CHAPTER 4  Molecular Docking studies of new 4\{[(Aryl) methylene] amino\} - 2,5,6-Substituted-thieno[2,3-\textit{d}]pyrimidine derivatives

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4.1 Introduction to COX inhibitors

Cyclooxygenase also known as prostaglandin H synthase, is a key enzyme involved in the biosynthetic cascade of prostanoids that result in the formation of prostaglandins, prostacyclins and thromboxanes from poly unsaturated fatty acid. Cox catalyzes the conversion of arachidonic acid into an intermediate PGH₂, a precursor of variety of biologically important prostanoids. Two isoforms of membrane bound cyclooxygenase are identified as COX-1 and COX-2 with distinct catalytic properties. COX-1 plays a major role in the maintenance of cellular and vascular homeostasis, where as COX-2 is important in the biosynthesis of prostanoids which induce and regulate inflammation.

Cyclooxygenase inhibitors are well established as non steroidal anti-inflammatory drugs that are therapeutically useful in the treatment and management of inflammation and pain. This class of drugs has been widely used in the treatment of variety of inflammatory conditions such as osteoarthritis, rheumatoid arthritis, post operative pain and orthopedic injuries. However the conventional use of NSAIDs has been restricted due to their severe gastrointestinal toxicities.

Research has been focused to find novel anti-inflammatory agents with less unwanted effects. The discovery of distinct isoforms of cyclooxygenase (COX-1 and COX-2) led the scientist to understand about the mechanism behind NSAID’s toxicity and to design novel anti-inflammatory drugs as selective COX-2 inhibitors. Today there are several NSAIDs are in the market as selective COX-2 inhibitors called coxib’s with less gastric irritation.
4.1.1 Cyclooxygenase-2 inhibitors

The development of emerging technologies in the field of biological screening, chemical analysis geared up the research to find new potent COX-2 inhibitors. Recent studies have revealed the involvement of COX-2 in a variety of disease conditions such as oncogenesis, Alzheimer's, and Parkinsonism.
The crystal structures of COX-1 and COX-2 complexed with inhibitors such as flurbiprofen and sc558 has been determined, which led to study in detail about the binding interactions of substrate and inhibitor molecules in the active site.

The combination of computer aided drug design and x-ray crystallography has become an important tool in the drug design and development, and has been successfully employed in the design of selective COX-2 inhibitors. Several crystal structures of COX-2 complexed with a variety of ligands has been determined, among the reported inhibitors SC558 is the highly selective COX-2 inhibitor, similarly COX-1 co-crystallized complex with a moderate inhibitor Flurbiprofen has also been determined\textsuperscript{87,88} which are available in the RCSB Protein Data Bank.

### 4.1.2 Development of selective COX-2 inhibitors

Comparison of the active sites of these two co-crystallized structures revealed that, both active sites comprises a long hydrophobic channel with high electron density that interact with aromatic ring system of inhibitor and polar residue Arg 120 and Glu 524 that may form a salt bridge at the mouth of the enzyme. But the binding of sc558 in COX-2 active site hydrophobic channel forms a cavity that accept bromophenyl residue and the sulfonamide moiety stretches into the side pocket that is formed by the residue at position 523. It was evident from these studies that the only variation in the structure of cox isoform is presence of amino acid residue at 523, in COX-1 it is Ile and incase of COX-2 it is Val523. Moreover, the substitution of Isoleucine 523 in COX-1 by the Valine in COX-2 offers a side pocket adjacent to the central hydrophobic channel, which is a prerequisite for the COX-2 selectivity. In addition to this the sulfonamide moiety is further
stretches to COX-2 polar surface and probably interacts with glutamine 192, histidine 90 and arginine 513.

Designing of potential COX-2 inhibitors is still attracting attention. Enormous research has been done to design new selective COX-2 inhibitors, as a result two drugs Celecoxib and Rofecoxib are now in clinical use. Apart from their anti-inflammatory effect by increased selectivity to COX-2 enzyme, other clinical applications are under investigation.

4.2. Literature on molecular docking of Cyclooxygenase-2 (COX-2)

The availability of advanced computation tools and the knowledge of protein and its binding behavior would be helpful in the development of newer selective COX-2 inhibitors. However, prediction of the binding energy (hence affinity) and geometry of ligands at their target macromolecule remains a very difficult problem, but one of great interest is the design and development of novel drugs. Various approaches for the design and development of selective COX-2 inhibitors have appeared in the past literature and some of them are discussed hereunder.

Kurumbail et al\[89\], described about the structural basis for the selective inhibition of COX-2 by studying the conformational changes in the active site of murine COX-2 complexed with inhibitor molecules such as SC558, Indomethacin and Flurbiprofen. They reported the stabilization of carboxylate group of classical NSAIDs by the Arg120, a charged residue in the COX-2 hydrophobic channel. They proposed that due to the lack of carboxylate group in SC558, shows selectivity towards COX-2. The complex of SC558 with COX-2 cleaves the salt bridge between the residues of Arg120 and Glu240. They also described the time dependent inhibition of COX-2 with selected inhibitors, the
reason for such inhibition could be due to the interaction of inhibitor into another pocket that formed after induction of enzyme.

![SC-558 (69)](image)

Patcharawee et al, investigated the binding modes and molecular selectivity of twelve natural product derived compounds on COX-1 and COX-2 as possible anti-inflammatory agents. Free binding energies and inhibitory constants were determined by employing Autodock 4.2.0. From docking results selectivity index was estimated for each tested molecule. They finally found that among tested natural compounds γ-mangostin has got lowest selectivity index comparable to Rofecoxib that could be useful in the designing of new NSAIDs with good safety profiles.

![Mangostin (70)](image)
Madeswaran et al\textsuperscript{91}, evaluated the cyclooxygenase inhibitory activity of flavonoids using \textit{in silico} docking studies. Ten structurally diverse flavonoids were selected and studies were carried out using Autodock 4.2.0 employing LGA. Selected compounds binding energies were determined those were ranging from -8.77kcal/mole to -6.24kcal/mole and compared with the standard Celecoxib. Intermolecular energies and inhibition constants were also determined; finally it was concluded that structural features of flavonoids impart their cyclooxygenase inhibitory activity. This docking analysis could be useful in the development of safer and effective anti-inflammatory agents.

Zheng \textit{et al}\textsuperscript{92}, reported the essential structural profile of a dual functional inhibitor of COX-2 and 5-LOX. Molecular docking and 3D-QSAR studies were carried out with twenty one dual inhibitors of 7-tertiarybutyl-2,3-dihydro-3,3-dimethyl benzo furan(DHDMBF’s 104) on COX-2 and 3D model of 5-LOX. Binding orientations and conformations of selected compounds were determined with in the active sites of COX-2 and 5-LOX. CoMFA model were constructed based on the binding conformations on COX-2 and 5-LOX. From the docking and QSAR analysis it was concluded that, for a dual inhibitor of COX-2 and 5-LOX a moderately bulky group at R$_2$ position and negatively charged group at carboxyl group are important.

![Chemical structure of t-Butyl](71)
Rosati et al\textsuperscript{93}, reported the synthesis, docking and \textit{in vivo} anti-inflammatory activity of tetrahydro indazole derivatives. 2,3-disubstituted tetra hydro 2H indazoles were regioselectively synthesized in \(\alpha\)-Zirconium sulfophenyl phosphonate-methane phsphonate. Docking studies were carried out using the Autodock 3.0 into the catalytic site of COX-2 for the synthesized compounds to identify potential anti-inflammatory lead molecules. Two indazole derivatives were chosen based on the docking results for further \textit{in vivo} anti-inflammatory evaluation in two experimental models. This study proved the anti-inflammatory action of selected indazoles.

Zarghi et al\textsuperscript{94}, described about the synthesis, biological evaluation and molecular docking studies of new 4-carboxyl quinoline derivatives as cyclooxygenase-2 inhibitors. A group of 4-carboxy quinolines that possess methylsulfonyl at para position of the C2 phenyl ring were designed, synthesized and evaluated for \textit{in vitro} cyclooxygenase inhibition activity. Among the tested 4-carboxy quinolines, 7,8,9,10 tetra hydro 2-(4-methylsulfonyl)phenyl)benzo(h)quinoline\textsuperscript{73} (Figure 4.1) found to show high selectivity towards COX-2, more potent than standard Celecoxib. Molecular docking study of compound \textbf{106} into the active site of COX-2 showed that para methylsulfonyl group on the C2 phenyl ring oriented with P2 site and carboxyl group is interacted with
Arg120. Further structure activity relationship studies revealed that lipophilic groups on the C7 and C8 of quinoline imparts COX-2 inhibitory activity.

Figure 4.1 Docking of 7,8,9,10-tetrahydro-2-(4-(methylsulfonyl) phenyl) benzo[H]quinoline-4-carboxylic acid (73) in the active site of murine COX-2.

Zarghi et al\textsuperscript{95}, synthesized a new group of 2,3-diaryl quinoline derivatives (Figure 4.2) and evaluated as selective COX-2 inhibitors. All the designed drugs possess a methylsulfonyl pharmacophore at the para position of the C2 phenyl group. Structure activity relationship studies were also performed for selective COX-2 inhibition by varying the substituent at C4 quinoline group. Among the tested 2,3-diaryl quinoline derivatives, compound with 4-carboxyllic acid exhibited greater potency and selectivity for COX-2 inhibition and was more selective than standard drug Celecoxib. Docking of compound 107 in to the binding site of COX-2 revealed that methyl sulfonyl group is oriented in the vicinity of COX-2 Arg513, Val523 and Phe518 binding pocket, 4-carboxyllic group can interact with Ser530. This investigation has stated the importance of C4 substituent on quinoline for COX-2 inhibition activity.
Eren et al.\textsuperscript{96}, reported the synthesis of three novel series of diaryl heterocyclic derivatives, 2-oxo furan, 2-oxo 1,3-oxazole and pyrazoles moieties as central heterocyclic ring systems. Synthesized compounds were evaluated for their \textit{in vitro} inhibitory activity on cox-1 and COX-2. Compound (75) 2-oxo-5H-furan derivative exhibited greater potency with selectivity towards COX -1 and COX-2 with IC\textsubscript{50} values 0.061\textmu M and 0.325\textmu M respectively. In pyrazole series (76) showed highest inhibition, more potent than standard Rofecoxib with IC\textsubscript{50} value for COX-2 was 0.011\textmu M and 0.398\textmu M respectively, but did not show any selectivity for COX-2. Compound 108 demonstrated greater and selective COX-2 inhibition. Molecular docking studies further revealed the binding interactions for the selectivity and potency of compounds with pyrazoles.
El-sayed et al., reported the synthesis of new pyrazole and pyrazoline derivatives and evaluated their ability to inhibit ovine COX-1 and COX-2 isoforms using in vitro cyclooxygenase inhibition assay. Among the tested compound, new pyrazole derivative showed optimal COX-2 inhibition activity IC$_{50}$ 0.261µM comparable with standard drug Celecoxib. Further in vivo anti-inflammatory studies were carried out employing rat paw edema model for the selected compounds which showed COX-2 selective inhibition in in vitro studies. Molecular docking studies were also performed using MOE 2008 to identify the binding interactions in the active site of COX-2, this study revealed that tested compounds showed similar binding modes to SC558, a highly selective COX-2 inhibitor.
Figure 4.4 Superimposition of docked conformations of pyrazole derivatives in the active site of COX-2

Upasana et al., synthesized and evaluated 3-chloro-4 substituted azatidinones as possible analgesic and anti-inflammatory agents. In vivo anti-inflammatory and analgesic activities were carried out by employing carrageenan induced rat paw edema model and acetic acid induced writhing in mice respectively. Compound 77 exhibited potent analgesic and anti-inflammatory activity, as compared to the reference drug Nimesulide. Binding mode and binding affinities of synthesized compounds were studied by docking into the active site of COX-2 using molecular design suite (MDS), a good correlation was found between docking and pharmacological screening.

3-chloro-4 substituted azatidinone(77)
Sayed et al., reported the synthesis of a series of aminobenzene sulfonamide possessing a methylsulfonyl amino moiety at para position of the one phenyl ring. The synthesized compounds were evaluated for *in vitro* COX-2 inhibition assay. Structure activity relationship studies were carried out varying substituents on second phenyl ring, it was found from the studies that compound 78 with 4-methoxy substituent as potent COX-2 inhibitor. Docking studies were also performed by using MOE-Dock into the active site of COX-2 for the synthesized compounds. Docking studies revealed the favorable interactions of compound 78 within the primary binding site of COX-2 similar to selective inhibitor SC558.

![Aminobenzene sulfonamides(78)](image)

Rajeev kumar *et al*. described about the design of new series of pyrrole derivatives as selective COX-2 inhibitors. Docking studies were performed by employing Autodock 4.2.0 to identify the potential lead molecules from the designed pyrrole derivatives. The derivatives with good binding energy were selected for further synthesis and biological evaluation to support the docking results with practical data. Finally compounds 79 exhibited best binding energy with good *in vivo* analgesic activity.
Pyrrole derivatives (79)

Afshin zarghi et al\textsuperscript{101}, reported the design and synthesis of 2,5-substituted 1H indole derivatives as selective COX-2 inhibitors. Compounds were synthesized and evaluated for their ability to selectively inhibit the COX-2 over COX-1 isoform. Structure activity relationship of compounds with two different pharmacophore groups (azido or methylsulfonyl) on the para position of C2 phenyl ring of 1H indole was studied. It was found that compound 80 a methoxy group at C5 position and para methyl sulfonyl phenyl ring at C2 position showed better selectivity. When the same compound was docked in to the active site of COX-2, favorable binding modes were observed with in secondary pocket of cyclooxygenase-2.

2,5-substituted 1H indole (80)

Praveen Rao et al\textsuperscript{102}, reported the design and synthesis of new Rofecoxib analogs as selective COX-2 inhibitors. The methyl sulfonyl pharmacophore of Rofecoxib was replaced by N-acetyl sulfonamide bioiostere to design Rofecoxib analogs. Analogos were synthesized and evaluated for their \textit{in vitro} cyclooxygenase inhibition activity. From the acquired data structure activity relationship was established and was found that all the
tested compounds selectively inhibit cyclooxygenase-2. Docking studies of compound 81 in the active site of COX-2 has revealed the binding affinities which was comparable to Rofecoxib.

![Structure of Rofecoxib analogue (81)](image)

**Structure of Rofecoxib analogue (81)**

Chakraborti *et al*\(^{103}\), described about the molecular docking studies for a set of eighty-two ligands, which are structurally diverse inhibitors of cyclooxygenase isoforms including traditional NSAIDs and newer selective COX-2 inhibitors. Flexx docking software was employed and flexible docking was carried out, the free binding energies of ligand-protein complex was determined. The performance of Flexx was proved from the results as excellent reproducibility of the experimental conformations was obtained.

### 4.3 Present study

The present study details the application of a molecular docking procedure to discriminate between the active and inactive COX-2 inhibitors. Four compounds from each series of 2, 5, 6-substituted thieno pyrimidine Schiff bases were selected for the virtual screening purpose. Initially, the docking parameters were optimized, for the selected protein under study (1CX2) and for the selected ligands. The docking study
determines the binding nature of selected ligands with the active site of COX-2 and also identifies the ligands with selectivity towards COX-2. From the docking results the binding interactions of ligands with several amino acid residues in the active site can be visualized. In the present investigation the docking of selected ligands in the active site of COX-2 was carried out to identify the active ligands from binding pose and binding energies of ligands with in the macromolecule.

4.4 Molecular Docking Methodology

Autodock\textsuperscript{104} was successfully employed for the molecular docking studies of selected thieno[2,3-\textit{d}]pyrimidine derivatives into the active site of cyclooxygenase-2. Docking has been performed systematically by using molecular docking tools following the below mentioned steps.

a. Ligands preparation
b. Protein preparation
c. Assigning grid parameters
d. Assigning docking parameters
e. Docking run
f. Docking analysis.

**Software used in the present study**

<table>
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<td>MGL tools</td>
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4.4.1 Ligands preparation

Sixteen thieno pyrimidines were selected as ligands, and the structures were generated in Sybyl 7.0 and the energies were minimized for 1000 iterations using convergent method with 0.01 kcal/mole. The coordinates were then saved as separate .pdb file. Autodock 4.2.1 was used for further preparation of ligand molecules, the kollaman charges were added and the non polar hydrogens were merged. The number of torsion angles for ligands were set for each ligand and PDBQTs were generated.

4.4.2 Protein preparation

The coordinates of target protein cyclooxygenase-2 (Pdb code: 1cx2) co-crystallized with a highly selective inhibitor sc558 was downloaded from Brokeheaven protein data bank. The inhibitor molecule was deleted from the binding site of COX-2. Then the structure of macromolecule was checked, polar hydrogens were added in Autodock, non polar hydrogens were merged and atomic charges (Gastiger charges) were added. Finally the PDBQT of the macromolecule was generated.

4.4.3 Assigning grid parameters

The 3D grid parameters were assigned by using Autogrid algorithm to study the binding energies between enzyme and inhibitors. A separate grid parameter file (gpf) was generated for each ligand; the 3D grid box was set for the active site of cox-2 with grid points 40, 40 and 40 for x, y and z axis respectively.
4.4.4 Assigning docking parameters

Docking parameters were set to each inhibitor to define the docking type to be carried out and to set energy calculation, evaluations to be performed while docking by Autodock. The Lamarkian genetic algorithm method was chosen and atom types were set for macromolecule and ligand molecules. The number of docking runs, energy evaluations and number of generation were set separately for each inhibitor 50, 27000 and 250000000 respectively. Docking parameters thus assigned were saved as dpf file.

4.4.5 Docking run

Each ligand molecule from thirty six inhibitor molecule and SC558 were separately docked into the active site of protein molecule by using command line interface. Initially Autogrid was run from the grid parameter file using the command line in terminal window. Later Autodock was run from the command line, docking run generated a dlg file which gave different evaluation parameters such as intermolecular binding energies, docking score and docking pose.

4.4.6 Docking analysis

In the present investigation docking analysis was carried out to determine the best docked conformation or pose of each ligand in the active site of COX-2(1CX2) in a predetermined grid box. The docking log files (.dlg) thus generated in the docking run were used to visualize the top ten conformation of each docked ligand of the test set and docking scores in terms of binding energies were also recorded and presented in the Table 4.1. The best ranked conformation of each ligand has taken in to account to study the interactions with amino acid residues in the active site and the images are presented in the Figure s 4.5 to 4.20.
Table 4.1. Binding energies (kcal/mole) of top ten ranked conformations of docked ligands

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Figure 4.5. Binding interactions of SC558 with the active site of COX-2

Figure 4.6. Binding interactions of top ranked ligand ANB6 with in the active site of COX-2
Figure 4.7. Binding interactions of top ranked ligand ANB7 with in the active site of COX-2

Figure 4.8. Binding interactions of top ranked ligand ANB9 with in the active site of COX-2
Figure 4.9. Binding interactions of top ranked ligand ANC6 with in the active site of COX-2.

Figure 4.10. Binding interactions of top ranked ligand ANC7 with in the active site of COX-2.
Figure 4.11. Binding interactions of top ranked ligand ANC8 with in the active site of COX-2

Figure 4.12. Binding interactions of top ranked ligand ANC9 with in the active site of COX-2
Figure 4.13. Binding interactions of top ranked ligand BNB6 with in the active site of COX-2

Figure 4.14. Binding interactions of top ranked ligand BNB7 with in the active site of COX-2
Figure 4.15. Binding interactions of top ranked ligand BNB8 with in the active site of COX-2.

Figure 4.16. Binding interactions of top ranked ligand BNB9 with in the active site of COX-2.
Figure 4.17. Binding interactions of top ranked ligand BNC6 with in the active site of COX-2.

Figure 4.18. Binding interactions of top ranked ligand BNC7 with in the active site of COX-2.
Figure 4.19. Binding interactions of top ranked ligand BNC8 with in the active site of COX-2

Figure 4.20. Binding interactions of top ranked ligand BNC9 with in the active site of COX-2
4.5 Results and Discussion

Molecular docking studies were performed for sixteen compounds using Autodock 4.2.1, Autodock tools 1.5.4 was used to visualize the molecules three dimensionally and to set several parameters for docking studies. Cyclooxygenase-2 (Pdb code: 1CX2) was taken as target protein to study the binding interactions of the test set with in the active site. The co-ordinates of 1CX2 along with co-crystallized inhibitor molecule SC558 was obtained from protein data bank and SC558 was successfully deleted from the active site of COX-2 using Pymol, the same was used for the whole docking study.

Initially docking method for the active site of COX-2 (1CX2) was validated by re-docking with SC558 and the re-docking results were comply with the reported method in terms of free binding energy and binding pose.

Further each ligand of test set was docked into the active site of 1CX2 by applying the same set of parameters throughout the study. From the dlg file of each ligand the free binding energy and binding interactions of top 10 confirmations were determined and the best conformation was taken to study insight into the active site.

Ligand ANB6 docking conformation with least binding energy -8.67kcal/mole (Figure 4.6) in which the dimethoxy phenyl group was embedded into the binding pocket of residues Arg120, Val349, Leu359 and Tyr355 shown similarity with the binding modes of triflouromethyl group of SC558. Most importantly the imino bridge of ANB6 tends to interact with Val523 which is essential for COX-2 selectivity. Whereas the 2-methyl group was oriented towards Ser353 and Leu352 mimic binding mode of phenyl
sulfonyl moiety of SC558. Pyrimidine portion of the ligand ANB6 was tend to interact with the Ala527 and Gly526 similar with bromophenyl group of SC558.

The best docked conformation of ANB7 showed a free binding energy -8.96 kcal/mole (Figure 4.7), exhibited similar binding modes as seen with ANB6. The methoxy portion was fit into the binding pocket formed by Arg120, Val349, Tyr348 and Tyr355, the imino bridge was in close contact with Val523. The 2-methyl group along with pyrimidines portion of ligand oriented towards Ser530, and Leu531. The thienopyrimidine ring was in close proximity to Gly526.

The least energy conformation of ANB8 with a free binding energy of -9.12 kcal/mole was in good complementarity to the cox-2 active site and presented more favourable binding modes. The hydrophobicity and extended ring system of ANB8 fit into the binding pocket of Arg120 and Val349; moreover, the nitrogen of the indole nucleus is in close proximity to val523 and fit into the side pocket.

The docked conformation of the ligand ANB9 with least binding energy -8.42 kcal/mole was taken to analyze the docking interactions (Figure 4.8). It was found that the ANB9 showed different binding patterns than the ANB6 and ANB7. In this Thiophene ring occupied the binding pocket with residues His90, Leu352, ser353 and Val349. However the 5-methyl and imino portions are in close proximity with Val523, Meth522 and Phe518. The pyrimidines portion and 2-methyl group was embedded inside the Ser530. The 5-methyl group was in contact with Phe518, His90 and Tyr357, whereas 6-methyl portion was in hydrophobic pocket of residues Tyr387, Trp386 and Leu384.
The thienopyrimidines was interacting with the same residues Gly526 and Leu527 as seen in ANB6 and ANB7.

The best conformation of ANC6 gave binding energies -9.79kcal/mole (Figure 4.9) and showed similarity in binding modes as seen with ANB6 except the binding of cyclohexynyl portion. The dimethoxy phenyl portion was oriented in such away that it interact with Arg120, Leu359 and Val116; the imino bridge was in close proximity to Val523 and Meth522 and the pyrimidines portion with 2-methyl group oriented towards Ser530, Leu531 and Val 349. Finally the cyclohexynyl group was firmly fit into the hydrophobic core formed by Leu384, Tyr385, Trp387 and Phe518.

Similarly the ligand ANC7 with binding energy -9.35kcal/mole occupied same binding regions (Figure 4.10) as seen in ANB7. The para methoxy group was in the pocket Arg120, Val116, Leu359. The imino portion was oriented towards Met522 and Val523, Ser353, Tyr355 and Val348, the pyrimidine portion with 2-methyl group associated within the pocket formed by residues Ser530, Leu531 more closely than the otherportion of ANB7. The cyclohexynyl portion embedded inside the pocket with residues Tyr385, Leu384 and Trp387.

The ligand ANC8 showed a least binding energy conformation -9.86kcal/mole (Figure 4.11) and bound differently than the other compounds. The indolyl ring portion tends to interact with Leu384, Tyr 385 and Phe381; the imino bridge shares same binding mode as observed with other ligands with residues Met522 and Val523. Interestingly the imino group oriented towards Gly526 and Ala527. The pyrimidine portion with 2-methyl group was interacting with Val349, Ser 353, Leu352 and His90 which was not observed
in other ligands. This is may be due to the presence of large aromatic ring attached to imino group. However the binding of cyclohexanyl is similar as seen with ANC6 and ANC7 interacting with Ser530 and Leu531. Hence little variation could be observed in the binding modes of ANC8 compared with ANC6 and ANC7.

The binding of least energy conformation of ANC9 showed rather better binding modes than the ANB9 (Figure 4.12). The thienyl moiety was embedded in Arg120, Ser353 and Tyr355 and the imino bridge was oriented towards the Val523, Met522 and Phe518. Whereas 2-methyl and pyrimidines part of the ligand was in close proximity to Ser530, Leu531 and Val349. Finally the cyclohexanyl portion of the ligand occupied the same pocket of residues Trp387, Tyr385 as seen in ANC6, ANC7 and ANC8. The thienopyrimidine ring was oriented towards Ala527.

Best binding conformation of BNB6 has free binding energy -10.18kcal/mole shown binding interaction similar to SC558 (Figure 4.13). The dimethoxy phenyl group binds to the side pocket more firmly with residues Val523, Ile527, Ala516, Phe515, Arg513, His90 and the 2-phenyl ring occupies Arg120 and Val116. The thienopyrimidine nucleus was oriented towards Gly526 and Ala527 as seen with most of the ligands of test set. The 5-methyl group was embedded into the Leu352, Ser353 and 6-methyl group interacting with Val349 and Tyr385. Among the above binding interactions of BNB6, the binding into the side pocket through Val523 and Arg513 is an important feature of this ligand which is essential for Cylooxygenase-2 selectivity. Moreover the presence of an imine bridge and most importantly an aromatic nucleus may contribute for this interaction.
The ligand BNB7 of this series bound to the active site of COX-2 with binding energy of -9.84 kcal/mole shown similarities (Figure 4.14) with BNB6 binding. The imino bridge is interacting with hydrophilic Val523 and is in close proximity to Ser530 and Leu532. The paramethoxy groups opened into the side pocket of Arg513, Phe518, Ala516, His90 and Ala192. The phenyl group binds to Arg120, Tyr355 through Val349, Where as thienopyrimidine nucleus is interacting with Ser530 and Ser531. The 6-methyl group is in contact with Gly526 and Ala527.

Ligand BNB8 bound to active site in a manner different from other ligands and showing (Figure 4.15) maximum binding energy of -11.17 kcal/mole which is less than the binding energy of SC558 with COX-2. In this the imino bridge is oriented towards Leu352, Ser353 and the indole ring was opened into the residue Arg120, Val349. The 5 and 6 methyl groups were embedded into the hydrophobic core formed by Tyr385, Leu384, eventually and the thienopyrimidine nucleus oriented to Val523, Met522 and Phe518.

Another ligand of this series BNB9 bound to the active site of COX-2 with a free binding energy of -9.95 kcal/mole (Figure 4.16). Iminio bridge of BNB9 is in close proximity to Val349, Ser530 and Leu531 and the thienyl group was oriented towards Arg120. The pyrimidines portion and 2-phenyl group is opened into Tyr355, His90, Ser353, Leu352 and Phe518, whereas the thiophene portion is in contact within hydrophillic pocket formed by Phe518, Val523, Met522, Phe381 and Leu384 and the 5-methyl group was oriented towards Gly526 and Ala527.
Ligands BNC series have extra methylene carbons which contributes hydrophobicity and rigidity to the structure. The ligand BNC6 was bound to active site of COX-2 with free binding energy of -9.9 kcal/mole (Figure 4.17). In this binding the imino group was interacting with Ser530, Leu531 and the dimethoxy phenyl group was embedded into Tyr345, Val349 and Leu534. The phenyl group was in contact with Phe387, Tyr385, Phe518, Tyr386 and Trp387 and oriented towards the hydrophilic pocket Val523 and Met522. However the thienopyrimidine ring portion is oriented towards leu352 and ser353, whereas cyclohexynyl portion is in contact with Tyr355 and Leu359.

The ligand binding pattern of BNC7 to COX-2 active site is quite different compare with other ligands (Figure 4.18). In this the bridging imino group is in contact with Arg120 and paramethoxy phenyl group was closely associated with Tyr355, Ser530, Arg513, Ala516, Ile352 and Ser353. Interestingly the 2-phenyl group is embedded with in hydrophobic region formed by the residues Met522, Val523, Tyr387 and Gly526. Although the thienopyrimidine ring is in contact with Val349 and Leu531, the nitro and sulfur positions of thienopyrimidines and cyclohexanyl portions are oriented towards Leu531.

The ligand BNC8 is bound to the active site of COX-2 (Figure 4.19) with a least free binding energy of -11.54 kcal/mole which is lower than binding energies of all of the test ligands. This showed the fair fitness of the BNC8 to active site of the enzyme. However the binding interactions are slightly different from the interactions of other ligands. The imino group is in close proximity to Arg120 and the adjacent indole group is resides in the binding pocket of Arg513, Ala516, His90 and Leu352. Where as the 2-
phenyl group is oriented towards Val523 and in contact with other residues such as Met522 and Phe518. The thienopyrimidine nucleus is interacting with Val349 and the cyclohexenyl portion is contact with Tyr355.

Finally the ligand BNC9 bound to active site of COX-2 (Figure 4.20) with a free binding energy of -10.17 kcal/mole with the binding of imino group towards Ser530 and Leu531, where as thienyl group towards Tyr348 and Val349. The 2-phenyl group is oriented towards Met522, Tyr385, Trp387 and Leu384. The thienopyrimidine was oriented towards Val349 and also be in contact with Ala527 and Gly526 and the cyclohexynyl group is in close proximity to Leu359 and Tyr355.
### Chapter 5  Summary and conclusions

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<td>Conclusions</td>
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5.1 SUMMARY

**Chapter-1 Introduction**

**Thienopyrimidines–Chemistry, synthesis, reactivity, biological and pharmacological importance**

Thienopyrimidines and its derivatives have been generally associated with various biological and pharmacological properties. The synthesis of a large number of thienopyrimidines derivatives has been directed to obtain potentially active medicinal compounds. Many such compounds have been found to be promising; a few even have clinical application also. An exhaustive literature search has been done on the different methods of synthesis, reactivity, biological and pharmacological activities of thienopyrimidines and has been presented briefly in this chapter.

The need for the present work, its aims and objectives are specifically outlined in the chapter.

A gist of the work presented in subsequent chapters has been included at the end of the chapter. An exhaustive list of relevant references forms the concluding part of this chapter.

**Chapter-2**

**Synthesis and characterization of new 4{[(Aryl)methylene]amino}-2,5,6-substituted thieno[2,3-d]pyrimidine derivatives**

In the introduction part of this chapter a brief emphasize has given on the recent medicinal chemistry approaches in the discovery of potential lead molecules. Further a brief account on synthesis of thienopyrimidines has been outlined. A brief and important literature has been presented on the synthesis of variety of thieno[2,3-d]pyrimidines that was reported recently.
A need for the present investigation has been clearly indicated. It is followed by the text of the present work on the title compounds. Synthesis of the title compounds has been achieved as outlined in Scheme-1.

The present investigation synthesis of seven series of Schiff bases of 4-amino-2, 5, 6-substituted thienopyrimidines derivatives (ANB1-9, ANC1-9, ANP1-9, ANH1-9, BNB1-9, BNC1-9 and BNP1-9) has been carried out by following the Scheme-1.

The starting material 2-amino-3-cyano-thiophenes were prepared by following the reaction described by Gewald et al., according to this procedure each of the four ketones viz butanone, cyclohexanone, pentanone and heptanone were made to react with elemental sulphur and malenonitrile in ethanol using mild basic condition. This resulted in the formation of four 2-amino-3-cyano-thiophenes B, C, P and H. These compounds were further confirmed by comparing their melting point and TLC with the literature data.

All the four thiophenes were subjected to synthesis of 4-amino-2,5,6-substituted thieno[2,3-d]pyrimidines (ANB, ANC, ANP, ANH, BNB, BNC and BNP). This was achieved by the reactions of each of 2-amino-3-cyano-thiophene B, C, P and H with either acetonitrile or benzonitrile in sodium methoxide. This resulted in the cyclization of amino and nitrile groups at C2 and C3 of thiophene with nitrile group of alkyl or aryl nitriles and afforded seven different thieno[2,3-d]pyrimidines-4-amines viz ANB, ANC, ANP, ANH, BNB, BNC and BNP with varied substitutions at C2, C5 C6 positions. The compounds were obtained in good yields and purity was determined by their melting
Ketones Malononitrile

\[ \text{R}_1 \text{R}_2 \text{C} = \text{O} + \text{H}_2 \text{C} \text{N} \text{N} \rightarrow \text{B}, \text{C}, \text{P} \text{ and H} \]

sulphur

\[ \text{Ma} \text{lenonitrile} \]

NaOCH\text{3} \text{R}_3 \text{CN}

ANB, ANC, ANP, ANH, BNB, BNC and BNP

Ar = \[ C_6\text{H}_5, p-\text{Cl-C}_6\text{H}_4, p-\text{Br-C}_6\text{H}_4, \]

\[ p-N(\text{CH}_3)\text{C}_6\text{H}_4, p-N(\text{C}_2\text{H}_5)\text{C}_6\text{H}_4, \]

\[ p,m-(\text{OCH}_3)\text{C}_6\text{H}_4, p-(\text{OCH}_3)\text{C}_6\text{H}_4, \]

\[ 3\text{-indolyl, 3-Thienyl} \]

Ethanol Acetic acid Ar-CHO

ANB\text{1-9}, ANC\text{1-9}, ANP\text{1-9}, ANH\text{1-9}, BNB\text{1-9}, BNC\text{1-9} \text{ and BNP}\text{1-9}

SCHEME-I
Further the compounds obtained were purified by recrystallization from ethanol and used for the next step.

Each of 4-amino-2, 5, 6-substituted thieno[2,3-d]pyrimidines were then condensed with nine different aromatic aldehydes viz. benzaldehyde, para chloro benzaldehyde, para bromobenzaldehyde, para N,N-dimethyl amino benzaldehyde, para N,N-diethyl amino benzaldehyde, para methoxy benzaldehyde, 3,4-dimethoxy benzaldehyde, indolyl 3-aldehyde, theinyl-3-aldehyde in ethanol with a catalytic amount of glacial acetic acid. This resulted in the formation of seven series of 2,5,6 substituted thieno[2,3-d]pyrimidine schiff bases in good yield. The purity of the compounds was confirmed by their melting points and TLC. The title compounds were further characterized by their elemental, proton NMR, Infra red and Mass spectral data.

The spectral characterization of some of the synthesized compounds has been discussed in the results and discussion section.

**Chapter- 3**

**Biological evaluation of new 4[(Aryl)methylene]amino]-2,5,6-Substituted-thieno[2,3-d]pyrimidine derivatives**

As an introduction to this chapter the biological and pharmacological importance of thieno[2,3-d]pyrimidine derivatives reported in the literature so far has been presented in nutshell. In the next section to introduction standard procedures employed for the biological evaluation of all the synthesized new thieno[2,3-d]pyrimidine derivatives was discussed.
Table 5.1. Structure and substituents of new 4\{[(aryl)methylene]amino\}-2-methyl-5,6-substituted-thieno[2,3-d]pyrimidinederivatives (ANB1-9 and ANC1-9)

![Structural formula](image)

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Table 5.2. Structure and substituents of new 4-[(aryl)methylene]amino)-2-methyl-5,6-substituted-thieno[2,3-d]pyrimidinederivatives (ANP1-9 and ANH1-9)

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Table 5.3. Structure and substituents of new 4{[(aryl)methylene]amino}-2-phenyl-5,6-substituted-thieno[2,3-d]pyrimidinederivatives (BNB1-9 and BNC1-9)

![Chemical Structure](image)

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Table 5.4. Structure and substituents of new $4\{[(aryl)methylene]amino\}$-2-phenyl-5,6-substituted-thieno[2,3-$d$]pyrimidinederivatives (BNP1-9)

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All the sixty three thieno[2,3-$d$]pyrimidine derivatives were evaluated for antibacterial, antifungal and antioxidant activities. Among all the sixty three compounds thirty six compounds of four series have been screened for their ability to inhibit the ovine COX-2 (in vitro COX-2 inhibition assay). The detailed experimental methods, observations and results obtained in the above said experiments have been presented in this section.

The tabulated results of all the activities screened for the synthesized compounds have been discussed in results and discussion section of this chapter. Further the potential
activity trends were noted, some significant results obtained from the biological evaluation are recorded.

**Antibacterial activity**

All the sixty three compounds have been evaluated for *in vitro* antibacterial activity of against two gram positive bacteria viz., *S.aureus, B.subtilis* and two gram negative bacteria *E.coli* and *P.aeruginosa* by employing disc diffusion method. Ampicillin sodium was taken as standard to compare the results of antibacterial activity.

The antibacterial data of new 4{[(aryl)methylene]amino}-2,5,6-substituted-thieno[2,3-\textit{d}]pyrimidine derivatives indicate that some of the compounds ANC8, ANC9, ANB5, ANB8, ANB9, ANP2, ANP5, ANP9, ANH2, BNC8, BNC9, BNB5, BNB8, BNB9, BNP2, BNP5 and BNP9 exhibited potent antibacterial activity against all the bacterial strains employed in the present study. Whereas few of the compounds ANC6, ANC7, ANB3, ANP3, ANP7, ANP8 exhibited potent activity specifically on gram negative bacterial strains *E.coli* and *P.aeruginosa*. However compounds ANC2, ANB2, and BNC2 were found to show potent antibacterial activity on gram positive bacteria *S.aureus* and *B.subtilis*. It was observed that most of the tested compounds exhibited good to moderate antibacterial activity on tested bacterial strains.

**Antifungal activity**

All the sixty three compounds of seven series of new 4{[(aryl)methylene]amino}-2,5,6-substituted-thieno[2,3-\textit{d}]pyrimidine derivatives have been evaluated for *in vitro* antifungal activity against four fungal strains, two from *Candida* species and two from
Aspergillus species. The results thus obtained are compared with Fluconazole as standard drug.

The results of antifungal activity of tested compounds revealed that compounds ANC5, ANC8, ANC9, ANB5, ANB8, ANB9, ANH5, ANH6, ANH8, ANH9, BNC2, BNC3, BNC4, BNC8, BNC9, BNP3, BNP4 and BNP5 exhibited potent antifungal activity against tested fungal strains. Whereas compounds ANC4, ANB6, ANB7, ANH3, ANH7, BNC5, BNC6, BNC8, BNC9, BNP3, BNP4 and BNP5 exhibited good to moderate antifungal activity. It was also observed from the results that the compounds ANP6 and ANH4 were found to show potent activity on Aspergillus species, compounds ANP2, ANP9 and ANH6 exhibited potent antifungal activity on Candida species.

Antioxidant activity

All the sixty three compounds have been evaluated for their antioxidant activity by employing DPPH (1,1-diphenyl-2-picryl-hydrazide) method. The results of the evaluation have been compared by taking Ascorbic acid as the standard one. The IC$_{50}$ values of the test compounds have been compared with the IC$_{50}$ value of standard ascorbic acid.

The IC$_{50}$ values of antioxidant activity of new 4{[(aryl)methylene]amino}-2,5,6-substituted-thieno[2,3-\textit{d}]pyrimidine derivatives revealed that all the tested compounds exhibited very good antioxidant activity, however most significant of them were found to be compounds ANC7 and ANB9. Potent antioxidant activity has also been observed with compounds ANC2, ANC4, ANC9, ANB1, ANP7, ANP9, ANH7, ANH9, BNC3, BNC9,
BNB1, BNB4 and BNP9. Whereas all the other tested compounds exhibited good antioxidant activity.

**In vitro COX-2 enzyme inhibition assay**

All thirty six compounds of four series ANB, ANC, BNB and BNC have been evaluated for *in vitro* cyclooxygenase-2 enzyme inhibition assay. Celecoxib was taken as standard to review the results of tested compounds. IC$_{50}$ values were determined by considering percentage of enzyme inhibition versus standard concentrations.

The results of *In vitro* Cyclooxygenase-2 enzyme inhibition assay revealed that compounds ANB6, ANB9, ANC6, ANC8, ANC9, BNB6, BNC6 and BNC8 and exhibited highest and potent *in vitro* COX-2 inhibition than the other tested compounds indicate the ability of these compounds as anti-inflammatory agents. However compounds ANB2, ANB3, ANB7, ANB8, ANC7, BNB7, BNB8, BNB9, BNC1, BNC7 and BNC9 showed good inhibition on *ovine* COX-2 enzyme.

**Chapter - 4**

**Molecular Docking studies of new 4[(Arylmethylene]amino]-2,5,6-Substituted-thieno[2,3-d]pyrimidine derivatives**

This chapter describes about the molecular docking studies of new thieno[2,3-d]pyrimidine derivatives as cyclooxygenase-2 inhibitors. In the introduction of this chapter a brief account on cyclooxygenase enzymes and its isoforms and their physiological and pharmacological importance has been discussed. A little emphasize has been given on the structural basis for the selective COX-2 inhibition and also on the
structural features of COX-1 and COX-2. A brief literature reported on the molecular docking studies on COX-2 has been presented to support the present study.

Sixteen compounds of ANB, ANC, BNB and BNC series to carry out molecular docking studies on COX-2. This study was carried out using Autodock software version 4.2.1 on Ubuntu platform. The software used, detail docking procedure employed and the results obtained in the molecular docking studies have been presented in the molecular docking methodology section of this chapter. The binding energies of top ten conformations of docked ligands were tabulated and presented.

The results of docking studies in terms of binding energies and binding interactions of best conformation of each ligand in the active site of COX-2 has been discussed in result and discussion section. From the results it was observed that the ligands BNB6, BNB8, BNB9, BNC8 and BNC9 were fit into the active site of COX-2 with least binding energies than the other docked ligands. Also favorable binding interactions required for the selective inhibition of COX-2 have been observed with the above ligands.
5.2 CONCLUSIONS

Broadly, the following conclusions could be drawn from the results of the investigations.

1. Synthetic work of the studies could go positive as per the planning and as such in all the reactions carried out, the expected compounds alone could be obtained.

2. The antibacterial data of new 4\{[(aryl)methylene]amino\}-2,5,6-substituted-thieno[2,3-d]pyrimidine derivatives revealed that compounds ANC8, ANC9, ANB5, ANB8, ANB9, ANP2, ANP5, ANP9, ANH2, BNC8, BNC9, BNB5, BNB8, BNB9, BNP2, BNP5 and BNP9 exhibited potent and significant antibacterial activity against all the tested bacterial strains. The compounds with \textit{para} chlorophenyl, indol-3-yl and 3-thienyl substituents may contribute for this activity. Further more investigations are required to explore the broad spectrum antibacterial activity of these derivatives.

3. The results of antifungal activity of tested compounds revealed that compounds ANC5, ANC8, ANC9, ANB5, ANB8, ANB9, ANH5, ANH6, ANH8, ANH9, BNC2, BNC3, BNC4, BNB2, BNB7, BNB8 and BNB9 exhibited potent antifungal activity against tested fungal strains. Hence this molecular frame work could be considered as lead molecule for future antifungal agents.

4. The antioxidant activity of new 4\{[(aryl)methylene]amino\}-2,5,6-substituted-thieno[2,3-d]pyrimidine derivatives revealed that all the tested compounds exhibited strong antioxidant activity, however most of them were found to be compounds ANC7 and ANB9. Potent antioxidant activity has also been observed
with compounds ANC2, ANC4, ANC9, ANB1, ANP7, ANP9, ANH7, ANH9, BNC3, BNC9, BNB1, BNB4 and BNP9.

5. The results of *In vitro* COX-2 enzyme inhibition assay showed that compounds ANB6, ANB9, ANC6, ANC8, ANC9, BNB6, BNC6 and BNC8 and exhibited strong and potent *in vitro* COX-2 inhibition. Hence these molecules could be recommended for *in vivo* anti-inflammatory studies to further explore their anti-inflammatory property.

6. Moreover the docking studies of these compounds also supported their ability in inhibiting the COX-2 efficiently. From the docking studies it was found that the ligands BNB6, BNB8, BNB9, BNC8 and BNC9 were fit into the active site of COX-2 with least binding energies and also favorable binding interactions required for the selective inhibition of COX-2 have been observed. This binding affinity and pose may contribute for their inhibition ability and hence they could be considered as lead molecules for the development of future anti-inflammatory agents.

7. It has been felt necessary from the results of the preliminary investigations that there is a necessity for further advanced and indepth studies. Moreover, the results of the investigations could helpful in establishing SAR and QSAR studies to find novel and potential lead molecule in the thieno[2,3-d]pyrimidine series. However, more precise, systematic screening of the potent compounds could helpful in further development and then could directed to preclinical to clinical studies.
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