2. REVIEW OF LITERATURE

2.1 COX Isoenzymes, inhibition and related studies

2.1.1 The discovery of COX-II and its distribution

Needleman et at[1,2] postulated that cyclooxygenase (COX) enzyme exists in two different and distinct isoforms; COX-I, a house keeping enzyme responsible for basic level of prostaglandins (PGs) and COX-II, an inducible enzyme activated by different stimuli. This was confirmed in 1991 when the inducible form of the enzyme, COX-II was cloned[3,4]. But, it is now known that this inducible isoform is also constitutively expressed[5].

Under basal conditions the constitutive enzyme COX-I is expressed in nearly all tissues including the stomach, colon, kidney, spleen, liver, lung, heart and brain. PGs synthesized by COX-I in the kidney and stomach act as vasodilators. In the kidney these PGs help to maintain renal plasma flow and glomerular filtration rate during periods of systemic vasoconstriction. COX-I in platelets generates thromboxanes which play a key role in mediating platelet aggregation[6].

On the other hand COX-II expression is largely undetectable unless induced by inflammatory stimuli in cells such as synoviocytes, macrophages and endothelial cells. Such stimuli are proinflammatory cytokines (IL-1β, TNFα), lipopolysaccharides (LPS), mitogens and oncogens (e.g. phorbolic esters), growth factors (fibroblast growth factor, platelet derived growth factor, epidermal growth factor), hormones (e.g. LH) and disorders of water-electrolyte homeostasis[7-9].

COX-II is also known to be induced in the kidney in response to sodium depletion or in hyperfiltration states, in postsynaptic excitatory neurons in the brain after electroconvulsive stimulation and in colon adenoma and carcinoma cells. Animal studies, have suggested a broader spectrum of biological activity of COX-II[10].

2.1.2 Molecular Biology: COX-I Vs COX-II

Since COX-I and COX-II are isoenzymes, they are genetically independent proteins. Therefore the genes in humans for the two enzymes are located on different chromosomes and show different properties. COX-I gene is located on chromosome 9 while COX-II is encoded in a gene on chromosome 1[11,12]. The human COX-I gene contains 11 exons with 22 Kb genomic size whereas, the human COX-II gene containing only 10 exons has a relatively small genomic size of 8.3 Kb[13,14]. These isoenzymes are membrane-bound enzymes in the endoplasmic reticulum (ER). The cleavage of the signal peptide and insertion into the ER membrane i.e. post-translational modification produces the mature, glycosylated COX-I and COX-II proteins with the molecular weight of 67 and 72 KDa, respectively[15]. COX-II in the primary structure consists of a truncated signal peptide, and insertion of an additional 18 amino acid sequence acting as an epitope for specific COX-II antibodies[16].

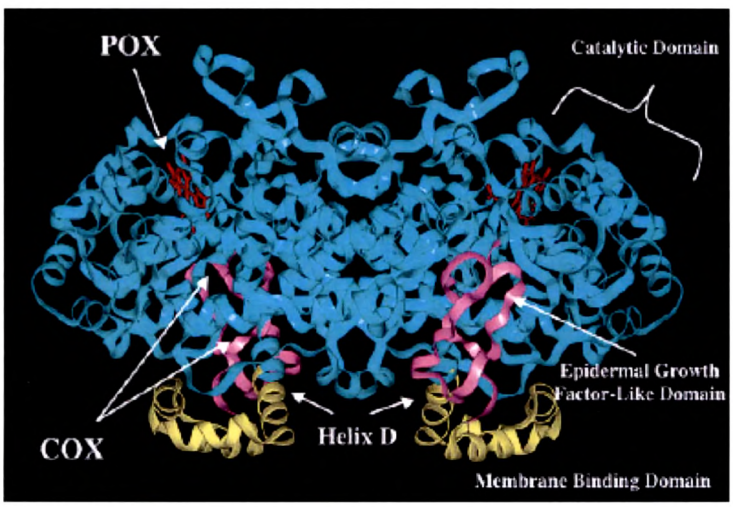


Figure 2.1.1 COX-II enzyme with different domains

The tertiary and quaternary structures of the COX enzymes contain three distinct domains: a short N-terminal epidermal growth factor (EGF) domain, a u-helical membrane-binding motif and a C-terminal catalytic site **(Figure 2.1.1)**. The COX and peroxidase active sites are located in the catalytic domain, which accounts for

approximately 80 % of the protein. The structure of COX-I and COX-II has approximately 60 % sequence identity. The significant difference between the two enzymes seems to be the existence of a comparatively much larger binding site in COX-II for NSAIDs[17] **(Figure 2.1.2).**

2.1.3 The binding sites of COX-I and COX-II

Picot et al[18] described the first X-ray crystal structure of the COX-I complexed with flurbiprofen, a nonselective NSAID. The 3D structure of the human COX-II enzyme was obtained by homology modeling[19] and by X-ray diffraction[19,20]. Kurumbail et al[17] published the 3D structure of murine COX-II, and the structures of the bovine COX-1 and murine COX-II complexed with several NSAIDs such as flurbiprofen, indomethacin and SC-558 (a selective COX-II inhibitor).

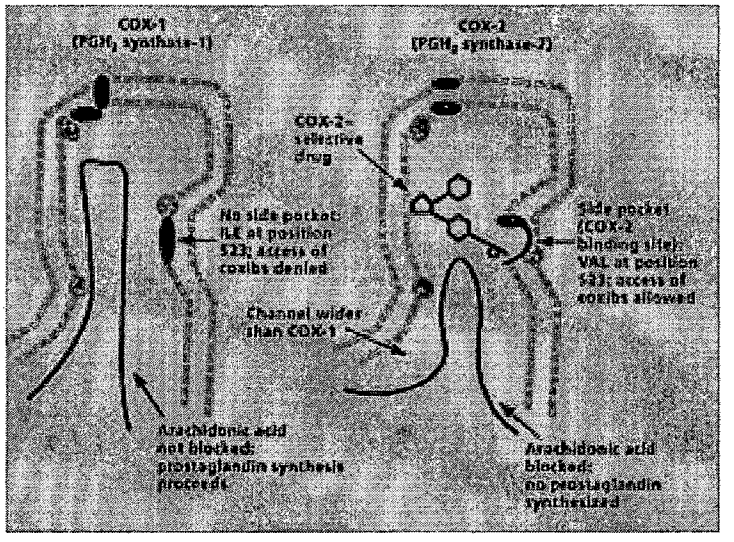


Figure 2.1.2 Comparison of the active sites of COX-I and COX-II

The COX active site consists of a long hydrophobic channel which is the binding site for NSAIDs. This channel contains areas with high electron density interacting with the aromatic system of NSAIDs. The carboxylic acid group of classic NSAIDs is directed towards tile mouth of the channel and interacts with the guanidinium group of arginine 120 (ARG 120). Glutamic acid 524 (GLU 524) which is located near ARG 120

(these are the onlv two residues which are polar in the hydrophobic channel) forms a salt bridge, blocking the mouth of tile enzyme. Thus, NSAIDs (flurbiprofen) inhibit the COX reaction by preventing arachidonic acid (AA) from invading the channel. Flurbiprofen is a slow-binding, time-dependent inhibitor of COX-I and COX-II. Indomethacin, another classic NSAID penetrates deeply into the channel and is stabilized by hydrophobic interactions but without any strong effect on COX-II[17-21].

SC-558, a selective COX-II inhibitor has a 1900 fold selectivity for COX-II over COX-I. The bromophenyl residue of the compound occupies the lobby while the entire phenylsulphonamide moiety stretches into the selective side pocket. In addition to this, the sulphonamide group extends into a region near the surface of the COX-II that is relatively polar and probably interacts with histidine 90 (HIS 90), glutamine 192 (GLN 192), and arginine 513 (ARG 513)[22]. SC-558 bound in COX-II active site is shown in **Figure 2.1.3**.

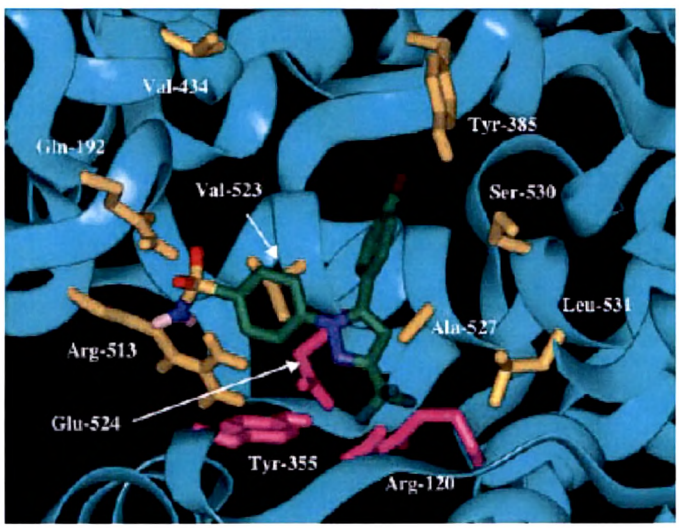


Figure 2.1.3 SC-558 bound in COX-II active site

Evidently, the exchange of a valine at position 523 in COX-II for a relatively bulky isoleucine residue in COX-I at the same position of the active site of the enzyme causes a structural modification. This modification in the COX-II enzyme-, allows access to an

additional side pocket, which is a pre-requisite for COX-II drug selectivity. Access to this side pocket is restricted in the case of COX-I. A further exchange of valine/isoleucine at position 434 is responsible for the formation of a 'gate'. In COX-II the less bulky valine is able to `swing' like gate because of less steric hindrance and offers enough space for the entry of compounds with room-filling substituents[22]. The comparison of the active sites of COX-I and COX-II is shown in **Figure 2.1.2.**

These studies are based on the static 3D structure of the enzyme and are helpful in understanding the enzyme inhibition mechanisms and in the design of selective compounds. However, enzymes are not static but dynamic. Hence, dynamic factors such as enzyme flexibility and rearrangement of the hydrogen bonding network at the entrance of the active site have been considered by Palomer et al[23]. They have investigated the selectivity of ketoprofen and its structural analogues by constructing molecular models which consider some implicit water molecules that participate in the dynamic hydrogen bonding network at the polar active site entrance with residues 355, 524, 120 and 513. These models suggest that active site hydration is essential for understanding inhibitor selectivity.

2.1.4 Physiological and Pathophysiological Role of COX-II

Recently, the multifaceted profile of COX-II enzyme and emerging therapeutic uses of COX-II inhibitors were thoroughly reviewed by various researchers[24,25].

2.1.4.1 Inflammation, pain and the CNS

Inflammation causes an increased synthesis of COX-II-dependent prostaglandins (PGs, mainly PGE_2), which sensitize peripheral nociceptor terminals and produce localized pain hypersensitivity. COX-II is expressed constitutively in the dorsal horn of the spinal cord and gets up-regulated briefly after trauma in the corresponding sensory segments of the spinal cord[26]. The widespread induction of COX-II expression

throughout endothelial cells within the CNS may underlie much of the PGE_2 production associated with hyperalgesia[27]. Intraspinal administration of a COX-II inhibitor decreases inflammation-induced central PGE_2 levels and mechanical hyperalgesia[28]. So, constitutive COX-II within the spinal cord appears to be important in pain perception and it can be induced within the spinal cord, reinforcing pain pathways. The selective COX-II inhibitors rofecoxib[29,30], celecoxib[30,31],and, valdecoxib[32] were reported to be analgesic when used in humans for post dental surgery pain, illustrating that COX-II was the target for the analgesic effects of NSAIDs even in acute pain conditions. Likewise, selective COX-II inhibitors such as valdecoxib and etoricoxib provide analgesia in primary dysmenorrhea[33,34] and rofecoxib when used preoperatively for relief from pain following knee replacement[35]. Evidently, COX-II plays an important role in the CNS in inflammation and pain and it can be deduced that therapeutic effects of COX inhibitors might be caused not only by peripheral enzyme inhibition but also at least partly, by central inhibition[31].

2.1.4.2 Eye (Glaucoma)

In patients suffering from primary open angle glaucoma (POAG) it was observed that there was either a complete loss of COX-II expression or only a few scattered COX-II expressing cells remained. Eyes of patients with either congenital juvenile or angle closure glaucoma showed COX-II expression indistinguishable from control eyes. Aqueous humor of eyes with POAG contained significantly less PGE_2 than control eyes. In normal eyes, ocular COX-I and COX-II expression were largely confined to the non-pigmented secretary epithelium of the ciliary body. COX-II expression was completely lost in the non-pigmented secretary epithelium of the ciliary body of the eyes with end stage POAG, whereas, COX-I expression remained unchanged[36,37].

2.1.4.3 Alzheimer's disease (AD)

COX-II is constitutively expressed in neurons and is upregulated in degenerative brain regions in AD such as the microglia of the cognitive centers within the hippocampus and cortex. Enhanced COX-II expression in the brain may be associated with β-amyloid protein deposition in the neuritic plaques of AD. This protein and its peptide precursors are thought to be a part of an inflammatory cascade in which microglia, a rich source of prostanoids, probably participates. The role of activated microglia, which expresses COX-II in cerebral inflarmmatory processes, was demonstrated in the rat[38].

2.1.4.4 Virus replication

PGE_2, the product of COX-II activity, increased transiently by a factor more than 50 in cultures of human fibroblasts infected with human cytomegalovirus[39]. Both specific and non-specific COX-II inhibitors could abrogate the virus mediated induction of PGE_2 accumulation. Levels of COX-II inhibitors that completely blocked the induction of COX-II activity, but did not compromise cell viability, reduced the yield of human cytomegalovirus in human fibroblast by a factor of >100 and the yield could be substantially restored by the addition of PGE_2 together with the inhibitory drugs. This finding[40] argued that elevated levels of PGE_2 were required for efficient replication of cytomegalovirus in fibroblasts. Another finding[41] suggests that combined expression of inducible nitric oxide synthase (iNOS) and COX-II may play an important role in prognosis of hepatitis C virus-positive hepatocellular carcinoma (HCC) patients.

2.1.4.5 Kidney

Like the CNS, the kidney contains constitutively expressed COX-II which seems to be involved into the regulation of the renin-angiotensin system[42,43]. In normotensive subjects neither blood pressure nor renal function is significantly affected by standard doses of celecoxib, rofecoxib, diclofenac, or naproxen[44,45]. However, in salt-depleted healthy subjects, selective inhibition of COX-II causes sodium and potassium retention,

demonstrating that an increased selectivity for COX-II does not spare the kidney under these conditions[46-48]. Also, celecoxib, rofecoxib, and naproxen cause reductions in glomerular filtration rate and urinary sodium excretion in elderly patients with compromised renal function[49,50]. In hypertensive elderly patients, COX-II-selective inhibitors may also promote edema formation and elevations in blood pressure[51,52].

2.1.4.6 Cardiovascular system

The activity of COX in endothelial cells is thought to be beneficial, contributing to the normal functioning of the cardiovascular system *via* the release of PGI_2. PGI_2 promotes vasodilatation, inhibits platelet aggregation and adhesion, and is an endogenous anti-lipidemic agent. COX-II is induced in animal and human blood vessels after physical damage or exposure to proinflammatory cytokines and appears to be associated with protective functions, inhibiting events such as cell proliferation, cytokine and endothelin-1 release, and adhesion receptor expression. Both celecoxib and rofecoxib reduces anti-aggregator PGI_2 production[53] and this could be prothrombotic.

It was noted in the Vioxx (rofecoxib) Gastrointestinal Outcome Research (VIGOR) study that there were significantly more myocardial infarctions, but not cerebral infarctions, with rofecoxib than with naproxen[54]. Direct analyses of the data from Celecoxib Long-term Arthritis Safety Study (CLASS) for cardiovascular events showed celecoxib and classic NSAIDs possess similar CVS adverse effects[55]. COX-II inhibition in certain cases, actually improves endothelial cell function in cardiovascular disease[56] and reduces subsequent cardiovascular events in patients with coronary syndromes without ST segment elevation admitted to a coronary care unit[57]. VIGOR used a population of rheumatoid arthritic patients, and it appears that rheumatoid arthritis is associated with a doubling in the risk of myocardial infarction, with no increase in the chances of stroke[58]. Naproxen reduces the risk of acute thromboembolic cardiovascular events (myocardial infarction, sudden death, and stroke) within the rheumatoid

population[59] for whom regular use of NSAIDs or paracetamol (but not aspirin) is associated with an increased incidence of hypertension[60]. In conclusion, the mass of data and analyses available appear to show that at standard recommended doses, COX-II-selective inhibitors do not increase the risks of thrombotic events and do not interfere with the anti-thrombotic effects of aspirin. They may even improve endothelium-dependent vasodilation and reduce low-grade chronic inflammation and oxidative stress in coronary artery disease and hypertension, and so could be beneficial in some patients with cardiovascular disease[61,62].

2.1.4.7 Cancer

The formation of new blood vessels by angiogenesis to provide blood supply is a major requirement for the growth of many tumors. The mature blood vessels express COX-I, while new angiogenic cells express COX-II. This shows that tumor-derived growth factor promotes angiogenesis by inducing the production of COX-II derived PGE_2. This is supported by the fact that PGs are known to be pro-angiogenic molecules and contribute to tumor growth by inducing the newly formed blood vessels (neoangiogenesis) that sustain tumor cell viability and growth[22].

Increased amounts of COX-II are commonly found in both premalignant tissues and malignant cancers of the head and neck, oesophagus and lung. COX-II expression is induced in a variety of cells leading to high levels of prostaglandin production. Such aberrant expression of COX-II has been reported in murine/human breast cancer, human colon cancer, lung cancer, head and neck cancer and pancreatic cancer.

There is extensive evidence, beyond the finding that COX-II is commonly over-expressed in tumors, to suggest that COX-II is mechanistically linked to the development of cancer. COX-II can affect multiple mechanisms that are important in carcinogenesis. It may aid in carcinogenesis by altering the normal cellular processes

like angiogenesis, cell proliferation, immunomodulation, carcinogenic metabolism and apoptosis[24,25].

2.1.4.8 Respiratory tract

COX-II is induced by cytokines in a number of airway cells, including the epithelium and underlying smooth muscles. Asthma and related diseases are characterized by excessive proliferation of airway cells, which contributes to airway narrowing in some patients. COX-II induction inhibits proliferation of human airway smooth muscle cells, suggesting a protective role of this enzyme in diseases such as asthma. Stevenson et al[63] showed that cross-reactivity between aspirin and rofecoxib/celecoxib does not occur in patients with aspirin-sensitive respiratory diseases. Celecoxib has been shown not to cause asthmatic attacks in 33 subjects who had previously responded to two or more NSAIDs[64]. In 15 patients with challenge-proven NSAID-induced cutaneous reactions, aspirin, nimesulide, and diclofenac[65] caused urticaria, angioedema, and nonurticarial rash whereas, rofecoxib produced no reaction[66]. This clearly suggests that it is inhibition of COX-I that underlies the cutaneous reaction to aspirin and the other NSAIDs in these patients, possibly due to the release of a prostanoid 'brake' on leukotriene production.

2.1.4.9 Reproductive tract

It has been well established that prostanoids are involved in ovulation, fertilization, blastocyst implantation and onset of labor[67]. COX-II is induced during human labor whereas COX-I is not. For the process of parturition it may well be that COX-I is required for at least normal entry into parturition since its absence leads to prepartum fetal death. As the COX-I gene is not strongly inducible, it may be that this isoform mediates the initial stages of labor onset, in which uterine contractions and early phases of cervical dilatation begin, and that the COX-II isoform induced by the action of proinflammatory cytokines coming from the decidua, trophoblast, or fetal membranes

might then generate the prostanoids that sustain myometrial contractility and cervical ripening leading to expulsion of the fetus. In human studies, the COX-II-selective inhibitor nimesulide has been shown to reduce preterm labor and rofecoxib, has been shown to have a negative local effect on human ovulation, resulting in delayed follicular rupture without affecting peripheral hormonal cyclicity[68].

2.1.4.10 Gastrointestinal tract

Celecoxib and rofecoxib were shown to cause a significantly lower incidence of upper gastrointestinal adverse effects (perforations, ulcers, and bleeds) than conventional NSAIDs. In the VIGOR study[54], treatment with rofecoxib at twice the approved maximal dose for long-term use resulted in significantly lower rates of clinically important upper gastrointestinal events and complicated upper gastrointestinal events than did treatment with a standard dose of naproxen. Moreover, the incidence of complicated upper gastrointestinal bleeding from beyond the duodenum was significantly lower among patients who received rofecoxib. In the CLASS, incidences of symptomatic ulcers and/or ulcer complications were not significantly different from patients taking celecoxib *versus* NSAIDs who were also taking concomitant low-dosage aspirin, indicating that the use of low-dose aspirin may abrogate the gastrointestinal-sparing effects of cclecoxib[69]. In contrast, analysis of non-aspirin users alone demonstrated that celecoxib, at a dosage 2- to 4-fold greater than the maximum therapeutic dosage, was associated with a significantly lower incidence of symptomatic ulcers and/or ulcer complications compared with NSAIDs. However, selective COX-II inhibitors are associated with some dyspepsia with an incidence less than that seen with NSAIDs but higher than that with placebo[70].

Despite these clinical findings, there is evidence that COX-II is protective within the gastrointestinal tract by influencing ulcer healing and the associated angiogenesis. Both COX-1 and COX-II are expressed in human gastric mucosa, and COX-II selective inhibitors suppress the formation of prostanoids from healthy samples of human

gastric[71] and colonic tissue[72]. Thus various lines of evidence suggest that COX-II is expressed throughout the human gastrointestinal tract although, it must be noted that in healthy subjects it appears that COX-II selective drugs have relatively little effect on *in vivo* gastric prostanoid production[73]. In animal models of ulceration and gastrointestinal damage, COX-II generated PGs actually promote gastrointestinal healing, and inhibition of both COX-I and COX-II is required to produce acute gastrointestinal damage[74]. However, in the presence of noxious stimuli it may well be that the action of COX-I is more important in protecting gastric integrity[75]. Inhibition of COX-II may also delay esophageal ulcer healing by reducing ulceration-induced esophageal epithelial cell proliferation[76].

2.1.4.11 Healing and inflammatory resolution

Selective inhibition of COX-II has been reported to impair ligament and fracture healing in rat[77,78]. Selective COX-II inhibitors impair glomerular capillary repair following glomerulonephritis[79]. In mice, COX-II inhibition may decrease the early inflammatory phase of epidermal wound healing and reduce scar tissue formation without disrupting reepithelialization or decreasing tensile strength[80]. In carrageenan-induced pleurisy in rats, a late burst of COX-II protein expression has been reported during the resolution period and COX-II inhibition was associated with significantly exacerbated inflammation at 48 h. These observations led the authors to conclude that COX-II is important in the processes underlying inflammatory resolution in this model[77].

2.1.5 Selective COX-II Inhibitors: Potential Applications

Selective COX-II inhibitors have primarily been developed as GIT-sparing anti-inflammatory drugs. However their role may not remain restricted to being anti-inflammatory drugs. Apart from the role of COX-II in inflammatory sites, it is also involved in numerous physiological and pathophysiological functions as discussed

above. Additionally, COX-II expression may be upregulated at certain sites such as in kidney during sodium restriction, in microglia of cognitive centers within the hippocampus and cortex in AD, and in intestinal adenomas and colon tumors[81,82]. Implication of COX-II in certain of these diseased states has opened new avenues for the potential use of in prophylaxis/control of these diseases.

In addition to their reduced gastrointestinal toxicity profiles, several *in vitro, in vivo,* and clinical studies have demonstrated that COX-2 selective inhibitors may prevent colorectal cancer[83,84]. Although the precise molecular mechanism involved in the chemopreventive action of these inhibitors is not entirely understood, the COX-2 isoenzyme has proven to play a central role in the development of colorectal cancer through the promotion of angiogenesis, increased invasiveness, and antiapoptotic effects[85].

The long-term cardiovascular safety of COX-2 selective inhibitors was recently called into question with the results of two trials: the Adenomatous Polyp Prevention on Vioxx trial (APPROVe, rofecoxib) and the Adenoma Prevention with Celecoxib trial (APC, celecoxib)[86,87]. Both trials, which were conducted to evaluate the use of COX-2 selective inhibitors for the prevention of recurrence of colorectal polyps, revealed a higher incidence of cardiovascular events (death, myocardial infarction, and stroke) in patients taking the drugs for an extended period of time. Of particular note, the APPROVe trial enrolled only those patients who did not have a prior history of cardiovascular disease and was halted prematurely because of the 2- to 3-fold increased risk of cardiovascular events among patients in the group that was taking 25 mg of rofecoxib compared to placebo. This led to the withdrawal of rofecoxib from the worldwide market in 2004. It has since been reported that other COX-2 selective inhibitors (celecoxib, etoricoxib (5-chloro-6-methyl-3-[4-(methylsulfonyl)phenyl]-2,3-bipyridine) and valdecoxib (4-(5-methyl-3-phenylisoxazol-4-yl)benzenesulfonamide) **(Figure 2.1.4)** and some nonselective classical NSAIDs also might pose a risk for increased cardiovascular events[88-91].

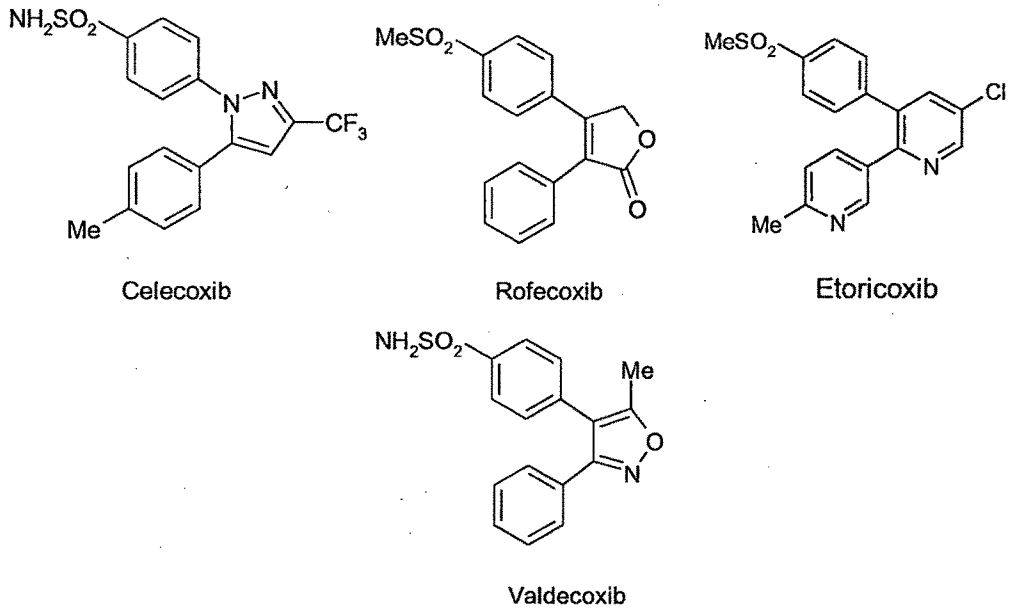


Figure 2.1.4 Selective COX-II inhibitors

Nevertheless, COX-II remains a very important pharmaceutical target for the treatment of debilitating diseases like rheumatoid arthritis and osteoarthritis and as a preventative agent for colon cancer. However, important questions remain unanswered concerning the benefit-risk profiles of traditional NSAIDs and both the diaryl heterocycle class of COX-II selective inhibitors and new, structurally distinct inhibitors like lumiracoxib (2-[(2-chloro-6-fluorophenyl)amino]-5-methylphenyl)acetic acid) that are selective for COX-II. There are several excellent reviews on the structure and mechanism of COX enzymes and the structure-function relationships of COX inhibitors[92-94].

2.1.5.1 Cancer

The studies carried out using various cancer cell lines showed the potent anti-angiogenic activity of COX-II inhibitors. Celecoxib demonstrated a potent anti-angiogenic activity in a rat model of angiogenesis, by suppression of corneal blood vessel formation[95,96]. Celecoxib suppressed induction of the colonic aberrant crypt foci (ACF) by azoxymethane (AOM) and inhibited colon tumor formation[95,96]. Moreover,

celecoxib has been approved for the treatment of patients with familial adenomatous polyposis[97,98]. COX-II inhibitors reduce the proliferation of gastric cancer cell lines and the formation of aberrant crypt foci in the rat. As far as colon is concerned, this effect may be directly associated with the inhibition of COX-II although, some studies have implicated other targets such as protein kinase C-β[99]. Rofecoxib reduces cell proliferation in Barrett's esophagus[100]. Celecoxib may enhance the anti-tumor efficacy of chemoradiation but, with increase in toxicity[101]. For more than 20 years NSAIDs have been known to reduce the proliferation of hepatoma cell lines. This effect is duplicated by selective COX-II inhibitors[102].

Rao *et al*[103] reported the over expression of the inducible isoform of nitric oxide synthase (NOS) and COX-II in colonic tumors of humans, as well as in colon tumors that developed in rats after the administration of the colon specific carcinogen. The combined administration of SC-51 and celecoxib inhibited the COX-II activity to a greater extent than the individual administration of both these agents. These findings showed that selective iNOS inhibitors may have chemopreventive properties and that their coadministration with a selective COX-II Inhibitor may have additional chemopreventive potential[103].

2.1.5.2 Alzheimer's disease (AD)

The fact that COX-II is elevated in areas related to memory (cortex, hippocampus) and the amount of COX-II correlates with the deposition of β-amyloid protein, representing a possible therapeutic benefit and a hopeful new strategy in the prevention or treatment of AD[104]. It has been suggested that some NSAIDs like ibuprofen, indomethacin and sulindac sulfide preferentially decrease the 42 amino acid form of β-amyloid protein[105,106]. But, disappointingly the most recent trial of rofecoxib and naproxen found no slowing of cognitive decline in patients with mild-to-moderate Alzheimer's disease over 12 months. Although the study failed to show a treatment benefit in people who already have AD, NSAIDs still could prove to be effective in

preventing the disease, therefore trials of NSAIDs for prophylactic use in AD are going on[107].

2.1.5.3 Glaucoma

Celecoxib and valdecoxib possess an unsubstituted arylsulfonmide moiety which is a common structural feature to many carbonic anhydrase (CA) inhibitors. Using enzyme kinetics and X-ray crystollography, Weber et al[108] demonstrated an unexpected nanomolar affinity of the COX-II specific arylsulfonamide-celecoxib and valdecoxib for isoenzymes of the totally unrelated carbonic anhydrase (CA) family such as CA-I, II, IV and IX whereas, the methylsulfone-bearing rofecoxib and valdecoxib lowered intraocular pressure, suggesting that these agents may have utility in the treatment of this disorder. The studies showed similarities between the sites of CA and COX-II enzymes, suggesting some potential to predict unexpected cross-reactivity with CAs may also contribute to difference in the pharmacological profiles, in particular with respect to glaucoma and anticancer therapy and may suggest new opportunities for use of these COX-II selective NSAIDs[108].

2.1.6 Molecular modeling studies

Chavatte et al[109] reported three-dimensional quantitative structure-activity relationship (3D-QSAR) studies using comparative molecular field analysis (CoMFA) of an extensive series of 305 varied diarylheterocyclic derivatives known as COX-2 selective inhibitors. X-ray crystal structure of COX-2 bound with SC-558, a selective COX-2 inhibitor, was used to derive the putative bioactive conformation of these inhibitors. A CoMFA study of COX-II inhibitors using receptor independent alignment was carried out by Datar and Co-workers[110]. They used a diverse set of 53 cyclooxygenase-2 inhibitors which were aligned in two different ways and subjected to CoMFA analysis. The first method of alignment of the molecules was based on the binding information sourced from the crystallographic study and the second mode of alignment was

generated by docking the inhibitors in the binding pocket using the DOCK and AFFINITY suite of programs.

Desiraju et al[111] reported CoMFA, CoMSIA and docking studies to optimize the structural requirements on the 1,2 diarylimidazole skeleton for their anti-inflammatory activities.

Recently, Shekhawat and group[112] analyzed the cyclooxygenase (COX) inhibition activities of the derivatives of 3-phenoxypyran-4-one through multiple-regression analysis (MRA). The derived significant correlation equation for COX-2 inhibition which suggested that the ortho-substituent with negative resonance parameter, the para-substituent with lower dipole moment and the meta-substituent having higher resonance parameter on the aryl ring were advantageous for the COX-II inhibitory activity.

QSAR studies of 2-acetoxyphenyl alkyl sulfides were performed using Bioloom, CAChe 6.1, and Dragon 3.0 for the COX-2 and COX-1 inhibition by Agrawal et al[113]. They suggested that lipophilicity affects both COX-1 and COX-2 inhibition in different manner and indicator variables like presence of aromatic ring and triple bond play an important role in COX-2 selectivity. Further, they also concluded that branching in the molecule and lesser number of carbonyl groups would be favorable for COX-2 inhibition.

Chaturvedi and Co-workers reported QSAR analysis of 2,3 diarylindoles[114], meclofenamic acid analogs[115], diarylthiophene derivatives[116], 1,5 diarylpyrazoles[117] and 2,3 diarylbenzopyran derivatives[118] as selective COX-II inhibitors.

Lee et al[119] reported Comparative molecular field analysis and comparative molecular similarity indices analysis on 25 analogs of pimarane COX-II inhibitor to optimize their

cyclooxygenase-2 (COX-II) selective anti-inflammatory activities.

Chakraborti and Thilagavathi[120] carried out 3D QSAR studies on a set of thirty five molecules of 1,3-diaryl-4,5,6,7-tetrahydro-2*H*-isoindoles endowed with selective COX-2 inhibitory activity using comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA). FlexX was further used to find out the binding orientation of this new class of 1,3-diaryl isoindoles in the active site of COX-2.

Garcia-Nieto and group[121] reported automated docking and molecular dynamics simulations of nimesulide in the active site of human COX-II. It was found that nimesulide exploits the extra space made available by the replacement at position 523 of an isoleucine residue in COX-1 by a valine in COX-2 and establishes electrostatic interactions with both Arg-106 and Arg-499 (Arg-120 and Arg-513 in PGHS-1 numbering).

Pedretti et al[122] reported binding interactions of some selective COX-II inhibitors with the COX-I active site. Their aim was to identify key interactions for COX-II selectivity. Moziconacci et al[123] optimized and validated a docking scoring protocol which was further used for the virtual screening of selective COX-II inhibitors.

2.2 Farnesyltransferase (FTase), inhibitors and related studies

2.2.1 Introduction

The most occurring mutations in cancer are alterations of the Ras genes [Harvey Ha), Kirtsen Ki) and N-Ras][124]. The Ras genes encode 21-kDa proteins, called p21 or Ras, which are localized to the inner face of the plasma membrane. Ras binds GTP and GDP and serves as a molecular switch, interfacing between receptors and intracellular

effector proteins. When Ras is stimulated by receptor activation to bind GTP, it promotes cell proliferation. The GTPase activity of Ras then turns off the biological event. The mutations in Ras genes most often found in cancer inhibit Ras GTPase activity, so Ras remains bound to GTP and constitutively stimulates cell growth. Alteration in the function of Ras gene products, particularly Ki4B-Ras and N-Ras, are found in many different tumors, including carcinomas of the colon, pancreas and lung, neurofibrosarcomas, and various leukemias, including both adult and juvenile chronic myelogenous leukemia[125,126].

Many efforts have been made to inhibit the function of oncogenic Ras and subsequent cellular transformation induced by mutant Ras[127,128]. Much emphasis has been given towards developing novel chemotherapeutics against Ras induced cell transformation and has centered on inhibiting the enzyme farnesyl protein transferase[129]. The discovery that, Ras required prenylation for activity led to the targeting of the enzyme farnesyl protein transferase[130,131]. In this regard, the posttranslational modifications have attracted much attention, because they are required for appropriate sub-cellular localization of Ras proteins in the plasma membrane[130]. Thus farnesyltransferase inhibitors were conceived as a rational way to treat cancer by inhibiting the function of the oncogene Ras.

2.2.1 Structure and Function of Farnesyltransferase

Protein farnesyltransferase, a metalloenzyme catalyzes the reaction between Farnesyl diphosphate (FPP) and the cysteine residue of a polypeptide's C-terminal CaaX motif where, C is Cys; a is usually an aliphatic amino acid; X is the C-terminal residue (typically Met) to give a farnesyl thioether **(Figure 2.2.1.1)**[132].

The enzyme is a heterodimer consisting of 48 kDa 'α 'and 46 kDa 'β' subunits. The X-ray structure of farnesyltransferase enzyme bound to a benzonitrile inhibitor is shown in **Figure 2.2.1.2** (pdb code: 1MZC). The former subunit is also a component of the

closely related enzyme geranylgeranyltransferase type I (GGTase-I), which utilizes geranylgeranyl diphosphate (GGPP) as a prenyl donor and has different CaaX specificity. Farnesylation is usually followed by additional modification of the prenylated protein, including proteolytic removal of the last three residues of the "CaaX box" and carboxymethylation of the new C-terminal farnesylated cysteine[133].

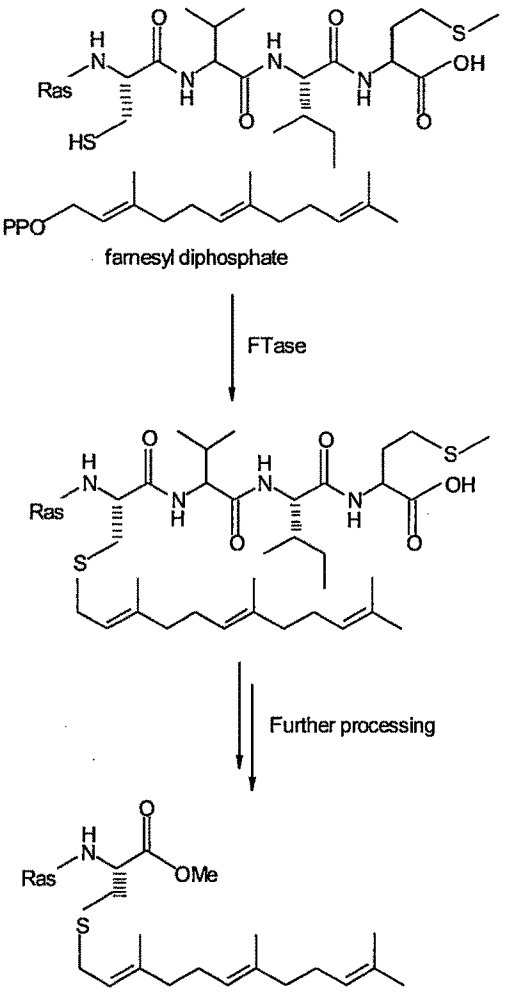


Figure 2.2.1.1 Post translational modification of Ras.

Crystallographic studies and enzymology has helped to understand the mechanistic details of catalysis by FTase[134]. Long and co-workers afforded an excellent overview of this enzyme catalyzed process[135]. The reaction proceeds *via* an ordered mechanism,

with FPP binding first, followed by CaaX substrate. The structures of ternary complexes using non reactive FPP or peptide analogs provide an explanation for this ordering. The isoprenoid moiety forms a substantial part of the binding surface for the CaaX peptide.

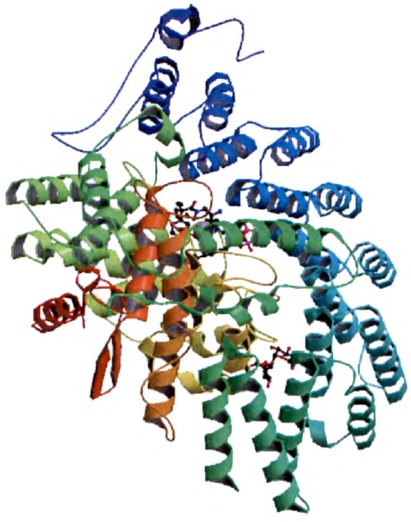


Figure 2.2.1.2 X-ray structure of farnesyltransferase bound to a benzonitrile inhibitor.

The peptide binds in an extended conformation with the cysteine sulfur coordinated to the active site zinc ion, which apparently lowers the pKa of the thiol, significantly increasing the local concentration of the reactive thiolate[136,137]. It is proposed[135] that a rotation of the FPP backbone brings its C_1 carbon into proximity with the thiolate, leading to a transition state that is consistent with previous mechanistic studies. Release of the farnesylated peptide product is slow and requires the addition of FPP[138]. The FPP displaces the product isoprenoid group to a new binding site, the 'exit groove', with concomitant change in the CaaX backbone from an extended to a β-turn conformation. Product release regenerates the FTase/FPP complex. The published structures have revealed that FTIs may inhibit the enzyme by blocking the peptide substrate site or by occupying part of the peptide site and the exit groove in a manner similar to the farnesylated peptide product[123].

2.2.2 Farnesyltransferase inhibitors: Mechanism of action

Due to the functional role of Ras farnesylation, FTase inhibition was thought to be a strategy for interfering with Ras-dependent transformation[139-143]. Farnesylation is catalyzed by FTase, which is responsible for catalyzing farnesylation of several cellular proteins characterized by a CaaX box, transferring a C-15 farnesyl moiety from FPP to Cysteine. The three main Ras proteins share high homology in the first 165 amino acids, but show difference in 25 amino acids of the carboxy-terminal region that constitutes the hypervariable region. The post-translational modifications, beginning from amino acid 186 (always cysteine), increase the hydrophobicity of the carboxy-terminal region of the protein. The post translational modifications are the farnesylation of C-186 by the cleavage of three downstream amino acids aaX, followed by methylation of C-186 and finally a palmitoylation of cysteine residues in the region 165-186. Cysteine mutation in the CaaX box prevents farnesylation and Ras function. Mammalian N- and H-Ras proteins contain additional cysteine near their carboxy-termini (181 for N-Ras; 181 and 184 for H-Ras), and these serve as palmitoylation sites. K-Ras4B lacks this upstream cysteine and is prenylated, proteolyzed and methylated but not palmitoylated. Instead, K-Ras4B has a cluster of 8 lysine residues near its carboxyl terminus that are thought to function by forming electrostatic contacts with negatively charged phospholipids[144-147].

Studies have shown that farnesylation of Ras is the obligatory, first step in a series of post-translational modifications that leads to membrane association which determines the switch from an inactive to an active Ras-GTP bound form. Upon receiving a signal output, Ras-GTP acts as a molecular switch that turns on downstream effectors[144-147].

Ras has guanine nucleotide-binding activity and GTPase activities. Thus, Ras has GDP-bound inactive and GTP-bound active forms, which are intercompatible by the GDP/GTP exchange and GTPase reactions. The GTPase reaction is regulated by GTPase-activating proteins (GAPs), such as p120, GAP, NF1, and Gap1. The GDP/GTP exchange is regulated by Guanine Nucleotide Exchange Factors (GEFs). Ras is known

to be the downstream molecule of receptor tyrosine kinases, such as Epidermal Growth Factor (EGF) and protein kinase C. The activation of these proteins results in the stimulation of GEFs and the conversion from the GDP-bound form to GTP-bound form of Ras. Another Ras effector is the GEFs family RalGDS, (RGL and Rlf/RGL2) that serves as activators of the Ral small monomeric GTPases[139].

The third Ras effector is the phosphatidylinositol 3-kinase (PI3-K). Ras-GTP can bind and activate the catalytic subunit of this enzyme and act directly like a second messenger, binding several cytoskeleton kinase proteins and modulating the activity by conformational changes and/or their membrane translocation[147].

These observations guide us to understand the complex relationship between Ras activation, cellular proliferation and apoptosis. Thus, Ras can induce simultaneously anti/pro apoptotic pathways in function of quality and intensity activating signals, in function of cell type and metabolic conditions, and by activation of other Ras-independent or dependent pathways. Further, understanding of these processes would help identify the factors directly responsible for cell cycle deregulation in several tumors and would help in the design of specific strategies for the control on the proliferation of neoplastic cells.

Mutation in Ras genes have all been found in human tumors and the frequency of Ras mutations were found to be highest among genes in human cancers[148]. Activating mutations of Ras are found in humans in nearly all pancreatic cancers, one half of colon and thyroid tumors and one-third of lung tumors[148]. A divergent association of Ras mutations with distinct neoplasm is also seen when compared in humans and rodents. For example, Ras mutations are strongly associated with the carcinogen-induced mammary carcinomas in mice and rats[149] but such mutations are rare in human breast cancers[148]. Activating point mutations of Ras genes occur in approximately 30 % of human cancers, with certain malignancies having a particular high incidence[148]. The incidence of K-Ras mutations in pancreatic cancers approaches 90 %, while mutations

in K-Ras are detected in 50 % colorectal carcinomas and 30 % Non Small Cell Lung Cancers (NSCLC).

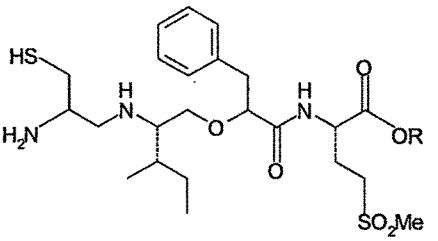
2.2.3 Development of farnesyltransferase inhibitors (FTIs)

Various structurally diverse classes of FTIs have been reviewed[150-153]. Here, emphasis is given only to the efforts that lead to development of various clinical candidates. The general approaches used for the development of FTIs are:

1. Design and synthesis of farnesyl diphosphate (FDP) analogs that compete with the substrate FDP for FTase.
2. Design and synthesis of peptide and non-peptide CaaX peptidomimetics that compete with the CaaX portion of Ras for FTase.
3. Design and synthesis of bisubstrate analogs that combine features of both FDP analogs and peptidomimetics.

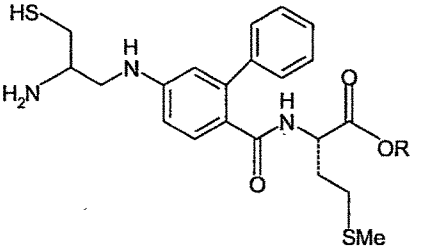
Even though potent FDP analogs have been discovered, much attention has been given to the analogs of the CaaX peptide[150]. Two of the most notable CaaX derived inhibitors are L-739,750 (**1**, Merck; FTase IC_{50} 1.8 nM)[154] and FTI-276 (**2**, Hamilton and Sebti group; FTase IC_{50} 0.6 nM)[155], and their ester prodrugs L-744,832 (**3**) and FTI-277 (**4**), respectively. L-744,832 was found to exhibit impressive efficacy in a transgenic mouse model of cancer[154]. Despite encouraging *in vivo* data, skepticism prevailed about the clinical utility of **2** because of its potential for thiol-based toxicity. Nonetheless, the AstraZeneca compound AZD3409 (**5**), which has both the key thiol and carboxylate groups blocked in a double prodrug strategy advanced to clinical stage[156]. An extensive effort to address the concern about the peptidomimetic mercaptan and carboxylate groups was undertaken by scientists at Merck. Truncation of the CaaX tetrapeptide and subsequent re-engineering gave rise to the piperazine based non-peptide thiol (**6**) (FTase IC_{50} 1 nM)[157].

Another early success was the identification of a general thiol replacement in 4-cyanobenzyl imidazole.



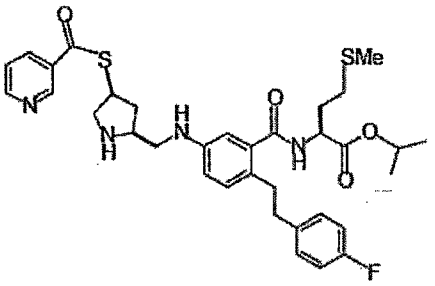
1 R = H; FTase IC_{50} = 1.8 nM

3 R = *i*-Pr

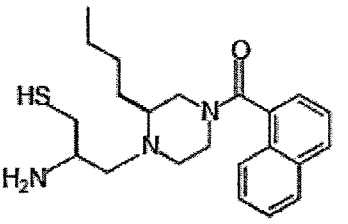


2 R = H; FTase IC_{50} = 0.6 nM

4 R = Me



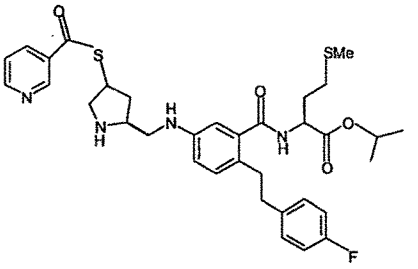
5 AZD 3409



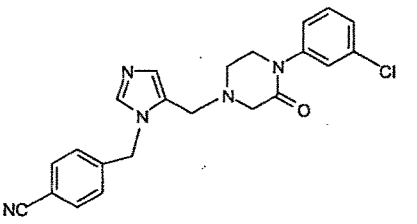
6; FTase IC_{50} = 1 nM

This was designed to simultaneously ligate the active site zinc ion and occupy a nearby hydrophobic binding site, which resulted in a potent peptidomimetic FTI like 7 (FTase IC_{50} 0.15 nM)[158]. Fusion of the non-peptide 6 with the thiol replacement in 7 and further optimization of the piperazine template led to piperazinone FTIs, which displayed significantly improved cell based activity compared with the earlier peptidomimetic FTIs[159]. For example the Merck clinical candidate L-778,123 (8, FTase IC_{50} 2 nM) inhibited the growth of H-Ras transformed cells with an IC_{50} value of 15 nM. Studies directed at optimization of such non-peptide cyanobenzylimidazoles utilized transferred Nuclear Overhauser Effect (NOE) data to design macrocyclic FTIs such as compound 9 (FTase IC_{50} 0.2 nM)[160]. Members of this class of FTIs exhibited the highest cell potency yet described for inhibitors of FTase. Researchers at Bristol-Myers Squibb sought analogues of thiol 6, in which the mercaptan was replaced by imidazole[161] and

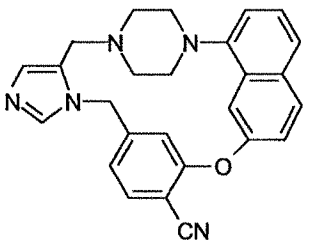
evolved their initial micromolar leads into a tetrahydrobenzodiazepine based series of compounds represented by **11** (FTase IC_{50} 24 nM)[162].



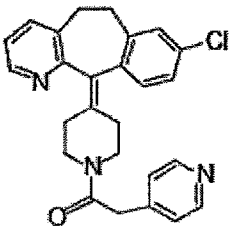
7; FTase IC_{50} = 0.15 nM



8; FTase IC_{50} = 2 nM

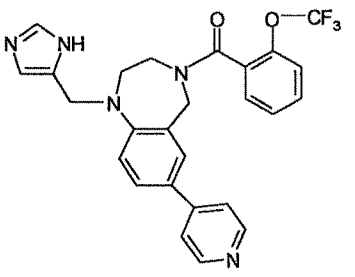


9; FTase IC_{50} = 0.2 nM

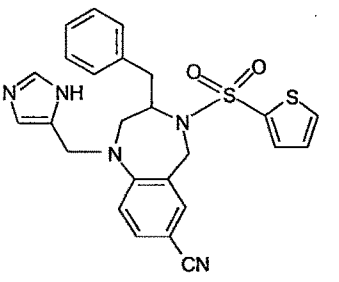


10; SCH-44342; FTase IC_{50} = 250 nM

It was discovered that the potency of analogues such as **11** was enhanced by addition of a phenylmethyl moiety at the 3-position and replacement of the 4-position amide with a sulfonamide.



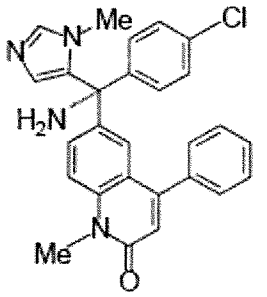
11; FTase IC_{50} = 24 nM



12; FTase IC_{50} = 1.4 nM

Incorporation of a 7-cyano substituent simultaneously improved the potency and aqueous solubility and these modifications led to the clinical candidate BMS-214662 (**12**, FTase IC_{50} 1.4 nM). Screening of chemical libraries at Schering-Plough led to the

identification of micromolar FTIs close[163]. Exploration of structure-activity relationships led to SCH-44342 (**10**), which exhibited significantly, enhanced potency (FTase IC_{50} 250 nM).



13, R115777; ZARNESTRA; FTase IC_{50} = 0.86 nM

R115777 (**13**, tipifarnib, ZARNESTRA; FTase IC_{50} 0.86 nM), is the first FTI to advance to human clinical trials[164]. Dalton[165] et al at Karolinska Institute, Stockholm in collaboration with Phillips University, Leibnz Institute and Ludwig-Maximilians University in Germany recently reported benzophenone-based FTase inhibitors, highly active against Trypanosoma Cruzi. These inhibitors can be helpful in the treatment of Chaga's disease. Researchers at the University of Washington also identified Tipifarnib as a potent inhibitor of Trypanosoma Cruzi with ED_{50} of 4 nM.

2.2.4 Molecular modeling studies

Very few *in silico* attempts have been made to explore the structural requirements responsible for farnesyltransferase inhibition. Corey and co-workers had earlier reported the crystal structure of farnesyl protein transferase complexed with a CaaX peptide and farnesyl diphosphate analog[166]. He reported in the ternary complex, the bound substrates are within van der Waals contact of each other and the farnesyl protein transferase (FPT) enzyme. α-Hydroxyfarnesylphosphonic acid (α-HFP) binds in an extended conformation in the active site cavity where positively charged side chains and solvent molecules interact with the phosphate moiety and aromatic side chains

pack adjacent to the isoprenoid chain. The backbone of the bound CaaX peptide adopts an extended conformation, and the side chains interact with both FPT and α-HFP. The cysteine sulfur of the bound peptide coordinates the active-site zinc. Pete and co-workers proposed a model for substrate binding[167]. They concluded that both farnesyl diphosphate and peptide substrates can be accommodated in the hydrophobic active-site barrel, with the sole charged residue inside the barrel, Arg202 of the β-subunit, forming a salt bridge with the negatively charged carboxy terminus of peptide substrates.

In order to clarify how PFTase discriminates between FPP and larger prenyl diphosphates, Tammy and his co-workers have examined the interactions between the enzyme and several isoprenoid analogs, GGPP, and the farnesylated peptide product using a combination of biochemical and structural methods. Comparison of the GGPP binding mode with the binding mode of the farnesylated peptide product suggests that the bulkier isoprenoid cannot rearrange to convert to product without unfavorable steric interactions with the acceptor protein. They proposed a 'second site exclusion model' in which PFTase binds larger isoprenoids in a fashion that prevents the subsequent productive binding of the acceptor protein[168].

Markus and co-workers using benzophenone based farnesyltransferase inhibitors reported two novel aryl binding sites. One is located in close proximity to zinc ion and is defined by the aromatic side chains Try 300β, Trp 303β, Tyr 361β and Tyr 365β. The second aryl binding site is defined by the side chains of Try 300β, Leu 295β, Lys 294β, Lys 353β and Lys 356β. This second aryl binding site has been used for the design of non-thiol farnesyltransferase inhibitors[169].

Modeling of binding modes and inhibition mechanism of some natural ligands of farnesyltransferase using molecular docking was carried out by Alessandro and co-workers. Their aim was to show that the molecular docking analysis can be successfully used to underline the inhibition mechanism of the natural ligands. They used BioDock

program for detailed docking analysis and the results were compared with the binding sites for natural substrates reported earlier. They have shown how the binding modes of the compounds can be related to dipole values, which are appreciably less for peptidomimetics than for FPP mimetic and reveal a straightforward method to predict and understand the farnesyltransferase inhibition mechanism[170].

In an effort to develop isoprenoid diphosphate-based FTase inhibitors, striking variations have been observed in the ability of modified analogs to bind to enzyme. For example 2Z-GGPP is an alternative substrate with high binding affinity, while GGPP is not an alternative substrate. Using this view Brian and co-workers have used pharmacophore and docking studies to elucidate a new binding pocket for isoprenoid analogs. The unique conformations between the first two isoprene units of 2Z-GGPP, but not GGPP, allows 2Z-GGPP to exploit this new binding pocket. These studies suggest that ligand conformational flexibility may be an important design consideration for the development of both inhibitors and alternative substrates of FTase[171]. Further using this information, Perola and co-workers have successfully applied virtual screening methodology for developing new leads as FTase inhibitors[172].

Polly and co-workers for the first time reported a QSAR study of the potency and selectivity of a large, diverse data set of farnesyltransferase inhibitors. They used novel molecular descriptors based on binned atomic properties and invariants of molecular matrices and a robust, nonlinear QSAR mapping paradigm, the Bayesian regularized neural network. They were successful in building robust QSAR models for farnesyltransferase and geranylgeranyltransferase inhibition. Further, they have derived a novel selectivity index that allows us to model potency and selectivity simultaneously and have built robust QSAR models using this index that have the potential to discover new potent and selective farnesyltransferase inhibitors[173].

Using pharmacophore modeling Schlitzer developed a pharmacophore model for CaaX-peptidomimetics. On the basis of this model, bisubstrate analogs were developed

as one class of non-thiol farnesyltransferase inhibitors. In most nonthiol farnesyltransferase inhibitors known in literature nitrogen containing heterocycles are used as cysteine replacements assuming that they coordinate the enzyme bound zinc. However, Schlitzer have shown that nitrogen heterocycles can be replaced by aryl residues lacking the ability to coordinate metal atoms, an observation which led to the postulation of two hitherto unknown aryl binding sites. Using flexible docking of model compounds and GRID analysis they were able to locate these postulated aryl binding sites. Subsequently, they used one of this aryl binding sites for the structure based design of highly active nonthiol farnesyltransferase inhibitors[174]. Recently, Equbal et al developed a pharmacophore model using CATALYST Hypogen program with a set of 22 structurally diverse farnesyltransferase inhibitors which were carefully selected with great diversity in both molecular structure and bioactivity for discovering new potent inhibitors[175].

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Aims and objectives

New drug discovery is a complex time and money consuming process. There is no set procedure for discovering a new drug molecule. More of intuition and less of science has remained in discovery of new drug molecules in the past. Slowly the situation is changing and more of scientific reasoning is getting involved in the process of drug discovery. Rational drug design is a part of drug discovery process involving more and more of scientific logic in the process. The advent of computers in the drug discovery process has revolutionized medicinal chemistry in understanding the enzyme/receptor-drug binding interactions. Hence it was planned to apply both two-dimensional and three-dimensinal QSAR techniques to explore the structure activity relationship studies for selective COX-II and farnesyltransferase inhibitors.

As COX-II is involved in diseases like cancer, inflammation, arthritis, glaucoma, it was one of our target of choice for carrying out the *insilico* experiments. Till date many selective COX-II inhibitors have been reported and blockbuster drug rofecoxib have been withdrawn from the market. Celecoxib is also under scrutiny for its side effects. Hence it was planned to explore the structural activity relationship for selective COX-II inhibitors using the powerful techniques like CoMFA and CoMSIA.

Farnesyltransferase was chosen as a second target for the *in silico* studies due to its involvement in cancer and it has been less explored. Most of the farnesyltransferase inhibitors failed in clinical trials. Hence it was planned to perform in silico studies and provide structural insights into the designing of more potent farnesyltransferase inhibitors.

Studies on selective COX-II inhibitors:

- Two-dimensional QSAR studies on diarylimidazoles as selective COX-II inhibitors to identify parameters that govern COX-II inhibitory activity and selectivity.

- To explore structure activity relationship for furanone derivatives as selective COX-II inhibitors using 3D QSAR CoMFA and CoMSIA techniques.
- To study the effect of partial charges and alignment rules on the statistical quality and predictivity ability of CoMFA models.
- To analyze the effect of additional descriptor (binding energy) on the predictive power of CoMFA model.
- To develop a universal 3D CoMFA model for COX-II inhibitors.

Studies on farnesyltransferase inhibitors:

- Develop CoMFA model for benzonitrile derivatives as farnesyltransferase inhibitors.
- Understand the structure activity relationship between tricyclicpiperazinyl derivatives and its farnesyltransferase inhibitory activity using CoMFA and CoMSIA studies.
- Develop 3D QSAR CoMFA and CoMSIA models for aminopyrridinone, aminonicotinonitrile and imidazolyl ether derivatives as farnesyltransferase inhibitors. Overlap the contour maps on the active site to identify possible sites of interaction.