MSc. Biotechnology REETIKA SINGH 2021

COMPARATIVE ANALYSIS OF DIFFERENT CURCUMIN ANALOGUES TO INHIBIT TLR4 EXPRESSION IN BREAST CANCER- AN IN-SILICO STUDY

A DISSERTATION
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CANDIDATE'S DECLARATION

I hereby certify that the work which is presented in the Major Project-II/Research Work entitled "Comparative Analysis Of Different Curcumin Analogues To Inhibit Tlr4 Expression In Breast Cancer- An In-Silico Study," in fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi is an authentic record of my own, carried out during a period from June 2020-May 2021, under the supervision of Dr. Asmita Das.

The matter presented in this report has not been submitted by me for the award of any other degree of this or any other Institute/University. The work has been communicated in with the following details:

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CERTIFICATE

To the best of my knowledge, the above work has not been submitted in part or in full for any Degree or Diploma to this University or elsewhere. I, further certify that the publication and indexing information given by the student is correct.

Place: Delhi

Date: 19-05-2021

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ABSTRACT

Chronic inflammation is closely related to the emergence of a number of cancers, including Breast cancer. Inflammation causes damage to the cell's DNA which leads to its abnormal growth and formation of tumor mass. One of the most commonly known receptor responsible for inflammatory reactions is Toll-like receptor 4 (TLR4). It is activated majorly by bacterial LPS. Its activation further activates Cyclooxygenase enzyme that catalyzes the conversion of arachidonic acid into prostaglandins that lead to inflammation-like conditions. COX2 has also been correlated to the promotion of tumor growth. It enhances metastasis, neoplasia, lymphangiogenesis, etc., and is also related to poor prognosis in the breast cancer patients. Curcumin derived from turmeric is a proven inhibitor of COX2. In my project I have aimed to analyse and compare the inhibitory properties of other analogues of curcumin that have previously been known to inhibit COX2. The experimental layout began with screening the molecules on the basis of drug-likeness using Lipinski rule of five. The suitable ligand molecules were further subjected to other experiments, i.e., ligand docking and drug potential assessment. After all the experiments, out of the five selected Curcumin analogues, Isoeugenol (extracted from clove) was determined as the best fit molecule. The druglikeliness and drug potential assessment results further validate its use as a potential inhibitor and can further be tested for in-vivo efficacy. This drug can further be used in the 1st line therapy of locally advanced and metastatic breast cancer patients as it will inhibit COX2 that promotes metastasis of cancer cells. Isoeugenol extracted from Eugenia caryophyllus (Cloves) can further be proven as a better COX2 inhibitor than its chemical counterparts, as it is a natural compound and will therefore have significantly less side effects.

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LIST OF SYMBOLS, ABBREVIATIONS

TLR	Toll-like Receptors
TME	Tumor Microenvironment
BC	Breast Cancer
NK Cells	Natural Killer Cells
CAF	Cancer-associated Fibroblasts
TAM	Tumor-associated Macrophages
COX2	Cyclooxygenase 2
EP1	Prostaglandin 1
TNF	Tumor Necrosis Factor
PAMPs	Pathogen-associated Molecular Patterns
DAMPs	Damage-associated Molecular Patterns
VEGF	Vascular Endothelial Growth Factor
SNPs	Small-nuclear Polymorphisms

ABSTRACT

Chronic inflammation is closely related to the emergence of a number of cancers, including Breast cancer. Inflammation causes damage to the cell's DNA which leads to its abnormal growth and formation of tumor mass. One of the most commonly known receptor responsible for inflammatory reactions is Toll-like receptor 4 (TLR4). It is activated majorly by bacterial LPS. Its activation further activates Cyclooxygenase enzyme that catalyzes the conversion of arachidonic acid into prostaglandins that lead to inflammation-like conditions. COX2 has also been correlated to the promotion of tumor growth. It enhances metastasis, neoplasia, lymphangiogenesis, etc., and is also related to poor prognosis in the breast cancer patients. Curcumin derived from turmeric is a proven inhibitor of COX2. In my project I have aimed to analyse and compare the inhibitory properties of other analogues of curcumin that have previously been known to inhibit COX2. The experimental layout began with screening the molecules on the basis of drug-likeness using Lipinski rule of five. The suitable ligand molecules were further subjected to other experiments, i.e., ligand docking and drug potential assessment. After all the experiments, out of the five selected Curcumin analogues, Isoeugenol (extracted from clove) was determined as the best fit molecule. The druglikeliness and drug potential assessment results further validate its use as a potential inhibitor and can further be tested for in-vivo efficacy. This drug can further be used in the 1st line therapy of locally advanced and metastatic breast cancer patients as it will inhibit COX2 that promotes metastasis of cancer cells. Isoeugenol extracted from Eugenia caryophyllus (Cloves) can further be proven as a better COX2 inhibitor than its chemical counterparts, as it is a natural compound and will therefore have significantly less side effects.

CHAPTER 1: INTRODUCTION

In humans, Cyclo-oxygenase (COX)-2 expression in breast cancer stimulates cancer cell migration and invasive property, enhances vascular endothelial growth factor production, and causes lymphangiogenesis in situ. All this is mainly from endogenous PGE2-mediated stimulation activity of prostaglandin E 1 & 4 receptors, presenting these as potential therapeutic targets to control lymphatic metastasis.

It showed other effects like rapid tumor growth, angiogenesis, metastasis to the inguinal and axillary lymph nodes, and the lungs. It has also been observed that chronic oral administration of COX-1/COX-2 inhibitor like indomethacin, COX-2 inhibitor like celecoxib, and an EP4 antagonist like ONO-AE3-208, except an EP1 antagonist ONO-8713 at nontoxic concentrations, markedly reduced tumor growth, angiogenesis, lymphangiogenesis, and metastasis to lymph nodes and lungs. Other residual tumors revealed reduced VEGF-C, D proteins, Akt phosphorylation, and increased apoptotic and proliferative cell ratios consistent with blockade EP4 signaling. This suggested that EP4 antagonists deserve further clinical testing for chemo-intervention of lymphatic metastasis in breast cancer.

COX2 is present as a downstream molecule in the TLR4 signaling pathway. Upon activation with agonists, for example, LPS, the TLR signaling pathway activates the COX2. TLRs are expressed on several cells in the tumor microenvironment, including the tumor cells and immune cells. For this reason, it becomes essential to inhibit the tumor-promoting molecules involved in the pathway.

1.1 LITERATURE REVIEW

Toll-like receptors are an integral component of innate immune system and adaptive immune system. Thirteen different types of TLRs have been recognized so far to have functional roles in the human system. TLRs are characterized for their pattern-recognition ability. TLRs are referred to as ‘sensors’ that recognize PAMPs and elicit an antagonist response against the pathogen by stimulating the release of various chemokines, cytokines, interferons, etc. The role of TLRs is not just limited to normal immune cells but can also be seen in malignant cells. Malignant growth is seen in tumor cells which is uncontrolled and non-directional, and ultimately leads to the formation of a mass of cells. Characteristics of a tumor is defined to a large extent by its surrounding environment which is referred to as a Tumor microenvironment (TME). A TME is basically composed due to the interplay of tumor cells and non-tumor cells (normal cells) surrounding the tumor. It creates a tumor-promoting environment, for instance, creating hypoxic conditions. TME contains all the immune cells like NK cells, macrophages, T-lymphocytes, B-lymphocytes, etc. But the roles immune cells play in TME have been observed to be greatly modulated. Some immune cells are known to be Tumor-suppressing while others are modulate their normal function and prove to be an agonist for tumor growth. In my thesis I have tried to highlight how TLRs and the immunomodulation of the Tumor microenvironment can be effectively used to design immunotherapies for various cancers. It has been devised that activation of TLRs can be used as a means to kill tumor cells. For this TLR agonists have been synthetically designed as anticancer drugs. One such is TLR7 agonist imiquimod, which is used as a drug for superficial basal cell carcinoma. TMEs can be targeted by targeting of the tumor

vasculature (that provides nutrition to growing tumor cells) that involves targeting the pathways that induce “angiogenic switch”[26], for example, VEGF signaling, FGF signaling, PDGF signaling and EGFR signaling. Another method is by targeting Cancer inflammation, affecting communication between cancer cells and TME, targeting the hypoxia in the TME is also considered to be instrumental to control tumor growth. ‘Avastin’ is the first drug approved by FDA that specifically targets TME in cancer.

1.1.1. TOLL LIKE RECEPTORS (TLRs)

Toll-like Receptors

TLRs are the prime member of the Pattern Recognising Receptors (PRRs) family.[1] This class of molecules is sensitized by several ligands. Both exogenous ligands (PAMPs) and endogenous ligands (DAMPs) are the targets for toll-like receptors[2]. The most commonly known TLR is TLR4; TLR4-mediated inflammation is correlated with several cancers and chronic diseases, thus, having a vital role as an amplifier of the inflammatory response towards a potentially harmful substance.[3][1][4]

TLRs belong to the class of Type 1 transmembrane glycoproteins. Their molecular structure has mainly three components, namely, an extracellular domain, a transmembrane helix and, a TIR domain (role in downstream signaling) located intracellularly[5]. Ten different types of TLRs have been identified in humans so far. These receptors can be localized, either intracellularly or extracellularly. Intracellularly placed TLRs like TLR3, 7, 8 and, 9 recognize Danger Associated

Molecular Patterns (DAMPs) that arise endogenously[6]. While the extracellularly localized TLRs like TLR1, 2, 5, 6 and, 10 are specialized to recognize the ligands that occur exogenously and are called Pathogen Associated Molecular Patterns (PAMPs), for example, bacteria, viruses and, other non-self components. TLR4 is peculiar because it is found intracellularly and extracellularly on the cell[6]. Another basis of classification is the homology in the amino-acid sequences at the peptide level of these receptors. Under this, the TLR family comprises two subfamilies, TLR2 subfamily comprising TLR1, 2, 6 and, 10, and TLR9 subfamily with TLR7, 8, and 9, respectively[7].

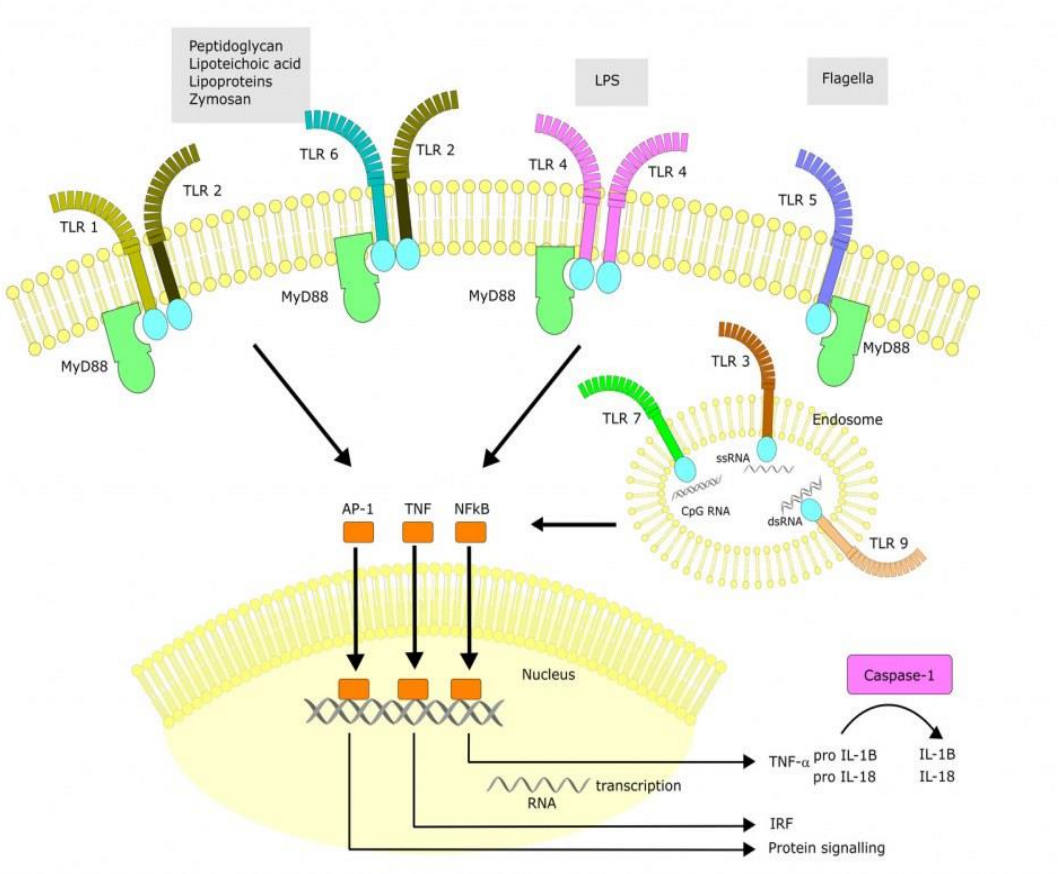

Fig. 1.1. The different Toll-like receptors are localized differently in the cell. They can be present extracellularly, intracellularly, or both.

(Ref: https://www.clinisciences.com/upload/tlrs-functions-tz3xaj.jpg)

Different types of TLR are activated by various ligands that may arise endogenously (DAMPs) or exogenously (PAMPs). Both the variety of ligands are capable of eliciting appropriate innate immune response post-activation of TLRs.[8] The first ligand to be defined was Liposaccharide (LPS),v an exogenous ligand and activates TLR4. A few other PAMPs are characterized, such as lipopeptides for TLR1, viral dsDNA for TLR3, flagellin for TLR5, and viral ssDNA TLR7, etc.[9] On the other side, a few identified DAMPs are HMGB1 and HSPs for TLR2, self dsRNA and mRNA for TLR3, self RNA for TLR7 and TLR8, etc.[9]. After binding to the receptor, endogenous and synthetic ligands induce the release of pro-inflammatory cytokines via a monocyte-macrophage system that acts as driver molecules of innate immunity[10], [11]. The different TLR agonists are important factors inducing macrophages that have tumor-regressing function.[12]

After activation with the defined PAMP or DAMP, the TLRs initiate a downstream signaling cascade to bring about the required immune response. The initial step of the activation process is ligand binding. This step causes the two receptor chains to join to form a dimer. In the intracellular TLRs, the TLRs' receptor chains are already present as inactive dimers activated by reorientating their TIR domain after the ligand attaches to the receptor[13]. The downstream signaling pathway involves five different adaptor proteins containing TIR domains, and these are, MyD88, TIRAP, TRIF, SARM, and TRAM[13], [14]. All TLRs carry out downstream signaling by utilizing the myD88-dependent pathway, except TLR3, which uses the TRIF-dependent pathway[7]. To summarise, the canonical path based on myD88[15] involves sequential activation of different downstream molecules—myD88 recruits IRAKs and TRAF6, which then causes the activation of TAK1. TAK1 induces the

activation via phosphorylation of the IKK complex, which ultimately leads to the final step: the release and translocation of transcription factor NF-κB to the nucleus. All the measures aid the release of pro-inflammatory compounds like TNF-α and IL-6, which trigger the immune response[16]–[18]. Signaling by TLR3 and, in some instances, TLR4 that is via TRIF adaptor molecule results in the secretion of Type 1 Interferons, which mediate the antiviral response by the immune system[19]. Orchestration of TLR activation with other signaling pathways causes the subsequent activation of different pathways like JNKs, p38, MAPK pathway, etc.[20]

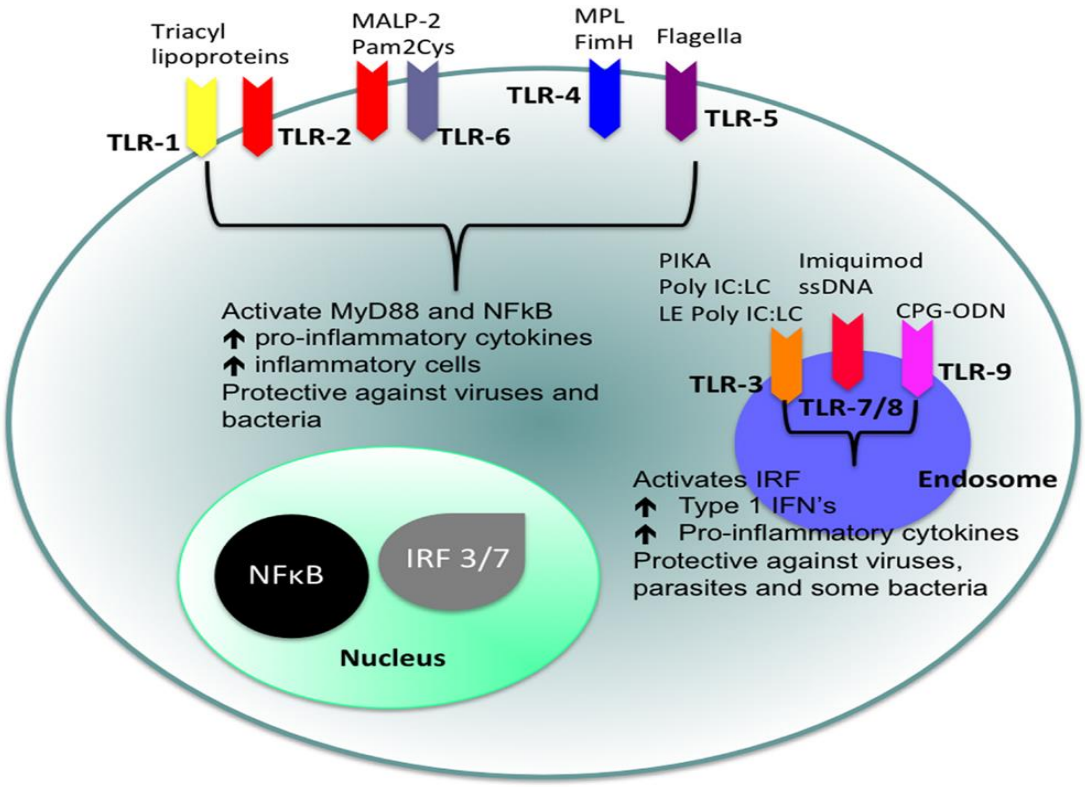

Figure 1.2. The different TLRs are activated by different ligand molecules that are specific to that TLR. The agonists can be either PAMPs or DAMPs.

Reference:https://www.frontiersin.org/files/Articles/76532/fimmu-05-00079-HTML/image_m/fimmu-05-00079-g001.jpg

TLRs play an instrumental role in diverse systems. Once the downstream signaling culminates successfully, it aids in recruiting leukocytes at the site of injury or infection. It also causes the uptake of microorganisms by phagocytic cells like monocytes, NK cells, etc.[21], [22] Ioannou et al. reported TLRs' role in regulating apoptosis by the presence of anti-apoptotic proteins and apoptotic inhibitors. This way, it maintains tissue homeostasis[23].

Due to these properties research on TLRs is now a days considered to be instrumental in disease recognition and also immunotherapy specific for the disease.

Normally 10-15 different types of TLRs have been observed for various species. For humans specifically, 10 functional TLRs have been recorded. The classification of TLRs is done on the basis of factors like their localization in the cell, whether intra- or extra-cellular. Also the signal transduction pathway each type of TLR uses and the structure. The structure of TLR determines the type of ligand that will bind to the TLR.

Apart from the diversity, all TLRs share some common structural features for instance, the N ectodomain, a single transmembrane segment and the C-cytsolic domain. Leucine-rich repeats are commonly found on the ecto-domain that is primarily involved in the ligand-recognition by TLR

Malignant T-cell transformation may also result due to continued stimulation of TLR by ligand. TLR pathway causes the activation of Nuclear factor κB (NFκB) and

JAK-STAT pathway that promote the survival, activation and proliferation of immune cells.

1.1.2. **ROLE OF TLRS IN IMMUNE CELLS**

Activation of TLR2 signalling to make anti-tumor macrophages

Acetylated glucomannan polymer with the degree of acetylation 1.8, abbreviated as acGM-1.8. This polymer was found to be adequate for the stimulation of macrophages, as it has structure similar to that microbial signals. These polymers mimic the mechanism of action, also they exhibit higher speficity and lower toxicity as compared to that of the natural microbial signal.

acGM-1.8 specifically reacts with TLR2 because it triggered the same response as Pam3CSK4 (agonist for TLR2). This was further proven by various microarray and ontology analysis that TLR signalling pathway was involved. This was evidented because many genes that are a part of TLR Signalling that are significantly upregulated.

acGM-1.8 is a safe TLR agonist as compared to other conventional TLR2 or TLR4 agonist. This was confirmed by treating mice with four classical molecules and comparing the results when treated with acGM-1.8. It is evidently observed that acGM-1.8 provided 90% and 100% survival rate at 20 mg/kg and 5 mg/kg concentration, respectively. Thus, highlighting the safety for in-vivo use, over the other conventional agonist.

TLRs further stimulate macrophages to release cytokines. These macrophages possesed anti-tumor properties. [27]

Increased expression of TLR mRNA in septic condition

Sepsis is a life-threatening implication of an infection. The main cause is the disregulation of host's immune response to an infection, chemicals released into the bloodstream to target infection cause inflammation throughout the body, which can lead to Multiple Organ Dysfunction Syndrome (MODS) [28]. It is a major cause of Acute Kidney Infection (AKI) [29]

The concentrations of serum biomarkers, for example creatinine and urea were analyzed and compared between control and septic groups in certain time periods. It is clearly evident that septic groups had high concentrations. To monitor the inflammation, NGAL and IL18 were measured. It also depicted significant increase in CLP (Caecal Ligation and Puncture) group with a peak at 72 hours. TLRs receive and intensify the received signals. Immunoflourescence studies depicted with much high expression of TLR in cells around kidney tubules.

Difference in the expression of different TLRs in various organs was also observed. It was seen that TLR2 and TLR4 in the intestinal tissue. While TLR3 will be higher in intestine than in kidney at 72 hours. The TLR7 expression was found to be significantly higher in intestinal tissue in 24 hour sepsis.

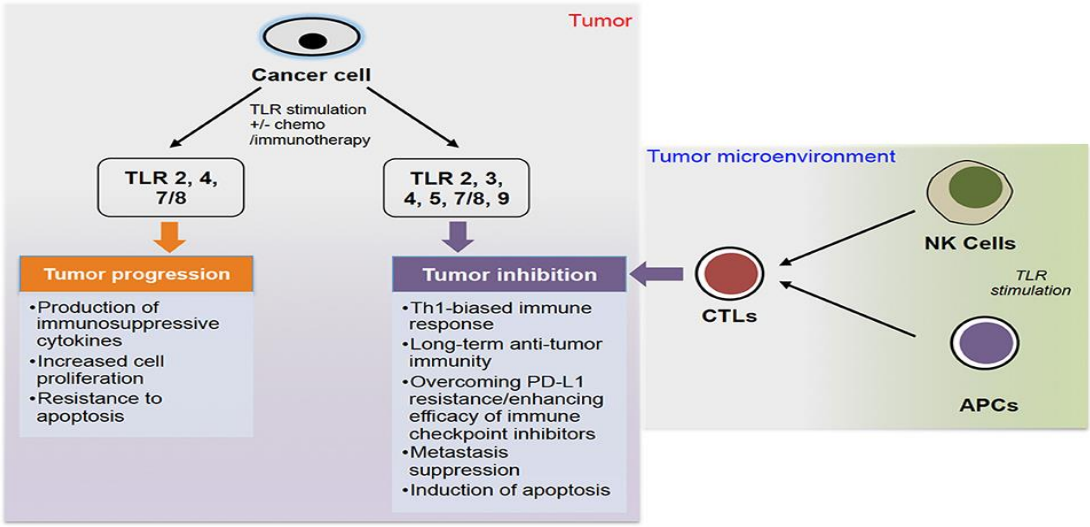

Figure 1.3. TLRs play diverse roles in the cancer immunity. Some TLRs promote tumor growth, while some restrict the tumor growth.

Reference: https://www.frontiersin.org/files/Articles/484631/fimmu-10-02388-HTML/image_m/fimmu-10-02388-g001.jpg

1.1.3. ROLE OF TLRs IN TUMOR CELLS

Role of TLR signalling in CLL

TLR signalling also plays a central role in B-cell malignant transformations that lead to Chronic Lymphocytic Lymphoma (CLL) an intermediate in all biological roles of MYD88. It was found that they exhibited inflammatory phenotype. This was concluded by analyzing the TLR expression profiles in three groups those were MYD88-mutated [also IGHV (Immunoglobulin Heavy Chain Variable region) mutated], MYD88-unmutated and MYD88-unmutated. The results from the three groups were relatable with high expression of TLRs. Apart from this, high

expression levels of gene sets related to cytokines and inflammation-associated Nuclear Factor Kappa B (NFκB) pathway and Signal Transducer and Activator of Transcriptor (STAT) signalling.

It reduced the B-cell activation markers that are normally upregulated by TLR stimulation. ND2158 also disrupts the activity of monocytes that are known for tumor-supporting abilities. This is because activity of monocytes depends upon TLR signalling. Post inhibitor treatment CD54, activation marker of monocytes was downregulated. All these observations highlight the relevance of TLR signalling in pathobiology of Chronic Lymphocytic Leukemia (CLL)[30].

Polymorphisms and haplotypes relation with cancer susceptibility

Single Nucleotide Polymorphisms in TLR genes are lately known for early detection of many carcinomas, including cervical cancer. Cervical cancer is listed below breast cancer for global cancer deaths with respect to gender-specific cases. Human Papilloma Virus (HPV) infection is found to be critical for the development and progress of cervical cancer, because HPV-DNA has been pointed in cervical tumors globally.

Two SNPs each of TLR4 and TLR9 genes were revealed by Linkage Disequilibrium (LD) analysis. In patients with TLR4 haplotype with sequence GCAG lead to decrease in risk while TLR9 haplotype GATC led to increased risk of acquiring HPV16 and HPV18 infection. Whereas TLR4 haplotype GCAG decreased risk of acquiring cervical cancer.[34]

1.1.4. ROLE OF VARIOUS IMMUNE CELLS IN TUMOR NICHE OR TUMOR MICROENVIRONMENT

TUMOR MICROENVIRONMENT

The tumor microenvironment comprises the tumor cells and various other cells like stromal cells and immune cells found in its proximity. Different kinds of inflammatory cells largely infiltrate human tumors.[32], [33]This infiltration is related to the body's responsiveness towards the tumor and is known as Immune Surveillance.[34] It has been found that modeling the tumor microenvironment can cease further tumor progression.

Effector cells of both immune systems are present in the tumor niche. These include, macrophages, polymorphonuclear leukocytes (PMNLs), B cells, T cells, dendritic cells, and rare NK cells.[35] The functin or mechanism of these immune cells' defense action differs significantly in the tumor niche when present in other regions of the body. Many mechanisms can lead to this altered function.

Ineffective immune surveillance has been the major promoting factor for developing immunotherapies that aim to regress tumor growth. Some examples of tumor immunotherapies are anti-tumor vaccines, direct targeting of cytotoxic T cells to the tumor sites, modifications to enhance immune reaction, cytokine delivery, etc. Considering all this, researchers have focussed on potential therapeutic strategies that can modulate the tumor microenvironment.

Cells ruptured by tissue injury or chemo-/radiation therapy release various DAMP proteins associated with diseases and function through diverse signaling pathways. These proteins result in chronic inflammation that induces an immunosuppressive tumor microenvironment, where DAMP proteins activate TLRs on inhibitory immune cells with suppressive characteristics.[36] This is one of how the tumor microenvironment is modulated to curb tumor growth.

Tumor Microenvironment (TME) is composed by the interaction of malignant cells with normal cells (non-malignant cells). Normal cells present in TME have the property of promoting carcinogenesis. Intercellular communication is driven by chemokines and cytokines. Various immune cells are modified, their inflammatory and wound-healing processes are down-regulated by mutation that promote cancer.[35]

COMPOSITION OF TME

The various cells of TME are distinguished by specific markers (Joyce and Polland, 2009). Several variations are observed in morphology and functions of various immune cells when present in the TME. Following is the description of various immune cells and their distinguished function in TME.

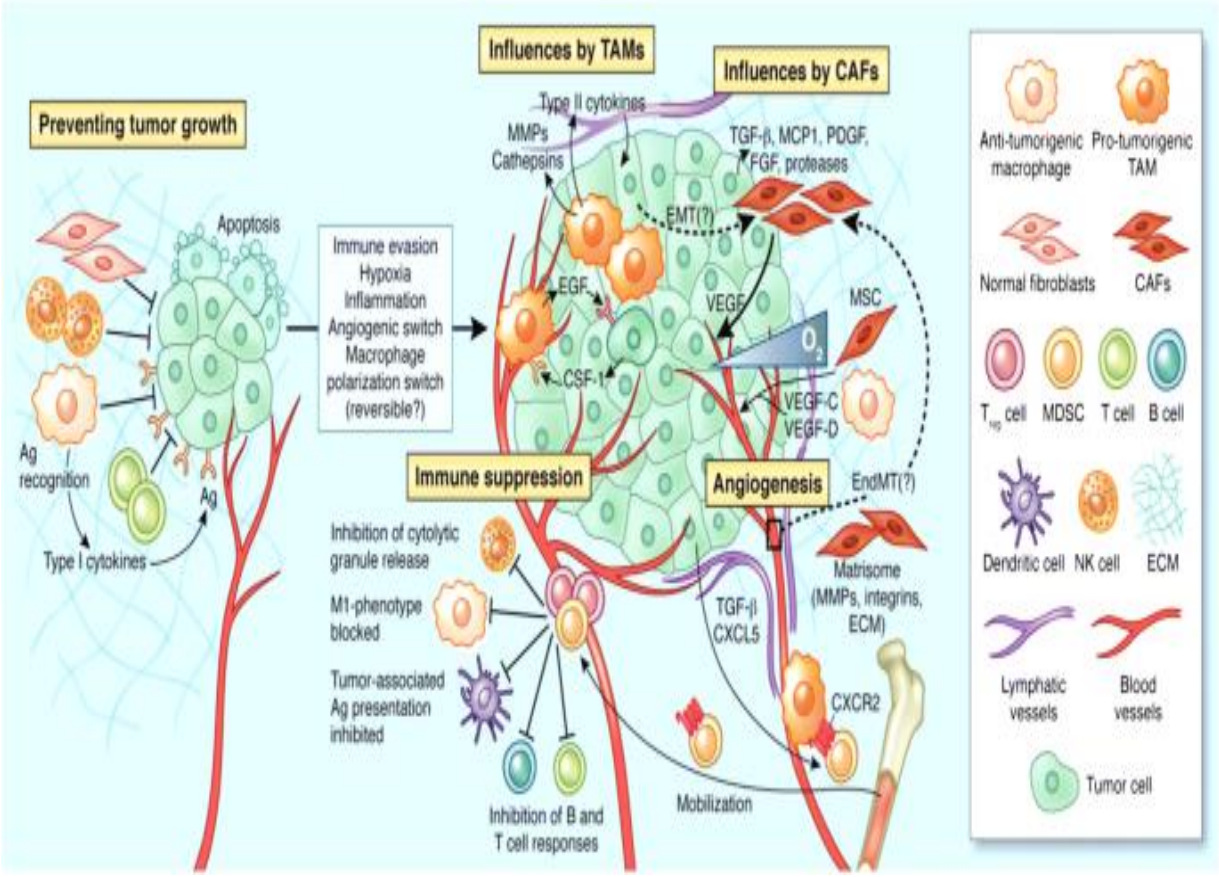

Figure 1.4. The composition of TLR Microenvironment is very dynamic with a variety of immune cells expressing their immune-activity that can either be promotive or regressive.

Reference:https://media.springernature.com

T Lymphocytes

Different types of T-cell populations are found in Tumor regions. For example, CD8+ memory T cells (are linked with good prognosis and are capable of killing tumor cells[36]. CD8+TH1 cells produce Interleukin-2 and Interferon-γ, production of these two compounds is also linked with strong prognosis of tumor state. [36] Other CD4+ cells such as TH2, producing other Interleukins (support B-cell response) or produced by TH2 cells favor tissue inflammation, thus thought to be

tumorigenic [36], along with the negative outcomes their positive result has been recorded for Breast cancer[37] and also in TH17 cells in oesophageal cancers. [38]

T-regulatory (Treg) cells are described as most tumor-promoting type [39]. They cause the suppression of immune function by the production molecules that inhibit recognition and hence killing of tumor cells.[40] Therefore, they result in worse prognosis. Tumor-suppressive role is also known for Treg cells, it is related with good prognosis.[41][42][43]

Γδ T-cells have characteristics of innate immune system rather than usual adaptive immune response and have potent cytotoxic activity against a great range of malignant cells, including cancerous stem cells.[42][44] Some studies reveal the immune surveillance associated with these cells. Thus, the role of these cells in prognosis is not yet certain.

B Lymphocytes

They are commonly known to drain the lymph structures adjacent to TME. This is linked to good prognosis for cancers.[45][46] However, reverse results linked with suppression of cytotoxicity have also been recorded in mouse models.[47] More recent data reveals its tumor- promoting action demonstrated in mouse model for skin cancers.[48][49] Breg cells or B10 cells producing IL10 are known for immunosuppressive action.[50] They are also known to increase metastasis in breast cancer.[51] Antibodies against CD20 are also inhibited by Breg cells.[52] All these results have been obtained in mouse models and are yet to be proven as responses in cases of human carcinomas.

Tumor Associated Macrophages (TAMs)

They are pro-tumorigenic and are most abundant in TME.[53] Most TAMs have high IL10 and low IL12 phenotype with expression of scavenger receptor class A and mannose receptor.[35][54] Abundance of TAMs is linked to poor prognosis.[54] They are known to be highly angiogenic.[55][56]

Interaction of TAM and TME shapes tumor environmental conditions. Tumor areas create conditions suitable for TAM propagation, for instance, creating a hypoxic environment for TAM growth..[57] In humans, hypoxia-induced angiogenesis promoting macrophage phenotype has been identified.[58], [59]

Myeloid-derived Suppressor Cells

Currently known to increase in numbers in human and mouse cancers.[61][62] These cells are known to differentiate to TAMs. MDSCs inhibit activation of CD8+ T cell through expression of Nitric Acid Synthase 2 and Arginase.[61] Treg development promoted by these cells [63].

Dendritic Cells (DCs)

In TME, Dendritic Cells are considered defective, that is they do not stimulate the response against tumor antigens. A new transcription factor ZBTB46 is found in human and mice DCs. This work suggested that unique cell lineage that will help us to understand Dendritic Cells in TME.[65]

Neutrophils

Their action as either tumorigenic or tumor-suppressor is still controversial. Tumor-growth activity has been observed for mouse cancer models.[66] This is done by promoting angiogenesid [67] and suppression of immune system.[68] Antitumor activity of these cells have been observed by immunological and cytokine activation. This is either done directly by eliminating disseminated tumor cells also by inhibition of TGFβ. These are found abundantly in TMEs (Sugimoto et al., 2006). [69], [70]

Cancer-associated Fibroblasts (CAFs)

CAFs secrete various growth factors and Insulin-like growth factor I (IGF1), which are mitogenic. TGFβ induces an immune-suppressive microenvironment by release of Epithelial-Mesenchymal Transition (EMT) contributes to suppression of immune system in TME. CAFs are also known to form dense desmoplastic stroma around the malignant cell target.[71], [72]

Lymphatic Endothelial Cells

High concentration of VEGFC or VEGFD increase the invasion of tumor cells in Lymphatic vessels, which causes extensive sprouting of Lymphatic vessels, enlargement of collecting lymph vessel anf lymphoangiogenesis in lymph nodes. They mechanically modulate the TME and also by modulates host response to tumor.[77]

Role of Extra-cellular Matrix (ECM) in TME

ECM plays a functional role in metastasis, specially as the adhesion of a cell to ECM is responsible for movement into and outside of the TME. It has angiogenic

molecules and chemokines and gives elasticity, tensile and compressive strength. The stiffness of tumors as compared with surrounding normal tissues can be accounted to the CAFs deposition in ECM. They cause the reorientation and cross-linking of collagen and elastin fibres resulting in more rigid fibrils.[71], [78] Malignant cells, TAMs and CAFs release MMPs that cause degradation of extracellular matrix proteins. Another protease activated in TME is large class of cysteine protease called Cathepsins. [79]

Interaction between Tumor cells and their microenvironment

The solid tumors are not homogenous malignant cells, rather they are in a close interplay with the adjoining cells that surround the tumor mass. This interaction creates a microenvironment that is suitable for tumor progression. There has been an ongoing research to understand how the TAC and TC transcriptomes inter-relate and the role of Tumor-adjacent cells (TACs) play in tumor initiation, progression and response to treatment. In this regard, cancer mRNA abundance profiles for TCs and TACs were purified using in silico techniques, this is known as deconvolution algorithms.[80]

There is an increase in the development of biomarkers by using the deconvolution method for TCs and TACs mRNA. This has improved prognostic power of multi-gene biomarkers. Results obtained from TC and TAC profiling were found to heterogeneous population of cells with unique microenvironmental pressures, but the heterogeneity recorded was far less than the one recorded for bulk cell samples. An example of multiclonal tumor is that of a breast cancer. The TC mRNA abundances in this case are found to be the mixture of different sub-clonal groups of cells

differing in their individual prevalences. Similar mixture of clones is obtained after profiling of TAC mRNA abundances, composed of different immune cells and fibroblasts.[81]

Single cell sequencing is currently being proven to be an effective way to learn about the heterogeneity of TCs and TACs and also their interactions, thus predicting cancer aggression.

TLRs are expressed almost on all cells, not only in normal conditions but also tomourous conditions. Thus, the effect of modulating the innate and adaptive immune responses by means of changing the usual roles performed by toll-like receptors, it has been seen that effective anti-cancer drugs can be designed successfully. Growth in tumors is promoted by its surrounding environment that is commonly referred to as the tumor-microenvironment. Inhibiting the tumor-supporting activity of the tumor-microenvironment is ascertained to be useful as an anti-cancer drug. There has been an on-going research to target in the tumor-microenvironment. It can be effectively used to develop and design therapeutic agents with antagonistic effect on tumor-supporting activity of various immune cells. It has been observed that the functional and structural characteristics of immune cells change when they are present in the TME.

1.2. RECENT ADVANCES IN TME MODULATION

1.2.1. TUMOR-PROMOTING MODIFICATIONS IN TUMOR NICHE

Toll-like receptors expressed on the cancer cells are known to aid in tumor progression by various mechanisms. These mechanisms include an easy immune escape of the tumor mass, supporting metastasis and angiogenesis, etc. In recent years, intensive research has helped to understand these mechanisms. Out of various TLRs that tumor cells express, TLR4 is the one that predominantly exerts the tumor-supporting effect.

The set of experiments conducted by Chun et al. demonstrated that by reducing TLR4 and MYD88, mammary tumor growth regressed as it decreased CCL2 expression.[37] The difference in CCL2 expression in DCs and Tumor cells was analyzed when the surface TLRs were treated with LPS. Lack of IRAK-M (a negative regulator of TLR) was proven to be harmful as constitutive TLR expression in some cancer cells produced pro-inflammatory proteins leading to chronic inflammation that correlated to the occurrence of many cancers.[37] Another report demonstrated the tumor-elevating role of autophagic Cancer-associated Fibroblasts (CAFs) that secrete a leaderless protein HMGB1 (High Mobility Group Box-1), activating the TLR4 on the cancer cells by acting as its endogenous ligand. Hence, it plays the role of a pro-inflammatory cytokine.[38] indicates high metastasis and a higher relapse rate to a cancerous state, leading to decreased overall survival.[38] In lung adenocarcinoma cells, H3K9 demethylase (KDM3A) has been proven to affect TLR4 activation and subsequent enhanced immune escape positively.[39] TLR4 activation led to the enhanced secretion of inhibitory CKs like TGF-β, IL-35, and HO-1. These cytokines facilitate the tumor to escape the immune system by decreasing T effector cells and DCs. The expression of Fox p3 found on Treg cells also decreased post activation of TLR4.[39] Shuguang et al. proved S100A8 to have

a promoting role in the metastasis of the Cholangiocarcinoma cells.[40] S100A8 is a calcium-binding protein also referred to as myeloid-related protein 8. It is an endogenous ligand to TLR4 and is responsible for its activation. High expression of this protein is reported in bone-marrow-derived cells like Neutrophils.[41] S100A8 expands the VEGF expression on CCA cells via activation of the TLR4 pathway. VEGF played a role in angiogenesis. Another tumor-supportive role of TLR4 was researched by Rossana et al.; she pointed out that its activation stimulates the release of effective immune-suppressive exosomes. These exosomes allow tumor cells to escape immune surveillance and even supports metastasis.[42]

Thus, high S100A8 and high VEGF expression in CCA were proven to be a direct indication of poor prognosis.[40] Another role of TLR4 was found to be associated with cervical cancer cells.[43] Sexually transmitted HPV causes Cervical cancer. HPV can exist in two forms, HPV16 and HPV18. Only 1% of such infections turn cancerous. Its anatomical position makes it more prone to coming in contact with microbes, which leads to the chronic activation of inflammatory responses.[44] The expression of TLR4 was significantly more in HPV+ cells than the normal cells. The expression heightened in cells infected with HPV16 than HPV18.[43] Its tumor-supporting role depends on its ability to enhance cancer cell proliferation and resistance to apoptosis. The expression further increased and decreased upon treatment with LPS (known ligand for TLR4) and PDTC (inhibitor of TLR4), respectively.[43][45]

Mesenchymal Stem Cells (MSCs) infiltrate prostate cancer, constituting about 1% of the cells found in the tumor niche.[46]The study hypothesized the positive effects of

eliminating the MSC from the tumor niche. MSCs are previously known to have immunosuppressive functions that promote tumor invasion and angiogenesis.[47]These cells derived from prostate cancer were experimentally proven to suppress T-cell proliferation. The effect is reported to be dose-dependent. MSCs express PD-L1 and PD-L2 constitutively, with pro-inflammatory signals like IFNγ and TNFα, upregulation of these ligands was reported.[48]Increased immune checkpoint molecules on CD8+ T-cells that infiltrate the cancer tissue were observed in a study conducted by Xingzhe et al.[49]This expression was higher than on the T cells found in normal tissues. One reason that the study confirmed for this enhanced expression of PD-1 and 2B4 was cholesterol in the tumor. On the other hand, cholesterol decreased CD8+ T cells' motility and the secretion of inflammatory CKs like TNF-α, Granzyme-B, and IFNγ by the CD8+ cells.[49] TIL that takes up cholesterol gets exhausted in the tumor microenvironment, resulting in a dampened immune response. XBP1, an ER-stress-response gene, was upregulated in T cells present in the tumor's proximity. This increase in XBP1 was indicative of the presence of cholesterol.[49]

According to a meta-analysis report, the high TLR4 expression leads to lowered OS and DFS in cancer patients. This analysis further provided evidence that an increased TLR4 is predicts poor prognosis in various cancers.[50] Additional evidence also suggested that increased expression of TLR4 can also be linked with the increased metastasis, thus, promoting tumor progression. Another study aimed to demonstrate the role of TLR4 expression as a prognostic factor in HCC cancer cells. They found that the strong cytoplasmic and nucleic expression of TLR4 results in a reduced disease-specific and overall survival. This increase in TLR expression was attributed

to the conditions like cirrhosis and excessive alcohol consumption.[51] According to a study, Th17 cells are ‘inert’ in the tumor niche with no specific role.[52] Based on previous reports, increased glycolysis is indicative of enhanced tumor growth. Lactate affects the immune cells in the tumor niche locally.[53] Tumor acidification due to lactate led to T cells’ apoptosis and reduced these phagocytic cells’ concentration, enabling tumor evasion.[53]

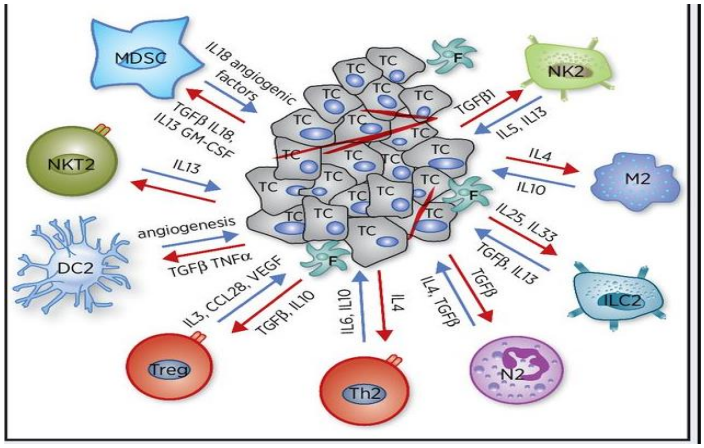

Figure 1.5. There are several immune cells that are altered by various factors that turn the tumor niche into a tumor-promoting environment;

Reference: https://cancerres.aacrjournals.org/content/canres/79/18/4557/F1.large.jpg

1.2.2. TUMOR-REGRESSING MODIFICATIONS IN TUMOR NICHE

Several alterations in the tumor niche that can regress the tumor mass growth are known over the years. Most of these mechanisms are related to increasing the expression of Toll-like receptors found on cancer cells by their respective ligands. Some other studies facilitated the immune attack on these tumor cells by enhancing the killing action of immune cells found in the tumor mass’s vicinity.

Imiquimod (a TLR7 agonist) downregulates Hepato carcinoma stem cells' malignant behavior. This feat is by affecting the IKK-NFκB-IL6 signaling in the nucleus of the cancer cells.[54] Post-treatment cancer stem cells displayed a stronger SNAIL (a marker of epithelial to mesenchymal transition (ENT)) signal than the differentiated tumor cells.[54] Another study negatively related the TLR4 expression to the stemness of cancer stem cells in glioblastoma.[55] TLR activation by DAMPs or PAMPs resulted in an immune attack on the tumor mass and seized proliferation.

This activation also led to the secretion of pro-inflammatory CKs. This inhibitory action on tumor cells leads to the down-regulation of RBBP5 expression. RBBP5 is a transcription factor with high expression in CSCs.[55] This enhanced expression is predictive of a more prolonged overall survival of melanoma patients.[56] Another study conducted by Aurobind et al. presented the reversion of M2 to M1 utilizing IFN-αβ signaling, thus preventing further tumor progression. He demonstrated the conversion of pro-tumorigenic M2a and M2c type macrophages back to the anti-tumorigenic M1 form by TLR3 activation. This activation was carried out by its ligand poly (I: C) in a dose-dependent manner.[57] This is proven by increased expression of markers associated with M1 type macrophages (CD40, CD80, CD86, and MHC2) and subsequent decrease in M2 markers (CD206, CD163, and TIM3). TLR3 stimulation of M2a and M2c macrophages increased these cells' capacity to proliferate CD4+ T cells and enhanced the antigen presentation.[57] Treg cells are present in abundance in pancreatic cancer and pancreatic cancer-associated lesions (PanIN).

The depletion of Treg cells from the tumor microenvironment of these cancers plays many tumor-supporting roles. Some of these are the increase myeloid cells that suppress immune system's function, increased expression of immune suppressive factors like Arg1 and chi3l3, and various immune checkpoint molecules PL-L1, etc.[58] The study also demonstrated an idea that contradicted the previous findings that stated that Treg cells' depletion also resulted in the tumor progression driven by CD4+ T cells. Along with the pro-tumorigenic effects, the study also highlighted the potential anti-tumorigenic impact of the inhibition of CCR1. CCR1 is a common receptor for various chemokines.[58]

Experiments on Colorectal Cancer models correlated immunoscore with tumor prognosis and analyzed the patient's response to immunotherapies involving anti-PD1 and PD-L1.[59] Results suggested that higher immunoscore was predictive of better survival because of lower invasion rates by lymph vessels into the tumor tissue and decreased metastasis. A better survival rate is due to a higher prevalence of CD3+ and CD8+ TILs in the tumor environment.[59]

SEP (a polysaccharide isolated from Strongylocentrotus nudus egg) was presented as a potential immunomodulatory molecule in the tumor microenvironment by Xin et al. The study suggested that SEP can be effectively used to enhance the cytotoxicity of NK cells present in the tumor microenvironment of pancreatic ductal carcinoma.[60] This increase was produced in several ways, like the upregulation of NKG2D/MICA expression on the cells. This increased expression was due to the high anti-tumor activity of NK cells by Acebes et al. in 2016.[61] SEP increased the

surface TLR2 and TLR4 expression via an increase in phosphorylation of molecules involved in the signaling pathway.[60]

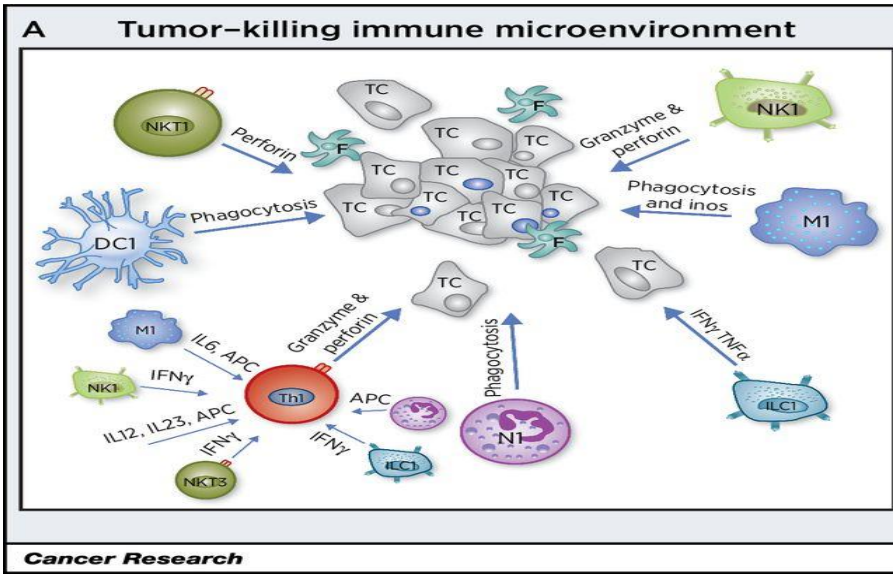

Figure 1.7. Several modifications make the tumor niche very aggressive that leads to killing or abrogation of the spread of tumor cells. These modications create a Tumor-regressing tumor microenvironment;

Reference: https://cancerres.aacrjournals.org/content/canres/79/18/4557/F1.large.jpg

1.2.3. RECENT THERAPEUTIC APPROACHES

After studying the tumor-regressing and tumor-promoting modulations in the tumor microenvironment, specific therapeutic approaches that are currently employed are pointed. Some of these include the activation of specific Toll-like Receptors by their respective agonists. Other strategies include specific molecules that upregulate or downregulate different tumor niche components or the tumor cells' receptors.

Still, several researchers have found out compounds like NF-kB/p65-activating factors and are known to enhance progranulin synthesis, thus, promoting STAT3 activation.[64] IRAK-M, a negative regulator of TLR4 and MYD88, was assumed by Chun et al. to regress Breast Cancer Cells' growth.[37] Another study presented Imiquimod (a TLR7 agonist) to downregulate HCC stem cells' malignant behavior by affecting the IKK-NFκB-IL6 signaling in the nucleus of the cancer cell.[54] Activation of TLR4 leads to reduced tumor progression in glioblastoma. This effect mediates an enhanced immune attack on cancer cells.[55] Another positive impact of TLR4 on tumor regression in the case of lung cancer is also known. Its activation using Polygonatum sibricum polysaccharides causes the increased production of pro-inflammatory cytokines, leading to an immune attack on tumor cells.[65] Activation of TLR3 by poly (I:C) restricts tumor growth in a dose-dependent manner. There is a reversion of pro-tumorigenic M2a and M2c type of macrophages to anti-tumorigenic M1 form by IFN-αβ signaling and prevents further tumor progression.[57]

The potential effect of degrading Mesenchymal Stem Cells that infiltrate the prostate tumor suppressed the tumor's growth. This degradation increases immune targeting by various means like cytotoxic T cell activity and immune checkpoint inhibitor expression, which results in the inhibition of tumor growth.[48] Inhibition of CCR1, which is the common receptor for various chemokines, produces an anti-tumorigenic effect in the case of Pancreatic Cancers.[58] Another research report suggested using flavonoids as an anti-proliferative agent for thyroid cancer, thus, recommending it as a remedial agent in its management.[66] SEP, a polysaccharide isolated from Strongylocentrotus nudus egg, has been presented as a potential immunomodulatory molecule in the tumor microenvironment of pancreatic ductal carcinoma[60]. The

experiments suggested that SEP is effectively used to enhance NK cells' cytotoxicity. There is a significant increase in mRNA level expression for various cytokines like TNF-α, IL-2, and IFN-γ.[60] Other sets of experiments demonstrated that Immune Checkpoint Therapy (ICT) could successfully enhance the infiltration of both bone and subcutaneous castrate-resistant prostate cancer (CRPC) tumor T cells. No significant effect in abrogating the growth of tumors related to the bone is known.[52] The reason attributed to this resistance to ICT was T cells' polarization into Th17 and Treg cells instead of Th1 cells. This immune inhibitory polarization is due to the high level of TGFβ (caused by excessive bone remodeling and mineralization) in the CRPC with bone metastases.[52] This decrease is circumvented in the study by performing a combination therapy employing anti-CTLA4 and anti-TGFβ; the result obtained had restored the concentration of Th1 cells and cytotoxic T cells in the tumor niche. It also demonstrated the organ specificity linked to the polarization of T cells and subsequently the effectiveness of ICT on the cancer of that organ.[52]

Oxaliplatin is an essential constituent of chemotherapy against Colorectal cancer. Its treatment leads to reduced tumor volume. There is an increased effect by the co-administration of Resiquimod (R848), a TLR 7/8 agonist. R848 is known to reverse the resistance to Oxaliplatin.[67] It has been known that increased glycolysis is indicative of enhanced tumor growth. Almut et al. revealed that T and NK cells turn ineffective on tumor cells infiltrated with Myeloid cells. This infiltration occurs due to high lactate content.[53] Lactate affects the immune cells in the tumor niche. With inhibition of lactate dehydrogenase (LDHA) enzyme, the IFN gamma concentration and granzyme-producing CD8+ T cells and NK cells increased in such tumor masses,

thus leading to an immune attack on tumor cells.[53] Over the years, several evidences have indicated the tumor-suppressive role for SHP-1 and SHP-2.68]–[70][71]

Nanotechnology is gaining acceptance for various roles like the more targeted delivery of the concerned drug. One such example is Single-Walled Carbon Nanotubes (SWCNTs). These SWCNTs further result in signal transduction using MyD88. In an experiment, NF-kb signalling leads to the secretion of inflammatory chemokines at the tumor site. TLR2 and TLR4 activation was demonstrated experimentally by Qin Zeng1 et. al.[12]

To conclude, the effectiveness of modulating the tumor microenvironment is intensively researched. The different types of cells constituting the tumor microenvironment are correlated to a specific role in either progressing or regressing tumor growth. The toll-like receptors that are found on the surface of immune cells and tumor cells are regulated to get the desired results. The effect of TLRs in the tumor niche is highly variable. Post-activation, TLRs may aid the growth of a tumor or even abolish it. The receptors found on innate cells are activated by their agonists synthesized artificially. Activation of TLRs further leads to the coordinated immune attack that kills the tumor cells.

Other compounds like cholesterol and lactic acid lead to an enhanced tumor progression. TLR4 mainly predominantly affects tumor responses. It enhances tumor progression by the release of pro-inflammatory cytokines and other growth-promoting factors. Mesenchymal Stem Cells (MSCs) that infiltrate the tumor mass

increase the tumor size. The release of DAMPs like HMGB1 is by the infiltration by MSCs. In the future, the researchers can further target the suppression of various tumor-promoting TLRs. Other potential therapies are based on identifying and eliminating compounds or metabolites that aid in angiogenesis and metastasis.

Certain TLRs like TLR7, TR3, etc., have been observed to cause tumor growth abrogation. Subsequent activation of these TLRs is considered therapy. Specific molecules enhance the anti-tumor activity of different immune cells found in the tumor microenvironment. One such molecule is SEP that further enhanced the action of NK cells.

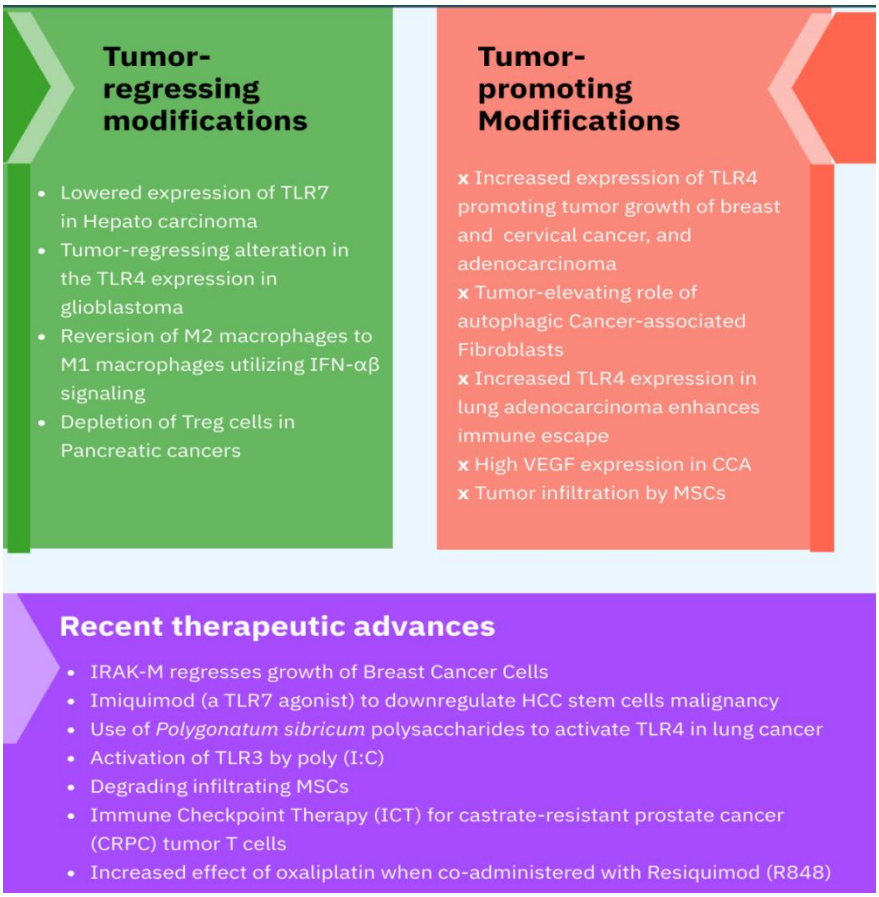

Figure 1.5: The image provides an overview of the several modifications that are present or can be made in the tumor microenvironment in order to stop tumor growth.

Reference: Singh & Das, Manuscript Communicated

CHAPTER 2: EXPERMENTATION AND RESULTS

EXPERIMENTATION LAYOUT

A total of five curcumin analogues were selected from literature. After selection, they were analyzed for drug-likeness property, subjected to molecular ligand docking, and also several in silico methods for drug potential assessment. These experiments were conducted to identify the best inhibitor for the selected target molecule, that is the cyclooxygenase 2 (COX2), that is expressed downstream of the TLR4 signalling pathway. The tumor of focus for the conducted experiment is the breast cancer. The rationale for the tumor of choice and the receptor of choice is entirely based on the correlation found while conducting the literature survey.

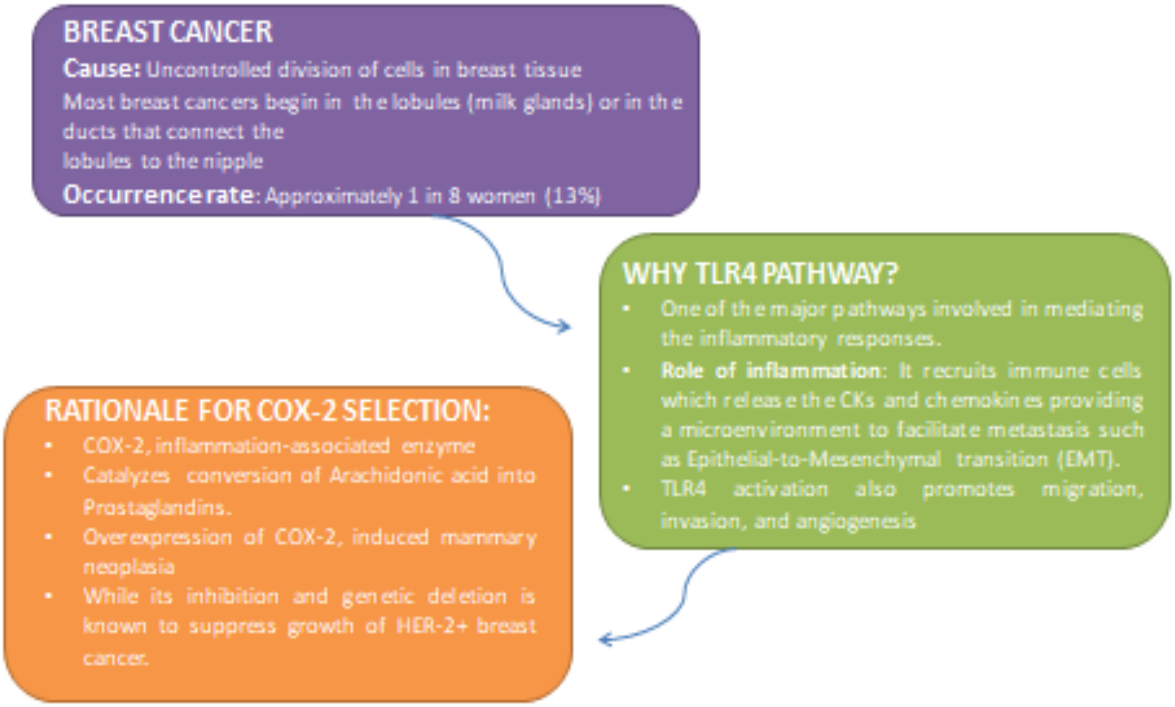

Figure 2.1. The key components of the conducted experiment is the tumor of choice, i.e. Breast cancer, TLR4 molecule, and COX2 that is specifically targeted in the experiment.

Reference: Singh and Das, manuscript under-preparation

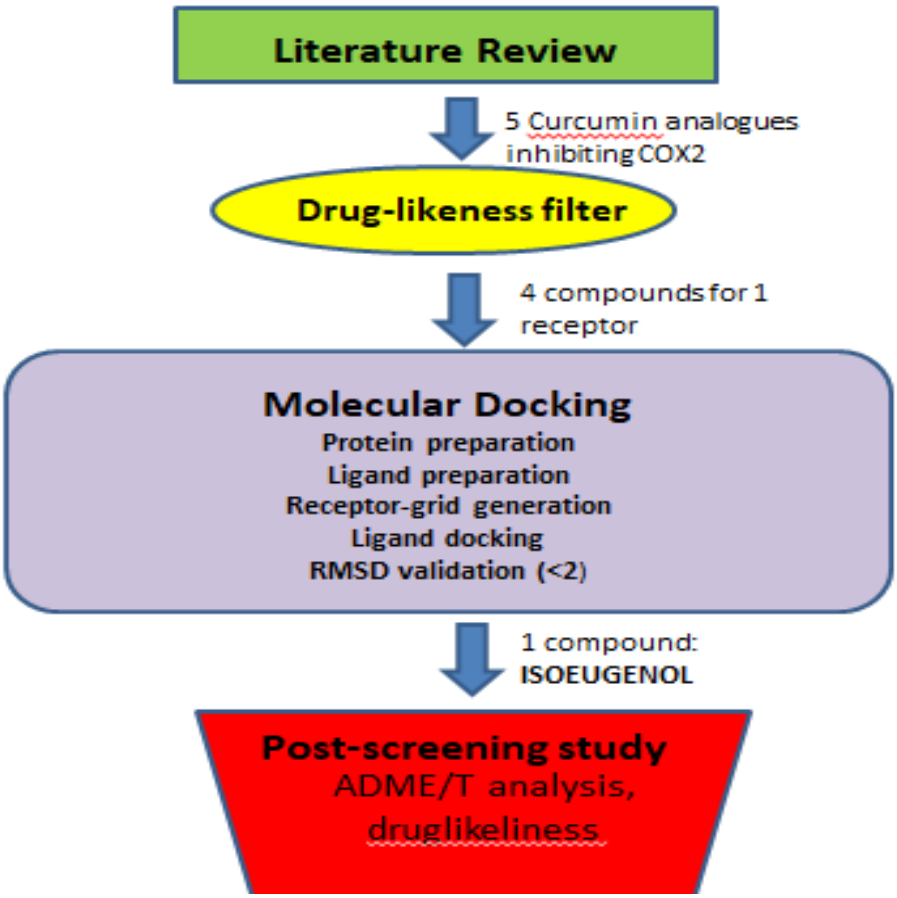

Figure 2.1. The experiment is mainly divided into three parts, i.e., Drug-likeness filter, ligand-docking, and post-screening study.

Reference: Singh and Das, Manuscript under-preparation

2.1. ANAKYSIS OF DRUG-LIKENESS PROPERTY

Choosen ligands were then analyzed if they are in accordance with Lipinski's rule of five. The violation of this rule states that a drug has poor bioavailability and low permeation. To conduct this experiment canonical smiles of each ligand is obtained from PubChem database and then analyzed using Molinspiration Cheminformatics

server for different parameters that determine drug-likeness property. Cassumunin A violated the rule, this states that this ligand molecule will have unlikely bioavailability and lesser permeation if developed as a drug. So, this molecule was not subjected to further experimental steps.

Sl. No.	Compound Name	Source	MW (g/mol)	HBA	HBD	miLogP	Nrotb	TPSA (Å2)	Lipinski Violation
Rule			<500	<10	<5	< or = 5	< or = 10		
1	Cassumunin A	Ginger (*Zingiber cassumunar*)	558.63	8	2	4.96	13	111.53	1
2	6-Gingerol	Ginger (*Zingiber officinale Roscoe*)	294.39	4	2	3.22	10	66.76	0
3	Isoeugenol	Cloves (*Eugenia caryophyllus*)	164.2	2	1	2.38	2	29.46	0
4	Dibenzoylmethane	Licorice (*Glycyrrhiza echinata*)	224.26	2	0	2.88	4	34.14	0
5	Yakuchinone A	Galanga (*Alpinia officinarum*)	312.41	3	1	4.24	9	46.53	0

Table 2.1. Results from drug-likeness analysis step revealed that Cassuminin A violates the Lipinski's rule of five and thus, cannot be carried forward for further steps.

Reference: Singh and Das, Manuscript under preparation

2.2. LIGAND DOCKING EXPERIMENT

2.2.1. Preparing the protein molecule

3D crystal structure of Human COX2 (PDB ID: 5F1A), was downloaded in the PDB format from Protein Data Bank. The structure was then prepared and subjected to further processing using the Protein Preparation Wizard of Maestro Schrodinger Suite 12.8 version. The different modicfications made to the protein molecule (COX2) are, assignment of bond orders to the structure, hydrogen to heavy atoms, water molecules removed from the atoms, addition of missing side chains to the protein backbone via Prime, and generation of het states with Epik at the pH range of 7 ± 2. In the last preparation step, the structure was refined and minimized using Optimized Potentials for Liquid Simulations (OPLS_2005) as a force field. The minimization step was performed by setting the greatest substantial particle RMSD to 30 Å and water molecules that were under 3H-bonds to non-water components were eradicated

.

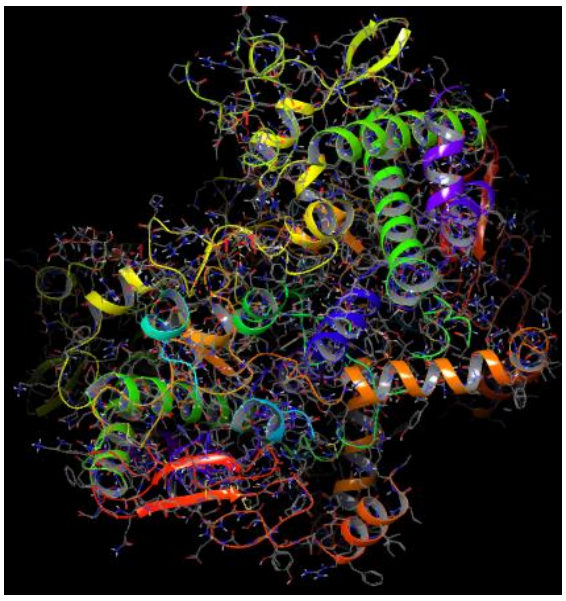

Figure 2.1 : The image of the processed COX2 molecule after all the said modifications steps were carried out using protein preparation wizard.

2.2.2. Preparation of ligand molecules

A total of four ligand structures except the one that violated Lipinski's rule of five were downloaded from PubChem database as a SDF file.

Sl. No. Rule	Compound Name	Source
1	Cassumunin A	Ginger (*Zingiber cassumunar*)
2	6-Gingerol	Ginger (*Zingiber officinale Roscoe*)
3	Isoeugenol	Cloves (*Eugenia caryophyllus*)
4	Dibenzoylmethane	Licorice (*Glycyrrhiza echinata*)
5	Yakuchinone A	Galanga (*Alpinia officinarum*)

Table 2.2. The following ligands were modified using the LigPrep wizard of Maestro.

Reference: Singh & Das, manuscript under preparation

These all structures were then subjected to processing steps using the LigPrep tool. Their minimized structures were processed using Epik2.2 within pH 7.0 ± 2.0. Minimization carried out at OPLS_2005 force field generating 32 possible stereoisomers. All the ligand molecules to the above mentioned steps. All these steps were conducted to make the ligand molecule suitable for fitting into the receptor molecule that is COX2. These modifications were decided on the basis of literature review and also the characteristics of the pre-existing ligand in the COX2 molecule, that was removed before proceeding to the ligand docking step.

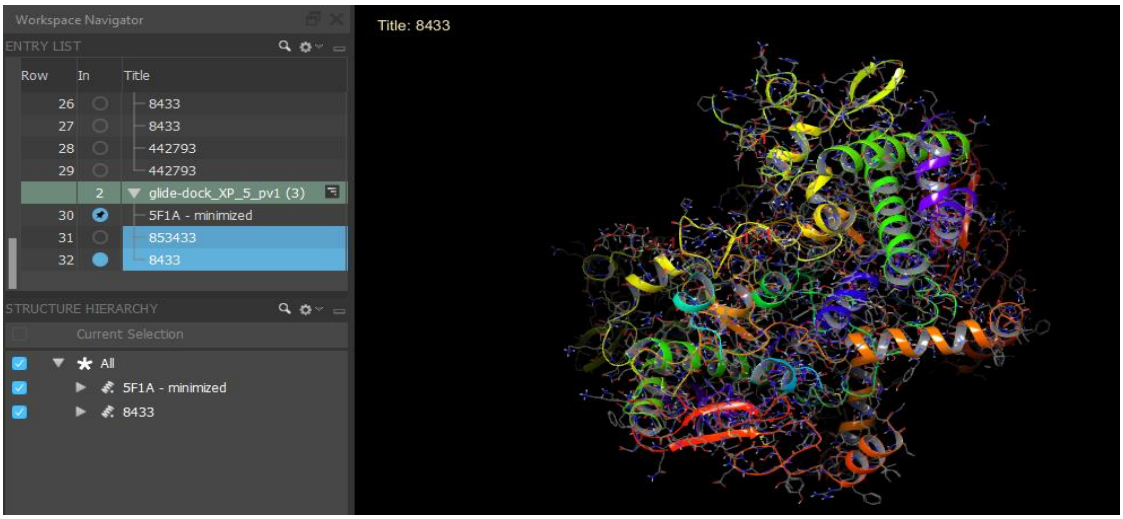

Figure 2.2. Isoeugenol molecule was modified according to the mentioned steps in order to make it a suitable ligand to be docked with the receptor.

Reference: Singh & Das, manuscript under preparation

2.2.3. Generation Of The Receptor Grid

The purpose of this grid is usually to confine the active sites that are specific regions of the target protein for ligand molecule for it to dock specifically in that space only. Receptor grid was then generated utilizing the default Van der Waals (VdW) radius scaling factor of 1.0 and charge cutoff of 0.25 which was then subjected to OPLS_2005 force field for minimized structure in the Glide docking feature.

2.2.4. Ligand Docking Using Glide

XP ligand docking is known to work more accurately where there are less number of ligand molecules than the standard precision ligand docking that is usually recommended for very large compound libraries. Both of these docking methods were then applied for the ligand molecules and intended target molecule to make the comparison among the different docking parameters. Van der Waals radius scaling factor and the charge cutoff were kept as the default, 0.80 and 0.15, respectively, for

the ligand molecules under experimentation. Best poses and type of ligand-receptor interactions were analyzed minutely using Discovery Studio Visualizer version 4.5.

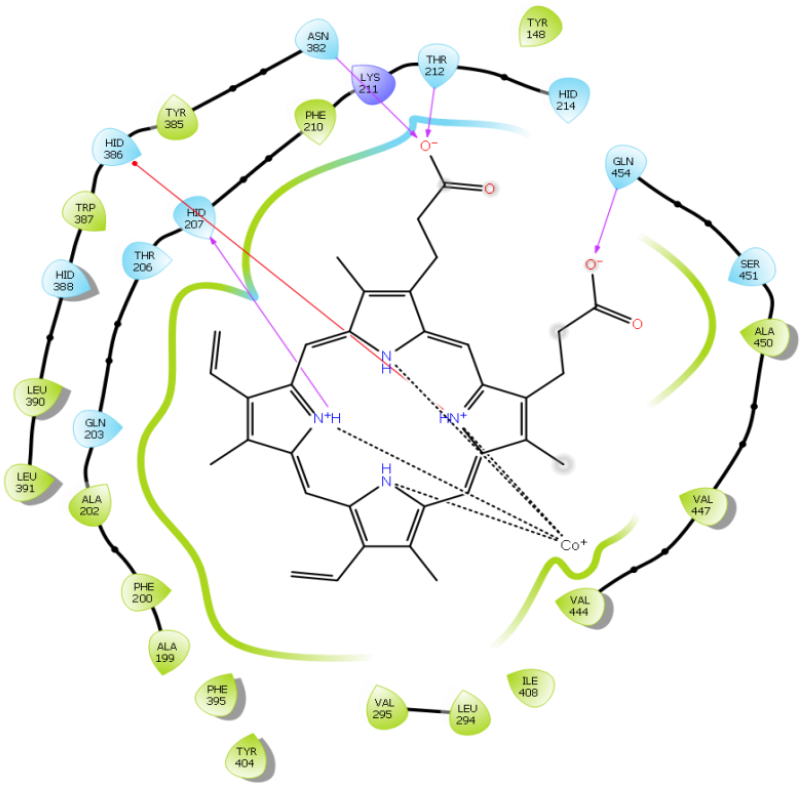

Figure 2.3: The Ligand (Isoeugenol) interacted with several amino acids of the Receptor (COX2) via hydrogen and hydrophobic bonds, when in the binding pocket. Reference: Singh & Das, Manuscript under preparation

Out of all the four ligand molecules that underwent ligand docking using glide, Isoeugenol was the only molecule that was found to be suitable. The suitability was decided on a number of factors. Isoeugenol was the only ligand molecule with a RMSD less than 2, minimal values of Ionization and state penalties, and a negative docking score. Hence, this ligand molecule came out to the best fit molecule.

Receptor Name	Compund Name	Source	Ionization Penalty	State Penalty	Docking score	Glide gscore	Glide emodel
COX2 (*Cyclooxygenase 2*)	**Isoeugenol**	*Eugenia caryophillus*	0.001	0.0006	-5.674	-5.675	-24.19

Table 2.2. Isoeuenol is the best found ligand molecule with suitable characteristics.

Reference: Singh & Das, manuscript under preparation.

Characteristics of the best fit molecule	
PubChem CID	853433
Molecular formula	$C_{10}H_{12}O_2$
IUPAC name	2-methoxy-4-[(E)-prop-1-enyl]phenol
SMILE	CC=CC1=CC(=C(C=C1)O)OC
Molecular Weight	164.2
Physical properties	Appearance: pale yellow oily liquid Odour: Spice-clove odor Boiling point: 511 °F at 760 mm Hg Freezing point: 14°F Density 1.08 g / cm3 Solubility: Slightly soluble

Table 2.3. Isoeugenol has almost all the characteristics that increase the drug likeability, thus, making it the best fit molecule.

Reference: Singh & Das, manuscript under preparation.

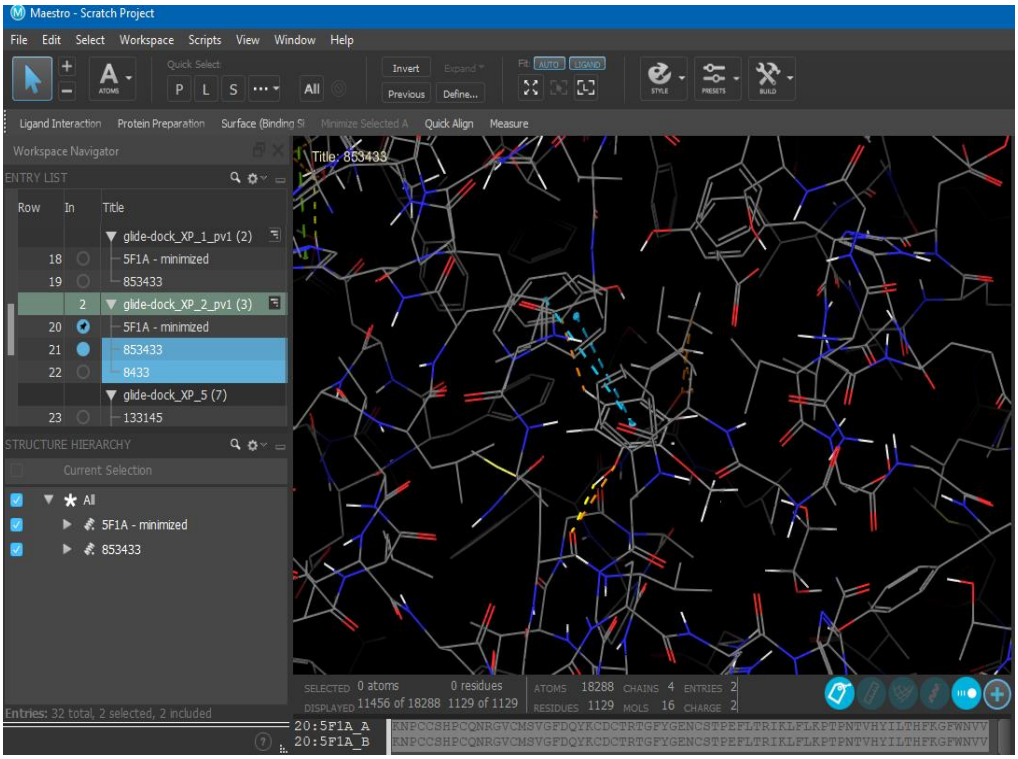

Figure 2.4. Glide docking results for docking between Isoeugenol and COX2 revealed the position of the ligand in the binding pocket of the receptor molecule.

Reference: Singh & Das, Manuscript under preparation

2.3. DRUG-POTENTIAL ASSESSMENT

In silico assessment of ADME/T characteristics for the candidate ligand molecule is helpful to enhance the chances of its success in the drug discovery steps. For this, canonical smiles of the ligand was used to predict its drug-like potential and also approximate pharmacokinetic (PK) and pharmacodynamic (PD) parameters. The ADME/T profile and also other physical attributes of the ligand molecule were predicted using maestro version 12.8 and also SwissADME database that is available online.

Compound: **ISOEUGENOL**	
Property	Value
Pharmacokinetics	
GI Absorption	High
Log Kp (skin permeation)	-5.14 cm/s
Human oral absorption	3
% human oral absorption	100
Druglikeness	
Lipinski	Yes; 0 violation
Ghose	Yes
Veber	Yes
Egan	Yes
Muegge	No; 1 violation: MW<200
Bioavailability Score	0.55

Table 2.4. ADME/T, PK, and PD results of the Isoeugenol molecule further validate its drug potential and increased chances of its success as a drug candidate.

Reference: Singh & Das, Manuscript under preparation

CHAPTER 3: DISCUSSION AND CONCLUSION

In silico assessmemt of the drug-likeliness helps to find the compounds that have poor drug success chances. These are the ones with poor PK, and PD properties. The violation of Lipinski's rule of five by a prospected drug molecule is indicative of the fact that the compound is more likely to fail if taken to the final stages of a actual drug discovery. For the current experiment, the ligand molecules were analyzed using the Lipinski's rule of five. Cassumunin A was found to violate the rule and was not considered in other experimental steps. The four ligand molecules were analyzed using the molecular ligand docking experiment.

Molecular docking is one of the commonly used methods for computer-aided drug designing (CADD). This method uses specific algorithm that assigns a binding energy to the docked complex after ligand docking which in turn reflects upon the binding affinity of a ligand to its molecular target. The lowest or the most negative binding energy of ligand-receptor complex confirms the higher affinity, that is, they remain in contact for more time. In the experiment, Glide ligand docking was carried out using Maestro v12.8 for making the comparison between various docking parameters of different ligand molecules with COX2. However, the best ligand for one receptor was selected based on the lowest docking scores. Isoeugenol showed the best binding free energy than the rest ligand molecules. Upon continuous exploration using the different methods of molecular docking, Isoeugenol was confirmed as the best inhibitor of COX-2. Hydrogen bonding and the hydrophobic interactions play significant role to strengthen the ligand-receptor interaction. The selected ligand formed multiple hydrogen bonds and other hydrophobic interactions in the binding

cleft of respective receptor molecule. It formed hydrophobic and several hydrogen interactions with many amino acids of both the active sites of COX-2. Thus, the selected best compound is expected to interfere with the normal functioning of the target protein COX2. Insilico analysis of ADME/T (absorption, distribution, metabolism, excretion, and toxicity) is crucial to determine if a drug is likely to survive in the later stages of drug development and this data helps to reduce the time and total cost of drug discovery by assisting in vitro assays. PerOral is the most commonly used route of administration. The delivered drug then migrates via the digestive tract to the intestine and so the drug undergoing investigation is expected to be the one that is highly absorbed in the human intestine. P-glycoproteins are cell membrane glycoproteins responsible to facilitate transport of drugs through the cell membrane and so their inhibition by candidate drug might affect the normal drug transport in the human body.

As per all the conducted experiments, Isoeugenol has been characterized as the most suited ligand molecule to target COX2 enzyme in the TLR4 signalling pathway. The druglikeliness and drug potential assessment results further validate its use as a potential inhibitor and can further be tested for in-vivo efficacy. Isoeugenol extracted from Eugenia caryophyllus (Cloves) can further be proven as a better COX2 inhibitor than its chemical counterparts, as it is a natural compound and will therefore have significantly less side effects. This drug can further be used in the 1st line therapy of locally advanced and metastatic breast cancer patients as it will inhibit COX2 that promotes metastasis of cancer cells. Thus, abrogating the growth of tumor mass with minimal toxicity.

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