aluminum and amyloid cascade hypotheses. *Int J Alzheimers Dis* **8**, 276393.

[81] Lenaz G, Fato R, Genova ML, Bergamini C, Bianchi C, Biondi A (2006) Mitochondrial Complex I: Structural and functional aspects. *Biochim Biophys Acta* **1757**, 1406-1420.

[82] Dröse S, Brandt U (2012) Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. *Adv Exp Med Biol* **748**, 145-169.

[83] Hsieh HL, Yang CM (2013) Role of redox signaling in neuroinflammation and neurodegenerative diseases. *Biomed Res Int* **2013**, 484613.

[84] von Bernhardi R, Eugenín J (2012) Alzheimer's disease: Redox dysregulation as a common denominator for diverse pathogenic mechanisms. *Antioxid Redox Signal* **16**, 974-1031.

[85] Hochstrasser T, Weiss E, Marksteiner J, Humpel C (2010) Soluble cell adhesion molecules in monocytes of Alzheimer's disease and mild cognitive impairment. *Exp Gerontol* **45**, 70-74.

[86] Massaad CA, Klann E (2011) Reactive oxygen species in the regulation of synaptic plasticity and memory. *Antioxid Redox Signal* **14**, 2013-2054.

[87] Jang SS, Chung HJ (2016) Emerging link between Alzheimer's disease and homeostatic synaptic plasticity. *Neural Plast* **2016**, 7969272.

[88] Belmeguenai A, Hansel C (2005) A role for protein phosphatases 1, 2A, and 2B in cerebellar long-term potentiation. *J Neurosci* **25**, 10768-10772.

[89] Chakroborty S, Kim J, Schneider C, West AR, Stutzmann GE (2015) Nitric oxide signaling is recruited as a compensatory mechanism for sustaining synaptic plasticity in Alzheimer's disease mice. *J Neurosci* **35**, 6893-6902.

[90] Sunico CR, González-Forero D, Domínguez G, García-Verdugo JM, Moreno-López B (2010) Nitric oxide induces pathological synapse loss by a protein kinase G-, Rho kinase-dependent mechanism preceded by myosin light chain phosphorylation. *J Neurosci* **30**, 973-984.

[91] Roh SE, Woo JA, Lakshmana MK, Uhlar C, Ankala V, Boggess T, Liu T, Hong YH, Mook-Jung I, Kim SJ, Kang DE (2013) Mitochondrial dysfunction and calcium deregulation by the RanBP9-cofilin pathway. *FASEB J* **27**, 4776-4789.

[92] Bedse G, Di Domenico F, Serviddio G, Cassano T (2015) Aberrant insulin signaling in Alzheimer's disease: Current knowledge. *Front Neurosci* **9**, 204.

[93] Jha NK, Jha SK, Kumar D, Kejriwal N, Sharma R, Ambasta RK, Kumar P (2015) Impact of insulin degrading enzyme and neprilysin in Alzheimer's disease biology: Characterization of putative cognates for therapeutic applications. *J Alzheimers Dis* **48**, 891-917.

[94] Solito E, Sastre M (2012) Microglia functions in Alzheimer's disease. *Front Pharmacol* **3**, 14.

[95] Teich AF, Nicholls RE, Puzzo D, Fiorito J, Purgatorio R, Fa' M, Arancio O (2015) Synaptic therapy in Alzheimer's disease: A CREB-centric approach. *Neurotherapeutics* **12**, 29-41.

[96] Faulconbridge LF, Hayes MR (2011) Regulation of energy balance and body weight by the brain: A distributed system prone to disruption. *Psychiatr Clin North Am* **34**, 733-745.

[97] Hasselmo ME (2006) The role of acetylcholine in learning and memory. *Curr Opin Neurobiol* **16**, 710-715.

[98] Resende RR, Adhikari A (2009) Cholinergic receptor pathways involved in apoptosis, cell proliferation and neuronal differentiation. *Cell Commun Signal* **7**, 20.

[99] Placzek AN, Zhang TA, Dani JA (2009) Nicotinic mechanisms influencing synaptic plasticity in the hippocampus. *Acta Pharmacol Sin* **30**, 752-760.

[100] Mufson EJ, Counts SE, Perez SE, Ginsberg SD (2008) Cholinergic system during the progression of Alzheimer's disease: Therapeutic implications. *Expert Rev Neurother* **8**, 1703-1718.

[101] Tammimäki A, Horton WJ, Stitzel JA (2011) Recent advances in gene manipulation and nicotinic acetylcholine receptor biology. *Biochem Pharmacol* **82**, 808-819.

[102] Feduccia AA, Chatterjee S, Bartlett SE (2012) Neuronal nicotinic acetylcholine receptors: Neuroplastic changes underlying alcohol and nicotine addictions. *Front Mol Neurosci* **5**, 83.

[103] Posadas I, López-Hernández B, Ceña V (2013) Nicotinic receptors in neurodegeneration. *Curr Neuropharmacol* **11**, 298-314.

[104] Ni R, Marutle A, Nordberg A (2013) Modulation of α7 nicotinic acetylcholine receptor and fibrillar amyloid-β interactions in Alzheimer's disease brain. *J Alzheimers Dis* **33**, 841-851.

[105] Beaulieu JM, Gainetdinov RR (2011) The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol Rev* **63**, 182-217.

[106] Martorana A, Koch G (2014) Is dopamine involved in Alzheimer's disease? *Front Aging Neurosci* **6**, 252.

[107] Moreno-Castilla P, Rodriguez-Duran LF, Guzman-Ramos K, Barcenas-Femat A, Escobar ML, Bermudez-Rattoni F (2016) Dopaminergic neurotransmission dysfunction induced by amyloid-β transforms cortical long-term potentiation into long-term depression and produces memory impairment. *Neurobiol Aging* **41**, 187-199.

[108] Koch G, Di Lorenzo F, Bonní S, Giacobbe V, Bozzali M, Caltagirone C, Martorana A (2014) Dopaminergic modulation of cortical plasticity in Alzheimer's disease patients. *Neuropsychopharmacology* **39**, 2654-2661.

[109] Liang SL, Carlson GC, Coulter DA (2006) Dynamic regulation of synaptic GABA release by the glutamate-glutamine cycle in hippocampal area CA1. *J Neurosci* **26**, 8537-8548.

[110] Winsky-Sommerer R (2009) Role of GABAA receptors in the physiology and pharmacology of sleep. *Eur J Neurosci* **29**, 1779-1794.

[111] Limon A, Reyes-Ruiz JM, Miledi R (2012) Loss of functional GABA (A) receptors in the Alzheimer diseased brain. *Proc Natl Acad Sci U S A* **109**, 10071-10076.

[112] Li Y, Sun H, Chen Z, Xu H, Bu G, Zheng H (2016) Implications of GABAergic Neurotransmission in Alzheimer's disease. *Front Aging Neurosci* **8**, 31.

[113] Zhuo M (2009) Plasticity of NMDA receptor NR2B subunit in memory and chronic pain. *Mol Brain* **2**, 4.

[114] Guntupalli S, Widagdo J, Anggono V (2016) Amyloid-β-induced dysregulation of AMPA receptor trafficking. *Neural Plast* **2016**, 3204519.

[115] Behnisch T, Yuanxiang P, Bethge P, Parvez S, Chen Y, Yu J, Karpova A, Frey JU, Mikhaylova M, Kreutz MR (2011) Nuclear translocation of jacob in hippocampal neurons after stimuli inducing long-term potentiation but not long-term depression. *PLoS One* **6**, e17276.

[116] Pozueta J, Lefort R, Shelanski ML (2013) Synaptic changes in Alzheimer's disease and its models. *Neuroscience* **251**, 51-65.

[117] Xu J, Kurup P, Nairn AC, Lombroso PJ (2012) Striatal-enriched protein tyrosine phosphatase in Alzheimer's disease. *Adv Pharmacol* **64**, 303-325.

[118] Snigdha S, Smith ED, Prieto GA, Cotman CW (2012) Caspase-3 activation as a bifurcation point between plasticity and cell death. *Neurosci Bull* **28**, 14-24.

[119] Blandina P, Munari L, Provensi G, Passani MB (2012) Histamine neurons in the tuberomamillary nucleus: A whole center or distinct subpopulations? *Front Syst Neurosci* **6**, 33.

[120] Nuutinen S, Panula P (2010) Histamine in neurotransmission and brain diseases. *Adv Exp Med Biol* **709**, 95-107.

[121] Cacabelos R, Torrellas C, Fernández-Novoa L, López-Muñoz F (2016) Histamine and immune biomarkers in CNS disorders. *Mediators Inflamm* **2016**, 1924603.

[122] Medhurst AD, Roberts JC, Lee J, Chen CP, Brown SH, Roman S, Lai MK (2009) Characterization of histamine H3 receptors in Alzheimer's disease brain and amyloid over-expressing TASTPM mice. *Br J Pharmacol* **157**, 130-138.

[123] Gannon M, Che P, Chen Y, Jiao K, Roberson ED, Wang Q (2015) Noradrenergic dysfunction in Alzheimer's disease. *Front Neurosci* **9**, 220.

[124] Marien MR, Colpaert FC, Rosenquist AC (2004) Noradrenergic mechanisms in neurodegenerative diseases: A theory. *Brain Res* **45**, 38-78.

[125] Hammerschmidt T, Kummer MP, Terwel D, Martinez A, Gorji A, Pape HC, Rommelfanger KS, Schroeder JP, Stoll M, Schultze J, Weinshenker D, Heneka MT (2013) Selective loss of noradrenaline exacerbates early cognitive dysfunction and synaptic deficits in APP/PS1 mice. *Biol Psychiatry* **73**, 454-463.

[126] Combarros O, Warden DR, Hammond N, Cortina-Borja M, Belbin O, Lehmann MG, Wilcock GK, Brown K, Kehoe PG, Barber R, Coto E, Alvarez V, Deloukas P, Gwilliam R, Heun R, Kölsch H, Mateo I, Oulhaj A, Arias-Vásquez A, Schuur M, Aulchenko YS, Ikram MA, Breteler MM, van Duijn CM, Morgan K, Smith AD, Lehmann DJ (2010) The dopamine β-hydroxylase -1021C/T polymorphism is associated with the risk of Alzheimer's disease in the Epistasis Project. *BMC Med Genet* **11**, 162.

[127] Chen Y, Peng Y, Che P, Gannon M, Liu Y, Li L, Bu G, van Groen T, Jiao K, Wang Q (2010) α (2A) adrenergic receptor promotes amyloidogenesis through disrupting APP-SorLA interaction. *Proc Natl Acad Sci U S A* **111**, 17296-17301.

[128] Wang D, Fu Q, Zhou Y, Xu B, Shi Q, Igwe B, Matt L, Hell JW, Wisely EV, Oddo S, Xiang YK (2013) β2 adrenergic receptor, protein kinase A (PKA) and c-Jun N-terminal kinase (JNK) signaling pathways mediate tau pathology in Alzheimer disease models. *J Biol Chem* **288**, 10298-10307.

[129] Arreola R, Becerril-Villanueva E, Cruz-Fuentes C, Velasco-Velázquez MA, Garcés-Alvarez ME, Hurtado-Alvarado G, Quintero-Fabian S, Pavón L (2015) Immunomodulatory effects mediated by serotonin. *J Immunol Res* **2015**, 354957.

[130] Fidalgo S, Ivanov DK, Wood SH (2013) Serotonin: From top to bottom. *Biogerontology* **14**, 21-45.

[131] Bantick RA, Deakin JF, Grasby PM (2001) The 5-HT1A receptor in schizophrenia: A promising target for novel atypical neuroleptics? *J Psychopharmacol* **15**, 37-46.

[132] Thathiah A, De Strooper B (2011) The role of G protein-coupled receptors in the pathology of Alzheimer's disease. *Nat Rev Neurosci* **12**, 73-87.

[133] Cochet M, Donneger R, Cassier E, Gaven F, Lichtenthaler SF, Marin P, Bockaert J, Dumuis A, Claeysen S (2013) 5-HT4 receptors constitutively promote the non-amyloidogenic pathway of APP cleavage and interact with ADAM10. *ACS Chem Neurosci* **4**, 130-140.

[134] Kovacs GG, Klöppel S, Fischer I, Dorner S, Lindeck-Pozza E, Birner P, Bötefür IC, Pilz P, Volk B, Budka H (2003) Nucleus-specific alteration of raphe neurons in human neurodegenerative disorders. *Neuroreport* **14**, 73-76.

[135] Garcia-Alloza M, Gil-Bea FJ, Diez-Ariza M, Chen CP, Francis PT, Lasheras B, Ramirez MJ (2005) Cholinergic-serotonergic imbalance contributes to cognitive and behavioral symptoms in Alzheimer's disease. *Neuropsychologia* **43**, 442-449.

[136] Hoozemans JJ, Chafekar SM, Baas F, Eikelenboom P, Scheper W (2006) Always around, never the same:

Pathways of amyloid beta induced neurodegeneration throughout the pathogenic cascade of Alzheimer's disease. *Curr Med Chem* **13**, 2599-2605.

[137] Yun HM, Kim S, Kim HJ, Kostenis E, Kim JI, Seong JY, Baik JH, Rhim H (2007) The novel cellular mechanism of human 5-HT6 receptor through an interaction with Fyn. *J Biol Chem* **282**, 5496-5505.

[138] Hendricksen M, Thomas AJ, Ferrier IN, Ince P, O'Brien JT (2004) Neuropathological study of the dorsal raphe nuclei in late-life depression and Alzheimer's disease with and without depression. *Am J Psychiatry* **161**, 1096-1102.

[139] Stranahan AM, Erion JR, Wosiski-Kuhn M (2013) Reelin signaling in development, maintenance, and plasticity of neural networks. *Ageing Res Rev* **12**, 815-822.

[140] Xiong Z, Thangavel R, Kempuraj D, Yang E, Zaheer S, Zaheer A (2014) Alzheimer's disease: Evidence for the expression of interleukin-33 and its receptor ST2 in the brain. *J Alzheimers Dis* **40**, 297-308.

[141] Guzman SJ, Gerevich Z (2016) P2Y receptors in synaptic transmission and plasticity: Therapeutic potential in cognitive dysfunction. *Neural Plast* **2016**, 1207393.

[142] Venkatachalam K, Montell C (2007) TRP channels. *Annu Rev Biochem* **76**, 387-417.

[143] Wang J, Jackson MF, Xie YF (2016) Glia and TRPM2 channels in plasticity of central nervous system and Alzheimer's diseases. *Neural Plast* **2016**, 1680905.

[144] Marcello E, Epis R, Saraceno C, Di Luca M (2012) Synaptic dysfunction in Alzheimer's disease. *Adv Exp Med Biol* **970**, 573-601.

[145] Kihara T, Shimohama S (2004) Alzheimer's disease and acetylcholine receptors. *Acta Neurobiol Exp* **64**, 99-105.

[146] Giuffrida ML, Tomasello F, Caraci F, Chiechio S, Nicoletti F, Copani A (2012) Beta-amyloid monomer and insulin/IGF-1 signaling in Alzheimer's disease. *Mol Neurobiol* **46**, 605-613.

[147] Vaarmann A, Kovac S, Holmström KM, Gandhi S, Abramov AY (2013) Dopamine protects neurons against glutamate-induced excitotoxicity. *Cell Death Dis* **4**, e455.

[148] Zhang Y, Li P, Feng J, Wu M (2016) Dysfunction of NMDA receptors in Alzheimer's disease. *Neurol Sci* **37**, 1039-1047.

[149] Akhtar MW, Sanz-Blasco S, Dolatabadi N, Parker J, Chon K, Lee MS, Soussou W, McKercher SR, Ambasudhan R, Nakamura T, Lipton SA (2016) Elevated glucose and oligomeric β-amyloid disrupt synapses via a common pathway of aberrant protein S-nitrosylation. *Nat Commun* **7**, 10242.

[150] Accardi MV, Daniels BA, Brown PM, Fritschy JM, Tyagarajan SK, Bowie D (2014) Mitochondrial reactive oxygen species regulate the strength of inhibitory GABA-mediated synaptic transmission. *Nat Commun* **5**, 3168.

[151] Chadwick W, Brenneman R, Martin B, Maudsley S (2010) Complex and multi dimensional lipid raft alterations in a murine model of Alzheimer's disease. *Int J Alzheimers Dis* **2010**, 604792.

[152] Koch G, Di Lorenzo F, Bonní S, Ponzo V, Caltagirone C, Martorana A (2012) Impaired LTP- but not LTD-like cortical plasticity in Alzheimer's disease patients. *J Alzheimers Dis* **31**, 593-599.

[153] Fidzinski P, Korotkova T, Heidenreich M, Maier N, Schuetze S, Kobler O, Zuschratter W, Schmitz D, Ponomarenko A, Jentsch TJ (2015) KCNQ5 K^+ channels control hippocampal synaptic inhibition and fast network oscillations. *Nat Commun* **6**, 6254.

[154] Hong S, Beja-Glasser VF, Nfonoyim BM, Frouin A, Li S, Ramakrishnan S, Merry KM, Shi Q, Rosenthal A, Barres BA, Lemere CA, Selkoe DJ, Stevens B (2016) Complement and microglia mediate early synapse loss in Alzheimer mouse models. *Science* **352**, 712-716.

[155] Liu Y, Yoo MJ, Savonenko A, Stirling W, Price DL, Borchelt DR, Mamounas L, Lyons WE, Blue ME, Lee MK (2008) Amyloid pathology is associated with progressive monoaminergic neurodegeneration in a transgenic mouse model of Alzheimer's disease. *J Neurosci* **28**, 13805-13814.

[156] Meltzer CC, Smith G, DeKosky ST, Pollock BG, Mathis CA, Moore RY, Kupfer DJ and Reynolds CF 3rd. (1998) Serotonin in aging, late-life depression, and Alzheimer's disease: The emerging role of functional imaging. *Neuropsychopharmacology* **18**, 407-430.

[157] Zecharia AY, Yu X, Götz T, Ye Z, Carr DR, Wulff P, Bettler B, Vyssotski AL, Brickley SG, Franks NP, Wisden W (2012) GABAergic inhibition of histaminergic neurons regulates active waking but not the sleep-wake switch or propofol-induced loss of consciousness. *J Neurosci* **32**, 13062-13075.

[158] Panula P, Rinne J, Kuokkanen K, Eriksson KS, Sallmen T, Kalimo H and Relja M (1998) Neuronal histamine deficit in Alzheimer's disease. *Neuroscience* **82**, 993-997.

[159] Lussier AL, Weeber EJ, Rebeck GW (2016) Reelin proteolysis affects signaling related to normal synapse function and neurodegeneration. *Front Cell Neurosci* **10**, 75.

[160] Herring A, Donath A, Steiner KM, Widera MP, Hamzehian S, Kanakis D, Kölble K, ElAli A, Hermann DM, Paulus W, Keyvani K (2012) Reelin depletion is an early phenomenon of Alzheimer's pathology. *J Alzheimers Dis* **30**, 963-979.

[161] Cuchillo-Ibañez I, Balmaceda V, Mata-Balaguer T, Lopez-Font I, Sáez-Valero J (2016) Reelin in Alzheimer's disease, increased levels but impaired signaling: When more is less. *J Alzheimers Dis* **52**, 403-416.

[162] Sanz JM, Chiozzi P, Ferrari D, Colaianna M, Idzko M, Falzoni S, Fellin R, Trabace L, Di Virgilio F (2009) Activation of microglia by amyloid beta requires P2X7 receptor expression. *J Immunol* **182**, 4378-4385.

[163] Parvathenani LK, Tertyshnikova S, Greco CR, Roberts SB, Robertson B, Posmantur R (2003) P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. *J Biol Chem* **278**, 13309-13317.

[164] Erb L, Cao C, Ajit D, Weisman GA (2015) P2Y receptors in Alzheimer's disease. *Biol Cell* **107**, 1-21.

[165] Fu AK, Hung KW, Yuen MY, Zhou X, Mak DS, Chan IC, Cheung TH, Zhang B, Fu WY, Liew FY, Ip NY (2016) IL-33 ameliorates Alzheimer's disease-like pathology and cognitive decline. *ProcNatl Acad Sci U S A* **113**, E2705-E2713.

[166] Yamamoto S, Wajima T, Hara Y, Nishida M, Mori Y (2007) Transient receptor potential channels in Alzheimer's disease. *Biochim Biophys Acta* **1772**, 958-967.

[167] Martorana A, Mori F, Esposito Z, Kusayanagi H, Monteleone F, Codecá C, Sancesario G, Bernardi G, Koch G (2009) Dopamine modulates cholinergic cortical excitability in Alzheimer's disease patients. *Neuropsychopharmacology* **34**, 2323-2328.

[168] West AE, Greenberg ME (2011) Neuronal activity-regulated gene transcription in synapse development and cognitive function. *Cold Spring Harb Perspect Biol* **3**, 6.

[169] Kulkarni SK, Dhir A (2010) An overview of curcumin in neurological disorders. *Indian J Pharm Sci* **72**, 149-154.

[170] Pohanka M (2014) Inhibitors of acetylcholinesterase and butyrylcholinesterase meet immunity. *Int J Mol Sci* **15**, 9809-9825.

[171] Grossberg GT (2003) Cholinesterase inhibitors for the treatment of Alzheimer's disease: Getting on and staying on. *Curr Ther Res Clin Exp* **64**, 216-235.

[172] Inestrosa NC, Godoy JA, Vargas JY, Arrazola MS, Rios JA, Carvajal FJ, Serrano FG, Farias GG (2013) Nicotine prevents synaptic impairment induced by amyloid-β oligomers through α7-nicotinic acetylcholine receptor activation. *Neuromolecular Med* **15**, 549-569.

[173] Fernández-Ruiz J, Hernández M, Ramos JA (2010) Cannabinoid-dopamine interaction in the pathophysiology and treatment of CNS disorders. *CNS Neurosci Ther* **16**, e72-e91.

[174] Gutierres JM, Carvalho FB, Schetinger MR, Marisco P, Agostinho P, Rodrigues M, Rubin MA, Schmatz R, da Silva CR, de P, Cognato G, Farias JG, Signor C, Morsch VM, Mazzanti CM, Bogo M, Bonan CD, Spanevello R (2014) Anthocyanins restore behavioral and biochemical changes caused by streptozotocin-induced sporadic dementia of Alzheimer's type. *Life Sci* **96**, 7-17.

[175] Wang S, Yu L, Yang H, Li C, Hui Z, Xu Y, Zhu X (2016) Oridonin attenuates synaptic loss and cognitive deficits in an Aβ1-42-induced mouse model of Alzheimer's disease. *PLoS One* **11**, e0151397.

[176] Wang S, Huang R, Zhang S, Wei L, Zhuo L, Wu X, Tang A, Huang Q (2013) Beneficial effects of asiaticoside on cognitive deficits in senescence-accelerated mice. *Fitoterapia* **87**, 69-77.

[177] Patel S, Grizzell JA, Holmes R, Zeitlin R, Solomon R, Sutton TL, Rohani A, Charry LC, Iarkov A, Mori T, Echeverria Moran V (2014) Cotinine halts the advance of Alzheimer's disease-like pathology and associated depressive-like behavior in Tg6799 mice. *Front Aging Neurosci* **6**, 162.

[178] Winblad B (2005) Piracetam: A review of pharmacological properties and clinical uses. *CNS Drug Rev* **11**, 169-182.

[179] Wang DM, Yang YJ, Zhang L, Zhang X, Guan FF, Zhang LF (2013) Naringin enhances CaMKII activity and improves long-term memory in a mouse model of Alzheimer's disease. *Int J Mol Sci* **14**, 5576-5586.

[180] Zhang Z, Liu X, Schroeder JP, Chan CB, Song M, Yu SP, Weinshenker D, Ye K (2014) 7, 8-dihydroxyflavone prevents synaptic loss and memory deficits in a mouse model of Alzheimer's disease. *Neuropsychopharmacology* **39**, 638-650.

[181] Wang X, Blanchard J, Grundke-Iqbal I, Iqbal K (2015) Memantine attenuates Alzheimer's disease-like pathology and cognitive impairment. *PLoS One* **10**, e0145441.

[182] Li J, Deng J, Sheng W, Zuo Z (2012) Metformin attenuates Alzheimer's disease-like neuropathology in obese, leptin-resistant mice. *Pharmacol Biochem Behav* **101**, 564-574.

[183] Casarejos MJ, Perucho J, Gomez A, Muñoz MP, Fernandez-Estevez M, Sagredo O, Fernandez Ruiz J, Guzman M, de Yebenes JG, Mena MA (2013) Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy. *J Alzheimers Dis* **35**, 525-539.

[184] Gupta K, Hardingham GE, Chandran S (2013) NMDA receptor-dependent glutamate excitotoxicity in human embryonic stem cell-derived neurons. *Neurosci Lett* **543**, 95-100.

[185] Palygin O, Lalo U, Pankratov Y (2011) Distinct pharmacological and functional properties of NMDA receptors in mouse cortical astrocytes. *Br J Pharmacol* **163**, 1755-1766.

[186] Sanchez PE, Zhu L, Verret L, Vossel KA, Orr AG, Cirrito JR, Devidze N, Ho K, Yu GQ, Palop JJ, Mucke L (2012) Levetiracetam suppresses neuronal network dysfunction and reverses synaptic and cognitive deficits in an Alzheimer's disease model. *Proc Natl Acad Sci U S A* **109**, E2895-E2903.

[187] Lee BY, Ban JY, Seong YH (2005) Chronic stimulation of GABAA receptor with muscimol reduces amyloid beta protein (25-35)-induced neurotoxicity in cultured rat cortical cells. *Neurosci Res* **52**, 347-356.

[188] Marcade M, Bourdin J, Loiseau N, Peillon H, Rayer A, Drouin D, Schweighoffer F, Désiré L (2008) Etazolate, a neuroprotective drug linking GABA (A) receptor pharmacology to amyloid precursor protein processing. *J Neurochem* **106**, 392-404.

[189] Yoshiike Y, Kimura T, Yamashita S, Furudate H, Mizoroki T, Murayama M, Takashima A (2008) GABA (A) receptor-mediated acceleration of aging-aassociated memory decline in APP/PS1 mice and its pharmacological treatment by picrotoxin. *PLoS One* **3**, e3029.

[190] Prickaerts J, van Goethem NP, Chesworth R, Shapiro G, Boess FG, Methfessel C, Reneerkens OA, Flood DG, Hilt D, Gawryl M, Bertrand S, Bertrand D, König G (2012) EVP-6124, a novel and selective α7 nicotinic acetylcholine receptor partial agonist, improves memory performance by potentiating the acetylcholine response of α7 nicotinic acetylcholine receptors. *Neuropharmacology* **62**, 1099-1110.

[191] Paul P, de Belleroche J (2014) The role of D-serine and glycine as co-agonists of NMDA receptors in motor neuron degeneration and amyotrophic lateral sclerosis (ALS). *Front Synaptic Neurosci* **6**, 10.

[192] Paula-Lima AC, Brito-Moreira J, Ferreira ST (2013) Deregulation of excitatory neurotransmission underlying synapse failure in Alzheimer's disease. *J Neurochem* **126**, 191-202.

[193] Shankar GM, Li S, Mehta TH, Garcia-Munoz A, Shepardson NE, Smith I, Brett FM, Farrell MA, Rowan MJ, Lemere CA, Regan CM, Walsh DM, Sabatini BL, Selkoe DJ (2008) Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med* **14**, 837-842.

[194] Paula-Lima AC, Louzada PR, De Mello FG, Ferreira ST (2003) Neuroprotection against Abeta and glutamate toxicity by melatonin: Are GABA receptors involved? *Neurotox Res* **5**, 323-327.

[195] Alawdi SH, El-Denshary ES, Safar MM, Eidi H, David MO, Abdel-Wahhab MA (2016) Neuroprotective effect of nanodiamond in Alzheimer's disease rat model: A pivotal role for modulating NF-κB and STAT3 signaling. *Mol Neurobiol*. doi: 10.1007/s12035-016-9762-0

[196] Mohameda AR, Solimana GY, Ismailb CA, Mannaac HF (2015) Neuroprotective role of vitamin D3 in colchicine-induced Alzheimer's disease in rats. *Alexandria J Med* **51**, 127-136.

[197] Singla N, Dhawan DK (2016) Zinc improves cognitive and neuronal dysfunction during aluminium-induced neurodegeneration. *Mol Neurobiol*. doi: 10.1007/s12035-015-9653-9

[198] Li W, Yu J, Liu Y, Huang X, Abumaria N, Zhu Y, Huang X, Xiong W, Ren C, Liu XG, Chui D, Liu G (2013) Elevation of brain magnesium prevents and reverses cognitive deficits and synaptic loss in Alzheimer's disease mouse model. *J Neurosci* **33**, 8423-8441.

[199] Duarte AI, Moreira PI, Oliveira CR (2012) Insulin in central nervous system: More than just a peripheral hormone. *J Aging Res* **2012**, 384017.

[200] Severini C, Petrella C, Calissano P (2016) Substance P and Alzheimer's disease: Emerging novel roles. *Curr Alzheimer Res* **13**, 964-972.

[201] Hegde AN (2010) The ubiquitin-proteasome pathway and synaptic plasticity. *Learn Mem* **17**, 314-327.

[202] Rodrigues EM, Scudder SL, Goo MS, Patrick GN (2016) Aβ-induced synaptic alterations require the E3 ubiquitin ligase Nedd4-1. *J Neurosci* **36**, 1590-1595.

[203] Gerges NZ, Tran IC, Backos DS, Harrell JM, Chinkers M, Pratt WB, Esteban JA (2004) Independent functions of hsp90 in neurotransmitter release and in the continuous synaptic cycling of AMPA receptors. *J Neurosci* **24**, 4758-4766.

Journal of Alzheimer's Disease 48 (2015) 891–917
DOI 10.3233/JAD-150379
IOS Press

Review

Impact of Insulin Degrading Enzyme and Neprilysin in Alzheimer's Disease Biology: Characterization of Putative Cognates for Therapeutic Applications

Niraj Kumar Jha[a,1], Saurabh Kumar Jha[a,1], Dhiraj Kumar[a,1], Noopur Kejriwal[a], Renu Sharma[a], Rashmi K. Ambasta[a] and Pravir Kumar[a,b,*]
[a]*Molecular Neuroscience and Functional Genomics Laboratory, Department of Biotechnology, Delhi Technological University (Formerly DCE), Shahbad Daulatpur, Delhi, India*
[b]*Department of Neurology, Tufts University School of Medicine, Boston, MA, USA*

Accepted 14 July 2015

Abstract. Alzheimer's disease (AD) is a neurodegenerative process primarily characterized by amyloid-β (Aβ) agglomeration, neuroinflammation, and cognitive dysfunction. The prominent cause for dementia is the deposition of Aβ plaques and tau-neurofibrillary tangles that hamper the neuronal organization and function. Aβ pathology further affects numerous signaling cascades that disturb the neuronal homeostasis. For instance, Aβ deposition is responsible for altered expression of insulin encoding genes that lead to insulin resistance, and thereby affecting insulin signaling pathway and glucose metabolism in the brain. As a result, the common pathology of insulin resistance between Type-2 diabetes mellitus and AD has led AD to be proposed as a form of diabetes and termed 'Type-3 diabetes'. Since accumulation of Aβ is the prominent cause of neuronal toxicity in AD, its clearance is the prime requisite for therapeutic prospects. This purpose is expertly fulfilled by the potential role of Aβ degrading enzymes such as insulin degrading enzyme (IDE) and Neprilysin (NEP). Therefore, their molecular study is important to uncover the proteolytic and regulatory mechanism of Aβ degradation. Herein, (i) *In silico* sequential and structural analysis of IDE and NEP has been performed to identify the molecular entities for proteolytic degradation of Aβ in the AD brain, (ii) to analyze their catalytic site to demonstrate the enzymatic action played by IDE and NEP, (iii) to identify their structural homologues that could behave as putative partners of IDE and NEP with similar catalytic action and (iv) to illustrate various IDE- and NEP-mediated therapeutic approaches and factors for clearing Aβ in AD.

Keywords: Alzheimer's disease, amyloid-β, insulin degrading enzyme, Neprilysin, therapeutics

INTRODUCTION

Alzheimer's disease (AD) is a neurological disorder that is characterized by neuronal death, which is caused by the abnormal burden of amyloid-β (Aβ) in the brain resulting in memory loss and cognitive decline [1–3]. The cognitive collapse in AD occurs due to neuronal dysfunction that is attributable to the extracellular Aβ

[1]These authors contributed equally to this work.

*Correspondence to: Dr. Pravir Kumar, MSc (BHU), PhD (Germany), PDF/Faculty (USA), Associate Professor, FW4TF3, Department of Biotechnology, Delhi Technological University (Formerly Delhi College of Engineering), Shahbad Daulatpur, Bawana Road, Delhi 110042, India. Tel.: +91 9818898622; E-mail: Pravir.Kumar@tufts.edu; pravirkumar@dce.edu.

ISSN 1387-2877/15/$35.00

aggregates and the intraneuronal aggregates of tau protein, which further forms Aβ plaques and neurofibrillary tangles, respectively [4–7]. Apart from Aβ deposition and neurofibrillary tangles, other characteristic abnormalities include dystrophic neurites, impaired energy metabolism, non-dynamic equilibrium between Aβ production and its clearance, chronic oxidative stress, mitochondrial dysfunction, DNA damage, elevated pro-apoptotic genes, and signaling pathways [8–11]. Currently researchers have reported that insulin and insulin-like growth factor resistance is another prominent cause provoking the progression of AD [12]. In addition, production of insulin in the brain, in addition to the pancreas, led to the proposal of another form of diabetes, termed 'Type-3 diabetes' for AD.

IS ALZHEIMER'S DISEASE A 'TYPE-3 DIABETES'? A MISNOMER OR A FACT

Insulin, insulin receptor (IR), and insulin–like growth factor-1 (IGF-1) play a prominent role in the brain via regulating brain metabolism, neuronal growth, and differentiation [13–15]. Any hindrance in the cross-talk between insulin and neuronal glucose metabolism may slow down ATP synthesis that culminates in neuronal apoptosis. This is in context with the dysfunction of energy metabolism linked with stress-induced fluctuations in the brain IR signaling cascade and the parallel death signals that caused apoptotic death [16, 17]. Mounting conclusive evidence uncovered the fact that diabetes and altered insulin signal may have a direct and indirect profound impact on progression of AD [18]. Diabetes mellitus is one of the most widespread metabolic disorders, primarily governed by interference in insulin signaling cascade, with prevalence in aged individuals. It is linked with gradually and highly progressive end-organ injury to the brain. Moreover, mild to moderate alterations of cognitive function have been clearly stated in both type 1 and type 2 diabetic (T1D and T2D, respectively) patients [19, 20]. The cardinal defect in T2D is insulin resistance, whose roots are relative insulin deficiency, and falls in the context of a cluster of metabolic and vascular risk factors, that is termed "metabolic syndrome." In this framework, a growing amount of evidence directly links insulin to cognitive decline and dementia in T2D [19, 20]. Furthermore, under dementia-type disorders, T2D has been increasingly linked with AD [21]. The connections between T2D and AD comprise high-cholesterol levels, aging-related processes, peripheral and central nervous system (CNS) insulin resistance, dysfunctional IR and IR signaling cascades, decreased glucose transport, and neurodegeneration. As a result of recurrent hyperinsulinemia/hypoglycemia in T2D, cognitive impairment promotes AD onset. Moreover, several epidemiology studies reported that T2D increased the risk of AD, and it is plausible that hyperinsulinemia plays a decisive role in AD progression [22–24].

Furthermore, de la Monte and colleagues presented compelling evidence for abnormalities in insulin/IR signaling in the brains of AD patients. These abnormalities greatly reduced CNS expression of genes encoding insulin, its receptors, and related signaling molecules in AD brain. Further, this situation promoted the researchers to consider sporadic AD as a "neuroendocrine disorder" that shared features with T1D and T2D, and they referred to it as "Type-3 diabetes", which closely resembles diabetes mellitus but still differs from it [12, 25, 26]. However, diabetic patients are highly prone (nearly 50 to 60%) to AD development due to insulin resistance in the brain, but non-diabetic patients are also developing AD. This reflects that in spite of the common features of insulin resistance in both T2D and AD (Fig. 1), they differ in their exact mechanism of occurrences. Still, it is debatable to consider AD as "Type-3 diabetes" and requires intense in-depth studies to unravel the mystery.

In AD brains, synaptic plasticity and neuronal survival are directly influenced by insulin resistance or indirectly by insulin degrading enzyme (IDE), which has been strongly advocated to be a key player in Aβ catabolism that catabolizes insulin and IGF-1, and also degrades Aβ [27, 28]. Moreover, insulin raises the extracellular concentration of Aβ via two independent mechanisms that include inhibition of extracellular degradation of Aβ by IDE or stimulation of Aβ secretion by the augmentation of its trafficking from the endoplasmic reticulum to the plasma membrane [29, 30]. Since, IRs in the brain do not desensitize itself, IDE creates a negative feedback loop to control insulin action. In fact, insulin exposure stimulates PI3-K/Akt-dependent upregulation of IDE, thereby preventing insulin signaling and promoting Aβ clearance in neurons. This evidence supports the existence of a therapeutic window allowing insulin treatment to adjust the levels of IDE in AD, without provoking direct competitive inhibition of Aβ breakdown. Insulin can be toxic for brain, if present in high levels, and creates both its positive and negative impact on brain metabolism, thus acting as a two-edged knife on the brain [31–33]. Apart from IDE, Aβ plaque formation is also downregulated by another degradative enzyme called Neprilysin (NEP) that is known to prevent AD progression.

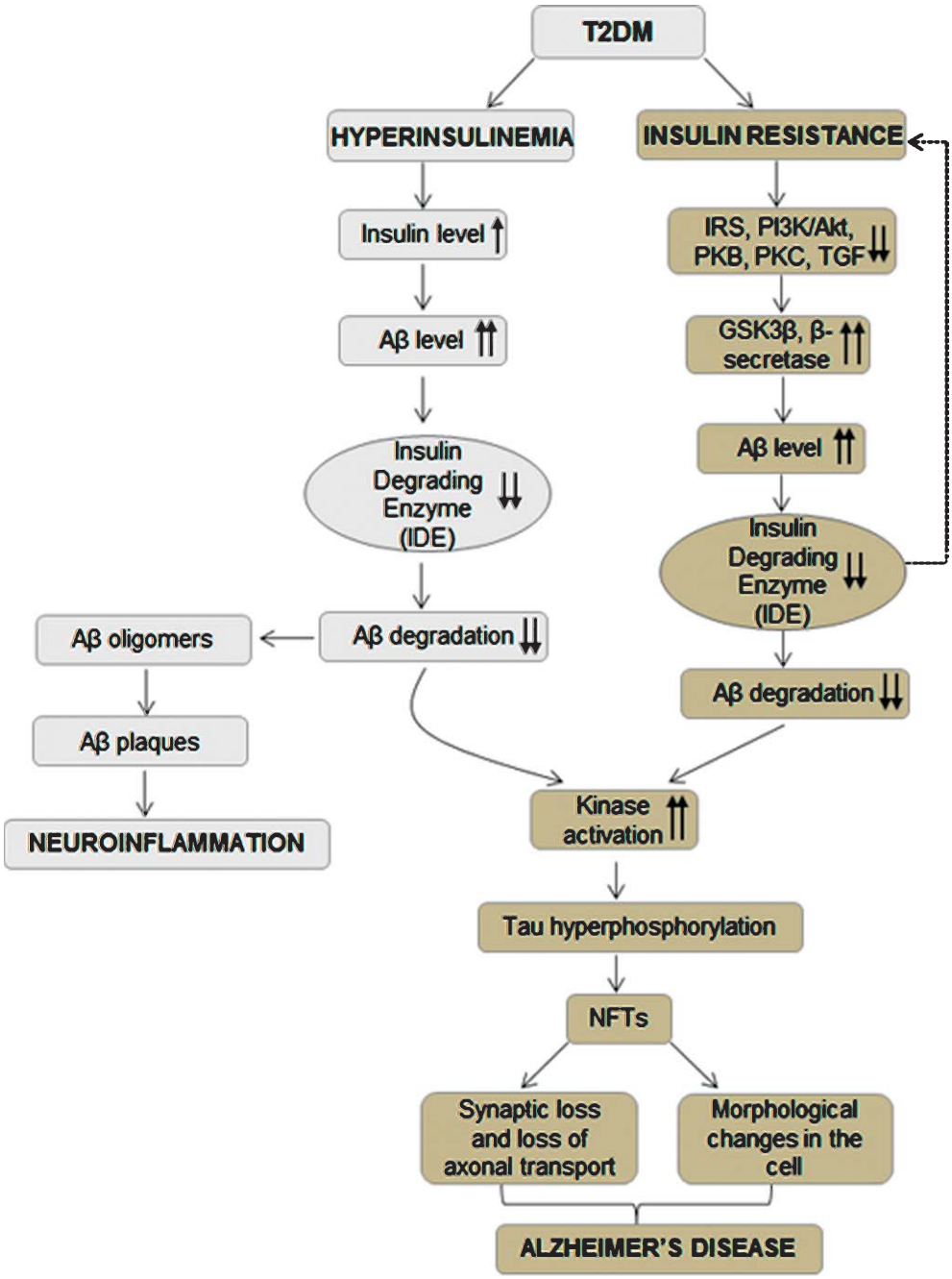


Fig. 1. Association of insulin degrading enzyme with the clinical symptoms of Alzheimer's disease as well as to the risk of type-2 diabetes mellitus.

NEPRILYSIN: NATURE'S AGENT FOR AD PREVENTION

NEP is a single-pass type II transmembrane protein, which has the single polypeptide chain of 750aa residues that is encoded by 2250 bp membrane metalloendopeptidase (MME) gene in humans [34, 35]. NEP has a molecular weight ranging from 85 to 110 kDa depending on its glycosylated isoforms [36, 37]. Among the human genome, the NEP gene is mainly located on chromosome 3 and exists in a single copy that covers >80 kb. Furthermore, it is composed of 24 exons and is found to be highly conserved across mammalian species [38]. It is a thermolysin-like zinc

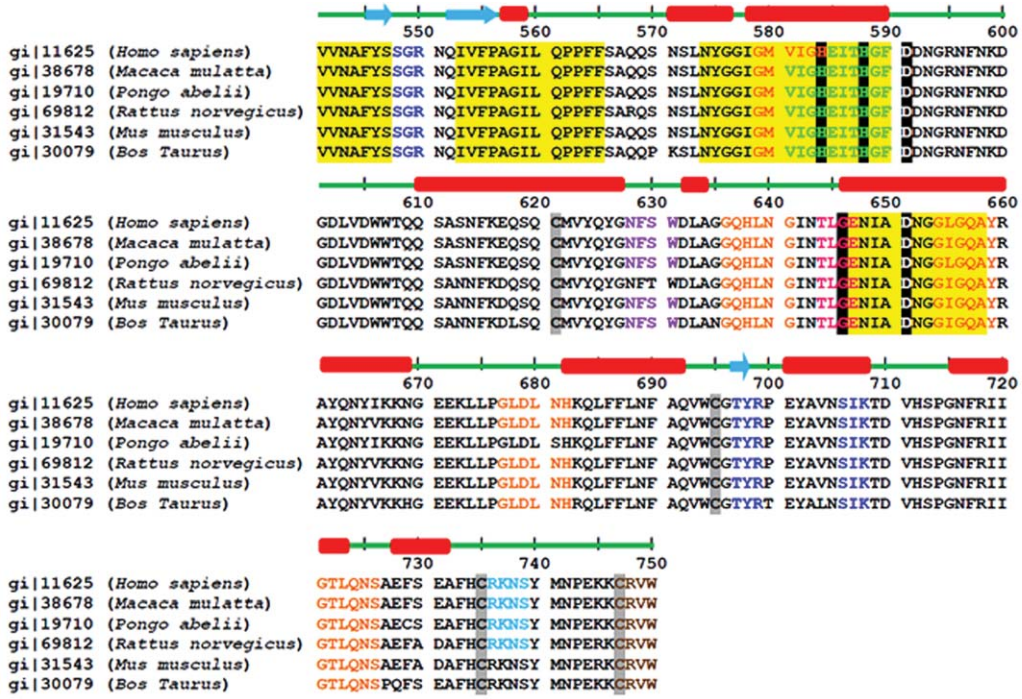
metalloendopeptidase that requires one Zn^{2+} ion per subunit as a cofactor for its catalytic activity [39–41]. Moreover, a His-Glu-X-X-His (Zinc consensus sequence) is responsible for catalytic activity of NEP [42, 43]. NEP is also known as enkephalinase, kidney brush border neutral proteinase, endopeptidase, and neutral endopeptidase [44]. Previously, it has been identified as major atrial natriuretic peptide degrading enzyme expressed in kidney cell signifying its role in blood volume and pressure regulation [45]. However, its expression is much lower in several other tissues such as brain, where it is found on neuronal membranes (both pre- and post-synaptically), nigrostriatal bundle areas, hippocampal, and temporal cortex region, and is responsible for Aβ degradation in AD patients [46–48]. Moreover, in the case of the CNS, NEP is mainly expressed by neurons, activated astrocytes, and microglial cells, while in case of peripheral tissues, it is transiently expressed at the surface of certain haematopoietic cells [49–52].

NEP plays a crucial role in ceasing regulatory peptides (∼30 aa) that are actively involved in maintaining physiological homeostasis by altering the metabolic pathways of mammalian nervous, cardiovascular, immune, and inflammatory systems [53, 54]. Moreover, it possesses an ectoenzyme-like activity, which is involved in hydrolyzing extracellular oligopeptides at the N-terminal of amino acid residues that make it suitable for the degradation of the small hydrophobic $A\beta_{40\text{-}42}$ peptide [55]. The Aβ peptide degrading ability of NEP was first demonstrated by Howell et al. *in vitro* and later confirmed through *in vivo* experiments [56, 57]. NEP can digest both monomeric and toxic oligomeric forms of Aβ in the brain, and it has been demonstrated that irregular function of gene encoding NEP causes a two-fold increase in the endogenous toxic $A\beta_{40\text{-}42}$ levels (high plaque load) in different regions within the brain and thereby causing impaired synaptic plasticity and cognitive abnormalities that ultimately increase the risk of AD [58–60]. Further, it has also been observed that individuals with certain polymorphisms in the NEP gene have been associated with AD; for instance, a meta-analysis study has identified the association between NEP variants (rs989692 and rs3736187) and AD. These meta-analysis data also highlight that rs3736187 (A/G) polymorphisms might be a beneficial single nucleotide polymorphism (SNP), which is linked with a decreased risk in AD progression [61]. Interestingly, under hypoxic stress conditions such as cerebral ischemia, NEP activity gets affected due to altered AβPP processing by elevated expression of hypoxic products such as HNE [62]. This confirmed one possible mechanism by which NEP loses its activity and accumulation of Aβ in AD pathology and thus can correlate the role of NEP in Aβ clearance [63]. Apart from Aβ clearance, NEP also degrades several neuropeptides at the synapses such as enkephalins, substance P, tachykinins, neuropeptide-Y, and bradykinin. Besides the amyloid-degrading function of NEP, it is also involved in various functions within the brain which include memory and motor functions, synaptic plasticity, circadian rhythms, locomotion, sleep, anxiety, pain, blood-brain barrier (BBB) integrity, hyperalgesia, fatigue, water homeostasis, and neuroinflammation. Furthermore, its role has also been reported in the progression of a number of cancers, including renal, prostate, and lung cancer [64–66]. Since elucidation of the mechanistic role of any protein depends on its unique sequence and structure, its sequential and structural analyses have been done in order to gain additional molecular insights.

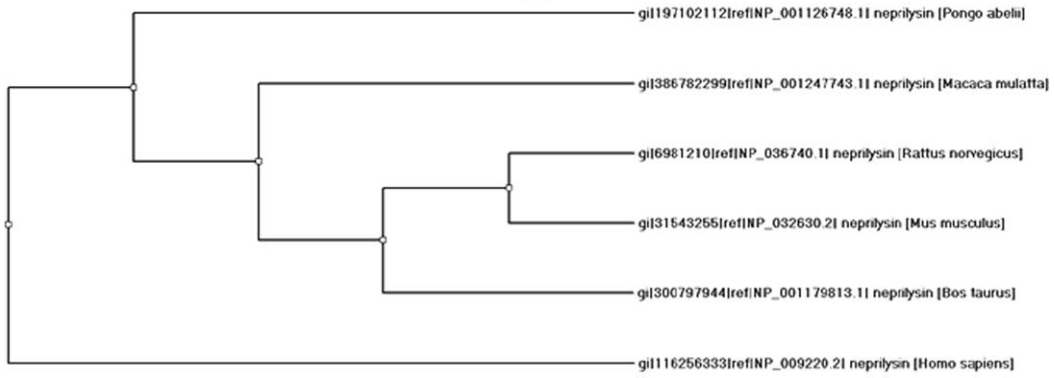
IN SILICO SEQUENTIAL AND STRUCTURAL ANALYSIS OF NEPRILYSIN

Primary structure analysis of NEP revealed it as a M13 family metallopeptidase (Source: InterPro) that preferentially cleaves polypeptides between the hydrophobic residues, especially Phe or Tyr. In order to understand the potential evolutionary relatives of NEP, BLAST tool has been used [67] and their sequence identity with NEP [*Homo sapiens*] is found to be (99%) NEP [*Macaca mulatta*], (99%) NEP [*Pongo abelii*], (94%) NEP [*Rattus norvegicus*], (94%) NEP [*Mus musculus*], and (91%) NEP [*Bos taurus*]. Moreover, multiple sequence alignments of these sequences (Fig. 2a) by ClustalW [68] revealed conserved zinc co-ordinating ligands at His584, His588, Glu646 [44], and active site aspartate residues at Asp591 and Asp651 [69] as well as 12 conserved cysteine residues that participate in the formation of six intra-subunit disulfide bonds (Cys57-Cys62, Cys80-Cys735, Cys88-Cys695, Cys143-Cys411, Cys234-Cys242, and Cys621-Cys747) [70]. Phylogenetic analysis of close relatives of NEP was performed by the neighbor-joining method [71] to construct a phylogenetic tree (Fig. 2b) that reflect evolutionary conservation between NEP [*Rattus norvegicus*] and NEP [*Macaca mulatta*], while NEP [*Homo sapiens*] as an out-group. The data obtained using ScanProsite [72] revealed one neutral zinc metallopeptidase (zinc-binding region) signature, which is important for its catalytic activity [73], 11 casein kinase II phosphorylation site, 11 protein kinase-C phosphorylation sites, one cAMP- and cGMP-dependent protein


10 20 30 40 50 60
gi|11625 (Homo sapiens) MGKSESQMDI TDINTPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|38678 (Macaca mulatta) MGRSESQMDI TDINTPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|19710 (Pongo abelii) MGKSESQMDI TDINTPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|69812 (Rattus norvegicus) MGRSESQMDI TDINAPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|31543 (Mus musculus) MGRSESQMDI TDINAPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
gi|30079 (Bos Taurus) MGRSESQMDI TDINTPKPKK KQRWTPLEIS LSVLVLLLTI IAVTMIALYA TYDDGICKSS
70 80 90 100 110 120
gi|11625 (Homo sapiens) DCIKSAARLI QNMDATTEPC TDFFKYACGG WLKRNVIPET SSRYGNFDIL RDELEVVLKD
gi|38678 (Macaca mulatta) DCIKSAARLI QNMDATAEPC ADFFKYACGG WLKRNVIPET SSRYGNFDIL RDELEVVLKD
gi|19710 (Pongo abelii) DCIKSAARLI QNMDATAEPC TDFFKYACGG WLKRNVIPET SSRYGNFDIL RDELEVVLKD
gi|69812 (Rattus norvegicus) DCIKSAARLI QNMDASAEPC TDFFKYACGG WLKRNVIPET SSRYSNFDIL RDELEVILKD
gi|31543 (Mus musculus) DCIKSAARLI QNMDASVEPC TDFFKYACGG WLKRNVIPET SSRYSNFDIL RDELEVILKD
gi|30079 (Bos Taurus) ECIKSAARLI QNMDATVEPC TDFFKYACGG WLKRNVIPET SSRYSNFDIL RDELEVVLKD
130 140 150 160 170 180
gi|11625 (Homo sapiens) VLQEPKTEDI VAVQKAKALY RSCINESAID SRGGEPLLKL LPDIYGWPVA TENWEQKYGA
gi|38678 (Macaca mulatta) VLQEPKTEDI VAVQKAKTLY RSCINESAID SRGGEPLLKL LPDIYGWPVA TENWEQKYGA
gi|19710 (Pongo abelii) VLQEPKTEDI VAVQKAKTLY RSCINESAID SRGGEPLLKL LPDVYGWPVA TENWEQKYGA
gi|69812 (Rattus norvegicus) VLQEPKTEDI VAVQKAKTLY RSCINESAID SRGGQPLLTL LPDIYGWPVA SQNWEQTYGT
gi|31543 (Mus musculus) VLQEPKTEDI VAVQKAKTLY RSCINESAID SRGGQPLLKL LPDIYGWPVA SDNWDQTYGT
gi|30079 (Bos Taurus) VLQEPKTEDI IAVQKAKMLY RSCINETAIE SRGGEPLLRL LPDIYDWPVA LENWEQKYGS
190 200 210 220 230 240
gi|11625 (Homo sapiens) SWTAEKAIAQ LNSKYGKKVL INLFVGTDDK NSVNHVIHID QPRLGLPSRD YYECTGIYKE
gi|38678 (Macaca mulatta) SWTAEKAIAQ LNSKYGKKVL INLFVGTDDK NSVNHVIHID QPRLGLPSRD YYECTGIYKE
gi|19710 (Pongo abelii) SWTAEKAIAQ LNSKYGKKVL INLFVGTDDK NSVNHVIHID QPRLGLPSRD YYECTGIYKE
gi|69812 (Rattus norvegicus) SWTAEKSIAQ LNSKYGKKVL INFFVGTDDK NSTQHIIHFD QPRLGLPSRD YYECTGIYKE
gi|31543 (Mus musculus) SWTAEKSIAQ LNSKYGKKVL INFFVGTDDK NSTQHIIHFD QPRLGLPSRD YYECTGIYKE
gi|30079 (Bos Taurus) SWTAEKSIAQ LNSKYGKKVI INFFVGTDDK NSTNHIIHID QPQLGLPSRD YYECTAAYKE
250 260 270 280 290 300
gi|11625 (Homo sapiens) ACTAYVDFMI SVARLIRQEE RLPIDENQLA LEMNKVMELE KEIANATAKP EDRNDPMLLY
gi|38678 (Macaca mulatta) ACTAYVDFMI SVARLIRQEE RLPIDENQLA LEMNKVMELE KEIANATAKP EDRNDPMLLY
gi|19710 (Pongo abelii) ACTAYVDFMI SVARLIRQEE RLPIDENQLA LEMNKVMELE KEIANATAKP EDRNDPMLLY
gi|69812 (Rattus norvegicus) ACTAYVDFMI SVARLIRQEQ RLPIDENQLS LEMNKVMELE KEIANATTKP EDRNDPMLLY
gi|31543 (Mus musculus) ACTAYVDFMI SVARLIRQEQ SLPIDENQLS LEMNKVMELE KEIANATTKP EDRNDPMLLY
gi|30079 (Bos Taurus) ACTAYVDFMI SVAKLIRQER GLPIDENQLS LEMNKVMDLE KEIANATTKS EDRNDPMLLY
310 320 330 340 350 360
gi|11625 (Homo sapiens) NKMTLAQIQN NFSLEINGKP FSWLNFTNEI MSTVNISITN EEDVVVYAPE YLTKLKPILT
gi|38678 (Macaca mulatta) NKMTLAQLQS NFSLEINGKP FSWLNFTNEI MSTVNISITN EEDVVVYAPE YLTKLKPILT
gi|19710 (Pongo abelii) KKMTLAQIQN NFSLEINGKP FSWLNFTNEI MSTVNISITN EEDVVVYAPE YLTKLKPILT
gi|69812 (Rattus norvegicus) NKMTLAKLQN NFSLEINGKP FSWSNFTNEI MSTVNINIQN EEEVVVYAPE YLTKLKPILT
gi|31543 (Mus musculus) NKMTLAKLQN NFSLEVNGKS FSWSNFTNEI MSTVNINIQN EEEVVVYAPE YLTKLKPILT
gi|30079 (Bos Taurus) NKMTLAEVQN KFSLEFSGKP FSWSNFTNEI MSTVNINIPH EEEVIVYAPE YLTNLKLILA
370 380 390 400 410 420
gi|11625 (Homo sapiens) KYSARDLQNL MSWRFIMDLV SSLSRTYKES RNAFRKALYG TTSETATWRR CANYVNGNME
gi|38678 (Macaca mulatta) KYSARDLQNL MSWRFIMDLV SSLSRTYKES RNAFRKALYG TTSETATWRR CANYVNGNME
gi|19710 (Pongo abelii) KYSARDLQNL MSWRFIMDLV SSLSRTYKES RNAFRKALYG TTSETATWRR CANYVNGNME
gi|69812 (Rattus norvegicus) KYSPRDLQNL MSWRFIMDLV SSLSRNYKES RNAFRKALYG TTSETATWRR CANYVNGNME
gi|31543 (Mus musculus) KYSPRDLQNL MSWRFIMDLV SSLSRNYKES RNAFRKALYG TTSETATWRR CANYVNGNME
gi|30079 (Bos Taurus) KYSARDLQNL MSWRFIMDLV SSLSRNYKES RNAFRKALYG TTSETATWRR CANYVNGNME
430 440 450 460 470 480
gi|11625 (Homo sapiens) NAVGRLYVEA AFAGESKHVV EDLIAQIREV FIQTLDDLTW MDAETKKRAE EKALAIKERI
gi|38678 (Macaca mulatta) NAVGRLYVEA AFAGESKHVV EDLIAQIREV FIQTLDDLTW MDAETKKRAE EKALAIKERI
gi|19710 (Pongo abelii) NAVGRLYVEA AFAGESKHVV EDLIAQIREV FIQTLDDLTW MDAETKKRAE EKALAIKERI
gi|69812 (Rattus norvegicus) NAVGRLYVEA AFAGESKHVV EDLIAQIREV FIQTLDDLTW MDAETKKKAE EKALAIKERI
gi|31543 (Mus musculus) NAVGRLYVEA AFAGESKHVV EDLIAQIREV FIQTLDDLTW MDAETKKKAE EKALAIKERI
gi|30079 (Bos Taurus) NAVGRLYVEA AFPGDSKHVV EDLIAQIRAV FIQTLDDLTW MDAETKKKAE EKALAIKERI
490 500 510 520 530 540
gi|11625 (Homo sapiens) GYPDDIVSND NKLNNEYLEL NYKEDEYFEN IIQNLKFSQS KQLKKLREKV DKDEWISGAA
gi|38678 (Macaca mulatta) GYPDDIVSND NKLNNEYLEL NYKEDEYFEN IIQNLKFSQS KQLKKLREKV DKDEWISGAA
gi|19710 (Pongo abelii) GYPDDIVSND NKLNNEYLEL NYKEDEYFEN IIQNLKFSQS KQLKKLREKV DKDEWISGAA
gi|69812 (Rattus norvegicus) GYPDDIISNE NKLNNEYLEL NYKEEEYFEN IIQNLKFSQS KQLKKLREKV DKDEWISGAA
gi|31543 (Mus musculus) GYPDDIISNE NKLNNEYLEL NYREDEYFEN IIQNLKFSQS KQLKKLREKV DKDEWISGAA
gi|30079 (Bos Taurus) GYPDDIISND DKLNKEYLEL SYQEEEYFEN IIQNLKFGQN RQLKKLREKV DKDEWISGAA



2(a)



2(b)

Fig. 2. a) Multiple sequence alignments of neprilysin (NEP) from different organisms with high sequence similarity. Motifs for NEP are highlighted in yellow color and conserved cysteine residues are highlighted in light grey color that is taking part in disulfide bond formation. Moreover, conserved zinc coordinating ligands His584, His588 and Glu646, and active site aspartate residues Asp591 and Asp651 are highlighted in black. Furthermore, secondary structural elements of NEP (*Homo sapiens*) obtained from DSSP are shown on the top of amino acid sequence as loop residues (green line), α-helices (red rectangles), and β-strands (blue arrows). Lastly, post translational modification sites are shown as colored amino acid residues with neutral zinc metallopeptidase region (light green), casein kinase II phosphorylation sites (pink), protein kinase-C phosphorylation sites (blue), N-myristoylation sites (orange), N-glycosylation sites (purple), amidation site (red), cAMP- and cGMP-dependent protein kinase phosphorylation site (light blue), and prenyl group binding site (brown). b) Phylogenetic relationship obtained by Neighbor-joining method using ClustalW. There is a single major cluster depicting evolutionarily highest conservation between NEP [*Rattus norvegicus*] and NEP [*Macaca mulatta*] with NEP [*Homo sapiens*] as out-group.

kinase phosphorylation site depicting their role in functional regulation of NEP via controlling their cellular locations, protein-protein interactions, and degradation by proteases. Moreover, there are 8 N-myristoylation sites and 6 N-glycosylation sites suggesting their role in membrane targeting and in imparting molecular stability to NEP, respectively [74, 75]. Lastly, one amidation site and one Prenyl group binding site (CAAX box) is also distributed well on the primary structure of NEP.

Table 1
List of sequence motifs present on Neprilysin identified by FingerPRINTScan

S. No.	Motif Name	Signature sequence	Id Score	Pf Score	p value	Sequence position
1	NEPRILYSIN (Neprilysin metalloprotease (M13) family signature)	WISGAAVVNAFYS	55.44	388	2.95E-05	535 to 548
		IVFPAGILQPPFF	82.23	567	1.81E-09	553 to 566
		NYGGIGMVIGHEITHGF	77.28	790	5.52E-12	574 to 591
		ENIADNGGLGQA	79.89	466	3.34E-07	647 to 659
2	CUATPASEI (Copper-transporting ATPase 1 signature)	TVNISITNEEDVVVYAPE	20	258	2.46E-03	333 to 351
		GAAVVNAFYSSGRNQIVFPAGILQ	17.5	180	6.27E-02	538 to 562
3	NRPEPTIDEY5R (Neuropeptide Y5 receptor signature)	EDEYFENIIQNLKFSQSKQL	34	223	5.20E-02	504 to 524
		DLVDWWTQQSASNFKEQSQCMVYQ	21.67	264	4.15E-03	602 to 626
4	RECA (RecA protein signature)	LKDVLQEPKTEDIVAVQKAKALYRSCINES	21.86	183	3.16E-02	118 to 148
		LGQAYRAYQNYIKKNGEE	25.28	191	1.17E-02	655 to 673
5	MTABOTROPICR (Metabotropic glutamate receptor signature)	FENIIQNLKFSQS	26.32	158	3.66E-02	508 to 521
		GGIGMVIGHEITHGFD	27.63	167	1.48E-02	576 to 592
6	DELTATUBULIN (Delta-tubulin signature)	DFMISVARLIRQEERLPIDEN	23.81	225	1.14E-02	247 to 268
		VGRLYVEAAFAGESKHVVED	22.5	155	8.11E-02	423 to 443
7	TNFC (GTP-binding elongation factor signature)	LVLLLTIIAVTMIALYATYDDG	27.27	200	3.24E-02	34 to 56
		EQKYGASWTAEKAIAQLNSKYGK	21.74	190	3.49E-02	175 to 198
8	TSHRECEPTOR (Thyrotropin receptor precursor signature)	SDCIKSAARLIQNMDATTE	20.18	190	8.36E-02	60 to 79
		GTLQNSAEFSEAFHCRKNSYMNPEKK	17.31	232	1.78E-02	721 to 747
9	SPLICEFRBRR1 (Pre-mRNA-splicing factor BRR1 signature)	AQLNSKYGKKVLINL	21.21	218	4.87E-02	189 to 204
		TKLKPILTKYSARDL	29.7	180	3.67E-02	353 to 368
10	AQUAPORIN10M (Mammalian aquaporin-10 signature)	RCANYVNGNME	36.36	230	3.30E-02	410 to 421
		SGAAVVNAFY	50	183	5.46E-02	537 to 547

Further, sequence motif analysis by FingerPRINTScan [76], identified motifs in 10 different families (Table 1) that belong to certain proteases, receptors, and factors signifying their involvement in cellular metabolic biological processes, such as modulation of peptides and regulation of signal transduction.

Furthermore, structural analysis of NEP (PDBID: 1R1H) revealed the presence of 43 α-helices (57%) and 15 β-strands (7%), which suggest that NEP is an α-rich protein (Source: DSSP) [77]. Structural classification of proteins or SCOP [78] analysis of NEP sequence suggested that it belonged to class of α and β proteins, with a Zinc-like fold and neutral endopeptidase family, and are classified as hydrolases (EC3.4.24.11). It is comprised of three domains viz. cytoplasmic (2–28), transmembrane (29–51), and extracellular (52–750). The structural topology of NEP is illustrated in Fig. 3, which is important to understand the protein unfolding mechanisms during its catalytic activity. NEP carries His583, His587, and Glu646 as coordinating ligands for Zn^{2+} ion [44, 79] and Asp590, Asp650 is assumed to form an active site triad along with coordinating histidine residues for its peptidase activity [69]. Moreover, homologous structures of NEP in the protein data bank have been identified by DALI server [80] (Table 2) that displayed its close structural similarity with endothelin-converting enzyme 1 (PDBID-3DWB) with Zscore: 49.6 and RMSD: 1.7. The obtained putative partners belong to proteases and co-proteases indicating their role in peptide cleavage or degradation.

INSULIN DEGRADING ENZYME: A THERAPEUTIC AGENT FOR Aβ DEGRADATION

IDE is an intracellular protease of 1019aa synthesized from a large reading frame of 3416 bp [81]. It is a single polypeptide chain of 110 kDa that naturally exists as a homodimer and requires one Zn^{2+} ion per subunit as a cofactor for its proteolytic activity [82, 83]. It is a thiol dependent, zinc metalloprotease that ubiquitously highly expressed in the brain, testes, liver, and muscles [84, 85]. IDE is principally located in cytosol, as well as on cell-surface [86, 87], although it has also been spotted within sub cellular organelles like mitochondria, endosomes, and peroxisomes, where its degradative role has been seen against oxidized proteins [88–90]. It is commonly known as insulysin or insulinase due to its well-known action against insulin and is located on chromosome 10q24, which is genetically linked with late-onset of AD genes [91, 92]. IDE possesses His-X-X-Glu-His (a zinc binding motif) at its active site that is common in a number of other eukaryotic and prokaryotic zinc peptidases [93]. Moreover, IDE has the potential to recognize the secondary and tertiary structures present in its substrates that enable it to recognize and cleave numerous small peptides, which include biologically active hormones and disease-related peptides, such as soluble amyloidogenic peptides, glucagon, calcitonin, amylin, atrial natriuretic peptide, insulin, and IGF-1

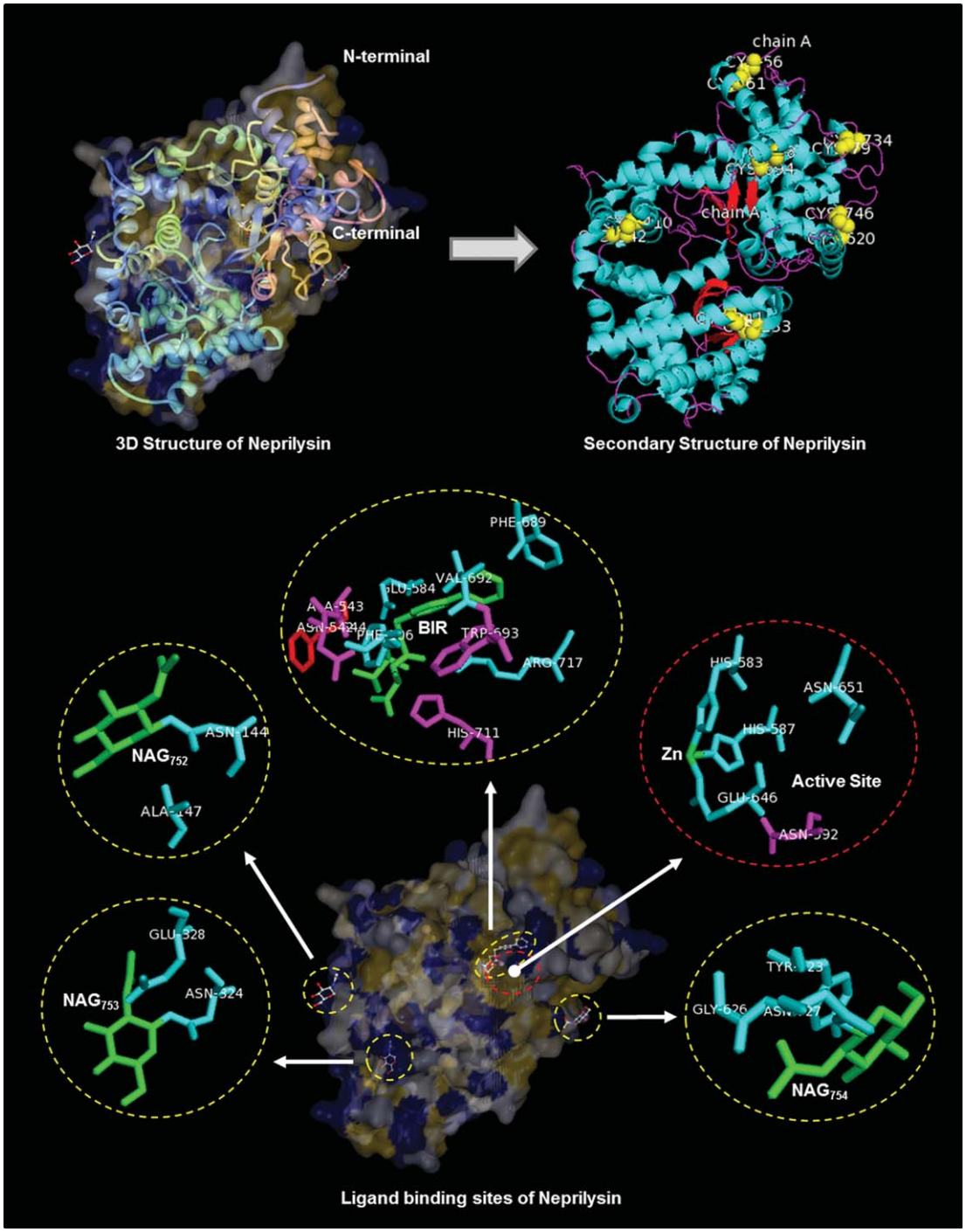


Fig. 3. 3D architecture of Neprilysin (PDB ID: 1R1H). Secondary structure of protein has 43 helices (blue), 15 strands (red), and loops (pink) where disulfide bridges are shown as yellow spheres (Cys56-Cys61, Cys79-Cys734, Cys87-Cys694, Cys142-Cys410, Cys233-Cys241, and Cys620-Cys746). Ligand binding sites of Neprilysin: Ligands are shown in green while ligand binding residues are shown in blue (helix forming residues), red (strand forming residues), and pink (loop residues). NAG (N-acetyl-D-glucosamine)-NAG_{752} binding residues-Asn144, Ala147; NAG_{753} binding residues-Asn324, Glu328; NAG_{754} binding residues-Tyr623, Gly626, Asn627; BIR (N-[3-[(1-aminoethyl)(Hydroxy)Phosphoryl]-2-(1,1'-biphenyl-4-ylmethyl)Propanoyl]Alanine) binding residues-Phe106, Asn542, Ala543, Phe544, Glu584, Phe689, Val692, Trp693, His711, Arg717; Zinc metal binding residues-His583, His587, Glu646, and active site residues-Asp591, Asp650.

Table 2
Ten potential putative partners of Neprilysin identified by DALI server

Name of Protein	PDB Code	Number of residues		Z-Score	RMSD	Sequence Identity (%)
		Total	Super-imposed			
Neprilysin	1R1H	696	696	69	0	100
Endothelin-Converting Enzyme 1	3DWB	661	658	49.6	1.7	41
Endopeptidase, Peptidase Family M13	3ZUK	658	641	44	2.2	31
Neutral Endopeptidase	4IUW	630	618	42	2.6	23
Peptidase, M48 Family	3C37	222	143	6.9	4	18
Endoplasmic Reticulum Aminopeptidase 2	3SE6	870	195	6.7	5.2	12
Tricorn Protease Interacting Factor F3	1Z1W	780	184	6.6	4.9	13
Glutamyl Aminopeptidase	4KXB	875	205	6.5	5.1	9
Thermolysin	3EIM	316	150	6.4	3.4	16
Secreted Metalloprotease MCP02	3NGX	299	144	6.2	3.4	18

and 2 at physiological pH [87, 94–96]. Recently, IDE has been identified as the protease responsible for the conversion of β-endorphin to γ-endorphin, signifying its role in signal transduction in the brain [97, 98]. Although it identifies amyloidogenic peptides, it can cleave them only in their monomeric soluble form and not in their aggregated form [43, 99]. It also digests cytoplasmic and monomeric Aβ peptides [100]. However, these peptides contain different proportions of α-helix and β-sheet structure in solution, but upon enzyme action or self-aggregation, they assume the β-sheet conformation. Multiple studies indicate that IDE provides a surface for its substrates to assume a β-sheet, where the residues interacting with IDE are same that are responsible for oligomerization and fibrillization of amyloidogenic peptides [101]. Thus, IDE acts as an amyloid-scavenging enzyme, hampering the formation of amyloid plaques, and nullifies their toxic effects [102]. Further studies identified the consensus sequence responsible for IDE-substrate interaction, i.e., '*hnhhhpsh*' where h represents hydrophobic, n (small, neutral), p (polar), and s (polar and/or small) amino acid residues [96, 103, 104].

Typical AD pathology, such as formation of amyloid-plaques, is found to be exacerbated by insulin dysregulation in the brain where insulin resistance and low insulin levels have been seen. IRs occupy neuronal synapses and astrocytes in the memory-processing brain regions such as cerebral cortex, hippocampus, olfactory bulb, cerebellum, and hypothalamus [105]. At the molecular level, when insulin binds with IR, it triggers certain signaling cascade that is mainly associated with the formation of long-term memory and learning, which comprised of intracellular signaling molecules; for instance, Grb-r/SOS, shc, Ras/Raf, and MEK/MAP kinases. Another signaling cascade, which includes IRS-1, PI3K/Akt/GSK-3, protein kinase-B/C, and non-receptor tyrosine kinase-pp60c-src molecules are also coupled with memory processing [106–108] and further activate other factors like IGFs and transforming growth factors [109]. Any dysregulation in the level of these factors in the brain imparts insulin resistance to the IRs [110, 111]. In addition, when insulin levels reach to significantly high level, Aβ, a major culprit neurotoxin in AD, starts to accumulate in senile plaques, resulting in neuroinflammation. Further, it is evidenced by the fact that exaggerated plasma insulin levels lead to an increased burden of Aβ peptide in the cerebrospinal fluid, resulting in memory loss; therefore, AD is also characterized as "neuroendocrine disorder" associated with insulin signaling [8, 112]. Insulin plays a crucial role in controlling neurotransmitter release at the synapses and triggers signaling cascades associated with learning/long-term memory, energy metabolism, glucose utilization, and neuronal survival [113]. It not only regulates the blood-sugar level but also acts as a growth factor on all cells, including neurons in CNS; therefore, any disturbances in insulin signaling might hamper cellular repair mechanisms, cell growth/differentiation, and provoke numerous degenerative processes.

IN SILICO SEQUENTIAL AND STRUCTURAL ANALYSIS OF INSULIN DEGRADING ENZYME

Primary structure analysis of IDE revealed it as a metalloenzyme and M16 family metallopeptidase such as s-ribosyl homocysteinase and mitochondrial processing peptidase respectively (Source: InterPro). Potential evolutionary relatives of IDE [*Homo sapiens*] were obtained by BLAST [67] that revealed its sequence identity as follows: (99%) IDE [*Macaca mulatta*], (98%) IDE precursor [*Bos taurus*], (98%) IDE [*Bos mutus*], (95%) IDE [*Mus musculus*]. Further,

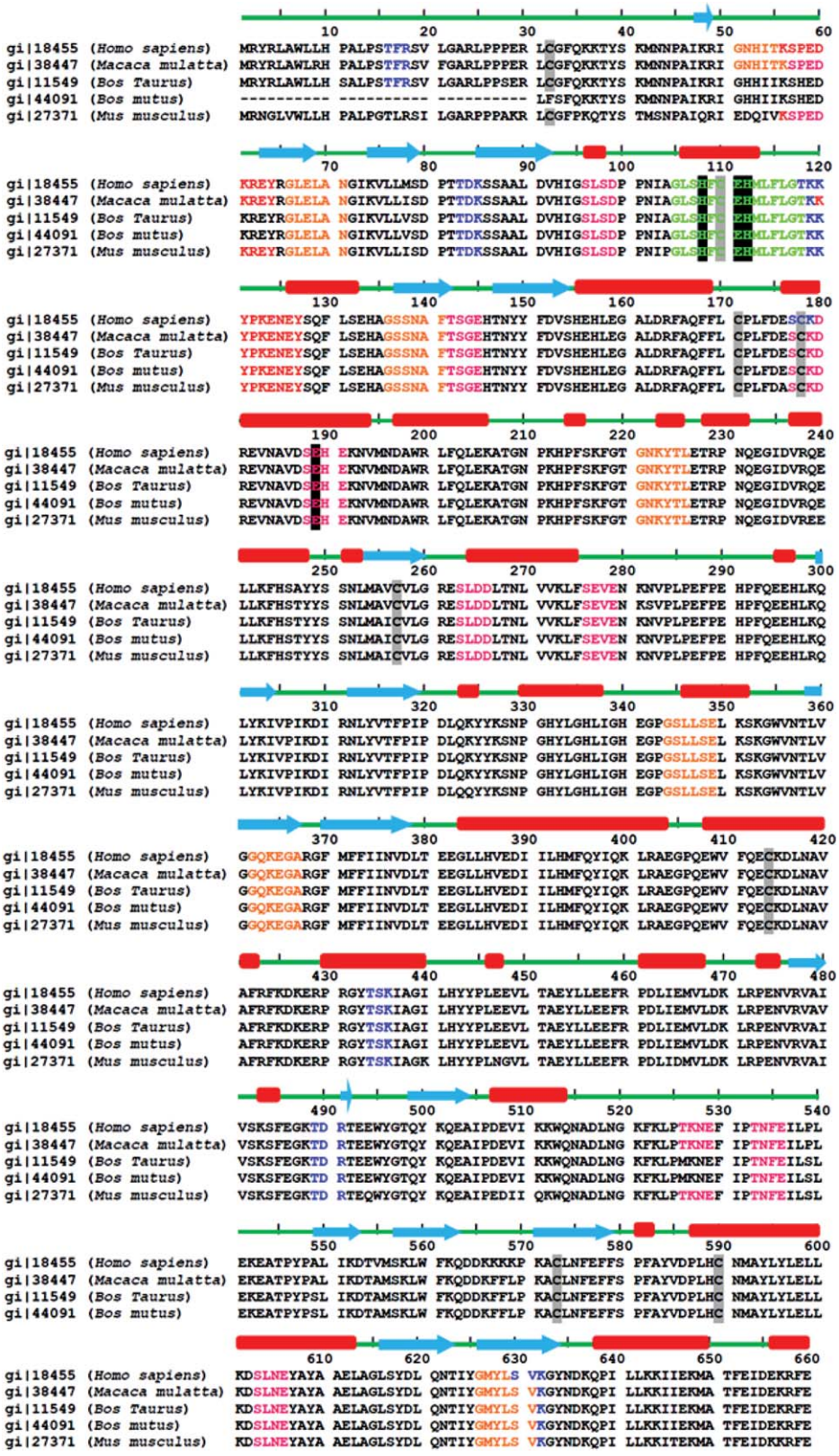
multiple sequence alignments of these sequences (Fig. 4a) by ClustalW [68] revealed 11 conserved cysteine residues signifying their role in regulation of IDE via oxidative or nitrosative processes; Zn^{2+} ion coordinating ligands at His108, His112, and Glu189 [114] and a conserved glutamate residue (Glu111) at the active site [83]. Phylogenetic analysis of a close relative of IDE was performed by the neighbor-joining method [71] to obtain a phylogenetic tree (Fig. 4b) that indicated evolutionary conservation between IDE precursor [*Bos taurus*] and IDE partial [*Bos mutus*], while IDE [*Homo sapiens*] as an out-group. Further, analysis using ScanProsite [72] identified one insulinase family (zinc-binding region) signature, which is important for its catalytic activity [115], 10 protein kinase-C phosphorylation sites, 5 tyrosine kinase phosphorylation sites, 16 casein kinase II phosphorylation sites, one cAMP- and cGMP-dependent protein kinase phosphorylation site depicting their role in functional regulation of IDE via controlling their cellular locations, protein-protein interactions, and degradation by proteases. Moreover, there are seven N-myristoylation sites and three N-glycosylation sites suggesting their role in membrane targeting and in imparting molecular stability to IDE, respectively [74, 75]. Finally, one microbodies C-terminal targeting the signal sites is also distributed well on the primary structure of IDE.

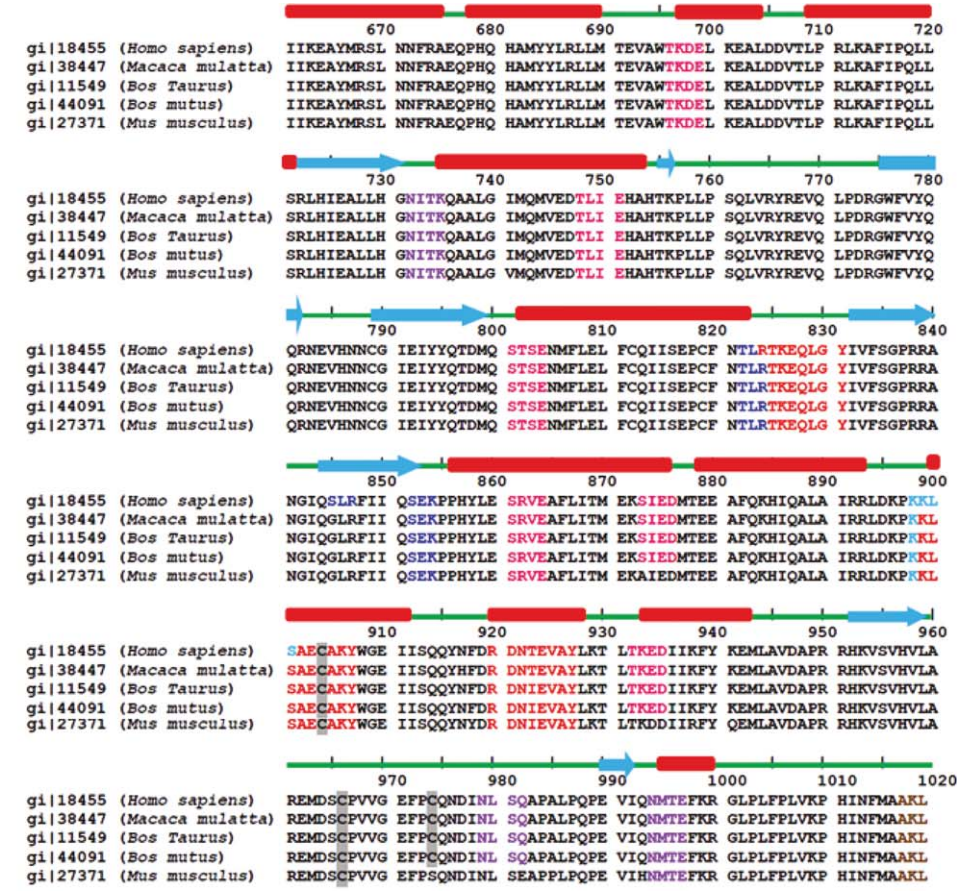
Further, sequence motifs analysis by FingerPRINTScan revealed motifs in nine different families of proteins (Table 3) that belong to certain transcription factors and nuclear receptors depicting their significant role in signaling processes.

Structural analysis of IDE (PDB ID: 4IFH) revealed the presence of 44 α-helices (41%) and 32 β-strands (19%), which suggest that IDE is an α-rich protein (Source: DSSP) [77] and belongs to the peptidase family M16 (EC 3.4.24.56) and has two isoforms: membrane associated and cytoplasmic. Further, its quaternary structure comprised of homodimers where it requires one Zn^{2+} ion per subunit for its peptidase activity. The structural topology of IDE is illustrated in Fig. 5, which is important for the understanding of protein unfolding mechanisms during its catalytic activity. IDE carries His108, His112, and Glu189 as coordinating ligands for Zn^{2+} ion [114] and Glu111 at active site contributing to its catalytic activity [83]. Moreover, homologous structures of IDE in the protein data bank have been identified by DALI server [80] (Table 4) that displayed its close structural similarity with Protease III (PDB ID: 1Q2L) with Z score: 30.8 and RMSD: 7.1. The obtained putative partners belong to different peptidases suggesting their role in peptide cleavage or degradation.

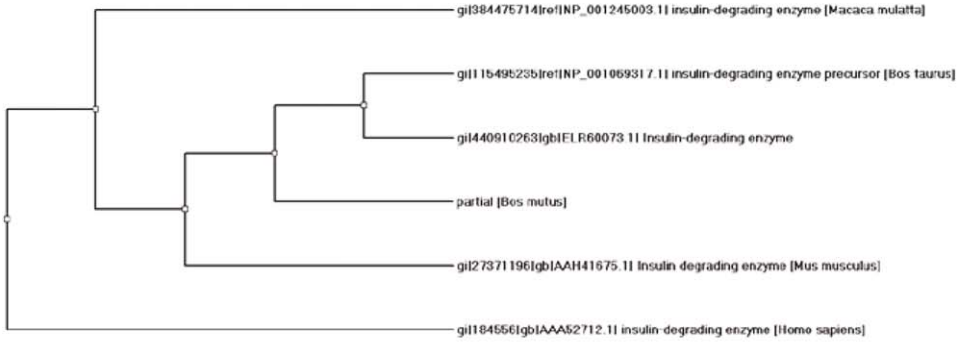
INSULIN DEGRADING ENZYME AND NEPRILYSIN MEDIATED Aβ CLEARANCE

Aβ accumulation is one of the characteristic features of AD that contributes to plaque formation and provokes the formation of toxic oligomeric Aβ complexes in the brain. The main component of plaques is the hydrophobic Aβ which is an approximately 4.2 kDa peptide formed mainly by proteolytic cleavage of the amyloid-β protein precursor (AβPP) by the action of proteolytic enzymes. The proteolytic cleavage is accompanied by two pathways, non-amyloidogenic (α-secretase) and amyloidogenic (β- and γ-secretase). The α-secretase cleavage site is mainly situated at position 16 within the Aβ sequence precluding the generation of Aβ, while amyloidogenic AβPP processing the main pathway of AβPP cleavage in cells that takes place at the plasma membrane. β- and γ-secretases produce various biologically active metabolites, including the Aβ and the AβPP intracellular domain (AICD) by virtue of proteolytic cleavage of AβPP [116]. Furthermore, the γ-secretase complex consists of four major proteins, including PS1 or PS2, Aph (anterior pharynx defective) 1a, presenilin enhancer2 (PEN2), and nicastrin. It cleaves its substrates within the membrane, a process often involved in important signaling cascades and termed regulated intramembrane proteolysis [35]. Since the catalytic subunit of γ-secretase is either PS1 or PS2, any mutations in AβPP or the multi-subunit protease complex γ-secretase components are one of the reasons for accumulation of Aβ that may lead to early-onset of familial AD [117]. Besides the *de novo* synthesis of Aβ caused by amyloidogenic processing of AβPP, Aβ levels are also highly dependent on Aβ degradation. Proteolytic cleavage by β- and γ-secretases following amyloidogenic cascade makes Aβ neurotoxic, while proteolytic cleavage by α-secretase makes soluble Aβ, which is neuroprotective in nature. There are two major proteases (IDE and NEP) involved in Aβ clearance that act on different targets responsible for Aβ deposition in the brain (Fig. 6). It has been observed that overexpression of IDE resulted in degradation of AICD and insulin to significantly reduced Aβ burden in AD patients [81, 118]. Furthermore, AICD binds to the NEP promoter and leads to transcriptional activation of NEP by competitive replacement with histone deacetylases. This transcriptional activation of NEP raises its expression in the brain and leads to Aβ clearance [119]. Recently, it has been identified that long isoform of IDE (IDE-Met1) is also involved in Aβ clearance following mitochondrial biogenesis path-


10 20 30 40 50 60
gi|18455 (Homo sapiens) MRYRLAWLLH PALPSTFRSV LGARLPPPER LCGFQKKTYS KMNNPAIKRI GNHITKSPED
gi|38447 (Macaca mulatta) MRYRLAWLRH PALPSTFRSV FGARLPPPER LCGFQKKTYS KMNNPAIKRI GNHITKSPED
gi|11549 (Bos Taurus) MRYRLAWLLH SALPSTFRSV LGARLPPSER LCGFQKKTYS KMNNPAIKRI GHHIIKSHED
gi|44091 (Bos mutus) ---------- ---------- ---------- LFSFQKKTYS KMNNPAIKRI GHHIIKSHED
gi|27371 (Mus musculus) MRNGLVWLLH PALPGTLRSI LGARPPPAKR LCGFPKQTYS TMSNPAIQRI EDQIVKSPED
70 80 90 100 110 120
gi|18455 (Homo sapiens) KREYRGLELA NGIKVLLMSD PTTDKSSAAL DVHIGSLSDP PNIAGLSHFL EHMLFLGTKK
gi|38447 (Macaca mulatta) KREYRGLELA NGIKVLLISD PTTDKSSAAL DVHIGSLSDP PNIAGLSHFL EHMLFLGTKK
gi|11549 (Bos Taurus) KREYRGLELA NGIKVLLVSD PTTDKSSAAL DVHIGSLSDP PNIAGLSHFL EHMLFLGTKK
gi|44091 (Bos mutus) KREYRGLELA NGIKVLLVSD PTTDKSSAAL DVHIGSLSDP PNIAGLSHFL EHMLFLGTKK
gi|27371 (Mus musculus) KREYRGLELA NGIKVLLISD PTTDKSSAAL DVHIGSLSDP PNIPGLSHFL EHMLFLGTKK
130 140 150 160 170 180
gi|18455 (Homo sapiens) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDESCKD
gi|38447 (Macaca mulatta) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDESCKD
gi|11549 (Bos Taurus) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDESCKD
gi|44091 (Bos mutus) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDESCKD
gi|27371 (Mus musculus) YPKENEYSQF LSEHAGSSNA FTSGEHTNYY FDVSHEHLEG ALDRFAQFFL CPLFDASCKD
190 200 210 220 230 240
gi|18455 (Homo sapiens) REVNAVDSEH EKNVMNDAWR LFQLEKATGN PKHPFSKFGT GNKYTLETRP NQEGIDVRQE
gi|38447 (Macaca mulatta) REVNAVDSEH EKNVMNDAWR LFQLEKATGN PKHPFSKFGT GNKYTLETRP NQEGIDVRQE
gi|11549 (Bos Taurus) REVNAVDSEH EKNVMNDAWR LFQLEKATGN PKHPFSKFGT GNKYTLETRP NQEGIDVRQE
gi|44091 (Bos mutus) REVNAVDSEH EKNVMNDAWR LFQLEKATGN PKHPFSKFGT GNKYTLETRP NQEGIDVRQE
gi|27371 (Mus musculus) REVNAVDSEH EKNVMNDAWR LFQLEKATGN PKHPFSKFGT GNKYTLETRP NQEGIDVREE
250 260 270 280 290 300
gi|18455 (Homo sapiens) LLKFHSAYYS SNLMAVCVLG RESLDDLTNL VVKLFSEVEN KNVPLPEFPE HPFQEEHLKQ
gi|38447 (Macaca mulatta) LLKFHSTYYS SNLMAVCVLG RESLDDLTNL VVKLFSEVEN KSVPLPEFPE HPFQEEHLKQ
gi|11549 (Bos Taurus) LLKFHSTYYS SNLMAICVLG RESLDDLTNL VVKLFSEVEN KNVPLPEFPE HPFQEEHLKQ
gi|44091 (Bos mutus) LLKFHSTYYS SNLMAICVLG RESLDDLTNL VVKLFSEVEN KNVPLPEFPE HPFQEEHLKQ
gi|27371 (Mus musculus) LLKFHSTYYS SNLMAICVLG RESLDDLTNL VVKLFSEVEN KNVPLPEFPE HPFQEEHLRQ
310 320 330 340 350 360
gi|18455 (Homo sapiens) LYKIVPIKDI RNLYVTFPIP DLQKYYKSNP GHYLGHLIGH EGPGSLLSEL KSKGWVNTLV
gi|38447 (Macaca mulatta) LYKIVPIKDI RNLYVTFPIP DLQKYYKSNP GHYLGHLIGH EGPGSLLSEL KSKGWVNTLV
gi|11549 (Bos Taurus) LYKIVPIKDI RNLYVTFPIP DLQKYYKSNP GHYLGHLIGH EGPGSLLSEL KSKGWVNTLV
gi|44091 (Bos mutus) LYKIVPIKDI RNLYVTFPIP DLQKYYKSNP GHYLGHLIGH EGPGSLLSEL KSKGWVNTLV
gi|27371 (Mus musculus) LYKIVPIKDI RNLYVTFPIP DLQQYYKSNP GHYLGHLIGH EGPGSLLSEL KSKGWVNTLV
370 380 390 400 410 420
gi|18455 (Homo sapiens) GGQKEGARGF MFFIINVDLT EEGLLHVEDI ILHMFQYIQK LRAEGPQEWV FQECKDLNAV
gi|38447 (Macaca mulatta) GGQKEGARGF MFFIINVDLT EEGLLHVEDI ILHMFQYIQK LRAEGPQEWV FQECKDLNAV
gi|11549 (Bos Taurus) GGQKEGARGF MFFIINVDLT EEGLLHVEDI ILHMFQYIQK LRAEGPQEWV FQECKDLNAV
gi|44091 (Bos mutus) GGQKEGARGF MFFIINVDLT EEGLLHVEDI ILHMFQYIQK LRAEGPQEWV FQECKDLNAV
gi|27371 (Mus musculus) GGQKEGARGF MFFIINVDLT EEGLLHVEDI ILHMFQYIQK LRAEGPQEWV FQECKDLNAV
430 440 450 460 470 480
gi|18455 (Homo sapiens) AFRFKDKERP RGYTSKIAGI LHYYPLEEVL TAEYLLEEFR PDLIEMVLDK LRPENVRVAI
gi|38447 (Macaca mulatta) AFRFKDKERP RGYTSKIAGI LHYYPLEEVL TAEYLLEEFR PDLIEMVLDK LRPENVRVAV
gi|11549 (Bos Taurus) AFRFKDKERP RGYTSKIAGI LHYYPLEEVL TAEYLLEEFR PDLIEMVLDK LRPENVRVAI
gi|44091 (Bos mutus) AFRFKDKERP RGYTSKIAGI LHYYPLEEVL TAEYLLEEFR PDLIEMVLDK LRPENVRVAI
gi|27371 (Mus musculus) AFRFKDKERP RGYTSKIAGK LHYYPLNGVL TAEYLLEEFR PDLIDMVLDK LRPENVRVAI
490 500 510 520 530 540
gi|18455 (Homo sapiens) VSKSFEGKTD RTEEWYGTQY KQEAIPDEVI KKWQNADLNG KFKLPTKNEF IPTNFEILPL
gi|38447 (Macaca mulatta) VSKSFEGKTD RTEEWYGTQY KQEAIPDEVI KKWQNADLNG KFKLPTKNEF IPTNFEILPL
gi|11549 (Bos Taurus) VSKSFEGKTD RTEEWYGTQY KQEAIPDEVI KKWQNADLNG KFKLPMKNEF IPTNFEILSL
gi|44091 (Bos mutus) VSKSFEGKTD RTEEWYGTQY KQEAIPDEVI KKWQNADLNG KFKLPMKNEF IPTNFEILSL
gi|27371 (Mus musculus) VSKSFEGKTD RTEQWYGTQY KQEAIPEDII QKWQNADLNG KFKLPTKNEF IPTNFEILSL
550 560 570 580 590 600
gi|18455 (Homo sapiens) EKEATPYPAL IKDTVMSKLW FKQDDKKKKP KACLNFEFFS PFAYVDPLHC NMAYLYLELL
gi|38447 (Macaca mulatta) EKEATPYPAL IKDTAMSKLW FKQDDKFFLP KACLNFEFFS PFAYVDPLHC NMAYLYLELL
gi|11549 (Bos Taurus) EKEATPYPSL IKDTAMSKLW FKQDDKFFLP KACLNFEFFS PFAYVDPLHC NMAYLYLELL
gi|44091 (Bos mutus) EKEATPYPSL IKDTAMSKLW FKQDDKFFLP KACLNFEFFS PFAYVDPLHC NMAYLYLELL
610 620 630 640 650 660
gi|18455 (Homo sapiens) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKIIEKMA TFEIDEKRFE
gi|38447 (Macaca mulatta) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKIIEKMA TFEIDEKRFE
gi|11549 (Bos Taurus) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKIIEKMA TFEIDEKRFE
gi|44091 (Bos mutus) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKIIEKMA TFEIDEKRFE
gi|27371 (Mus musculus) KDSLNEYAYA AELAGLSYDL QNTIYGMYLS VKGYNDKQPI LLKKITEKMA TFEIDKKRFE



4(a)



4(b)

Fig. 4. a) Multiple sequence alignment of insulin degrading enzyme (IDE) from different organisms with high sequence similarity. Conserved cysteine residues are highlighted in gray while Zn^{2+} ion coordinating ligands His108, His112, and Glu189, and a conserved active site glutamate (Glu111) residue is highlighted in black. Further, secondary structural elements of IDE (*Homo sapiens*) obtained from DSSP are shown on the top of amino acid sequence with loop residues (green line), α-helices (red rectangles), and β-strands (blue arrows). Moreover, potential sites are shown as colored amino acid residues as follows insulinase family (zinc binding region) signature (light green), casein kinase II phosphorylation sites (pink), protein kinase-C phosphorylation sites (blue), N-myristoylation sites (orange), N-glycosylation sites (purple), tyrosine kinase phosphorylation sites (red), cAMP- and cGMP-dependent protein kinase phosphorylation site (light blue), and microbodies C-terminal targeting signal (brown). b) Phylogenetic relationship obtained by Neighbor-joining method using ClustalW. There is a single major cluster depicting evolutionarily highest conservation between IDE precursor [*Bos taurus*] and IDE partial [*Bos mutus*] with IDE [*Homo sapiens*] as an out-group.

Table 3
List of sequence motifs present on IDE identified by FingerPRINTScan

S.No.	Motif Name	Signature sequence	Id Score	Pf Score	*p* value	Sequence position
1	LYMPHOTACTNR (Lymphotactin	LGRESLDDLTNLVV	39.29	295	4.36E-03	259 to 273
	receptor signature)	FRFKDKERPRG	40.91	215	6.09E-02	422 to 433
2	F138DOMAIN (FAM138 N-	GIKVLLMSDPTTDK	30.95	192	3.28E-03	72 to 86
	terminal domain signature)	LSDPPNIAGLSHFCEH	26.39	127	8.16E-02	97 to 113
3	LVIRUSORF2 (Luteovirus ORF2	HAGSSNAFTSGEHTNYYFDVSH	25.97	221	4.22E-02	134 to 156
	putative replicase 1 signature)	LIEMVLDKLRPENVRVAIV	15.79	178	2.03E-02	463 to 482
4	SIGMA70FCT (Major sigma-	NITKQAALGIMQMV	18.57	189	2.80E-02	732 to 746
	70 factor signature)	QKHIQALAIRRL	30.48	167	3.12E-02	883 to 894
5	CYTOCHROMEF (Cytochrome	LYKIVPIKDIRNLYVTFPIPD	23.4	174	9.01E-02	301 to 322
	F signature)	IEMVLDKLRPENVRVAIV	34.3	202	3.57E-02	464 to 482
6	NUCLEARECPTR (Orphan	EEFRPDLIEMVLDKLRPE	20.71	177	3.30E-02	457 to 475
	nuclear receptor (4A nuclear	CAKYWGEIISQQYNFD	29.55	218	9.99E-02	904 to 920
	receptor) family signature)					
7	FLGFLGJ (Flagellar	SPFAYVDPLHCNMAYLYLELLKDS	16.67	210	6.78E-02	580 to 604
	protein FlgJ signature)	KSIEDMTEEAFQKHIQAL	22.22	220	5.05E-02	872 to 890
8	COUPTNFACTOR (COUP transcription	NFEILPLEKEATPYPA	32.5	179	9.54E-02	534 to 550
	factor (2F nuclear receptor)	PRLKAFIPQLLSRL	26.19	180	4.91E-02	710 to 724
	family signature)					
9	NISCPROTEIN (Nisin biosynthesis	KDKERPRGYTSKIAGIL	29.41	240	9.03E-02	425 to 442
	protein NisC signature)	LIKDTVMSKLWFKQDDKKKKPKAC	25	230	7.99E-02	550 to 574

Table 4
Ten potential putative partners of IDE identified by DALI server

Name of Protein	PDB Code	Number of residues		Z-Score	RMSD	Sequence Identity (%)
		Total	Superimposed			
Insulin-Degrading Enzyme	4IFH	956	955	60.2	0	100
Protease III	1Q2L	937	684	30.8	7.1	28
Zinc Metalloprotease	2FGE	979	801	20.1	4.2	12
Falcilysin	3S5K	1053	808	17.8	4.3	13
Zinc Peptidase	3AMI	422	406	16.4	3.4	17
Ubiquinol-Cytochrome-C Reductase Complex Core Pro	2A06	442	412	15.6	3.5	17
NADH-Quinone Oxidoreductase Subunit 1	2YBB	442	412	15.5	3.5	17
Mitochondrial Processing Peptidase Alpha Subunit	1HR7	440	413	15.5	3.5	16
Peptidase M16 Inactive Domain Family Protein	3GWB	412	395	15.5	3.6	14
Cytochrome BC1 Complex	1BGY	446	403	14.7	3.5	15

way. IDE-Met1 links the mitochondrial biogenesis pathway with mitAβ levels and organelle functionality. Activation of PGC1-α by the effect of mitochondrial biogenesis stimuli promotes NRF-1 expression, which makes long (IDE-Met1) and the short (IDE-Met42) IDE isoforms. Furthermore, the long IDE isoform is involved in mitochondrial Aβ clearance without any toxic effect [120]. Another important candidate for Aβ clearance is ApoE, which acts within microglia and in the extracellular space to affect the clearance of Aβ through promoting its proteolysis by IDE and NEP. The endolytic degradation of Aβ peptides within microglia is facilitated by NEP, while extracellular Aβ is degraded by IDE. Since the ability of ApoE to promote Aβ degradation is dependent upon the ApoE isoform and its lipidation status, lipidated ApoE is formed by transfer of lipids to ApoE, which is accomplished mainly by ABCA1 and LXR activation in the nucleus. It has also been reported that one isoform of ApoE, ApoE4, is associated with higher risk of AD, while the ApoE2 and ApoE3 isoforms are associated with lower risk of AD compared with ApoE4 [121]. Furthermore, the receptor for the advanced glycation end products (RAGE) act as a transporter of Aβ across the BBB into the CNS where it deposits Aβ, while the low-density lipoprotein receptor-related protein (LRP) mediates extra burden of Aβ outside the brain. Both IDE and NEP are also involved in RAGE-mediated Aβ degradation inside the brain, while ApoE, ApoJ, and α_2-macroglobulin are involved in Aβ transportation outside the brain in liver for degradation through LRP complex [122]. Since RAGE assists in Aβ accumulation inside the neuron, IDE and NEP bind to RAGE and blocks their functions in order to maintain memory cognition and neuronal survival [123].

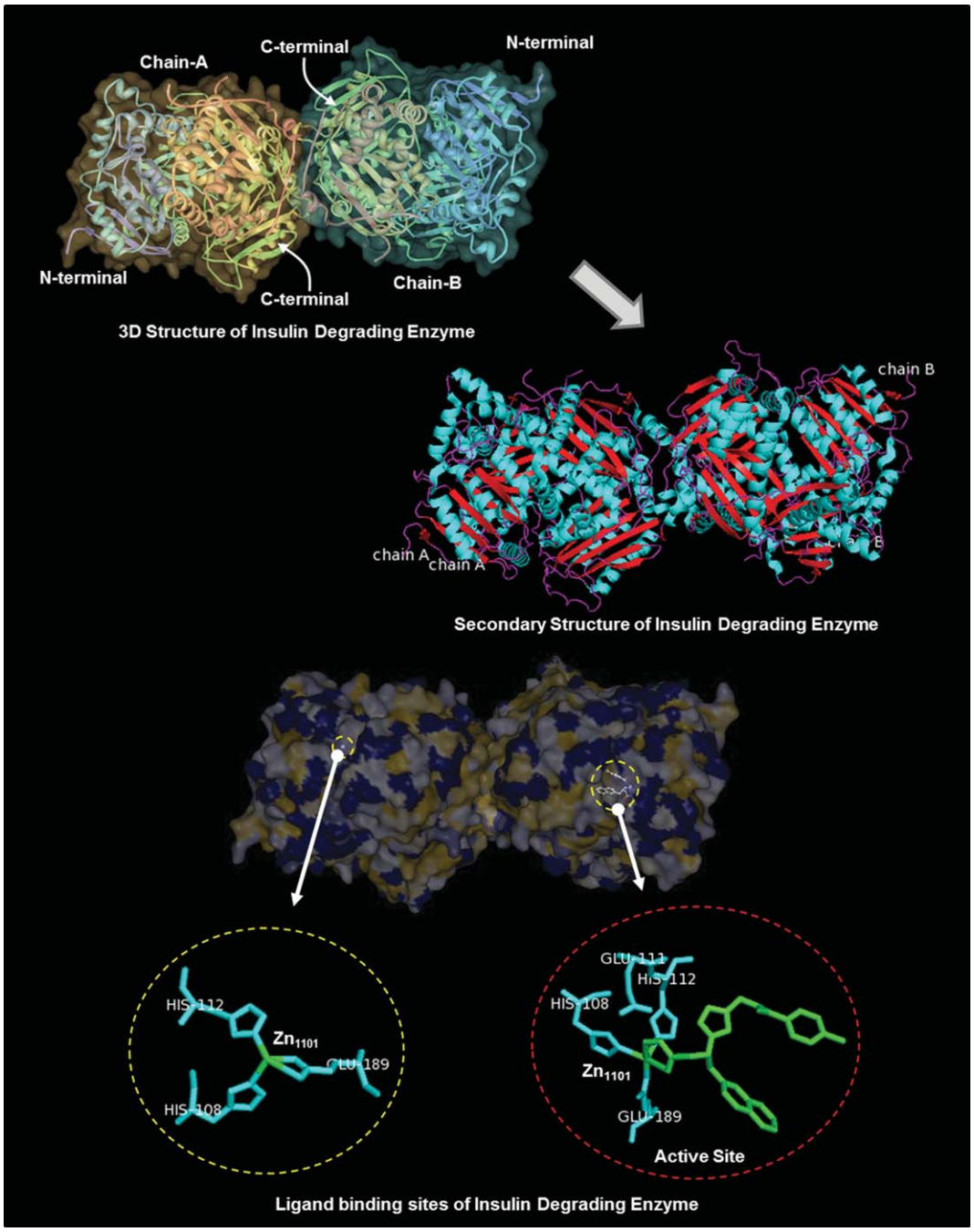


Fig. 5. 3D architecture of Insulin degrading enzyme (PDB ID: 4IFH). Secondary structure of protein has 44 helices (blue), 32 strands (red), and loops (pink) without any disulfide bridges. Ligand binding sites of IDE: Ligands are shown in green, while ligand binding residues are shown in blue (Helix forming residues). Chain A: Zinc metal binding residues- His108, His112, Glu189; Chain B: Zinc metal binding residues (His108, His112, and Glu189) and active site residue (Glu111).

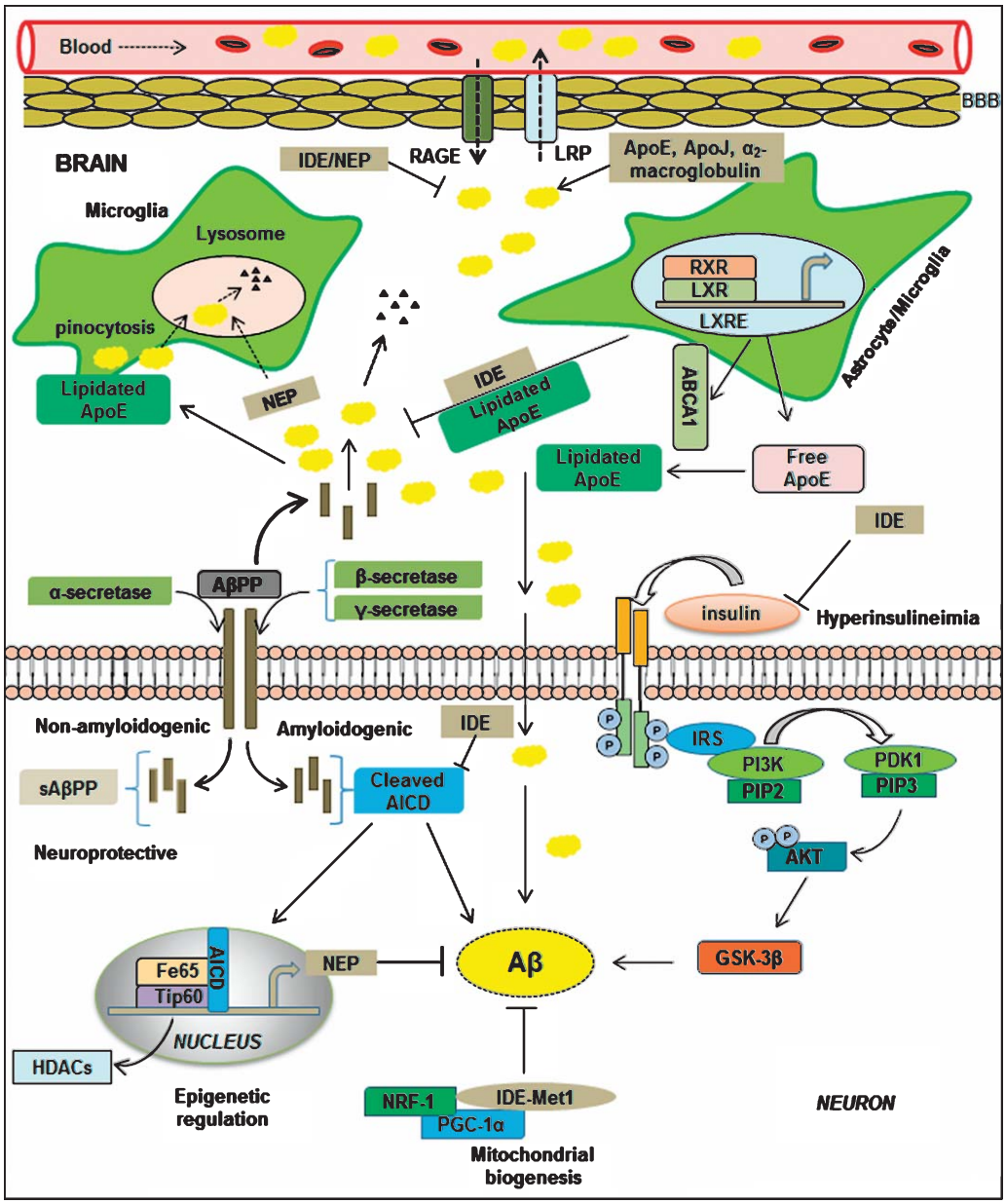


Fig. 6. Cross-talk between insulin degrading enzyme and neprilysin (NEP)-mediated Aβ degradation. Following Aβ aggregation via amyloidogenic pathway, simultaneously Aβ degradation is also critical for normal brain functionality. Here in this figure, IDE and NEP are playing a crucial role in order to reduce the toxic effect of Aβ. IDE is degrading AICD, insulin, and mitAβ, and also directs the extracellular degradation of Aβ together with lipidated ApoE, whereas NEP is epigenetically regulated by AICD raising its expression for Aβ clearance and also involved in intracellular Aβ degradation along with lipidated ApoE. Further, these enzymes are also participating in RAGE-mediated Aβ degradation inside the brain, while ApoE, ApoJ, and α_2-macroglobulin is assisting Aβ transportation outside the brain via LRP complex for degradation in liver.

INTEGRATIVE ROLE OF INSULIN DEGRADING ENZYME AND NEPRILYSIN IN AD THERAPEUTICS

In the case of AD, it is difficult with a single model to entirely mimic the disease progression in humans. Therefore, models are developed based on different proposed pathways for testing the pharmacodynamics of the drugs and also the biology of the disease and its development [124]. To date, extracellular accumulation of Aβ is found to be the main pathology involved in AD, thus reduction of Aβ deposition is the major

concern for neurobiologists. To achieve this goal, different strategies need to be formulated that could slow down the disease progression or even lead the way to discover its treatment. Such a method includes the degradation of Aβ with the action of proteolytic enzymes such as NEP and IDE [43]. Moreover, elevated concentration of Aβ has been observed in response to altered gene expression of NEP and IDE. In one study, overexpression of IDE and NEP in AβPP-transgenic mice was found to decrease Aβ levels in the brains of the transgenic mice as compared to the non-transgenic mice. Mice that expressed IDE showed 2-fold reduction in Aβ deposition, while mice that showed the 8-fold increase in NEP levels were free of Aβ deposits, thereby exhibiting its potential in reduction of lethal phenotype [125, 126]. These observations conclude that enhancement in the level of these enzymes would confer a protective effect against AD. Thus, supplying excess of degrading enzymes with the help of various approaches such as gene therapy or pharmacological drugs could be a potential therapy against AD [127–129]. The major hurdle impeding the development of novel therapies for AD is the ability to cross the BBB. However, this problem has recently been addressed in a number of ways as follows.

CURRENT STRATEGIES TO DELIVER INSULIN DEGRADING ENZYME AND NEPRILYSIN FOR THERAPEUTIC PURPOSES

At present, coupling of NEP with red blood cells is used to transport NEP from brain to plasma where it can effectively degrade Aβ. Further, lentiviral vector expressing NEP was fused with apolipoprotein B (ApoB) that binds with LDL-receptor to facilitate its transport to CNS and other parts of the brain, thereby decreasing Aβ levels in an AβPP-transgenic mice model [130, 131]. The reason behind this is its high cloning capacity and ability to form complex cassettes of lentiviral vector, and it is widely being used for long-term expression of NEP to reduce Aβ burden in the brain [132, 133]. Further, stereotaxic infusion of NEP-encoding viral vectors into the hippocampus has been shown to reduce Aβ burden in AD brain. However, a more competent and global delivery system is required to target widely distributed Aβ, and one such system is the adeno-associated viral vector (AAVV) that provides neuronal gene expression throughout the brain after peripheral administration; for instance, a single intra cardiac administration of the vector carrying NEP gene in an AD-mice model has been shown to elevate expression of NEP throughout the brain, thereby reducing Aβ oligomer formation [134, 135]. Another viral vector, Sindbis viral vector, has also been used to reduce Aβ burden in the brain where the NEP gene is inserted into the viral vector and then allowed to infect the neurons selectively and efficiently with elevated NEP levels [136, 137]. Moreover, intracranial viral vector delivery by injecting NEP directly into the brain has also been reported to reduce Aβ deposition, while intravenous infusion of a recombinant enzyme is a clinically desirable therapy [34]. However, earlier studies revealed the significance of NEP expressing viral vectors in reducing Aβ levels in AD brain, but it has several drawbacks regarding control of insert size, desired expression (short- or long-term), and their target cell type. Therefore, in order to overcome these drawbacks, recently recombinant soluble NEP expression vector from insect cells has been transfected into AD mice with the help of intracerebral injection, thereby ameliorating memory impairment in AD brain. In this way, protein therapy approaches might have been potential for development of alternative therapies for treatment of AD [138].

Another strategy for lowering Aβ formation is the downregulation of AβPP gene, which is possible with the newly emerging siRNA technology where siRNA against AβPP mRNA is used. These siRNA approaches have worked well in cell culture, but they lack enough potentiality to express their function inside the cells due to lack of efficient delivery systems. In order to achieve RNA interference effect, short hairpin RNAs (shRNA) is used in viral vectors such as retroviruses and adeno-associated viruses due to their good transduction efficiency along with the long-term expression of transgene in nervous system [139]. Another approach to facilitate enhanced clearance of Aβ, a replication-defective Herpes simplex virus (HSV) based vector has been developed that comprised of genes required for maintaining its latency state as well as transgene expression in the host. For instance, HSV-NEP vector is designed to express NEP that showed 5-fold reductions in Aβ levels, and this effect is found to be upregulated with the increased infection by viral vectors. Thus with the use of HSV vectors, the siRNA against AβPP can also be delivered into the host with the aim of lowering Aβ levels [140]. However, yet another strategy to eliminate Aβ peptide is the administration of auto-antibodies, where Aβ-cDNA expressing recombinant adeno-associated viral vectors (rAAV) is injected intramuscularly or

orally to induce auto-antibody production against Aβ [139, 141, 142].

Another major concern with the use of gene therapy in treating AD is the delivery of the gene into Aβ susceptible areas. This goal can be achieved by using monocytes, which are the major immune cells that migrate to the Aβ site. In this approach, genes can be transfected into bone marrow cells with a specific promoter that allows the expression of the genes in differentiated macrophages. For instance, $CD11b^{+}$ cells (monocytes) that are transfected with NEP, when injected twice a week in a transgenic mice model, stopped the amyloid deposition completely. The efficacy and safety of this method have been confirmed; therefore, this could be a more potent and permanent treatment for AD [143]. Further, evidence suggests a role of short-term neural stem cell (NSC) transplantation in ameliorating cognition in an AD transgenic mice model by improving endogenous synaptic connectivity. But,this approach has no longer the effect on the underlying neurofibrillary tangle and Aβ pathology. Therefore, combinatorial approaches of gene therapy are required for achieving long-term efficacy; for instance, in 3xTg-AD and Thy1-AβPP transgenic mice model, stem cell-mediated delivery of NEP showed significant reductions in Aβ pathology along with increased synaptic density. Remarkably, these Aβ plaque burdens are reduced not only in the hippocampus and subiculum adjacent to engrafted NSCs, but also within the amygdala and medial septum areas, that receive afferent projections from the engrafted region [144]. Another approach to deliver NEP is using human adipose tissue-derived mesenchymal stem cells (ADSCs) that release enzymatically active NEP carrying exosomes. Moreover, ADSC-derived exosomal delivery into neuroblastoma cells (N2a) resulted in the reduction of both secreted and intracellular Aβ levels [145]. Recently, a convection-enhanced delivery of NEP gene resulted into a 20-fold increase in NEP protein level with preserved enzyme activity in the striatum, thereby causing significant reduction in endogenous $A\beta_{40\text{-}42}$ levels [146, 147]. Most importantly, the delivery of insulin antibodies or insulin-inhibiting peptides into the brain can be a prominent therapeutic approach against toxic Aβ. However, fewer practical but augmented IDE levels in the CNS or disease prone site in the brain (via IDE infusion or gene therapy), would be of therapeutic interest [30]. Moreover, further investigation is to be needed to explore the gene therapy potential of IDE in the brain in order to nullify the toxic effect of Aβ. Since proper regulation of IDE and NEP is essential for their therapeutic applications, recently identified factors/agent controlling their expression level is discussed here.

RECENTLY IDENTIFIED THERAPEUTIC AGENTS REGULATING IDE AND NEP LEVELS IN THE BRAIN

Sex steroids are one of the therapeutic agents who ameliorate cognitive deficits in AD animal models by reducing Aβ levels in the brain due to elevated expression of IDE and NEP. For instance, administration of androgen receptor agonist (ACP-105) in association with estrogen receptor β (ERβ) agonist (AC-186) in male gonadectomized triple transgenic mice showed elevated levels and activity of IDE and NEP [148]. Another agent, nitric oxide synthase (NOS2) which is overexpressed in case of AD, was also found to be involved in the regulation of IDE and NEP. An *in vitro* study revealed that activity of IDE was inhibited by nitric oxide donor 'Sin-1', while NEP activity remains unaffected. However, an *in vivo* study showed downregulated activity of IDE in AβPP/PS1 mice, but not in AβPP/PS1/NOS2 (-/-) mice, signifying the role of NOS2 in preventing Aβ degradation through negative regulation of IDE. Thus loss of NOS2 activity would have been promising effect in positively triggering Aβ clearance in AD brain [149]. Recently, amyloid associated proteins, including ApoJ and ApoE, were found to modify Aβ uptake in human astrocytes where NEP expression is increased upon exposure to ApoE in combination with both Aβ preparations, i.e., Aβ-oligomer and Aβ-fibrillar, but NEP and IDE expression remain unaffected by exposure to Aβ-oligomeric or Aβ-fibrillar alone. It is important to note that Aβ alone indeed does not affect the astrocytic expression of IDE and NEP [102]. However, use of retinoic acid receptor (RARα) agonist has been reported to promote Aβ clearance by augmenting IDE and NEP activity in both microglia and diseased neurons [150]. Based on these observations, retinoic acid is a potential molecule, involved in the maintenance of synaptic plasticity of neurons, which may assist in keeping memory function intact.

Interleukin-34 (IL-34), a newly discovered cytokine, is found to overexpress IDE in microglia where it decreases oxidative stress without generating neurotoxic molecules, thus promoting the clearance of soluble oligomeric Aβ and preventing synaptic dysfunction and neuronal damage in AD [151]. Moreover, interleukin receptor-associated kinases (IRAKs; intracellular signaling molecules) have been reported

Table 5
List of agonists and antagonists regulating IDE and NEP expression level

S. No.	Agonists	Targets	Molecular Functions	References
1	DHA (Docosahexaenoic acid)	IDE	It is the most important fatty acid in the brain especially rich in the neurons and synaptosomes of the cerebral cortex. DHA significantly upregulates the expression of IDE in neural cells.	[163]
2	ATP (Adenosine triphosphate)	IDE	ATP facilitates the transition from the closed state to the open conformation. Biophysical analysis of ATP-induced conformational switch of IDE thereby showed the increased expression of IDE.	[164]
3	Valproic acid	NEP	Valproic acid attenuates the prenatal hypoxia-induced Aβ neuropathology, learning, and memory deficits via inhibiting the activation of histone deacetylase 1 (HDAC1), preventing the decrease in H3-Ace in the NEP promoter regions and reducing the down-regulation of NEP.	[165, 166]
4	Propranolol	IDE	It enhances the expression of IDE. Moreover, the expression of Akt, BDNF, and Tau hyperphosphorylation is decreased by propranolol treatment as shown by Tg2576 mice.	[167]
5	Neuropep-1	IDE & NEP both	It increases the level of IDE and NEP. However, Neuropep-1 treatment does not alter the expression of full-length AβPP, α-, β-, or γ-secretase.	[168]
6	Somatostatin (octreotide)	IDE	Somatostatin directly interacts with IDE. It binds to the active site of one IDE subunit, induces an enhancement of IDE proteolytic activity toward fluorogenic Aβ by another subunit.	[169, 170]
7	Imidazole-derived 2-[N-carbamoylmethyl-alkylamino] acetic acids	IDE	It acts as substrate-dependent modulators of IDE in Aβ hydrolysis.	[171]
8	Cysteine 904	IDE	Cysteine 904 is required for Maximal IDE Activity and Polyanion Activation.	[172]
9	BRI2 (British precursor protein)	IDE	BRI2 acts as a receptor protein that regulates IDE levels, and in turn influences AβPP metabolism.	[173]
10	Apomorphine (APO)	IDE	Apomorphine, a kind of dopamine receptor agonists responsible for promoting the intracellular Aβ degradation via activating IDE.	[174]
11	Leptin	IDE	Leptin enhances the expression level of IDE putatively by activating the Akt pathway.	[175, 176]
12	Suramin	IDE	Suramin increases the activity of the enzyme IDE by changing the turnover rate of the enzyme for its substrate.	[177, 178]
13	Estrogen	NEP	Estrogen positively regulates expression of NEP to promote degradation of Aβ and reduce the risk of AD.	[179]
14	Humanin (HN)	NEP	Humanin (HN), a 24-residue peptide act as a Neuroprotective factor, which shows anti-cell death activity against a wide spectrum of AD. It also increases the expression of NEP.	[180]
15	Trichostatin (TSA)	NEP	TSA treatment significantly enhances NEP expression by elevating the acetylation of histone H3 on NEP promoter.	[181]
16	5-aza-deoxycytidine (5-Aza-dc)	NEP	5-Aza-dc induces the demethylation of NEP gene and significantly increases its expression in a dose-dependent manner.	[181]
17	Ginsenoside Rg1 & Rg3	IDE&NEP both	It increases the intracellular level of both IDE and NEP in the hippocampus by upregulating PPARγ, thereby decreasing Aβ burden.	[182, 183]
18	Imatinib (Gleevec)	NEP	It is known tyrosine kinase inhibitors, which elevates AICD in H4 human neuroglioma cells and also increase the expression of NEP protein.	[184]
19	GW742	IDE & NEP both	A PPARdelta agonist reduces amyloid burden by enhancing the expression of IDE and NEP.	[185]

Table 5
(Continued)

S. No.	Agonists	Targets	Molecular Functions	References
20	EGCG	NEP	EGCG strongly increases the NEP activity, thus lead to Aβ degradation.	[186]
21	Curcumin	IDE & NEP both	It increases Aβ clearance by increasing both IDE and NEP activity and also prevents Aβ production by inhibiting PS2, a catalytic component of γ-secretase.	[187]
22	Resveratrol (RSV)	NEP	RSV significantly increases both the estradiol and NEP level that decrease Aβ deposition; by upregulation of estradiol level which consequently leads to increase the level of NEP, thus contribute to Aβ degradation.	[188]
23	Norepinephrine	IDE	Norepinephrine augments microglia to uptake and degrades Aβ peptides through upregulation of IDE.	[189]
24	Testosterone	NEP	Testosterone increases neuronal viability in cultured hippocampal neurons through the AR-dependent MAPK/ERK signalling pathway. Thus, it elevates the levels of NEP to facilitate Aβ clearance.	[190–192]
25	Androgens	NEP	Androgens increases NEP expression through the AR-dependent MAPK/ERK signalling pathway.	[193]
26	Nobiletin	NEP	It enhanced NEP activity both at the gene and protein level in time- and dose-dependent manner in SK-N-SH cells and thereby promoting Aβ clearance.	[194]
S. No.	Antagonists	Targets	Molecular Functions	References
1	Palmitic acid (PA)	IDE	PA treatment significantly reduces the expression level of IDE, an important protease responsible for the degradation of Aβ in neural cells.	[163]
2	Sevoflurane	IDE and NEP both	Sevoflurane alters the expression of receptors and enzymes involved in Aβ clearance, thereby reducing the levels of IDE and NEP in the brain.	[195]
3	Streptozotocin	NEP	It decreases the NEP activity in the hippocampus and cortex regions, thereby increasing Aβ level.	[196]
4	Thiorpan	NEP	Intracerebroventricular infusion of thiorphan, a NEP inhibitor raises cortical and cerebrospinal fluid (CSF) Aβ concentrations in the brain. It indicates that age-related decrease in NEP could lead to increased Aβ burden.	[197]
5	Tautomycetin	NEP	A specific inhibitor of protein phosphatase-1a, tautomycetin induces extensive phosphorylation of the Ser6 NEP intracellular domain, resulting into reduced cell surface NEP activity.	[153, 161]
6	Phosphoramidon	NEP	It acts as NEP inhibitor. Phosphoramidon induces a dramatic increase in Aβ levels, resulting into rapid plaque formation.	[198]
7	Nicastrin	NEP	Nicastrin deficiency drastically lowers NEP expression.	[199]
8	Ketamine	NEP	Ketamine suppresses the Aβ degradation of NEP by reducing p38 MAPK-mediated pathway activity.	[200]
9	Spinorphin	NEP	It is a heptapeptide, which inhibits dipeptidyl peptidases and angiotensin-converting enzyme activity as well as NEP activity.	[201, 202]
10	Sialorphin	NEP	It prevents spinal and renal NEP activity from breaking down its two physiologically relevant substrates, substance P and Met-enkephalin *in vitro*. It is a natural systemically active regulator of NEP.	[203]
11	Opiorphin	NEP	Opiorphin was a first NEP inhibitor isolated from saliva which had some pain-suppressive potency.	[204, 205]
12	Copper (Cu^{2+})	NEP	Copper down regulates NEP activity through modulation of NEP protein degradation.	[206]
13	Lead (Pb)	NEP	Pb causes both the over expression of AβPP and repression of NEP resulting in the build-up of Aβ.	[207]
14	Leptin	NEP	Leptin significantly decreases the expression of NEP through activation of ERK signalling cascade.	[208]

to trigger TLR signals that initiate the canonical pro-inflammatory signaling pathways, which further activate JNK-p38 and ERK-MAP kinases to generate reactive oxygen species. Therefore, evidence suggests that functional loss of IRAK4 promotes amyloid clearance mechanisms by raising IDE expression [152]. In a recent study, protein phosphatases have been implicated in the reduction of kinase-mediated Aβ deposition; for instance, elevated Aβ levels in AD patients have been reduced in response to activated protein phosphatase-1a that dephosphorylate NEP and result in enhanced cell-surface NEP activity [153]. Likewise, another candidate, mitochondria-targeted antioxidant catalase, has been identified that possesses a protective role against Aβ toxicity and prevents abnormal AβPP processing through increased IDE and NEP activity [154].

A recent study revealed that human placenta amniotic membrane-derived mesenchymal stem cells, which are known for their potent immune-adulatory and paracrine effects, also have a pivotal role in improving spatial learning via increasing NEP and IDE expression in the brain [155]. Furthermore, using human-induced pluripotent stem cell-derived macrophage cells harboring NEP2 demonstrated decreased levels of Aβ in the 5XFAD mice model [156]. Moreover, neural stem cells carrying NEP ameliorated AD pathology by restoring synaptic density and reducing Aβ load in transgenic mice models of AD via 3xTg-AD and Thy1-AβPP [157]. Recently, it has been identified that various drugs, e.g., MK8931, AZD3293, E2609, and TPP488, have been used to reduce Aβ burden in the brain by triggering endogenous expression of IDE and NEP. These drugs act as an inhibitor of different signaling molecules, mainly targets on BACE1, BACE2, and RAGE molecule. These signaling molecules are basically involved in AβPP cleavage and their processing that lead to increased Aβ concentration in the brain. Furthermore, MK8931, AZD3293, E2609, and TPP488 treatment have been noticed, signifying their major contribution in a dose-dependent attenuation of Aβ levels in plasma and cerebrospinal fluid [158–161]. Besides therapeutic potential of NEP and IDE, their elevated levels have also been found associated with several side effects; for instance, cAMP-responsive element-binding protein (CREBP) mediated transcription caused age-dependent axonal degeneration, and it is also involved in the progression of various cancers [65, 162]. Therefore, in order to escape the side effects, we need to adopt the combinatorial therapeutic approach by using their agonists and antagonists for targeting Aβ toxicity in the brain. Such agonists and antagonists of IDE and NEP have been elaborated in Table 5.

CONCLUSIONS

The biology behind Aβ deposition in the brain and the clearance mechanism is quite complicated, and thus much attention has been given in the past few decades to understand these phenomena and their significance in AD. The etiopathology of AD is caused by accumulation of toxic Aβ peptides that occurred due to several factors, mainly associated with proteolytic cleavage of AβPP by β-secretase and γ-secretase, that lead to the formation of AICD and toxic Aβ. Protective enzymes such as IDE and NEP are found to play a crucial role in degradation of amyloidogenic or toxic Aβ peptides in the brain, thus signifying their therapeutic potential. Both IDE and NEP are metalloendopeptidases, which require Zn^{2+} ion as a cofactor for their catalytic activity. Multiple sequence analyses of both IDE and NEP [*Homo sapiens*] have revealed a sequence identity with *Macaca mulatta* of 99%. Sequential analysis has also revealed the presence of different post-transcriptional modification sites, for instance, protein kinase-C phosphorylation sites, tyrosine kinase phosphorylation sites, and casein kinase II phosphorylation sites, depicting their role in functional regulation of IDE and NEP. Further, structural analysis of both IDE and NEP has shown the presence of amino acids (His, Glu, and Asp) at their zinc binding motif and active site, which are responsible for their proper functions. Importantly, closely associated putative partners of both IDE and NEP have been identified that have almost similar functions and sequence identity with this peptidase.

In addition to *in silico* study of IDE and NEP, its elevated expression in the brain is important for rendering the toxic effect of Aβ. For that, more competent and global delivery systems like lentiviral vector, adeno-associated viral vector, siRNA, and recombinant gene therapy based delivery of IDE and NEP is required to target widely distributed area in the Aβ affected brain. Furthermore, a convection-enhanced delivery system is also used to transfer these enzymes to Aβ laden areas in the brain to reduce its toxicity. Additionally, using different agonists and antagonists based combinatorial therapeutic approach has also been shown for proper homeostatic regulation of both IDE and NEP, as high levels of these peptidases cause some side effects. Moreover, any changes in the levels of IDE and NEP cause accumulation of Aβ which is highly toxic to the cell,

because the levels of these two peptidases are altered in an age-dependent manner. In older age due to its downregulation, it does not influence the proteolytic degradation of Aβ and thus accumulation of toxic Aβ hampers the normal functionality of neuronal cells and therefore causes changes in the morphology of the neurons, memory loss, and consequent cell death.

ACKNOWLEDGMENTS

The authors would like to thank Delhi Technological University management for support and encouragement.

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/15-0379r1).

REFERENCES

[1] Crews L, Masliah E (2010) Molecular mechanisms of neurodegeneration in Alzheimer's disease. *Hum Mol Genet* **19**, 12-20.

[2] Iqbal K, Grundke-Iqbal I (2002) Neurofibrillary pathology leads to synaptic loss and not the other way around in Alzheimer disease. *J Alzheimers Dis* **4**, 235-238.

[3] Trojanowski JQ, Lee VM (2000) "Fatal attractions" of proteins. A comprehensive hypothetical mechanism underlying Alzheimer's disease and other neurodegenerative disorders. *Ann NY Acad Sci* **924**, 62-67.

[4] Kumar P, Jha NK, Jha SK, Ramani K, Ambasta RK (2015) Tau phosphorylation, molecular chaperones, and ubiquitin E3 ligase: Clinical relevance in Alzheimer's disease. *J Alzheimers Dis* **43**, 341-361.

[5] Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del TK (2006) Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta Neuropathol* **112**, 389-404.

[6] Avila J, Lucas JJ, Perez M, Hernandez F (2004) Role of tau protein in both physiological and pathological conditions. *Physiol Rev* **84**, 361-384.

[7] Buee L, Bussiere T, Buee-Scherrer V, Delacourte A, Hof PR (2000) Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Res Brain Res Rev* **33**, 95-130.

[8] De la Monte SM (2012) Brain insulin resistance and deficiency as therapeutic targets in Alzheimer's disease. *Curr Alzheimer Res* **9**, 35-66.

[9] Moreira PI, Santos MS, Seiça R, Oliveira CR (2007) Brain mitochondrial dysfunction as a link between Alzheimer's disease and diabetes. *J Neurol Sci* **257**, 206-214.

[10] De la Monte SM, Wands JR (2005) Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: Relevance to Alzheimer's disease. *J Alzheimers Dis* **7**, 45-61.

[11] De la Monte SM, Wands JR (2006) Molecular indices of oxidative stress and mitochondrial dysfunction occur early and often progress with severity of Alzheimer's disease. *J Alzheimers Dis* **9**, 167-181.

[12] De la Monte SM, Wands JR (2008) Alzheimer's disease is type 3 diabetes-evidence reviewed. *J Diabetes Sci Technol* **2**, 1101-1113.

[13] Yang JW, Raizada MK, Fellows RE (1981) Effect of insulin on cultured rat brain cells: Stimulation of ornithine decarboxylase activity. *J Neurochem* **36**, 1050-1057.

[14] Gasparini L, Xu H (2003) Potential roles of insulin and IGF-1 in Alzheimer's disease. *Trends Neurosci* **26**, 404-406.

[15] Shah B, Hausman RE (1993) Effect of insulin on GABAergic development in the embryonic chick retina. *Brain Res Dev Brain Res* **72**, 151-158.

[16] Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, Feldman EL (2002) High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. *FASEB J* **16**, 1738-1748.

[17] Chi MM, Schlein AL, Moley K (2000) High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology* **141**, 4784-4792.

[18] Hoyer S (2002) The brain insulin signal transduction system and sporadic (type II) Alzheimer disease: An update. *J Neural Transm* **109**, 341-360.

[19] Li L, Holscher C (2007) Common pathological processes in Alzheimer disease and type 2 diabetes: A review. *Brain Res Rev* **56**, 384-402.

[20] Biessels GJ, Kappelle LJ (2005) Increased risk of Alzheimer's disease in Type II diabetes: Insulin resistance of the brain or insulin-induced amyloid pathology? *Biochem Soc Trans* **33**, 1041-1044.

[21] Rasgon N, Jarvik L (2004) Insulin resistance, affective disorders, and Alzheimer's disease: Review and hypothesis. *J Gerontol Biol Sci Med Sci* **59**, 178-183.

[22] Stolk RP, Breteler MM, Ott A, Pols HA, Lamberts SW, Grobbee DE, Hofman A (1997) Insulin and cognitive function in an elderly population. The Rotterdam Study. *Diabetes Care* **20**, 792-795.

[23] Luchsinger JA, Tang MX, Shea S, Mayeux R (2004) Hyperinsulinemia and risk of Alzheimer disease. *Neurology* **63**, 1187-1192.

[24] Zhu X, Perry G, Smith MA (2005) Insulin signaling, diabetes mellitus and risk of Alzheimer disease. *J Alzheimers Dis* **7**, 81-84.

[25] Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, De la Monte SM (2005) Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease–is this type 3 diabetes? *J Alzheimers Dis* **7**, 63-80.

[26] (2006) Alzheimer research forum live discussion: Is Alzheimer's type 3 diabetes? *J Alzheimers Dis* **9**, 349-353.

[27] Carro E, Trejo JL, Spuch C, Bohl D, Heard JM, Torres-Aleman I (2005) Blockade of the insulin-like growth factor I receptor in the choroid plexus originates Alzheimer's-like neuropathology in rodents: New cues into the human disease? *Neurobiol Aging* **27**, 1618-1631.

[28] Carro E, Torres-Aleman I (2004) The role of insulin and insulin-like growth factor 1 in the molecular and cellular mechanisms underlying the pathology of Alzheimer's disease. *Eur J Pharmacol* **490**, 127-133.

[29] Rhein V, Eckert A (2007) Effects of Alzheimer's amyloid beta and tau protein on mitochondrial function - role of glucose metabolism and insulin signaling. *Arch Physiol Biochem* **113**, 131-141.

[30] Moreira PI, Duarte AI, Santos MS, Rego AC, Oliveira CR (2009) An integrative view of the role of oxidative stress, mitochondria and insulin in Alzheimer's disease. *J Alzheimers Dis* **16**, 741-761.

[31] Zhao WQ, Chen H, Quon MJ, Alkon DL (2004) Insulin and the insulin receptor in experimental models of learning and memory. *Eur J Pharmacol* **490**, 71-81.
[32] van der Heide LP, Ramakers GMJ, Marten PS (2006) Insulin signaling in the central nervous system: Learning to survive. *Prog Neurobiol* **79**, 205-221.
[33] Cardoso S, Correia S, Santos RX, Carvalho C, Santos MS, Oliveira CR, Perry G, Smith MA, Zhu X, Moreira PI (2009) Insulin is a two-edged knife on the brain. *J Alzheimers Dis* **18**, 483-507.
[34] Spencer B, Verma I, Desplats P, Morvinski D, Rockenstein E, Adame A, Masliah E (2014) A neuroprotective brain-penetrating endopeptidase fusion protein ameliorates Alzheimer disease pathology and restores neurogenesis. *J Biol Chem* **289**, 17917-17931.
[35] Grimm MO, Mett J, Stahlmann CP, Haupenthal VJ, Zimmer VC, Hartmann T (2013) Neprilysin and Aβ clearance: Impact of the APP intracellular domain in NEP regulation and implications in Alzheimer's disease. *Front Aging Neurosci* **23**, 5-98.
[36] Relton JM, Gee NS, Matsas R, Turner AJ, Kenny AJ (1983) Purification of endopeptidase-24.11 ("enkephalinase") from pig brain by immunoadsorbent chromatography. *Biochem J* **215**, 519-523.
[37] Malfroy B, Kuang WJ, Seeburg PH, Mason AJ, Schofield PR (1988) Molecular cloning and amino acid sequence of human enkephalinase (neutral endopeptidase). *FEBS Lett* **229**, 206-210.
[38] D'Adamio L, Shipp MA, Masteller EL, Reinherz EL (1989) Organization of the gene encoding common acute lymphoblastic leukemia antigen (neutral endopeptidase 24.11): Multiple mini exons and separate 5′ untranslated regions. *Proc Natl Acad Sci U S A* **86**, 7103-7107.
[39] Webster CI, Burrell M, Olsson LL, Fowler SB, Digby S, Sandercock A, Snijder A, Tebbe J, Haupts U, Grudzinska J, Jermutus L, Andersson C (2014) Engineering neprilysin activity and specificity to create a novel therapeutic for Alzheimer's disease. *PLoS One* **9**, e104001.
[40] Oefner C, Roques BP, Fournie-Zaluski MC, Dale GE (2004) Structural analysis of neprilysin with various specific and potent inhibitors. *Acta Crystallog D* **60**, 392-396.
[41] Turner AJ, Isaac RE, Coates D (2001) The neprilysin (NEP) family of zinc metalloendopeptidases: Genomics and function. *Bioessays* **23**, 261-269.
[42] Gough M, Parr-Sturgess C, Parkin E (2011) Zinc metalloproteinases and amyloid Beta-Peptide metabolism: The positive side of proteolysis in Alzheimer's disease. *Biochem Res In* **2011**, 721463.
[43] Malito E, Hulse RE, Tang WJ (2008) Amyloid β-degrading cryptidases: Insulin degrading enzyme, presequence peptidase, and neprilysin. *Cell Mol Life Sci* **65**, 2574-2585.
[44] Roques BP, Noble F, Dauge V, Fournie-Zaluski MC, Beaumont A (1993) Neutral endopeptidase 24.11: Structure, inhibition, and experimental and clinical pharmacology. *Pharmacol Rev* **45**, 87-146.
[45] Nalivaeva NN, Belyaev ND, Zhuravin IA, Turner AJ (2012) The Alzheimer's amyloid-degrading peptidase, neprilysin: Can we control it? *Int J Alzheimers Dis* **2012**, 383796.
[46] Hellstrom-Lindahl E, Ravid R, Nordberg A (2008) Age-dependent decline of neprilysin in Alzheimer's disease and normal brain: Inverse correlation with A beta levels. *Neurobiol Aging* **29**, 210-221.
[47] Iwata N, Mizukami H, Shirotani K, Takaki Y, Muramatsu S, Lu B, Gerard NP, Gerard C, Ozawa K, Saido TC (2004) Presynaptic localization of neprilysin contributes to efficient clearance of amyloid-beta peptide in mouse brain. *J Neurosci* **24**, 991-998.
[48] Barnes K, Turner AJ, Kenny AJ (1992) Membrane localization of endopeptidase-24.11 and peptidyl dipeptidase A (angiotensin converting enzyme) in the pig brain: A study using subcellular fractionation and electron microscopic immune cytochemistry. *J Neurochem* **58**, 2088-2096.
[49] Hickman SE, Allison EK, El Khoury J (2008) Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. *J Neurosci* **28**, 8354-8360.
[50] Fisk L, Nalivaeva NN, Boyle JP, Peers CS, Turner AJ (2007) Effects of hypoxia and oxidative stress on expression of neprilysin in human neuroblastoma cells and rat cortical neurons and astrocytes. *Neurochem Res* **32**, 1741-1748.
[51] LeBien TW, McCormack RT (1989) The common acute lymphoblastic leukemia antigen (CD10). Emancipation from a functional enigma. *Blood* **73**, 625-635.
[52] Matsas R, Kenny AJ, Turner AJ (1986) An immunohistochemical study of endopeptidase-24.11 ("enkephalinase") in the pig nervous system. *Neuroscience* **18**, 991-1012.
[53] Silva AP, Cavadas C, Grouzmann E (2002) Neuropeptide Y and its receptors as potential therapeutic drug targets. *Clin Chim Acta* **326**, 3-25.
[54] Fischer HS, Zernig G, Schuligoi R, Miczek KA, Hauser KF, Gerard C, Saria A (2000) Alterations within the endogenous opioid system in mice with targeted deletion of the neutral endopeptidase ("enkephalinase") gene. *Regul Pept* **96**, 53-58.
[55] Wang DS, Dickson DW, Malter JS (2006) β-Amyloid degradation and Alzheimer's disease. *J Biomed Biotechno* **2006**, 58406.
[56] Howell S, Nalbantoglu J, Crine P (1995) Neutral endopeptidase can hydrolyze β-amyloid (1–40) but shows no effect on β-amyloid precursor protein metabolism. *Peptides* **16**, 647-652.
[57] Takaki Y, Iwata N, Tsubuki S, Taniguchi S, Toyoshima S, Lu B, Gerard NP, Gerard C, Lee HJ, Shirotani K, Saido TC (2000) Biochemical identification of the neutral endopeptidase family member responsible for the catabolism of amyloid β peptide in the brain. *J Biochem* **128**, 897-902.
[58] Saido TC (2013) Metabolism of amyloid β peptide and pathogenesis of Alzheimer's disease. *Proc Jpn Acad Ser B Phys Biol Sci* **89**, 321-339.
[59] Nalivaeva NN, Fisk LR, Belyaev ND, Turner AJ (2008) Amyloid-degrading enzymes as therapeutic targets in Alzheimer's disease. *Curr Alzheimer Res* **5**, 212-224.
[60] Iwata N, Higuchi M, Saido TC (2005) Metabolism of amyloid-beta peptide and Alzheimer's disease. *Pharmacol Ther* **108**, 129-148.
[61] Guo X, Tang P, Liu P, Liu Y, Hou C, Li RM (2014) Meta-analysis of the association between two neprilysin gene polymorphisms and Alzheimer's disease. *J Neurol Sci* **346**, 6-10.
[62] Oh-hashi K, Nagai T, Tanaka T, Yu H, Hirata Y, Kiuchi K (2005) Determination of hypoxic effect on neprilysin activity in human neuroblastoma SH-SY5Y cells using a novel HPLC method. *Biophys Biochem Res Commun* **334**, 380-385.
[63] Wang DS, Iwata N, Hama E, Saido TC, Dickson DW (2003) Oxidized neprilysin in aging and Alzheimer's disease brains. *Biochem Biophys Res Commun* **310**, 236-241.
[64] Gohring B, Holzhausen HJ, Meye A, Heynemann H, Rebmann U, Langner J, Riemann D (1998) Endopeptidase

24.11/CD10 is down-regulated in renal cell cancer. *Int J Mol Med* **2**, 409-414.
[65] Cohen AJ, Bunn PA, Franklin W, Magill-Solc C, Hartmann C, Helfrich B, Gilman L, Folkvord J, Helm K, Miller YE (1996) Neutral endopeptidase: Variable expression in human lung, inactivation in lung cancer, and modulation of peptide-induced calcium flux. *Cancer Res* **56**, 831-839.
[66] Papandreou CN, Usmani B, Geng Y, Bogenrieder T, Freeman R, Wilk S, Finstad CL, Reuter VE, Powell CT, Scheinberg D, Magill C, Scher HI, Albino AP, Nanus DM (1998) Neutral endopeptidase 24.11 loss in metastatic human prostate cancer contributes to androgen-independent progression. *Nat Med* **1**, 50-57.
[67] Stephen FA, Thomas LM, Alejandro A, Jinghui Z, Zheng Z, Webb M, David JL (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389-3402.
[68] Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.
[69] Le Moual H, Dion N, Roques BP, Crine P, Boileau G (1994) Asp650 is crucial for catalytic activity of neutral endopeptidase 24-11. *Eur J Biochem* **221**, 475-480.
[70] Oefner C, Pierau S, Schulz H, Dale GE (2007) Structural studies of a bifunctional inhibitor of neprilysin and DPP-IV. *Acta Crystallogr D Biol Crystallogr* **63**, 975-981.
[71] Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425.
[72] Castro DE, Sigrist CJ, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A, Hulo N (2006) ScanProsite: Detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Res* **34**, W362-W365.
[73] Le Moual H, Roques BP, Crine P, Boileau G (1993) Substitution of potential metal-coordinating amino acid residues in the zinc-binding site of endopeptidase-24.11. *FEBS Lett* **324**, 196-200.
[74] Martin DD, Beauchamp E, Berthiaume LG (2011) Post-translational myristoylation: Fat matters in cellular life and death. *Biochimie* **93**, 18-31.
[75] Sola RJ, Griebenow K (2009) Effects of glycosylation on the stability of protein pharmaceuticals. *J Pharm Sci* **98**, 1223-1245.
[76] McWilliam H, Li W, Uludag M, Squizzato S, Park YM, Buso N, Cowley AP, Lopez R (2013) Analysis tool web services from the EMBL-EBI. *Nucleic Acids Res* **41**, 597-600.
[77] Kabsch W, Sander C (1983) Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **22**, 2577-2637.
[78] Lo Conte L, Ailey B, Hubbard TJ, Brenner SE, Murzin AG, Chothia C (2000) SCOP: A structural classification of proteins database. *Nucleic Acids Res* **28**, 257-259.
[79] Roques BP, Noble F, Crine P, Fournie-Zaluski MC (1995) Inhibitors of neprilysin: Design, pharmacological and clinical applications. *Methods Enzymol* **248**, 263-283.
[80] Holm L, Rosenström P (2010) Dali server: Conservation mapping in 3D. *Nucleic Acids Res* **38**, W545-W549.
[81] Edbauer D, Willem M, Lammich S, Steiner H, Haass C (2002) Insulin-degrading enzyme rapidly removes the beta-amyloid precursor protein intracellular domain (AICD). *J Biol Chem* **277**, 13389-13393.
[82] Malito E, Ralat LA, Manolopoulou M, Tsay JL, Wadlington NL, Tang WJ (2008) Molecular bases for the recognition of short peptide substrates and cysteine-directed modifications of human insulin-degrading enzyme. *Biochemistry* **47**, 12822-12834.
[83] Shen Y, Joachimiak A, Rosner MR, Tang WJ (2006) Structures of human insulin-degrading enzyme reveals a new substrate recognition mechanism. *Nature* **443**, 870-874.
[84] Yfanti C, Mengele K, Gkazepis A, Weirich G, Giersig C, Kuo WL, Tang WJ, Rosner M, Schmitt M (2008) Expression of metalloprotease insulin-degrading enzyme insulysin in normal and malignant human tissues. *Int J Mol Med* **22**, 421-431.
[85] Duckworth WC (1988) Insulin degradation: Mechanisms, products, and significance. *Endocr Rev* **9**, 319-345.
[86] Schmitz A, Schneider A, Kummer MP, Herzog V (2004) Endoplasmic reticulum-localized amyloid beta-peptide is degraded in the cytosol by two distinct degradation pathways. *Traffic* **5**, 89-101.
[87] Farris W, Mansourian S, Leissring MA, Eckman EA, Bertram L, Eckman CB, Tanzi RE, Selkoe DJ (2004) Partial loss-of-function mutations in insulin-degrading enzyme that induce diabetes also impair degradation of amyloid beta-protein. *Am J Pathol* **164**, 1425-1434.
[88] Leissring MA, Farris W, Wu X, Christodoulou DC, Haigis MC, Guarente L, Selkoe DJ (2004) Alternative translation initiation generates a novel isoform of insulin-degrading enzyme targeted to mitochondria. *Biochem J* **383**, 439-446.
[89] Morita M, Kurochkin IV, Motojima K, Goto S, Takano T, Okamura S, Sato R, Yokota S, Imanaka T (2000) Insulin-degrading enzyme exists inside of rat liver peroxisomes and degrades oxidized proteins. *Cell Struct Funct* **25**, 309-315.
[90] Hamel FG, Mahoney MJ, Duckworth WC (1991) Degradation of intraendosomal insulin by insulin-degrading enzyme without acidification. *Diabetes* **40**, 436-443.
[91] Williamson J, Goldman J, Marder KS (2009) Genetic aspects of Alzheimer disease. *Neurologist* **15**, 80-86.
[92] Bertram L, Blacker D, Mullin K, Keeney D, Jones J, Basu S, Yhu S, McInnis MG, Go RC, Vekrellis K, Selkoe DJ, Saunders AJ, Tanzi RE (2000) Evidence for genetic linkage of Alzheimer's disease to chromosome 10q. *Science* **290**, 2302-2303.
[93] Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ (2008) MEROPS: The peptidase database. *Nucleic Acids Res* **36**, D320-D325.
[94] Qiu WQ, Zhu H (2014) Amylin and its analogs: A friend or foe for the treatment of Alzheimer's disease? *Front Aging Neurosci* **6**, 186.
[95] Valera Mora ME, Scarfone A, Calvani M, Greco AV, Mingrone G (2003) Insulin clearance in obesity. *J Am Coll Nutr* **22**, 487-493.
[96] Kurochkin IV (2001) Insulin-degrading enzyme: Embarking on amyloid destruction. *Trends Biochem Sci* **26**, 421-425.
[97] Asvadi NH, Morgan M, Herath HM, Hewavitharana AK, Shaw PN, Cabot PJ (2014) Beta-endorphin 1-31 biotransformation and cAMP modulation in inflammation. *PLoS One* **11**, e90380.
[98] Safavi A, Miller BC, Cottam L, Hersh LB (1996) Identification of gamma-endorphin-generating enzyme as insulin-degrading enzyme. *Biochemistry* **35**, 14318-14325.
[99] Chou YH, Kuo WL, Rosner MR, Tang WJ, Goldman RD (2009) Structural changes in intermediate filament networks alter the activity of insulin-degrading enzyme. *FASEB J* **23**, 3734-3742.

[100] Stargardt A, Gillis J, Kamphuis W, Wiemhoefer A, Kooijman L, Raspe M, Benckhuijsen W, Drijfhout JW, Hol EM, Reits E (2013) Reduced amyloid-β degradation in early Alzheimer's disease but not in the APPswePS1dE9 and 3xTg-AD mouse models. *Aging Cell* **12**, 499-507.

[101] Sarroukh R, Cerf E, Derclaye S, Dufrêne YF, Goormaghtigh E, Ruysschaert JM, Raussens V (2011) Transformation of amyloid β (1-40) oligomers into fibrils is characterized by a major change in secondary structure. *Cell Mol Life Sci* **68**, 1429-1438.

[102] Mulder SD, Veerhuis R, Blankenstein MA, Nielsen HM (2012) The effect of amyloid associated proteins on the expression of genes involved in amyloid-β clearance by adult human astrocytes. *Exp Neurol* **233**, 373-379.

[103] Hulse RE, Ralat LA, Wei-Jen T (2009) Structure, function, and regulation of insulin-degrading enzyme. *Vitam Horm* **80**, 635-648.

[104] Song ES, Juliano MA, Juliano L, Hersh LB (2003) Substrate activation of insulin-degrading enzyme (insulysin). A potential target for drug development. *J Biol Chem* **278**, 49789-49794.

[105] Lee HK, Kumar P, Fu Q, Rosen KM, Querfurth HW (2009) The insulin/Akt signaling pathway is targeted by intracellular beta-amyloid. *Mol Biol Cell* **20**, 1533-1544.

[106] Lee CC, Huang CC, Wu MY, Hsu KS (2005) Insulin stimulates postsynaptic density-95 protein translation via the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway. *J Biol Chem* **280**, 18543-18550.

[107] Bondy CA, Cheng CM (2004) Signaling by insulin-like growth factor 1 in brain. *Eur J Pharmacol* **490**, 25-31.

[108] Zhao WQ, Alkon DL (2001) Role of insulin and insulin receptor in learning and memory. *Mol Cell Endocrinol* **177**, 125-134.

[109] Daian T, Ohtsuru A, Rogounovitch T, Ishihara H, Hirano A, Akiyama-Uchida Y, Saenko V, Fujii T, Yamashita S (2003) Insulin-like growth factor-I enhances transforming growth factor-beta-induced extracellular matrix protein production through the P38/activating transcription factor-2 signaling pathway in keloid fibroblasts. *J Invest Dermatol* **120**, 956-962.

[110] Messier C, Teutenberg K (2005) The role of insulin, insulin growth factor, and insulin-degrading enzyme in brain aging and Alzheimer's disease. *Neural Plast* **12**, 311-328.

[111] Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C (2010) Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. *Neurobiol Aging* **31**, 224-243.

[112] Craft S (2009) The role of metabolic disorders in Alzheimer disease and vascular dementia: Two roads converged. *Arch Neurol* **66**, 300-305.

[113] Hoyer S (2004) Causes and consequences of disturbances of cerebral glucose metabolism in sporadic Alzheimer disease: Therapeutic implications. *Adv Exp Med Biol* **541**, 135-152.

[114] Manolopoulou M, Guo Q, Malito E, Schilling AB, Tang WJ (2009) Molecular basis of catalytic chamber-assisted unfolding and cleavage of human insulin by human insulin-degrading enzyme. *J Biol Chem* **284**, 14177-14188.

[115] Gehm BD, Kuo WL, Perlman RK, Rosner MR (1993) Mutations in a zinc-binding domain of human insulin-degrading enzyme eliminate catalytic activity but not insulin binding. *J Biol Chem* **268**, 7943-7948.

[116] Haass C, Kaether C, Thinakaran G, Sisodia S (2012) Trafficking and proteolytic processing of APP. *Cold Spring Harb Perspect Med* **2**, a006270.

[117] Murphy MP, LeVine H 3rd (2010) Alzheimer's disease and the amyloid-beta peptide. *J Alzheimers Dis* **19**, 311-323.

[118] Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, Eckman CB, Tanzi RE, Selkoe DJ, Guenette S (2003) Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain *in vivo*. *Proc Natl Acad Sci U S A* **100**, 4162-4167.

[119] Kerridge C, Belyaev ND, Nalivaeva NN, Turner AJ (2014) The Aβ-clearance protein transthyretin, like neprilysin, is epigenetically regulated by the amyloid precursor protein intracellular domain. *J Neurochem* **130**, 419-431.

[120] Leal MC, Magnani N, Villordo S, Buslje CM, Evelson P, Castaño EM, Morelli L (2013) Transcriptional regulation of insulin-degrading enzyme modulates mitochondrial amyloid β (Aβ) peptide catabolism and functionality. *J Biol Chem* **288**, 12920-12931.

[121] Jiang Q, Lee CY, Mandrekar S, Wilkinson B, Cramer P, Zelcer N, Mann K, Lamb B, Willson TM, Collins JL, Richardson JC, Smith JD, Comery TA, Riddell D, Holtzman DM, Tontonoz P, Landreth GE (2008) ApoE promotes the proteolytic degradation of Abeta. *Neuron* **58**, 681-693.

[122] Donahue JE, Flaherty SL, Johanson CE, Duncan JA 3rd, Silverberg GD, Miller MC, Tavares R, Yang W, Wu Q, Sabo E, Hovanesian V, Stopa EG (2006) RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease. *Acta Neuropathol* **112**, 405-415.

[123] Saito S, Ihara M (2014) New therapeutic approaches for Alzheimer's disease and cerebral amyloid angiopathy. *Front Aging Neurosci* **6**, 290.

[124] Citron M (2010) Alzheimer's disease: Strategies for disease modification. *Nat Rev Drug Discov* **9**, 387-398.

[125] Eckman EA, Adams SK, Troendle FJ, Stodola BA, Kahn MA, Fauq AH, Xiao HD, Bernstein KE, Eckman CB (2006) Regulation of steady-state beta-amyloid levels in the brain by neprilysin and endothelin-converting enzyme but not angiotensin-converting enzyme. *J Biol Chem* **281**, 30471-30478.

[126] Eckman EA, Eckman CB (2005) Abeta-degrading enzymes: Modulators of Alzheimer's disease pathogenesis and targets for therapeutic intervention. *Biochem Soc Trans* **33**, 1101-1105.

[127] Wata N, Tsubuki S, Takaki Y, Watanabe K, Sekiguchi M, Hosoki E, Kawashima-Morishima, M Lee, H J, Hama E, Sekine-Aizawa Y, Saido TC (2000) Identification of the major Abeta1-42-degrading catabolic pathway in brain parenchyma: Suppression leads to biochemical and pathological deposition. *Nat Med* **6**, 143-150.

[128] Citron M, Eckman CB, Diehl TS, Corcoran C, Ostaszewski BL, Xia W, Levesque G, St. George, Hyslop P, Younkin SG, Selkoe DJ (1998) Additive effects of PS1 and APP mutations on secretion of the 42-residue amyloid beta-protein. *Neurobiol Dis* **5**, 107-116.

[129] Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735-741.

[130] Amouri B, Marr RA, Gindi R, Potkar R, Michael S, Adame A, Rockenstein E, Verma IM, Masliah E (2011) Peripheral delivery of a CNS targeted, metalo-protease reduces aβ toxicity in a mouse model of Alzheimer's disease. *PLoS One* **6**, e16575.

[131] Mazur-Kolecka B, Frackowiak J (2006) Neprilysin protects human neuronal progenitor cells against impaired development caused by amyloid-beta peptide. *Brain Res* **1124**, 10-18.

[132] Guan H, Liu Y, Daily A, Police S, Kim MH, Oddo S, LaFerla FM, Pauly JR, Murphy MP, Hersh LB (2009) Peripherally expressed neprilysin reduces brain amyloid burden: A novel approach for treating Alzheimer's disease. *J Neurosci Res* **87**, 1462-1473.

[133] Kurz A, Perneczky R (2011) Amyloid clearance as a treatment target against Alzheimer's disease. *J Alzheimers Dis* **24**, 61-73.

[134] Iwata N, Sekiguchi M, Hattori Y, Takahashi A, Asai M, Ji B, Higuchi M, Staufenbiel M, Muramatsu S, Saido TC (2013) Global brain delivery of neprilysin gene by intravascular administration of AAV vector in mice. *Sci Res* **3**, 1472.

[135] Liu Y, Studzinski C, Beckett T, Murphy MP, Klein RL, Hersh LB (2010) Circulating neprilysin clears brain amyloid. *Mol Cell Neurosci* **45**, 101-107.

[136] Hama E, Shirotani K, Masumoto H, Sekine-Aizawa Y, Aizawa H, Saido TC (2001) Clearance of extracellular and cell-associated amyloid beta peptide through viral expression of neprilysin in primary neurons. *J Biochem* **130**, 721-726.

[137] Yoon SS, Jo SA (2012) Mechanisms of amyloid-β peptide clearance: Potential therapeutic targets for Alzheimer's disease. *Biomol Ther (Seoul)* **20**, 245-255.

[138] Park MH, Lee JK, Choi S, Ahn J, Jin HK, Park JS, Bae JS (2013) Recombinant soluble neprilysin reduces amyloid-beta accumulation and improves memory impairment in Alzheimer's disease mice. *Brain Res* **1529**, 113-124.

[139] Mandel RJ, Manfredsson FP, Foust KD, Rising A, Reimsnider S, Nash K, Burger C (2006) Recombinant adeno-associated viral vectors as therapeutic agents to treat neurological disorders. *Mol Ther* **13**, 463-483.

[140] Hong CS, Goins WF, Goss JR, Burton EA, Glorioso JC (2006) Herpes simplex virus RNAi and neprilysin gene transfer vectors reduce accumulation of Alzheimer's disease-related amyloid-beta peptide *in vivo*. *Gene Ther* **13**, 1068-1079.

[141] Worgall S, Sondhi D, Hackett NR, Kosofsky B, Kekatpure MV, Neyzi N, Dyke JP, Ballon D, Heier L, Greenwald BM, Christos P, Mazumdar M, Souweidane MM, Kaplitt MG, Crystal RG (2008) Treatment of late infantile neuronal ceroidlipofuscinosis by CNS administration of a serotype 2 adeno-associated virus expressing CLN2 cDNA. *Hum Gene Ther* **19**, 463-474.

[142] Manfredsson FP, Rising AC, Mandel RJ (2009) AAV9: A potential blood-brain barrier buster. *Mol Ther* **17**, 403-405.

[143] Lebson L, Nash K, Kamath S, Herber D, Carty N, Lee DC, Li Q, Szekeres K, Jinwal U, Koren J, Dickey CA, Gottschall PE, Morgan D, Gordon MN (2010) Trafficking CD11b-positive blood cells deliver therapeutic genes to the brain of amyloid-depositing transgenic mice. *J Neurosci* **30**, 9651-9658.

[144] Blurton JM, Spencer B, Michael S, Castello NA, Agazaryan AA, Davis JL, Müller FJ, Loring JF, Masliah E, LaFerla FM (2014) Neural stem cells genetically-modified to express neprilysin reduce pathology in Alzheimer transgenic models. *Stem Cell Res Ther* **5**, 46.

[145] Katsuda T, Tsuchiya R, Kosaka N, Yoshioka Y, Takagaki K, Oki K, Takeshita F, Sakai Y, Kuroda M, Ochiya T (2013) Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci Rep* **3**, 1197.

[146] Barua NU, Miners JS, Bienemann AS, Wyatt MJ, Welser K, Tabor AB, Hailes HC, Love S, Gill SS (2012) Convection-enhanced delivery of neprilysin: A novel amyloid-β-degrading therapeutic strategy. *J Alzheimers Dis* **32**, 43-56.

[147] Miners JS, Barua N, Kehoe PG, Gill S, Love S (2011) Aβ-degrading enzymes: Potential for treatment of Alzheimer disease. *J Neuropathol Exp Neurol* **70**, 944-959.

[148] George S, Petit GH, Gouras GK, Brundin P, Olsson R (2013) Nonsteroidal selective androgen receptor modulators and selective estrogen receptor β agonists moderate cognitive deficits and amyloid-β levels in a mouse model of Alzheimer's disease. *ACS Chem Neurosci* **4**, 1537-1548.

[149] Kummer MP, Hülsmann C, Hermes M, Axt D, Heneka MT (2012) Nitric oxide decreases the enzymatic activity of insulin degrading enzyme in AβPP/PS1 mice. *J Neuroimmune Pharmacol* **7**, 165-172.

[150] Goncalves MB, Clarke E, Hobbs C, Malmqvist T, Deacon R, Jack J, Corcoran JP (2013) Amyloid β inhibits retinoic acid synthesis exacerbating Alzheimer disease pathology which can be attenuated by an retinoic acid receptor α agonist. *Eur J Neurosci* **37**, 1182-1192.

[151] Mizuno T, Doi Y, Mizoguchi H, Jin S, Noda M, Sonobe Y, Takeuchi H, Suzumura A (2011) Interleukin-34 selectively enhances the neuroprotective effects of microglia to attenuate oligomeric amyloid-β neurotoxicity. *Am J Pathol* **179**, 2016-2027.

[152] Cameron B, Tse W, Lamb R, Li X, Lamb BT, Landreth GE (2012) Loss of interleukin receptor-associated kinase 4 signaling suppresses amyloid pathology and alters microglial phenotype in a mouse model of Alzheimer's disease. *J Neurosci* **32**, 15112-15123.

[153] Kakiya N, Saito T, Nilsson P, Matsuba Y, Tsubuki S, Takei N, Nawa H, Saido TC (2012) Cell surface expression of the major amyloid-β peptide (Aβ)-degrading enzyme, neprilysin, depends on phosphorylation by mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) and dephosphorylation by protein phosphatase 1a. *J Biol Chem* **287**, 29362-29372.

[154] Mao P, Manczak M, Calkins MJ, Truong Q, Reddy TP, Reddy AP, Shirendeb U, Lo HH, Rabinovitch PS, Reddy PH (2012) Mitochondria-targeted catalase reduces abnormal AβPP processing, amyloid β production and BACE1 in a mouse model of Alzheimer's disease: Implications for neuroprotection and lifespan extension. *Hum Mol Genet* **21**, 2973-2990.

[155] Kim KS, Kim HS, Park JM, Kim HW, Park MK, Lee HS, Lim DS, Lee TH, Chopp M, Moon J (2013) Long-term immunomodulatory effect of amniotic stem cells in an Alzheimer's disease model. *Neurobiol Aging* **34**, 2408-2420.

[156] Takamatsu K, Ikeda T, Haruta M, Matsumura K, Ogi Y, Nakagata N, Uchino M, Ando Y, Nishimura Y, Senju S (2014) Degradation of amyloid beta by human induced pluripotent stem cell-derived macrophages expressing Neprilysin-2. *Stem Cell Res* **13**, 442-453.

[157] Blurton-Jones M, Spencer B, Michael S, Castello NA, Agazaryan AA, Davis JL, Müller FJ, Loring JF, Masliah E, LaFerla FM (2014) Neural stem cells genetically-modified to express neprilysin reduce pathology in Alzheimer transgenic models. *Stem Cell Res Ther* **5**, 46.

[158] Hoglund K, Salter H, Zetterberg H, Andreason U, Olsson T, Alexander R, Kugler A, Cebers G, Ye N, Burdette D, Budd Haeberlein SL (2014) Monitoring the soluble amyloid precursor protein alpha (sAβPP) and beta (sAβPP) fragments in plasma and CSF from healthy individuals treated with BACE inhibitor AZD3293 in a multiple ascending dose study: Pharmacokinetic and pharmacodynamic correlate. *Alzheimers Dement* **10**, P447.

[159] Stone J, Kleijn HJ, Dockendorf M, Ma L, Palcza J, Tseng J, Tanen M, Forman M (2013) Consistency of Bace inhibitor-mediated brain amyloid production inhibition by MK-8931 in Alzheimer's disease patients and healthy young adults. *Alzheimers Dement* **9**, P690-P691.

[160] Sabbagh MN, Agro A, Bell J, Aisen PS, Schweizer E, Galasko D (2011) PF-04494700, an oral inhibitor of receptor for advanced glycation end products (RAGE), in Alzheimer's disease. *Alzheimer Dis Assoc Disord* **25**, 206-212.

[161] Lai R, Albala B, Kaplow JM, Aluri J, Yen M, Satlin A (2012) First- in-human study of E2609, a novel BACE1 inhibitor, demonstrates prolonged reductions in plasma beta-amyloid levels after single dosing. *Alzheimers Dement* **8**, P96.

[162] Iijima-Ando K, Hearn SA, Granger L, Shenton C, Gatt A, Chiang HC, Hakker I, Zhong Y, Iijima K (2008) Overexpression of neprilysin reduces Alzheimer amyloid-beta42 (Abeta42)-induced neuron loss and intraneuronal Abeta42 deposits but causes a reduction in cAMP-responsive element-binding protein-mediated transcription, age-dependent axon pathology, and premature death in Drosophila. *J Biol Chem* **283**, 19066-19076.

[163] Du J, Zhang L, Liu S, Wang Z (2010) Palmitic acid and docosahexaenoic acid opposingly regulate the expression of insulin-degrading enzyme in neurons. *Pharmazie* **65**, 231-232.

[164] Im H, Manolopoulou M, Malito E, Shen Y, Zhao J, Neant-Fery M, Sun CY, Meredith SC, Sisodia SS, Leissring MA, Tang WJ (2007) Structure of substrate-free human insulin-degrading enzyme (IDE) and biophysical analysis of ATP-induced conformational switch of IDE. *J Biol Chem* **282**, 25453-25463.

[165] Nalivaeva NN, Belyaev ND, Lewis D, Pickles AR, Makova NZ, Bagrova D, Dubrovskaya NM, Plesneva SA, Zhuravin IA, Turner AJ (2012) Effect of sodium valproate administration on brain neprilysin expression and memory in rats. *J Mol Neurosci* **46**, 569-577.

[166] Wang Z, Zhang XJ, Li T, Li J, Tang Y, Le W (2014) Valproic acid reduces neuritic plaque formation and improves learning deficits in AβPP(Swe) /PS1(A246E) transgenic mice via preventing the prenatal hypoxia-induced down-regulation of neprilysin. *CNS Neurosci Ther* **20**, 209-217.

[167] Dobarro M, Gerenu G, Ramírez MJ (2013) Propranolol reduces cognitive deficits, amyloid and tau pathology in Alzheimer's transgenic mice. *Int J Neuropsychopharmacol* **16**, 2245-2257.

[168] Shin MK, Kim HG, Baek SH, Jung WR, Park DI, Park JS, J, Kim DG, KL (2014) Neuropep-1 ameliorates learning and memory deficits in an Alzheimer's disease mouse model, increases brain-derived neurotrophic factor expression in the brain, and causes reduction of amyloid beta plaques. *Neurobiol Aging* **35**, 990-1001.

[169] Ciaccio C, Tundo GR, Grasso G, Spoto G, Marasco D, Ruvo M, Gioia M, Rizzarelli E, Coletta MJ (2009) Somatostatin: A novel substrate and a modulator of insulin degrading enzyme activity. *Mol Biol* **385**, 1556-1567.

[170] Tundo G, Ciaccio C, Sbardella D, Boraso M, Viviani B, Coletta M, Marini S (2012) Somatostatin modulates insulin-degrading-enzyme metabolism: Implications for the regulation of microglia activity in AD. *PLoS One* **7**, e34376.

[171] Charton J, Gauriot M, Guo Q, Hennuyer N, Marechal X, Dumont J, Hamdane M, Pottiez V, Landry V, Sperandio O, Flipo M, Buee L, Staels B, Leroux F, Tang WJ, Deprez B, Deprez-Poulain R (2014) Imidazole-derived 2-[N-carbamoylmethyl-alkylamino]acetic acids, substrate-dependent modulators of insulin-degrading enzyme in amyloid-β hydrolysis. *Eur J Med Chem* **22**, 184-193.

[172] Song ES, Melikishvili M, Fried MG, Juliano MA, Juliano L, Rodgers DW, Hersh LB (2012) Cysteine 904 Is required for maximal insulin degrading enzyme activity and polyanion activation. *PLoS One* **7**, e46790.

[173] Kilger E, Buehler A, Woelfing H, Kumar S, Kaeser SA, Nagarathinam A, Walter J, Jucker M, Coomaraswamy J (2011) BRI2 protein regulates β-amyloid degradation by increasing levels of secreted insulin-degrading enzyme (IDE). *J Biol Chem* **286**, 37446-37457.

[174] Ohyagi Y (2011) A drug targeting intracellular amyloid-β and oxidative stress: Apomorphine. *Rinsho Shinkeigaku* **51**, 884-887.

[175] Marwarha G, Ghribi O (2012) Leptin signaling and Alzheimer's disease. *Am J Neurodegener Dis* **1**, 245-265.

[176] Marwarha G, Dasari B, Prasanthi JR, Schommer J, Ghribi O (2010) Leptin reduces the accumulation of Abeta and phosphorylated tau induced by 27-hydroxycholesterol in rabbit organotypic slices. *J Alzheimers Dis* **19**, 1007-1019.

[177] Leissring MA, Farris W, Chang AY, Walsh DM, Wu X, Sun X, Frosch MP, Selkoe DJ (2003) Enhanced proteolysis of beta-amyloid in AβPP transgenic mice prevents plaque formation, secondary pathology, and premature death. *Neuron* **40**, 1087-1093.

[178] Perez A, Morelli L, Cresto JC, Castaño EM (2000) Degradation of soluble amyloid beta-peptides 1-40, 1-42, and the Dutch variant 1-40Q by insulin degrading enzyme from Alzheimer disease and control brains. *Neurochem Res* **25**, 247-255.

[179] Liang K, Yang L, Yin C, Xiao Z, Zhang J, Liu Y, Huang J (2010) Estrogen stimulates degradation of beta-amyloid peptide by up-regulating neprilysin. *J Biol Chem* **285**, 935-942.

[180] Niikura T, Sidahmed E, Hirata-Fukae C, Aisen PS, Matsuoka Y (2011) A humanin derivative reduces amyloid beta accumulation and ameliorates memory deficit in triple transgenic mice. *PLoS One* **6**, e16259.

[181] Deng Y, Lu X, Liu L, Li T, Zhang Y, Guo X, Yu G, Xi Bao, Yu Fen, Zi, Mian Yi, XueZa Zhi (2014) Expression of neprilysin gene is associated with methylation and histone modification on promoter in mouse neuroblastoma Neuro-2a cells. *Chinese* **30**, 810-813.

[182] Jang SK, Yu JM, Kim ST, Kim GH, Park da W, Lee do I, Joo SS (2015) An Aβ42 uptake and degradation via Rg3 requires an activation of caveolin, clathrin and Aβ-degrading enzymes in microglia. *Eur J Pharmacol* **5**, 1-10.

[183] Quan Q, Wang J, Li X, Wang Y (2013) Ginsenoside Rg1 decreases Aβ(1-42) level by upregulating PPARγ and IDE expression in the hippocampus of a rat model of Alzheimer's disease. *PLoS One* **8**, e59155.

[184] Bauer C, Pardossi PR, Dunys J, Roy M, Checler F (2011) γ-Secretase-mediated regulation of neprilysin: Influence of cell density and aging and modulation by imatinib. *J Alzheimers Dis* **27**, 511-520.

[185] Kalinin S, Richardson JC, Feinstein DL (2009) A PPARdelta agonist reduces amyloid burden and brain inflammation in

a transgenic mouse model of Alzheimer's disease. *Curr Alzheimer Res* **6**, 431-437.

[186] Melzig MF, Janka M (2003) Enhancement of neutral endopeptidase activity in SK-N-SH cells by green tea extract. *Phytomedicine* **10**, 494-498.

[187] Wang P, Su C, Li R, Wang H, Ren Y, Sun H, Yang J, Sun J, Shi J, Tian J, Jiang S (2014) Mechanisms and effects of curcumin on spatial learning and memory improvement in AβPPswe/PS1dE9 mice. *J Neurosci Res* **92**, 218-231.

[188] El-Sayed NS, Bayan Y (2015) Possible role of resveratrol targeting estradiol and neprilysin pathways in lipopolysaccharide model of Alzheimer disease. *Adv Exp Med Biol* **822**, 107-118.

[189] Kong Y, Ruan L, Qian L, Liu X, Le Y (2010) Norepinephrine promotes microglia to uptake and degrade amyloid beta peptide through upregulation of mouse formyl peptide receptor 2 and induction of insulin-degrading enzyme. *J Neurosci* **30**, 11848-11857.

[190] Pike CJ, Carroll JC, Rosario ER, Barron AM (2009) Protective actions of sex steroid hormones in Alzheimer's disease. *Front Neuroendocrinol* **30**, 239-258.

[191] Nguyen TV, Yao M, Pike CJ (2005) Androgens activate mitogen-activated protein kinase signaling: Role in neuroprotection. *J Neurochem* **94**, 1639-1651.

[192] Huang J, Guan H, Booze RM, Eckman CB, Hersh LB (2004) Estrogen regulates neprilysin activity in rat brain. *Neurosci Lett* **367**, 85-87.

[193] Yao M, Nguyen TV, Rosario ER, Ramsden M, Pike CJ (2008) Androgens regulate neprilysin expression: Role in reducing beta-amyloid levels. *J Neurochem* **105**, 2477-2488.

[194] Fujiwara H, Kimura J, Sakamoto M, Yokosuka A, Mimaki Y, Murata K, Yamaguchi K, Ohizumi Y (2014) Nobiletin, a flavone from Citrus depressa, induces gene expression andincreases the protein level and activity of neprilysin in SK-N-SH cells. *Can J Physiol Pharmacol* **l92**, 351-355.

[195] Liu Y, Gao M, Ma L, Zhang L, Pan N, Acta Scand A (2013) Sevoflurane alters the expression of receptors and enzymes involved in Aβ clearance in rats. *Acta Anaesthesiol Scand* **57**, 903-910.

[196] Liu Y, Liu L, Lu S, Wang D, Liu X, Xie L, Wang GJ (2011) Impaired amyloid β-degrading enzymes in brain of streptozotocin-induced diabetic rats. *Endocrinol Invest* **34**, 26-31.

[197] Newell AJ, Sue L, Scott S, Rauschkolb PK, Walker DG, Potter PE, Beach T (2003) Thiorphan-induced neprilysin inhibition raises amyloid beta levels in rabbit cortex and cerebrospinal fluid. *Neurosci Lett* **350**, 178-180.

[198] Marr RA, Spencer B (2010) NEP-like endopeptidases and Alzheimer's disease [corrected]. *J Curr Alzheimer Res* **7**, 223-229.

[199] Pardossi-Piquard R, Dunys J, Yu G, St George HP, Alves da CC, Checler F (2006) Neprilysin activity and expression are controlled by nicastrin. *J Neurochem* **97**, 1052-1056.

[200] Yamamoto N, Arima H, Naruse K, Kasahara R, Taniura H, Hirate H, Sugiura T, Suzuki K, Sobue K (2013) Ketamine reduces amyloid β-protein degradation by suppressing neprilysin expression in primary cultured astrocytes. *Neurosci Lett* **545**, 54-58.

[201] Honda M, Okutsu H, Matsuura T, Miyagi T, Yamamoto Y, Hazato T, Ono H (2001) Spinorphin, an endogenous inhibitor of enkephalin-degrading enzymes, potentiates leu-enkephalin-induced anti-allodynic and antinociceptive effects in mice. *Jpn J Pharmacol* **87**, 261-267.

[202] Nishimura K, Ueki M, Kaneto H, Hazato T (1993) Study of a new endogenous inhibitor of enkephalin-degrading enzymes; pharmacological function and metabolism of spinorphin. *Masui* **42**, 1663-1670.

[203] Rougeot C, Messaoudi M, Hermitte V, Rigault AG, Blisnick T, Dugave C, Desor D, Rougeon F (2003) Sialorphin, a natural inhibitor of rat membrane-bound neutral endopeptidase that displays analgesic activity. *Proc Natl Acad Sci U S A* **14**, 8549-8554.

[204] Rosa M, Arsequell G, Rougeot C, Calle LP, Marcelo F, Pinto M, Centeno NB, Jiménez-Barbero J, Valencia G (2012) Structure-activity relationship study of opiorphin, a human dual ectopeptidase inhibitor with antinociceptive properties. *J Med Chem* **55**, 1181-1188.

[205] Wisner A, Dufour E, Messaoudi M, Nejdi A, Marcel A, Ungeheuer MN, Rougeot C (2006) Human Opiorphin, a natural antinociceptive modulator of opioid-dependent pathways. *Proc Natl Acad Sci U S A* **103**, 17979-17984.

[206] Li M, Sun M, Liu Y, Yu J, Yang H, Fan D, Chui D (2010) Copper downregulates neprilysin activity through modulation of neprilysin degradation. *J Alzheimers Dis* **19**, 161-169.

[207] Huang H, Bihaqi SW, Cui L, Zawia NH (2011) *In vitro* Pb exposure disturbs the balance between Aβ production and elimination: The role of AβPP and neprilysin. *Neurotoxicology* **32**, 300-306.

[208] Yamamoto N, Tanida M, Ono Y, Kasahara R, Fujii Y, Ohora K, Suzuki K, Sobue K (2014) Leptin inhibits amyloid β-protein degradation through decrease of neprilysin expression in primary cultured astrocytes. *Biochem Biophys Res Commun* **445**, 214-217.

Ambasta *et al. J Transl Med (2015) 13:307*
DOI 10.1186/s12967-015-0665-z

JOURNAL OF TRANSLATIONAL MEDICINE

RESEARCH

Open Access

Comparative study of anti-angiogenic activities of luteolin, lectin and lupeol biomolecules

Rashmi K. Ambasta[1,2*], Saurabh Kumar Jha[1,2], Dhiraj Kumar[1], Renu Sharma[1], Niraj Kumar Jha[1,2] and Pravir Kumar[1,2,3]

Abstract

Background: Angiogenesis is a hallmark feature in the initiation, progression and growth of tumour. There are various factors for promotion of angiogenesis on one hand and on the other hand, biomolecules have been reported to inhibit cancer through anti-angiogenesis mechanism. Biomolecules, for instance, luteolin, lectin and lupeol are known to suppress cancer. This study aims to compare and evaluate the biomolecule(s) like luteolin, lupeol and lectin on CAM assay and HT-29 cell culture to understand the efficacy of these drugs.

Method: The biomolecules have been administered on CAM assay, HT-29 cell culture, cell migration assay. Furthermore, bioinformatics analysis of the identified targets of these biomolecules have been performed.

Result: Luteolin has been found to be better in inhibiting angiogenesis on CAM assay in comparison to lupeol and lectin. In line with this study when biomolecules was administered on cell migration assay via scratch assay method. We provided evidence that Luteolin was again found to be better in inhibiting HT-29 cell migration. In order to identify the target sites of luteolin for inhibition, we used software analysis for identifying the best molecular targets of luteolin. Using software analysis best target protein molecule of these biomolecules have been identified. VEGF was found to be one of the target of luteolin. Studies have found several critical point mutation in VEGF A, B and C. Hence docking analysis of all biomolecules with VEGFR have been performed. Multiple allignment result have shown that the receptors are conserved at the docking site.

Conclusion: Therefore, it can be concluded that luteolin is not only comparatively better in inhibiting blood vessel in CAM assay, HT-29 cell proliferation and cell migration assay rather the domain of VEGFR is conserved to be targeted by luteolin, lupeol and lectin.

Keywords: CAM assay, Flavonoids, HT-29 cell, Anti-angiogenesis, Luteolin, Lupeol, Lectin

Background

Angiogenesis process is regulated by several factors that have a critical role in governing the initiation and progression of tumour. Angiogenic factors such as bFGF, HGF, VEGF, hyluronatelyase, collagenase, MMP supports the formation of new blood vessels. In addition, cell cycle markers, for instance, cyclin A2, Cyclin Dependent Kinase-2, 6 and MAPK1, 14, 10 promote the tumour progression whereas caspase 3 inhibits the tumour progression. Mounting evidence is suggesting the critical role of cyclin inhibitors, and inducers of apoptotic markers in cancer therapy. Furthermore, several biomolecules elicit the anti-cancerous property such as, luteolin, lectin and lupeol but comparative studies in terms of anti-angiogenic activity remain unsettled.

Luteolin is a flavonoid; lupeol is a triterpene and lectin is a protein possessing carbohydrate. Flavonoids are polyphenols that play an important role in defending plant cells against microorganisms, insects, and UV

*Correspondence: rashmiambasta@gmail.com; rashmiambasta@dce.edu
[1] Department of Biotechnology, Delhi Technological University (Former Delhi College of Engineering), Delhi, India
Full list of author information is available at the end of the article

irradiation, luteolin sensitizes cancer cells to therapeutic-induced cytotoxicity through signaling pathways like PI3K/Akt [1], NF-kB [2], X-linked inhibitor of apoptosis protein and stimulating apoptosis pathways that induce p53. Luteolin has a C6–C3–C6 structure and possesses two benzene rings, a third, oxygen-containing (C) ring, and a 2–3 carbon double bond. Luteolin also possesses hydroxyl groups at carbons 5, 7, 3′, and 4′ positions. The hydroxyl moieties and 2–3 double bond are important structure features in luteolin that are associated with its biochemical and biological activities. Numerous studies have highlighted that luteolin is often glycosylated in plants, and the glycoside is hydrolyzed to free luteolin during absorption. Moreover, some portion of luteolin is converted to glucuronides when passing through the intestinal mucosa. Luteolin is heat stable and losses due to cooking are relatively low and may suppress VEGF [3, 4] expression by inhibiting transcription factor HIF-1α through p53-mediated proteasomal degradation.

Additionally, luteolin can suppress VEGF-induced signaling in endothelial cells. Luteolin effectively blocked activation of the VEGF receptor and its downstream molecule PI3K/Akt and PI3K/p70S6 kinase pathways, which may directly contribute to luteolin-induced anti-angiogenesis, resulting in suppression of proliferation and survival of human umbilical vein endothelial cells. Luteolin may also suppress angiogenesis by stabilizing hyaluronic acid, a neovascularization barrier. Hyaluronic acid is one of the most abundant constituents of the extracellular matrix that blocks neo vacuole formation and extension. An enzyme hyaluronidase catalyzes hyaluronic acid to break the barrier and to promote angiogenesis through the processed product. Further, oligosaccharides generated from hyaluronic acid bind to the CD44 receptor on the membranes of endothelial cells to trigger their proliferation, migration, and eventually angiogenesis. Luteolin is a strong inhibitor of hyaluronidase and maintains the neovascularization barrier. Moreover, tumor angiogenesis is dependent on the activity of MMPs where luteolin is a potent MMP inhibitor that attenuates MMP expression [5] through suppressing NF-κB or directly inhibiting MMP activity. These facts reflect that indeed luteolin is an important biomolecule for cancer therapy.

Another biomolecule, lupeol is a dietary triterpene that is important structural components of plant membranes, and free triterpenes serve to stabilize phospholipid bilayers in plant cell membranes just as cholesterol does in animal cell membranes. Most triterpenes contain 28 or 29 carbons and one or two carbon–carbon double bonds, typically one in the sterol nucleus and sometimes a second in the alkyl side chain. Triterpenes are natural components of human diets. The chemical formula of lupeol is $C_{30}H_{50}O$ and its melting point is 215–216 °C. Properties computed from the structure of Lupeol show that it has a molecular weight of 426.7174 (g/mol), H-Bond donor 1, H-Bond acceptor1, rotatable bond count 1, exact mass 426.386166. Studies have shown that topical application of Lupeol (40 mg/kg/three times a week) for 28 weeks can significantly decrease the tumor burden, its multiplicity and increase the latency period in the mouse model. The anti-tumor promotion effects of lupeol were observed to be associated with its potential to modulate signaling pathways such as nuclear factor kappa B (NFκB) and the phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B pathway) [6], which are reported to play an important role during tumorigenesis. Lupeol inhibits growth of highly metastatic tumors of human melanoma origin by modulating the ratio of Bcl-2 and Bax protein [7] levels in vitro and in vivo. Further, lupeol significantly inhibits the growth of metastatic melanoma cells harboring constitutive activation of Wnt/β-catenin [8, 9] signaling. Other reports have shown that lupeol administered orally in a dose of 2 g/kg has been reported to produce no adverse effects in rats and mice, and after 96 h of observation, no mortality was recorded. Moreover, Lupeol has also been reported to affect angiogenic gene like MMP and VEGF [10]. Taken together, these studies provide convincing evidence that lupeol is a non-toxic but highly potent chemo preventive and chemotherapeutic agent for cancer therapy.

Lectins are a group of glycoprotein playing a critical role in diagnosis of cancer [11–13] whereas targeting of lectins can increase the efficacy of anti-VEGF treatment [14]. On contrary, there are reports claiming that lectins promote angiogenesis [15]. Lectin in combination with RNAse called as Leczyme [16] has also been used for cancer therapy. Another feature of lectin that induces apoptosis and kills cancerous cells [17, 18] and thus lectin can qualify a good candidate for anti-cancer effect. It will be interesting to investigate which will be better in inhibiting angiogenesis.

In order to understand the anti-angiogenic efficacy of these drugs, chick chorio-allantoic-membrane (CAM) assay has been performed, which is one of the most acceptable and well characterized angiogenesis assays. Retinoic acid, a known inhibitor of VEGF and an angiogenesis marker that have been taken as positive control.

In this study, we investigate about the comparative anti-angiogenic effects of biomolecules i.e. luteolin, lectin and lupeol on CAM assay, HT-29 cell line and HT-29 cell migration assay. We also aim to investigate about the docking of these biomolecules with its molecular target site for inhibiting cancer.

Method

Structure of Biomolecules

The structure of the biomolecules for our study has been used from pubchem. Pubchem is a composite database (http://pubchem.ncbi.nlm.nih.gov/) that is backed up by three primary databases, i.e. Pcsubstance, Pccompund, PCBioAssay. Pubchem provides biological activity, chemical information of small molecules. Pcsubstance contains information about the substances; Pccompound contains information about chemical compounds, and PcBioAssay provides information about Bioassays.

CAM assay

The study have been approved from the ethical committee of Vellore Institute of Technology University(VITU), Vellore, India. Fertilized white leghorn chicken eggs were obtained from poultry farm. Eggs were incubated in an Incubator at 37 °C with 60 % humidity as mentioned in earlier [20]. A small window was made in the shell on day 3 of chick embryo development under aseptic conditions. The window was resealed with adhesive tape after drug application, and eggs were returned to the incubator until day 11 of chick embryo development. Photography was performed on the following day, and eggs were discarded after photography.

HT-29 cell culture

HT-29 cell culture was performed using DMEM, FBS, antibiotic and these cells were subcultured for biomolecule administration using standard protocol.

Cell migration assay

Cell migration assay was studied using cell scratch assay at 70 % confluency and measuring the distance of scratch after 24, 48 h of scratch in HT-29 cell.

Pharm Mapper

Chemical compounds have been downloaded from the Pubchem database (http://pubchem.ncbi.nlm.nih.gov/) in MDL SDF file format and have been loaded in PharmMapper server (http://59.78.96.61/pharmmapper/submit_file.php) with the options for searching of all potential candidates available in the PharmTargetDB. Furthermore, all the targets important for the process of angiogenesis have been identified, and their scores have been summarized in Fig. 1.

PharmMapper [19] is a web server for identifying the potential target candidates for a given probe (drugs, natural products). It adopts an alternative approach of pharmacophore mapping for potential drug targets. It possesses highly efficient and robust high-throughput mapping technique to identify target candidates from the databases within the short period of time. PharmMapper has been backed up by various databases that involve TargetBank, DrugBank, BindingDB and PDTD. PharmMapper accepts files in different formats in Tripos Mol2 or MDL SDF (http://59.78.96.61/pharmmapper/submit_file.php) that identifies best mapping poses and outputted top N potential drug targets against all targets in PharmTargetDB. With the submission of new molecules, fit scores have been calculated first, which is used to further calculate the normalized fit score. Moreover, every fit score of a specific pharmacophore has been compared to the fit score matrix to measure its score level among all the scores of the pharmacophore to find the Z′-score that adds more statistical meaning and confidence of comparison.

Colon cancer genome sequence analysis

Colon cancer genome sequence analysis information was taken from ICGC pooled data.

Molegro virtual docker (Version-6.0)

In this study protein–ligand, docking was performed with the help of Molegro Virtual Docker [21] for predicting the possible protein–ligand interactions (MVD).

Statistics

The statistics has been done using the excel software. Statistical significance was accepted at $p < 0.05$.

Result

Comparative chemical/structural/physical analysis and source of origin study of luteolin, lectin and lupeol

Retinoic acid can be naturally derived from carrot, pumpkin, squash, and sweet potatoes. Its IUPAC name is nona 2,4,6,8 tetraenoic acid with molecular mass is 300 g/mol, melting point is 180 °C, boiling point is 175 °C, pH = 4.9 and it is soluble in water. Another compound, luteolin can be naturally derived from olive oil, pepper, parsley. The molecular mass is 286 g/mol, melting point is 330 °C and boiling point is 660 °C, pH = 7.8 and it is soluble in alkaline solvent while lupeol can be naturally isolated from mango, aloe leaves, ginseng oil. The molecular mass of lupeol is 426 g/mol, melting point is 210 °C and boiling point is 488 °C, pH = 4.9 and it is soluble in water. Lectin is a glycoprotein with sugar chains, and its isoelectric point is 4.5; molecular mass is 104–112 kDa and it can be naturally isolated from peas, cherries and sweet pepper as mentioned in Table 1 in Fig. 2A.

The structure of luteolin has a hydroxyl group while lupeol has a methyl group like the positive control retinoic acid and lectin has multiple sugar chains. Therefore, we can summarize that based on structural analysis luteolin is the best in targeting angiogenesis.

a

S. No.	Target Name	Retinoic acid					Luteolin					Lupeol				
		PDB ID	No. of Feature	Fit Score	Normalized Fit Score	Z'-Score	PDB ID	No. of Feature	Fit Score	Normalized Fit Score	Z'-Score	PDB ID	No. of Feature	Fit Score	Normalized Fit Score	Z'-Score
1	MAPK 1	2OJJ	7	4.627	0.661	1.17939	_	_	_	_	_	2OJJ	7	3.142	0.4489	-0.49126
2	MAPK 14	1OUY	8	4.145	0.5181	0.19102	3E 92	10	3.736	0.3736	0.198107	1BL6	4	3.46	0.8651	0.678894
3	MAPK 10	1PMN	7	3.791	0.5415	-0.74694	_	_	_	_	_	1PMN	7	3.871	0.553	0.567501
4	Caspase-3	1RHR	8	4.618	0.5772	0.800748	1NMS	10	3.377	0.3377	-0.71819	1RHR	8	3.223	0.4029	-0.61965
5	bFGFR1	2FGI	8	4.483	0.5604	0.248995	3C4F	7	4.278	0.6111	2.74909	2FGI	8	3.433	0.4291	-0.28979
6	VEGFR2	3CP9	10	3.88	0.388	-0.28953	2P2I	6	3.466	0.5777	-0.0986	3CP9	10	3.42	0.342	0.048207
7	Hyaluronate lyase	1W3Y	7	4.379	0.6256	0.683456	1OJM	7	3.815	0.5449	0.747285	1W3Y	7	3.491	0.4987	0.183722
8	Prostaglandin G/H synthase 1	1DIY	6	4.004	0.6673	-0.29698	_	_	_	_	_	1DIY	6	3.769	0.6281	0.607966
9	Prostaglandin G/H synthase 2	4COX	7	4	0.5714	-0.05468	_	_	_	_	_	1CVU	7	3.471	0.4959	0.284178
10	Cell division protein kinase 2	1KE8	8	3.803	0.4754	-0.44082	1KE8	8	3.879	0.4849	1.19134	1R78	7	3.604	0.5148	0.480203
11	Cell division protein kinase 6	_	_	_	_	_	1XO2	5	4.433	0.8866	3.94576	_	_	_	_	_
12	Hepatocyte growth factor receptor	3F82	11	3.781	0.3437	-0.7395	3F82	11	3.748	0.3407	0.139748	3F82	11	3.935	0.3578	0.400731
13	Collagenase 3	1CXV	7	3.974	0.5677	-0.25227	3I7I	7	3.616	0.5165	0.967746	1FLS	6	3.024	0.504	-1.02511
14	Cyclin-A2	2IW6	7	3.903	0.5576	-0.25957	2IW8	7	3.89	0.5557	1.33083	2IW6	7	3.224	0.4606	-0.3645

b

VEGF	Mutation	Type of mutation	Chromosome	Cancer
VEGF A	C/T, A/T	Single base substitution	Chr 6	Colorectal
VEGF B	A/A, C/T	Single base substitution and deletion	Chr 11	Colorectal
VEGF C	C/T, G/A. G/T	Single base substitution	Chr 4	Colorectal

Fig. 1 **a** It demonstrates fit score for target molecule of biomolecules and **b** shows the pooled data for VEGF A, B, C from ICGC pooled data demonstrating identified point mutation in VEGF in colorectal cancer

Comparative anti-angiogenic analysis of biomolecules, luteolin, lectin and lupeol on CAM Assay

Chick chorio-allantoic membrane (CAM) assay is one of the most reliable angiogenesis assays. The biomolecules have been administered on CAM and visualized for its effect after 24, 48 h in a concentration and dose dependent manner as shown in Fig. 2B. The quantitation of angiogenesis was calculated based on thickness of the vessel, branching, sprouting of new vessels and the diameter using software and manual counting. We examined the effect of luteolin, lupeol, lectin and retinoic acid on CAM assay. It was observed that luteolin inhibited angiogenesis better compared to lectin and lupeol. Further, these biomolecules were administered on HT-29 cell culture to check its anti-cancerous property.

Comparative anti-cancerous analysis of biomolecules, luteolin, lectin and lupeol on HT-29 cell culture

The biomolecules luteolin, lectin and lupeol were administered on HT-29 cell culture and its effect was observed after 24 and 48 h as shown in Fig. 3a, b. Under these experimental conditions, the cells were photographed using a microscope and cell viability was performed using trypan blue analysis. The statistical analysis of cell viability demonstrates that luteolin is better in inhibiting cell growth at the lower dose compared to lectin and lupeol. In pilot study, we observed luteolin was the best amongst the compared drugs therefore; cell migration effect of luteolin was performed via scratch assay as shown in Fig. 3c, d and we observed that luteolin had the best efficacy to inhibit the cell migration at 80 μM in 48 h. In

summary, we can say that luteolin is better in inhibiting angiogenesis, cell proliferation and cell migration.

Comparative analysis of potential target signaling mechanism of biomolecules, luteolin, lectin and lupeol

We next investigated the target signaling molecule of these biomolecule using software analysis. Studies have demonstrated certain target sites for these biomolecules but in order to get a broader view about the target sites and decide future target sites; software analysis was performed using PharmMapper. PharmMapper is a web server for identifying the potential target candidates for a given probe (drugs, natural products). It adopts an alternative approach of pharmacophore mapping for potential drug targets. It possesses highly efficient and robust high-throughput mapping technique to identify target candidates from the databases within a short period of time. PharmMapper has been backed up by various databases that involve TargetBank, DrugBank, BindingDB and PDTD. PharmMapper accepts files in different formats in Tripos Mol2 or MDL SDF (http://59.78.96.61/pharmmapper/submit_file.php) that identifies best mapping poses and outputted top N potential drug targets against all targets in PharmTargetDB. With the submission of new molecules, fit scores have been calculated first, which is used to further calculate for normalized fit score. Moreover, every fit score of a specific pharmacophore has been compared to the fit score matrix to measure its score level among all the scores of the pharmacophore to find the Z′-score that adds more statistical meaning and confidence of comparison.

Table 2 of Fig. 1a enlists different potential downstream targets for the biomolecules of our interest. Luteolin target is bFGFR, VEGF, HGFR, Caspase 3, MAPK14, hyaluronatelyase and CDK6. The positive control retinoic acid has MAPK1, Caspase 3, bFGFR1, VEGF, hyaluronate lyase and CDK2 downstream target. The biomolecule lupeol also has the target candidate listed as MAPK10, HGFR, VEGF, hyaluronate lyase etc. The common target sites are VEGF, bFGFR1, HGFR, caspase3 and CDK2. Upon comparing the literature research output with the predicted targets, we find intriguing correlation between prediction and actual interest. Therefore, the best target site for future comparison can be VEGFR1, bFGFR1, HGFR, caspase3 and CDK2.

ICGC pooled data demonstrate that VEGF ligand is mutated in colon cancer tissues as shown in Table 3 of Fig. 1b. Hence the receptor of VEGF have been screened for conserved sequence domain in VEGFR-biomolecule(s) docking site.

These result suggests that one of the important target molecule of these biomolecule is VEGF and its receptor. Interestingly, VEGF ligand has been found to be mutated in colon cancer, indicating that VEGF is not only important for tumour development and progression but it can also act as a therapeutic target molecule for colon cancer.

Comparative analysis of protein–ligand docking affinity of biomolecules, luteolin, lectin and lupeol with VEGF

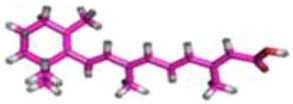
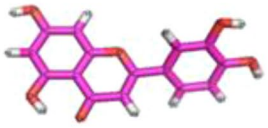
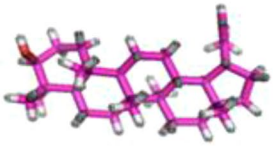
Numerous studies have shown that VEGFR1 is a marker for angiogenesis, therefore, the docking affinity of our biomolecules of interest has been checked against the VEGF receptors in Fig. 4a. In this study protein–ligand, docking was performed with the help of Molegro Virtual Docker for predicting the possible protein–ligand interactions (MVD).

To determine protein(VEGFR1)-ligand(Biomolecules) docking, VEGFR1 protein (PDB id: 1VPF) was used as a target molecule/macromolecule. Docking of target protein was performed with the four ligands namely; lupeol, lectin, retinoic acid and luteolin. The docked poses were analyzed individually, and the best pose was reported. The interactions between the ligands and target protein were represented pictorially. The final results of docking were predicted after evaluating all the possible conformations/orientations of the given ligands within the binding pocket of target protein in terms of the hydrogen bond energy, steric interactions. Further, comparative analyses of all the four ligands were carried out for the prediction of ligand with the highest potency towards target protein. Retinoic acid formed two hydrogen bonds with Cysteine 1018 residue of Target protein whereas; lupeol formed two hydrogen bonds with Cysteine 912 and Glycine 915 residues. The overall interaction energy, including hydrogen bond energy was also low for these two ligands. Though, lectin formed maximum number of hydrogen bonds with the target protein, it also depicted highest steric interactions but the overall interaction energy was low. It was observed that luteolin formed seven hydrogen bonds with the target protein at Ile1038, Val892, Glu878, Cys1018, Arg102 and two bonds with Ile1019, the steric interactions were less but the overall interaction energy was highest for luteolin. Therefore, this study proposes that luteolin has the highest inhibitory activity against target protein in terms of binding efficiency as compared to other three ligands that were used in this study.

As there are different types of VEGF receptors found on tissues, hence multiple alignments have been performed for the three VEGF receptors to check for the conserved sequence at docking sites of the biomolecules. Therefore, Sequence alignment has been done for VEGFR. The alignment shows conserved sequence at the docking sites as shown in Fig. 4b. As the docking sequence is conserved between the receptors, hence luteolin can be a good target for colon cancer therapy overcoming the hassles of non specific targeting.

Ambasta et al. 2015

A

NAME	MOL STRUCTUE	IUPAC NAME	PHYSICAL PROPERTIES	ORIGIN/SOURCES
RETINOIC ACID		(2E, 4E, 6E, 8E)-3, 7-dimethyl-9-(2, 6, 6-trimethylcyclohexan-1-yl)nona-2, 4, 6, 8-tetraenoic acid	Mol Formula: $C_{20}H_{28}O_2$ Mol Mass: 300.435g/mol Melting Point: 180∘C-182∘C Boiling Point: 175∘C PH: 4.97 Density: 1.07g/cm3 Storage: -20∘C Partition Coeffecient: 4.5 Solubility: In H_2O	Carrots, pumpkins, kale, butternut squash, cantaloupe, mangoes, spinach and sweet potatoes.
LUTEOLIN		2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxy-4H-1-benzopyran-4-on 3', 4', 5',7-tetrahydroxy-flavon	Mol Formula: $C_{15}H_{10}O_6$ Mol Mass: 286.24g/mol Melting Point: >330∘C Boiling Point: 616.1∘C PH: 7.8 Density: 1.66g/cm3 Storage: 2-8∘C Partition Coeffecient: 22.2 Solubility: In alkaline	Olive oil, celery, broccoli, green pepper, parsley, thyme, dandelion, perilla, chamomile tea, carrots, ,peppermint, rosemary, navel oranges, and oregano
LUPEOL		(1R,3aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol	Mol Formula: $C_{30}H_{50}O$ Mol Mass: 426.72g/mol Melting Point: 210∘C-215∘C Boiling Point: 488.11∘C PH: 4.97 Density: 0.98g/cm3 Storage: -20∘C Partition Coeffecient: ----- Solubility: In H_2O, $CHCL_3$, C_2H_5OH	Olive fruit, Mango fruit, Aloe Leaves, Elm Plant, Japanese Pear (shinko) and Ginseng Oil
LECTIN		———	PDB ID: 1LEM Organism: Lens culinaris Chain A: Length-181aa Chain B: Length-52aa Sugar specificity: αMan, αGlc Eluting sugar: MeαMan+MeαGlc Isoelectric point: 4.5-5.5 Mol weight: 104-112KDa Storage: -20∘C	Cherries, pomegranate, grape seeds, raspberry seeds, apples, watermelon, grapefruit, banana, lemon,Cocoa beans, mung bean, green peas and sweet peppers

B

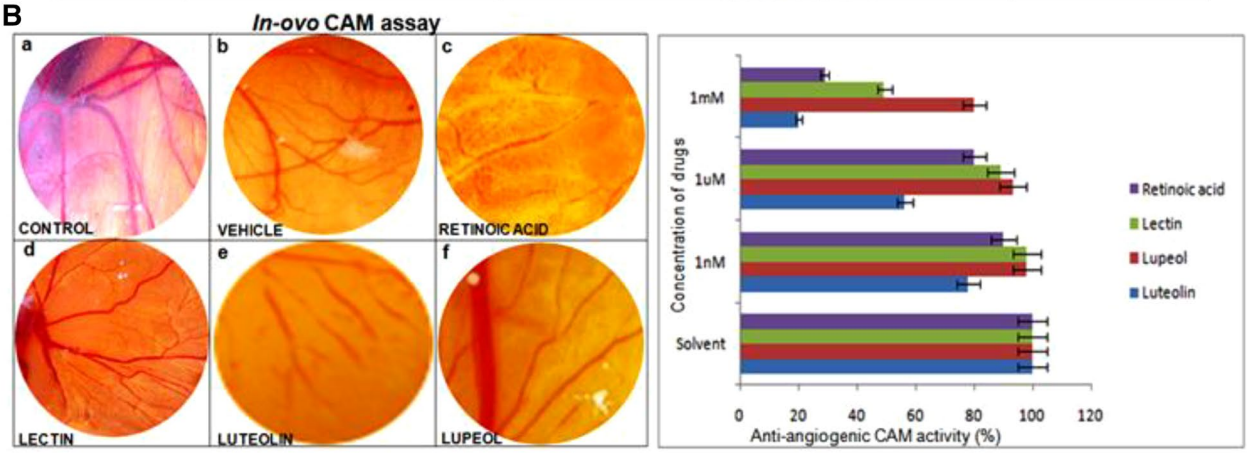
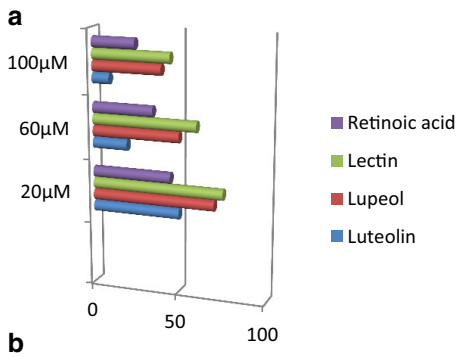
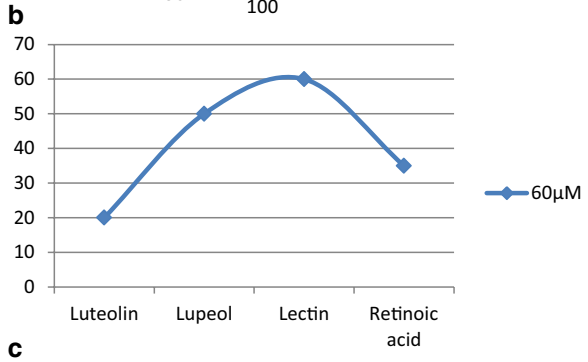


Fig. 2 **A** Structure, IUPAC name, physical properties and origin of Biomolecules in Table 1. **B** On the *left-hand side*, demonstrates CAM assay with biomolecules like luteolin, lupeol and lectin. Table 1**B** on the *right-hand side* shows time dependent and concentration dependent CAM assay with biomolecules





c

	10 µM	20 µM	40 µM	80 µM
Luteolin	3 cm	4 cm	4.5 cm	5 cm
Lectin	3 cm	3.2 cm	3.5 cm	4 cm
Lupeol	3 cm	3.1 cm	3.3 cm	3.5 cm
Retinoic Acid	3 cm	4.1 cm	4.6 cm	5.2 cm

Luteolin	**24 hr**	**48 hr**	**72 hr**
10 µM	3 cm	3 cm	3 cm
20 µM	3 cm	3.2 cm	4 cm
40 µM	3.1 cm	3.5 cm	4.5 cm
80 µM	3.3 cm	4 cm	5 cm

Fig. 3 **a** Demonstrates concenteration dependent administration of biomolecules on HT-29 cell while **b** shows the live cell at 60 µM. **c** shows the cell migration assay via scratch assay

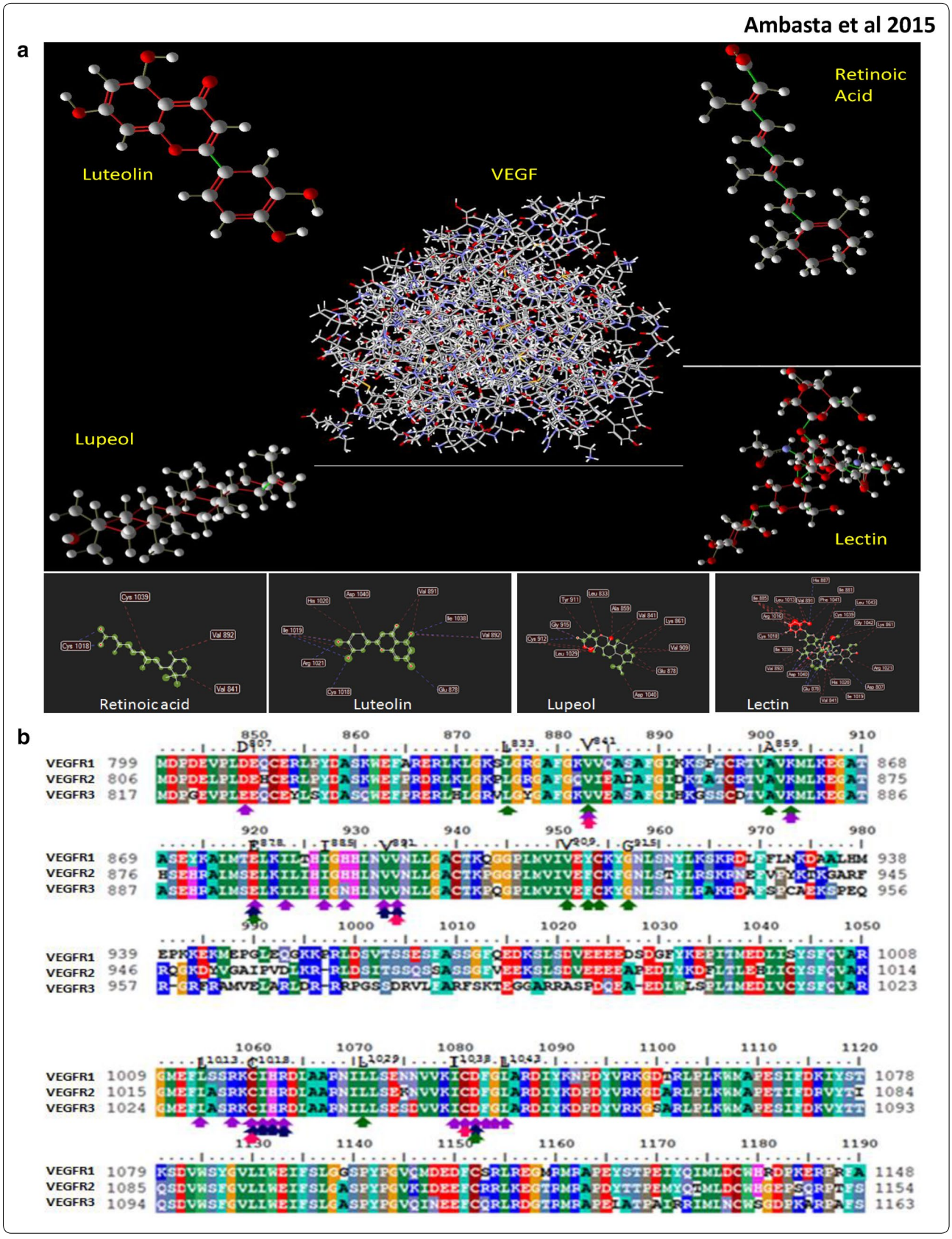
Discussion

In recent studies, several reports have been focused on individual role of luteolin, lupeol and lectin in tumour therapy but till date no study has compared these biomolecules. In this study, luteolin was found to be one of the best inhibitors of angiogenesis as compared with lectin and lupeol via CAM assay. In addition, Luteolin has been found to be comparatively better in its anti-cancerous effect as compared with lupeol and lectin as observed from HT-29 cell culture, cell migration assay and docking with VEGFR1. The purpose of this study was to compare the anti cancerous and anti-angiogenic role of biomolecules and analyze the docking domain of these biomolecules on the target site. Therefore, comparison analysis of the biomolecules have illuminated new facts that may contribute for future analysis.

To ensure the accuracy of comparison, quantitative analysis of CAM assay, HT-29 cell culture assay and cell migration assay has been done. Moreover another important question about the size, origin and chemical structure of the biomolecules have been addressed in Fig. 2. Due to the difference in the size of the biomolecules, it is expected that the steric hindrance will be different. Therefore, we compare a large protein versus small biomolecules for cancer therapy like luteolin, lupeol and positive control retinoic acid. We find that in our study, the large protein due to its size and steric hindrance poses several problems in its anti-angiogenic effect on CAM assay and docking with VEGF. Studies have reported that small molecule due to its size is better in permeabilization of the cell. Moreover in this study, the smaller biomolecules like luteolin and lupeol are better in inhibiting angiogenesis on CAM assay and docking with VEGF. Amongst the two small biomolecule lupeol and luteolin, luteolin has been found to be better in inhibiting angiogenesis and VEGF docking. Irrespective of the size of biomolecules, an obvious question arises about the target of these biomolecules in signaling for cancer progression.

Many reports demonstrates several target of these biomolecules but there is no platform where we can compare the target of the biomolecule using a score. Hence a software analysis has been used to predict the target site of these biomolecules. Importantly, the predicted result as shown in Fig. 1 demonstrates that the higher fit scores for retinoic acid are for bFGF, VEGFR2, hyaluronatelyase, MAPK and caspase 3. The higher fit scores for luteolin are bFGFR, VEGFR2, CDK2/6, hyaluronatelyase and MAPK14. The higher fit scores for lupeol are MAPK10, CDK2, HGFR, VEGFR2, hyaluoronate lyase. Several reports have confirmed the target site of lectin as VEGF, HGF, MAPK, hyaluronic acid. Hence it can be deduced that VEGF, HGF, MAPK and hyaluronate lyase can be a common target molecule for these biomolecules.

The new identified targets for cancer therapy in future using luteolin can be CDK2, CDK6, HGFR, MAPK14, FGF. Earlier reports demonstrated direct involvement of luteolin with VEGF [22, 23]. It has been confirmed both by prediction and actual reports that VEGFR have role in tumour development and progression. This study also confirms the mutated VEGF ligand in colorectal cancer. We have chosen VEGFR1 for docking analysis of different biomolecules as VEGF has been confirmed


Ambasta et al 2015
a
Luteolin
VEGF
Retinoic Acid
Lupeol
Lectin
Retinoic acid
Luteolin
Lupeol
Lectin
b
VEGFR1
VEGFR2
VEGFR3

(See figure on previous page.)
Fig. 4 **a** Demonstrates the structure of the biomolecule and structure of VEGFR1 along with the docking site of the biomolecule on VEGFR1 sequence. **b** Shows multiple alignment of VEGFR1, 2, 3 along with marked amino acid sequence for the biomolecules luteolin, lupeol and lectin. Multiple sequence alignments of vascular endothelial growth factor receptors (VEGFR1, VEGFR2, VEGFR3): amino acid residues with similar nature are highlighted with same colour. Further, active site residues of VEGFR1 identified upon docking with retinoic acid (V841, V892, C1018, C1039), luteolin (E878, V891, V892, C1018, I1019,H1020, R1021, I1038, D1040), Lupeol (L833, V841, A859, K861, E878, V909, Y911, C912,G915, L1029, D1040) and lectin (D807, V841, K861,E878, I881, I885, H887, V891, V892,L1013, R1016, C1018, I1019, H1020, R1021, I1038, C1039, D1040, F1041, G1042, L1043) are marked with *pink*, *dark blue*, *dark green* and *purple colour arrows* respectively

to be a positive regulator of angiogenesis. The fit score for VEGFR is highest for retinoic acid, known to inhibit angiogenesis, which is followed by luteolin and lupeol for its fit score. Therefore, this study demonstrates for the first time that amongst the biomolecules comprising of a large protein lectin and two small molecules, luteolin and lupeol along with the positive control retinoic acid, luteolin is better in inhibiting angiogenesis and docking with VEGF. Therefore, future cancer therapy can target signaling pathway with luteolin as compared with lupeol and lectin.

The anti-cancerous property of luteolin is good in HT-29 cells, but it is compromised in colon tumour cells. It may be due to presence of multi drug resistant (MDR) gene in colon cancer cells [24, 25].

Although, lectin has been reported to be anti-cancerous [26–29], but it is a large molecule compared with luteolin and lupeol. Luteolin and lupeol are small in size and have demonstrated to be better in inhibiting angiogenesis compared with lectin. Moreover, it has been shown by others that luteolin alone, and in combination with gefitinib or epigallocatechin-3-gallate [30, 31] can inhibit another cancer but till date, nobody has shown the comparative effects of luteolin, with lupeol or lectin in colon cancer. Hence this manuscript is showing for the first-time comparative anti-angiogenic effect of luteolin with lupeol and lectin.

Conclusion

Therefore, we can conclude that our study addressed a potentially important comparison criteria amongst the biomolecules lectin, luteolin and lupeol, luteolin has been found to be better in inhibiting angiogenesis and docking with VEGF receptors. The receptors are conserved at the docking sites, indicating that VEGF receptors can be safely targeted for cancer therapy via luteolin. Hence, it can be concluded that in future, luteolin can be preferred over lectin and lupeol for its anti-cancerous and anti-angiogenic property and docking site of these biomolecules are conserved at VEGFR domain.

Abbreviations

HT-29 cell: human Colorectal Adenocarcinoma cell line; PECAM: platelet endothelial cell adhesion molecule; Tie 1: tyrosine kinase with immunoglobulin like and EGF like domain; CAM: chick chorio allantoic membrane; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; bFGF: basal fibroblast growth factor; HGF: hepatocyte growth factor; MMPs: matrix metalloproteinase.

Authors' contributions

RKA designed, received funding as PI and wrote the manuscript. RKA and PK performed the HT-cell culture and cell migration assay. SKJ and NKJ helped in CAM assay. DK performed the software analysis for the target molecule table. RS helped to perform the docking software analysis. RKA and PK analyzed, coordinated and drafted the manuscript. All authors read and approved the final manuscript.

Author details

[1] Department of Biotechnology, Delhi Technological University (Former Delhi College of Engineering), Delhi, India. [2] School of Biosciences and Technology, Vellore Institute of Technology, University (VITU), Vellore, India. [3] Neurology Department, Adjunct Faculty, Tufts University School of Medicine, Boston, MA, USA.

Acknowledgements

The authors want to thank Science and Engineering Research Board (SERB, DST, India) Grant no. SR/FT/LS-62/2010 for funding the research work to Dr. Rashmi K Ambasta (RKA) as Principal Investigator and Young Scientist of the Project. The funders had no role in study design, data collection, analysis and preparation of the manuscript to decision to publish the manuscript. The authors also want to thank the management of Delhi Technological University, Delhi for the support,encouragement and excellent infrastructure provided.

Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

Received: 8 April 2015 Accepted: 8 September 2015
Published online: 18 September 2015

References

1. ParK CM, Song YS. Luteolin and luteolin-7-*O*-glucoside inhibit lipopolysaccharide-induced inflammatory responses through modulation of NF-kB/AP-1/PI3K-Akt signaling cascades in RAW 264.7 cells. Nutr Res Pract. 2013;7(6):423–9.
2. ParK SH, Kim JH, Lee DH, Kang JW, Song HH, Oh SR, Yoon DY. Luteolin 8-C-β-fucopyranoside inhibits invasion and suppresses TPA-induced MMP-9 and IL-8 via ERK/AP-1 and ERK/NF-kB signaling in MCF-7 breast cancer cells. Biochimie. 2013;95(11):2082–90.
3. Ansó E, Zuazo A, Irigoyen M, Urdaci MC, Rouzaut A, Martínez-Irujo JJ. Flavonoids inhibits hypoxia-induced vascular endothelial growth factor expression by a HIF-1 independent mechanism. Biochem Pharmacol. 2010;79(11):1600–9.

4. Bagli E, Stefaniotou M, Morbidelli L, Ziche M, Psillas K, Murphy C, Fotsis T. Luteolin inhibits vascular endothelial growth factor induced angiogenesis: inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3-kinase activity. Cancer Res. 2004;64(21):7936–46.
5. Kim HY, Jung SK, Byun S, Son JE, Oh MH, Lee J, Kang MJ, Heo YS, Lee KW, Lee HJ. Raf and PI3K are the molecular targets for the anti-metastatic effect of luteolin. Phytother Res. 2013;27(10):1481–8.
6. Saleem M, Afaq F, Adhami VM, Mukhtar H. Lupeol modulates NF-kB and PI3K/Akt pathways and inhibitsskin cancer in CD-1 mice. Oncogene. 2004;23(30):5203–14.
7. Kumari A, Kakkar P. Lupeol prevents acetaminophen-induced in vivo hepatotoxicity by altering the Bax/Bcl-2 and oxidative stress-mediated mitochondrial signaling cascade. Life Sci. 2012;90(15–16):561–70.
8. Tarapore RS, Siddiqui IA, Adhami VM, Spiegelman VS, Mukhtar H. The dietary terpenelupeol targets colorectal cancer cells with constitutively active wnt/β-catenin signaling. Mol Nutr Food Res. 2013;57(11):1950–8.
9. Tarapore RS, Siddiqui IA, Saleem M, Adhami VM, Spiegelman VS, Mukhtar H. Specific targeting of wnt/β-catenin signaling in human melanoma cells by a dietarytriterpenelupeol. Carcinogenesis. 2010;31(10):1844–53.
10. Avin BRV, Prabhu T, Ramesh CK, Vigneshwaran V, Riaz M, Jayashree K, Prabhakar BT. New role of lupeol in recticence of angiogenesis, the cellular parameter of neoplastic progression in tumorigenesis models through altered gene expression. BBRC. 2014;448(2):139–44.
11. Patil SA, Bshara W, Morrison C, Chandrasekaran EV, Matta KL, Neelamegham S. Overexpression of α2,3sialyl T-antigen in breast cancer determined by miniaturized glycosyltransferase assays and confirmed using tissue microarray immunohistochemical analysis. Glycoconj J. 2014;31(6–7):509–21.
12. Roy B, Chattopadhyay G, Mishra D, Das T, Chakraborty S, Maiti TK. On chip lectin microarray for glycoprofiling of different gastritis types and gastric cancer. Biomicrofluidics. 2014;8(3):034107.
13. Funasaka T, Raz A, Nangia-Makker P. Galectin-3 in angiogenesis and metastasis. Glycobiology. 2014;24(10):886–91.
14. Croci DO, Cerliani JP, Dalotto-Moreno T, Méndez-Huergo SP, Mascanfroni ID, Dergan-Dylon S, Toscano MA, Caramelo JJ, García-Vallejo JJ, Ouyang J, Mesri EA, Junttila MR, Bais C, Shipp MA, Salatino M, Rabinovich GA. Glycosylation dependent lectin receptor interactions preserve angiogenesis in anti-VEGF refractory tumours. Cell. 2014;156(4):744–58.
15. Piccolo E, Tinari N, Semeraro D, Traini S, Fichera I, Cumashi A, La Sorda R, Spinella F, Bagnato A, Lattanzio R, D'Egidio M, Di Risio A, Stampolidis P, Piantelli M, Natoli C, Ullrich A, Iacobelli S. LGALS3BP, lectingalactoside-binding soluble 3 binding protein, induces vascular endothelial growth factor in humanbreast cancer cells and promotes angiogenesis. J Mol Med (Berl). 2013;91(1):83–94.
16. Tatsuta T, Sugawara S, Takahashi K, Ogawa Y, Hosono M, Nitta K. Leczyme: a new candidate drug for cancer therapy. Biomed Res Int. 2014;2014:421415.
17. Tatsuta T, Hosono M, Takahashi K, Omoto T, Kariya Y, Sugawara S, Hakomori S, Nitta K. Sialic acid binding lectin (leczyme) induces apoptosis to malignant mesothelioma and exerts synergistic antitumour effects with TRAIL. Int J Oncol. 2014;44(2):377–84.
18. Tatsuta T, Hosono M, Sugawara S, Kariya Y, Ogawa Y, Hakomori S, Nitta K. Sialic acid binding lectin (leczyme)induces caspase-dependent apoptosis mediated mitochondrial perturbation in Jurkat cells. Int J Oncol. 2013;43(5):1402–12.
19. Liu Xiaofeng, Ouyang Sisheng, Biao Yu, Huang Kai, Liu Yabo, Gong Jiayu, Zheng Sisuan, Li Zhihua, Li Honglin, Jiang Hualiang. PharmMapper Server: a web server for potential drug target identification via pharmacophore mapping approach. Nucleic Acids Res. 2010;38:W609–14.
20. Kleibeuker EA, Schulkens IA, Castricum KC, Griffioen AW, Thijssen VL. Examination of the role of galectins during in vivo angiogenesis using the chick chorioallantoic membrane assay. Methods Mol Biol. 2015;1207:305–15.
21. Mishra V, Prasad CVSS. Ligand based virtual screening to find novel inhibitors against plant toxin Ricin by using the ZINC database". Bioinformation. 2011;7(2):46–51.
22. Ding H, Li D, Zhang Y, Zhang T, Zhu H, Xu T, Luo Y, Wang C. Luteolin inhibits smooth muscle cell migration and proliferation by attenuating the production of Nox4, p-Akt and VEGF in endothelial cells. Curr Pharm Biotechnol. 2014;14(12):1009–15.
23. Pratheeshkumar P, Son YO, Budhraja A, Wang X, Ding S, Wang L, Hitron A, Lee JC, Kim D, Divya SP, Chen G, Zhang Z, Luo J, Shi X. Luteolin inhibits human prostate tumor growth by suppressing vascular endothelial growth factor receptor 2-mediated angiogenesis. PLoS One. 2012;7(12):e52279.
24. Rao PS, Satelli A, Moridani M, Jenkins M, Rao US. Luteolin induces apoptosis in multidrug resistant cancer cells without affecting the drug transporter function—involvement of cell line specific apoptotic mechanisms. Int J Cancer. 2012;130:2703–14.
25. Chian S, Li YY, Wang XJ, Tang XW. Luteolin sensitizes two oxaliplatin—resistant colorectal cancer cell lines to chemotherapeutic drugs via inhibition of Nrf 2 pathway. Asian Pac J Cancer Prev. 2014;15(6):2911–6.
26. Cai Z, Zeng Y, Xu B, Gao Y, Wang S, Zeng J, Chen L, Huang A, Liu X, Liu J. Galectin-4 serves as a prognostic biomarker for the early recurrence/metastasis of hepatocellular carcinoma. Cancer Sci. 2014;105(11):1510–7.
27. Zhang CZ, Fang EF, Zhang HT, Liu LL, Yun JP. Moordica Charantialectin exhibits antitumour activity towards hepatocellular carcinoma. Invest New Drugs. 2014;33(1):1–11.
28. Hirao Y, Matsuzaki H, Iwaki J, Kuno A, Kaji H, Ohkura T, Togayachi A, Abe M, Nomura M, Noguchi M, Ikehara Y, Narimatsu H. Glycoproteomics approach for identifying glycobiomarker candidate molecules for tissue type classification of non small cell lung carcinoma. J Proteome Res. 2014;13(11):4705–16.
29. Kim YS, Kim SH, Shin J, Harikishore A, Lim JK, Jung Y, Lyu HN, Baek NI, Choi KY, Yoon HS, Kim KT. Luteolin suppresses cancer cell proliferation by targeting vaccinia-related kinase 1. PLoS One. 2014;9(10):e109655.
30. Sakurai MA, Ozaki Y, Okuzaki D, Naito Y, Sasakura T, Okamoto A, Tabara H, Inoue T, Hagiyama M, Ito A, Yabuta N, Nojima H. Gefitinib and luteolin cause growth arrest of human prostate cancer PC-3 cells via inhibition of cyclin G-associated kinase and induction of miR-630. PLoS One. 2014;9(6):e100124.
31. Gray AL, Stephens CA, Bigelow RL, Coleman DT, Cardelli JA. The polyphenols (−)-epigallocatechin-3-gallate and luteolin synergistically inhibit TGF-β-induced myofibroblast phenotypes through RhoA and ERK inhibition. PLoS One. 2014;9(10):e109208.