Chapter 1

INTRODUCTION

1

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Plant-derived salicylates have been used traditionally by mankind since long for the treatment of pain and fever[1]. After the development of aspirin in the late 1800's, numerous other drugs were discovered and used as antipyretics, analgesics and anti-inflammatories. These drugs were regarded as a group and became known as "aspirin-like drugs"[2,3,4]. The term nonsteroidal anti-inflammatory drugs (NSAIDs) were first applied to phenylbutazone after its introduction into the clinical practice in 1949, three years later after the demonstration of the anti-inflammatory properties of glucocorticoids[5].

Nonsteroidal anti-inflammatory drugs are among the most widely prescribed[6] and used drugs in the community for rheumatologic as well as non-rheumatologic conditions, which include acute and chronic pain, biliary and uteric colic, dysmennorrhoea, fever and other applications[7,8]. Worldwide, more than 13 million people consume NSAIDs daily. There are now more than 50 different NSAIDs in the market[9,10] Commonly used NSAIDs include aspirin, indomethacin, ketorolac, diclofenac, ibuprofen, naproxan, ketoprofen, flurbiprofen, mefenamic acid, piroxicam and many more in the ever-growing list of these drugs. But, none of them are ideal in controlling or modifying the sign and symptoms of inflammation, particularly in the common inflammatory joint diseases. The continuing research for the ultimate NSAIDs is driven by the high demand for better therapies for disabling musculoskeletal syndrome and by the dissatisfaction with the efficacy or gastrointestinal side effects of available NSAIDs. Although certain newer agents may offer safety and tolerability benefits over older agents, NSAIDs continue to be one of the most common group of drugs associated with serious side effects[11,12].

In 1971, Vane[13] reported the landmark finding that NSAIDs, including aspirin, inhibit prostaglandin biosynthesis Now it is evident that the therapeutic efficacy of NSAIDs is due to inhibition of cyclooxygenase (COX) and side effects are related to inhibition of COX-I, one of the two isoforms of COX, which exerts a cytoprotective action in the gastric mucosa and promotes normal renal function in the kidney[14-17].

1.1 BIOSYNTHESIS OF PROSTANOIDS AND LEUKOTRIENES

Two distinct families of autacoids that are derived from membrane phospholipids have been identified as the eicosanoids and platelet-activating factor (PAF).

Eicosanoids formed from certain polyunsaturated fatty acids (principally, arachidonic acid) include the prostaglandins, prostacyclin, thromboxane A_2 and the leukotrienes. In human, eicosanoids (Greek *eikosi*, twenty) are produced by the oxidation of 20-carbon fatty acids that are derived from the two essential fatty acids, linolenate and linoleate. Eicosanoids are potent local hormones that are released by most cells, act on same cells or nearby cells and then are rapidly inactivated. Their production increases in response to diverse stimuli. They produce a broad spectrum of biological effects[18,19].

In human beings, arachidonate is the most abundant precursor of eicosanoids. Arachidonate is either derived from dietary linoleic acid (9, 12-octadecadienoic acid) or ingested as a dietary component. Arachidonate is esterified to the phospholipids of cell membrane or other complex lipids. Thus, concentration of free arachidonate in the cell is very low. Biosynthesis of eicosanoids depends on availability of eicosanoid synthesizing enzymes i.e. phospholipase A_2 and diacylglycerol lipase[20]. The enhanced biosynthesis of eicosanoids occurs in response to physical, chemical and hormonal stimuli.

Hormones, autacoids and other substance augment the biosynthesis of eicosanoids by interacting with plasma membrane-bound receptors that are coupled to G proteins (GTP-binding regulatory proteins). Interaction of hormones and autacoids to plasma membrane bound receptors result in either direct activation of phospholipases (C and /or A_2) or in elevated cytosolic concentration of Ca^{+2}, which also activate these enzymes[21]. Physical stimuli are believed to cause an influx of Ca^{+2} by perturbing the cell membrane, thereby activating phospholipase A_2. Phospholipase A_2 hydrolyzes the sn-2 ester bond of membrane phospholipids. In contract, phospholipase C cleaves the phosphodiester bond, resulting in the formation of a 1,2-diglyceride. Arachidonate is then released from the diglyceride by the sequential actions of diglyceride lipase and monoglyceride lipase[22]. Once released, a portion of the arachidonate is metabolized rapidly to an oxygenated product by several distinct enzyme systems, including cyclooxygenases, lipoxygenases or cytochrome P450s.

Product of cyclooxygenases and lipoxygenases

Synthesis of PGs is accomplished in a stepwise manner by a ubiquitous complex of microsomal enzymes. The first enzyme in this synthetic pathway is fatty acid cyclooxygenase (COX), also called prostaglandin endoperoxide synthase or prostaglandin H synthase (PGHS). There are two isoforms of this enzyme, COX-I and COX-II[23-24]. COX-I is constitutively expressed in most cells. In contrast, COX-II is not normally present but may be induced by certain serum factors, cytokines and growth factors. COX-II can be inhibited by treatment with glucocorticoids such as dexamethasone.

The cyclooxygenase has two distinct activities:

(1) An endoperoxide synthase activity that oxygenates and cyclizes the arachidonic acid (AA) to form the cyclic endoperoxide PGG_2 and

(2) A peroxidase activity that converts PGG_2 to PGH_2 (Fig. 1)[25].

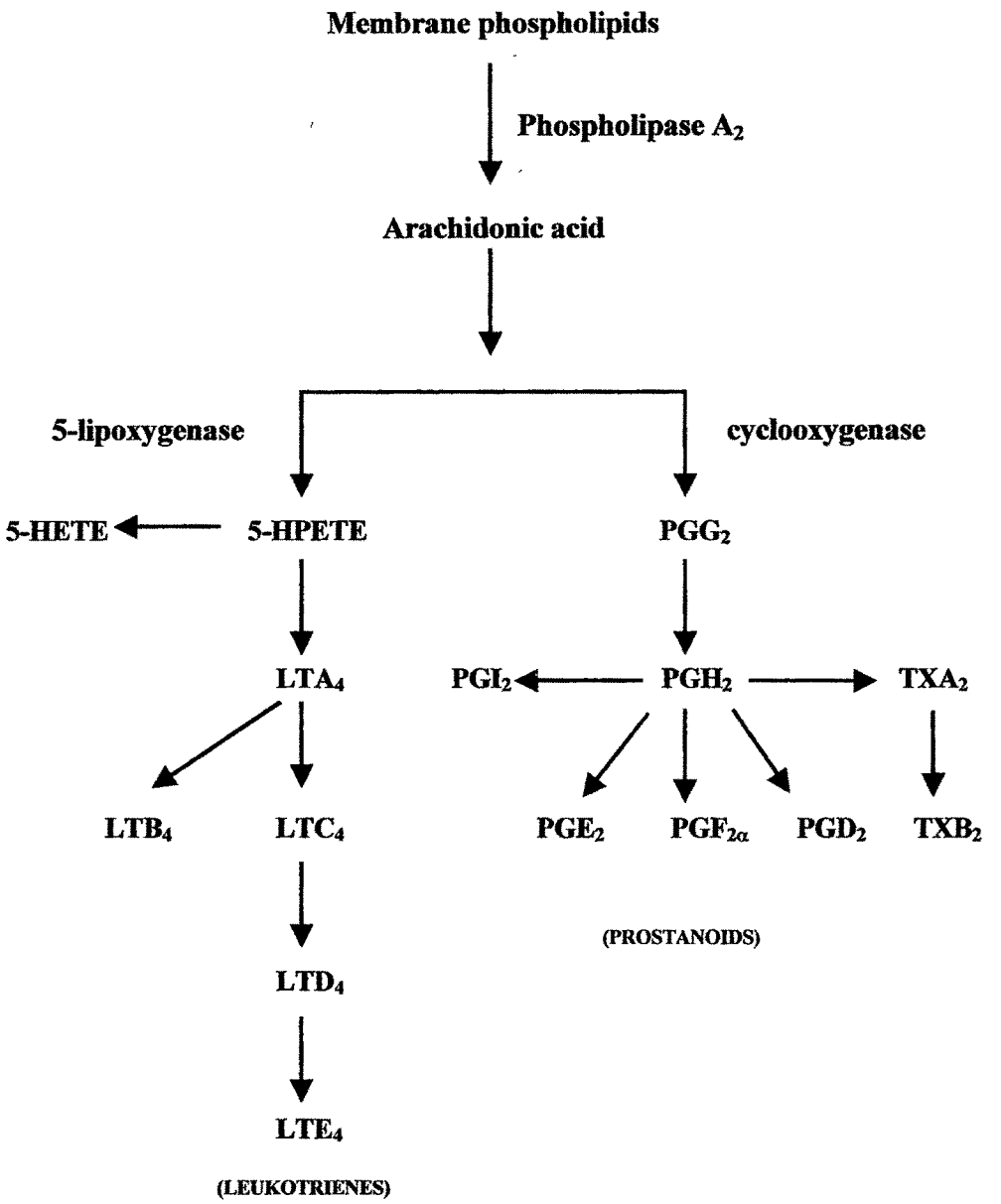


Fig. 1. Cyclooxygenase and lipoxygenase pathway.

PGG_2 and PGH_2 (cyclic endoperoxides) are chemically unstable, but can be transformed enzymatically into a variety of products, including PGI_2,

TXA_2, PGE_2, PGF_2 or PGD_2. Isomerases for synthesis of PGE_2 and PGD_2 have been identified. A 9-keto reductase catalyzes the inter conversion of PGE_2 and $PGF_{2\alpha}$ in some tissues[11,12].

The endoperoxide PGH_2 is also metabolized into two unstable and highly active compounds, TXA_2 and PGI_2. TXA_2 is formed by the action of thromboxane synthase. TXA_2 breaks down nonenzymatically ($t_{1/2}$ 30 sec) into stable, but inactive TXB_2. PGI_2 is formed from PGH_2 by prostacyclin synthase; it is hydrolyzed non-enzymatically ($t_{1/2}$ 3 min) into the inactive 6-keto, $PGF_1\alpha$[26-28].

Lipoxygenases are a family of cytosolic enzymes that catalyze the oxygenation of polyenic fatty acid to corresponding lipid hydroperoxides[10]. 5-lipoxygenase is the most important lipoxygenase in lipoxygenase pathway as it leads to synthesis of leukotrienes (LTs), LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4 (Fig. **1**).

1.2 THE ROLE OF PROSTANOIDS IN INFLAMMATION

The inflammatory response is always accompanied with the release of prostanoids and leukotrienes[10,29,30]. In areas of acute inflammation, mast cells release PGD_2. PGE_2 and PGI_2 are generated by the local tissues and blood vessels ($t_{1/2}$ = 5-10 min) due to inflammation. In chronic inflammation, monocytes and macrophages also release PGE_2.

The vasoconstriction that occurs immediately after injury is followed by a prolonged period of vasodilatation, mediated by PGE_2, PGI_2, PGD_2 and more weakly by LTB_4 and PAF[31,32]. PGE_2 and PGI_2 also have synergistic effect with histamine and bradykinin. The resultant increased blood flow due to dilation of blood vessels brings about erythema (redness) at inflammed area. This is the first symptom of inflammatory response.

Vasoconstriction effect is due to TXA_2. TXA_2 is an extremely short lived ($t_{1/2}$ = 30-40 sec) but potent vasoconstrictor of most vascular smooth muscle. TXA_2 appears to mediate the response of PAF_2, PGE_2 and LTB_4[33,34].

Dilatation of the arterioles by these pro-inflammatory mediators leads to elevated hydrostatic pressure in the venules and an increase in the vascular wall area. The net result is the development of vascular permeability. The exudation of plasma from the permeabilized blood vessels into connective tissue produces edema, the second symptom of inflammation. LTB_4 and PAF are produced by leukocytes and act with histamine and bradykinin to induce permeability in the dilated venules. Prostanoids do not directly increase the permeability of post capillary venules but they potentiate the effect of histamine and bradykinin. Similarly, prostanoids do not themselves produce pain but potentiate the effect of bradykinin by sensitizing afferent nerves[35].

PGs of the E series are also implicated in the production of fever, high concentrations of them are found in cerebrospinal fluid (CSF) infections and there is evidence that the increase in temperature generated by endogenous fever-inducing agents such as interleukin-1 is mediated by PGE_2 The antipyretic effect of NSAIDs is due partly to inhibition of the synthesis of PGE_2 in the hypothalamus[35].

It is thought that leukotrienes are the main mediators of both the early and late phases of asthma. LTB_4 can also be found in inflammatory exudates and is present in many inflammatory conditions, including rheumatoid arthritis, psoriasis and ulcerative colitis[36].

1.3 MECHANISM OF ACTION OF NSAIDs

Corticosteroids inhibit the activity of phospholipase A_2 and hence reduce the release of arachidonic acid and ultimately inhibit the formation of proinflammatory prostaglandins. Vane[27] made the seminal proposal in 1971 that in contrast to steroids, NSAIDs exerted their activity by inhibiting cyclooxygenase, a dual function enzyme[37,38]. Prostaglandins are formed by the oxidative cyclization of the central 5 carbons within the 20-carbon polyunsaturated fatty acid. The key regulatory enzyme, COX catalyses two sequential reactions: the initial cyclooxygenase reaction, which is also a target of NSAIDs, that is bis-oxygenation of arachidonic acid at C-11 and C-15 which gives PGG_2, while the subsequent peroxidase reaction converts PGG_2 to PGH_2 through endoperoxidation[25]. NSAIDs cannot inhibit peroxidase activity[39]. These two chemical reactions occur at two distinct active sites of the COX enzymes. In addition to prostaglandins, COX enzymes also generate minor amount of hydroxyeicosatetraenoic acids (HETEs), which are mono-oxygenated metabolites of arachidonic acid[40].

1.3.1 CYCLOOXYGENASE CATALYSIS

Substantial evidence supports the hypothesis that COX oxygenates arachidonic acid by a free radical mechanism (Fig. **2**).

Chemical steps involved in the conversion of arachidonic acid to PGG_2

The enzyme removes the 13-*pro(S)* hydrogen, which generates a pentadienyl radical with maximal electron density at C-11 and C-15. Trapping of the carbon radical at C-11 with O_2 produces a peroxyl radical, which adds to C-9 generating cyclic peroxide and a carbon-centered radical at C-8. The C-8 radical adds to the double bond at C-12, generating the bicyclic peroxide and an allylic radical with maximal

electron density at C-13 and C-15. Trapping of the carbon radical at C-15 with O_2 generates a peroxyl radical, which is reduced to PGG_2[41] (Fig. **2**).

The first step of removal of proton was found to be critical. The oxidant that removes 13-*pro(S)* hydrogen appears to be tyrosyl radical derived from Tyr 385 of cyclooxygenase[42]. This residue is interposed between the heme prosthetic group and cyclooxygenase active site (Fig. **3**) and is ideally positioned to interact with bound fatty acid molecule.

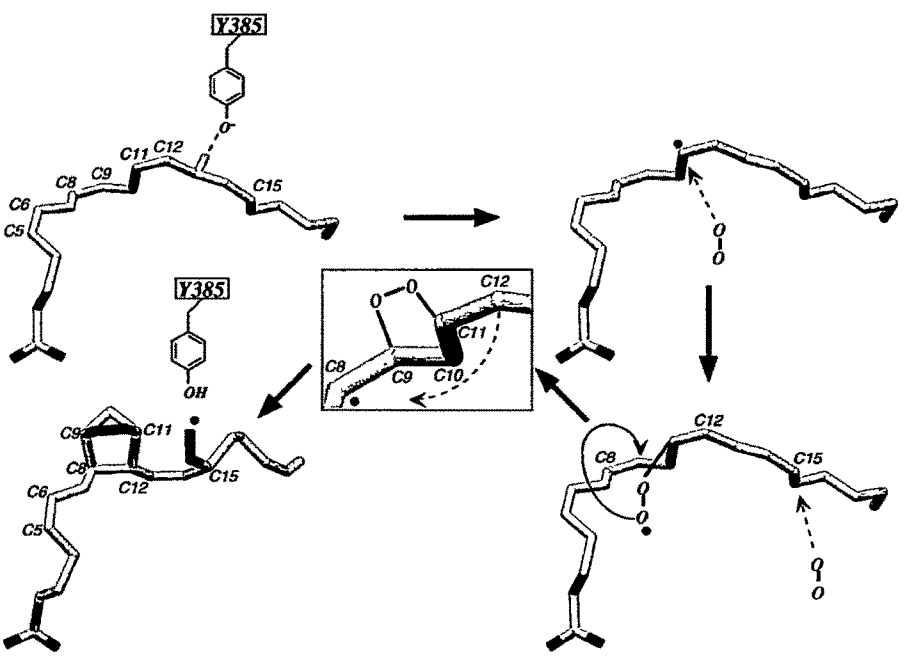


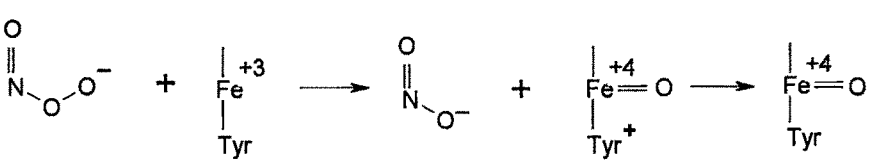
Fig. 2. Mechanistic sequence for converting AA to PGG_2. Abstraction of the 13-*pro(S)* hydrogen by tyrosyl radical leads to the migration of the radical to C-11 on AA. Attack of molecular oxygen produces a peroxyl radical, which adds to C-9 generating bicyclic peroxide and an allylic radical with maximal electron density at C-13 and C-15. Trapping of the carbon radical at C-15 with O_2 generates a peroxyl radical, which is reduced by Tyr 385 to PGG_2 and leads to regeneration of tyrosyl radical.

Protein radicals require an oxidant for their formation, which in most cases is a metal-containing prosthetic group[43]. COX is a homodimer of approximately 70 KDa subunits and each contains one molecule of heme[44].

The iron is ferric in the resting enzyme and is probably thermodynamically incapable of oxidizing Tyr 385 ($E_{1/2} = 0.9$ V for $Tyr^{\cdot} \rightarrow Tyr$ and $E_{1/2} = -0.2$ to $+0.2$ V for $Fe^{3+} \rightarrow Fe^{2+}$ for most hemes)[43,45]. Organic hydroperoxide or fatty acid hydroperoxide acts as an activator at the beginning of the reaction, which oxidizes the heme prosthetic group to ferryl oxo derivative that oxidizes Tyr 385 to tyrosyl radical. This tyrosyl radical then oxidizes arachidonic acid[46,47].

Peroxynitrite, the coupling product of nitric oxide and superoxide anion, is an excellent oxidant for the heme of COX and activates the enzyme even in the presence of concentrations of glutathione peroxidase and glutathione that inhibit activation by fatty acid hydroperoxides[48]. These findings provide a biochemical link between NO biosynthesis and prostaglandin biosynthesis and may explain the finding that NO synthase inhibitors reduce prostaglandin biosynthesis in inflammatory lesions *in vivo* (Equation **1**)[49]. Peroxynitrite activation of cyclooxygenase may be especially important in activated macrophages because inducible NO synthase and COX-II are immediate early genes that are dramatically expressed in response to exposure to inflammatory stimuli such as lipopolysaccharide.

Equation 1:



Tyr-385 radical is regenerated after the reduction of the peroxyl radical, precursor of PGG_2 at the end of each cyclooxygenase catalytic cycle (Fig. **2**). This leads to multiple turnovers per activation event and allows the accumulation of PGG_2[26,50-52].

All NSAIDs in clinical use have been shown to inhibit COX, leading to a marked reduction in PG synthesis[38]. The inhibition by aspirin is due to irreversible acetylation of the cyclooxygenase component of COX, leaving the peroxidase activity unaffected[53]. In contrast, NSAIDs like indomethacin or ibuprofen inhibit COX reversibly by competing with the substrate, arachidonic acid, for the active site of the enzyme[4]. All the activities of NSAIDs such as prevention of pathological overproduction of proinflammatory prostaglandins and the physiological formation of prostanoids are explained well by the postulate of inhibition of prostaglandin synthesis. The unwelcome ulcerogenic and renal side effects of NSAIDs such as aspirin and ibuprofen have been related to the inhibition of production of prostacyclin, which has a cytoprotective effect on the gastric mucosa and regulation of kidney function[15-17]. It thus appeared that the ulcerative effect of classical NSAIDs was an inevitable price to be paid for the desired anti-inflammatory activity, until the discovery that COX existed in two isoforms, COX-I and COX-II. More recently, the presence of a new isoform COX-III has been speculated upon[54].

1.4 CYCLOOXYGENASE ISOENZYMES: COX-I AND COX-II

1.4.1 MOLECULAR BIOLOGY

Two distinct COX enzymes exist. COX-I was first purified and characterized in the 1970s[13,55] and the gene was isolated in 1988[56,57]. The discovery and cloning of the second COX isoenzyme, COX-II, in 1991 initiated a revolution in the understanding of PGs and their functions in normal physiology and disease[58-60].The two enzymes are highly similar in structure and enzymatic activity. Both are homodimeric, heme-containing proteins with a molecular weight of roughly 70 Kda[44,61]. The genes for COX-I and COX-II are located on separate chromosomes, with COX-I on chromosome 9 and COX-II on chromosome 1[62]. The COX-II gene is

smaller than COX-I[68]. Exons 1 and 2 of COX-I (containing the translation site and original peptide) are condensed into a single exon in COX-II. The introns of COX-II are smaller than COX-I[63]. COX-II has a TATA box promoter and COX-I lacks a TATA box[64]. Lastly, the mRNA of COX-II contains long 3' untranslated regions containing several different polyadenylation signals and multiple "AUUUA" instability sequences that act to mediate rapid degradation of the transcript. These features differentiate the gene for COX-I into a gene consistent with rapid transcription and mRNA processing for processing a continuously transcribed stable message. It provides a constant level of enzyme in most cell types to synthesize prostaglandins responsible for homeostatic functions. In contrast, the features of the COX-II gene are those of an "immediate-early" gene that is not always present but is highly regulated and up-regulated during inflammation or pathological processes[65-68].

1.4.2 TISSUE EXPRESSION

COX-I and COX-II enzymes exhibit major differences in regulation and expression. Both COX-I and COX-II are inducible in certain situations but in general COX-I is expressed constitutively in most tissues and found throughout the body whereas COX-II is inducible[69]. COX-I is found in abundance in the gastrointestinal tract, where it produces prostaglandins (PGI_2 and PGE_2) that are considered cytoprotective. COX-I is also expressed in the kidneys and platelets. Thus, COX-I mediates homeostatic or "housekeeping" functions such as maintenance of vascular tone and mucosal integrity in the GI tract[15-17]. The human COX-I promoter region resembles that of the other housekeeping genes in that it lacks a TATA box and is generally not subject to transcriptional induction. However, COX-I expression is subject to developmental and inducible regulation under certain circumstances. For example, stem cell factor (SCF) treatment of immature murine bone marrow derived mast cells results in a 6–8 fold induction of COX-I mRNA and protein levels.

Estrogen-induced expression of COX-I is responsible for the increase in PGI_2 synthesis in perinatal pulmonary vascular beds and is partly responsible for the pulmonary vasodilation seen during this period[17,70].

In contrast, COX-II expression is undetectable in most normal tissues. Important exceptions to this rule are the brain and renal cortex where constitutive COX-II expression occurs. COX-II expression in many cell types is highly induced in response to proinflammatory stimuli such as IL-1, TNF, and bacterial lipopolysaccharide (LPS). COX-II gene expression is also subject to negative regulation. The anti-inflammatory cytokines IL-4, IL-10, and IL-13, and corticosteroids inhibit COX-II expression[17,70].

While it is up-regulated in response to certain stimuli, COX-II is expressed constitutively in some tissues. In most tissues where COX-II is constitutively expressed notably the brain and kidney, the enzyme is involved in biologic response to physiologic stress. In the kidney, the macula densa is an important component of the renin-angiotensin system that orchestrates sodium balance and fluid volume by monitoring salt concentration[71]. COX-II is constitutively expressed in the macula densa, and levels there are increased during salt deprivation, suggesting that prostaglandins produced by COX-II are important in sodium re-absorption in response to volume contraction[72]. In the brain, prostaglandins are involved in nervous system functions such as sleep-waking cycles, fever induction, and pain transmission. While COX-II is constitutively expressed in the brain, it is also up-regulated in parallel with fever and in response to seizures[16]. In addition to this, COX-II expression has also been observed in diseases like Alzheimer's[73,74] and various forms of cancer[75,76].

1.4.3 OVERALL STRUCTURE OF CYCLOOXYGENASE

COX-I and COX-II exist as homodimers with a molecular mass of approximately 70kDa per monomer (Fig. **3,4**)[44,77,78]. The tertiary and quaternary structures of the two enzymes are virtually identical. Each

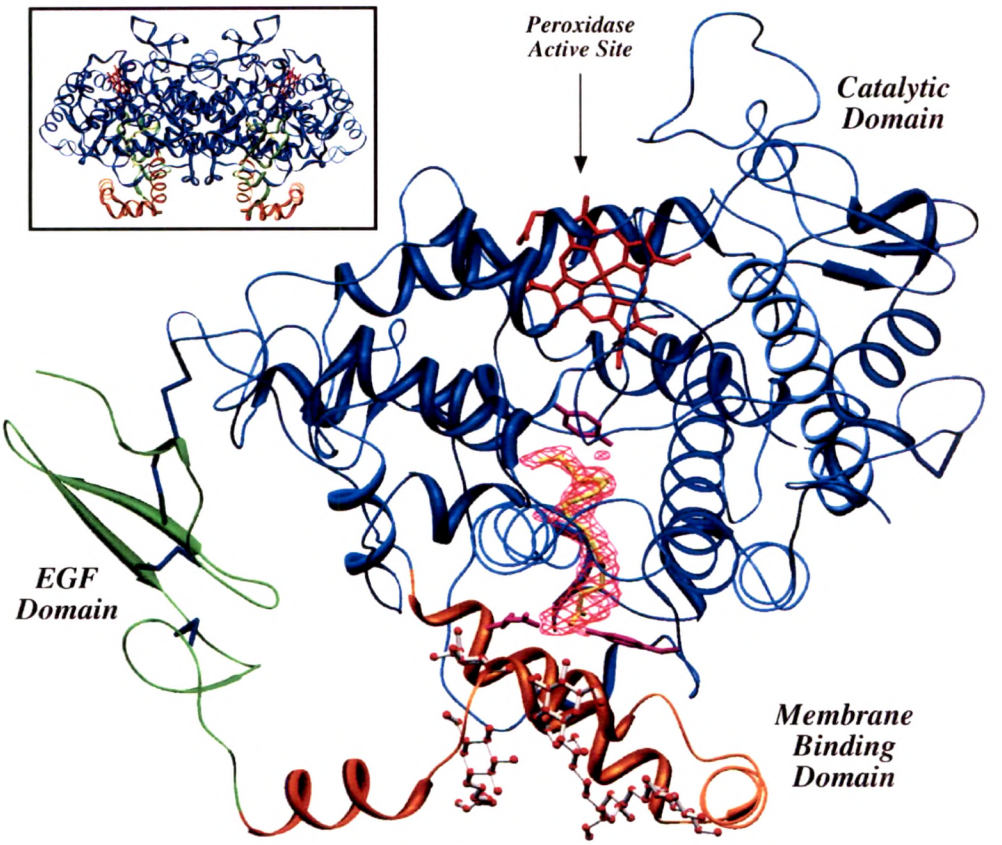


Fig. 3. A ribbon structure of COX-I monomer with AA bound in COX channel. The EGF domain, MBD and catalytic domain are shown in green, orange and blue respectively. The cofactor heme (red) marks the peroxidase active site. The COX active site is occupied by AA (yellow). Arg 120, Tyr 355 and Glu 524 comprise a H-bonding network that includes a constriction at the base of COX active site. They are depicted in (magenta). The volume beneath this constriction is termed lobby and is bordered on three sides by the MBD. The catalytic important Tyr 385 residue (magenta) is in between haem and AA.

monomer is composed of three distinct structural domains: a short N-terminal epidermal growth factor (EGF) domain, α-helical membrane-binding motif and a C-terminal catalytic domain (Fig. **3**)[79,80].

The first domain is highly similar to the epidermal growth factor (EGF) and is termed the EGF-like domain. The function of this domain in COX is poorly understood but is thought to facilitate recruitment and interaction with other cellular proteins[81].

The membrane-binding domain of COX-II consists of four short amphipathic α-helices that insert into the lipid bilayer, facing the lumen of the endoplasmic reticulum. Arachidonate derived from membrane lipids, is hydrophobic and approaches the heme via a channel extending from the membrane-binding domain of the enzyme[44,81]. In the image at right, the channel is occupied by an inhibitor, ibuprofen **(6)** (Fig. **4**).

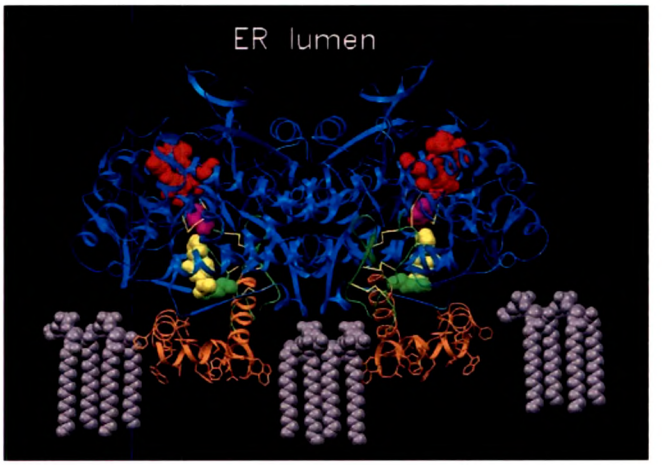


Fig. 4. A side view of prostaglandin synthase demonstrates an interaction of MBD with the lipid bilayer. Access into the hydrophobic COX channel is gained from the interior of the bilayer through the entrance formed by four amphipathic helices (orange). The channel is occupied by an ibuprofen (yellow).

COX-I and COX-II are bifunctional enzymes that carry out two sequential chemical reactions in spatially distinct but mechanistically coupled active sites. Both the cyclooxygenase and the peroxidase active sites are located in the catalytic domain, which accounts for approximately 80 % of the protein. The catalytic domain is highly homologous to mammalian peroxidases such as myeloperoxidase. The COX active site is

located at the end of a long hydrophobic channel that is broad near the membrane-binding domain (the lobby) and narrows as it extends toward the interior of the protein. A constriction composed of Arg 120, Tyr 355 and Glu 524 separates the lobby from the cyclooxygenase active site located above it. The cyclooxygenase active site may be subdivided into the main (substrate-binding) channel, which is largely hydrophobic, and a smaller amphipathic side pocket. The peroxidase active site is located on the surface of the protein near the heme cofactor[41,82,83] (Fig. 3). The volume of the COX-II active site is approximately 20% larger than that of the COX-I active site because of amino acid differences in the side pocket and a second shell of residues around the main channel. This is likely to be the reason for the increased promiscuity of COX-II for fatty acid substrates with different chain lengths and the ability of COX-II to metabolize larger, neutral derivatives of AA[80,84].

1.4.4 ROLE OF DIFFERENT AMINO ACIDS AT CYCLOOXYGENASE ACTIVE SITE

Eight amino acid residues play an important role for the substrate and inhibitor binding in the COX channel. The amino acid residues surrounding the active site were defined[85] as belonging to the inner 'first shell' (residues that are in direct contact with the inhibitors) or to the 'second shell' residues, which are not in direct contact with inhibitors. The amino acid numbering refers to the COX-I and COX-II enzyme coding (for the residues in COX-II one has to subtract 14 to reach the homologous amino acid residue in COX-I).

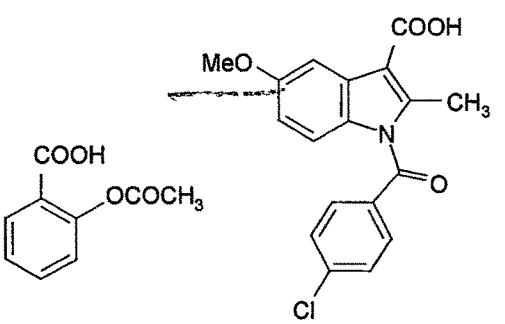
Catalytic Center

The amino acid Tyr 385 in COX-I (and the corresponding homologous amino acid Tyr 371 in COX-II) is located at the top of the channel and represents together with the heme group the catalytic center

(Fig. 3)[42,44]. The essential role of this Tyr 385 has been confirmed by site-directed mutagenesis[86].

Acylation Site

The hydroxyl group of serine in position 530 (Ser 516 in COX-II) is the target for the acetylation and the irreversible inhibition of COX-I by aspirin **(1)**[78]. X-ray analyses[80] and mutational experiments[87] have elucidated an additional function of Ser 530. This polar amino acid is involved in the binding of inhibitors with a benzoyl group, such as indomethacin **(2)**, or with an anilino NH, such as diclofenac **(3)** and meclofenamic acid **(4)**.



Aspirin **(1)** Indomethacin **(2)** Diclofenac **(3)**

R_1=H, R_2= CH_2COOH

Meclofenamic acid **(4)**

R_1=CH_3, R_2= COOH

Side Pocket and Extra Space in COX-II

Two structural features of COX-II account for the sensitivity of the binding of selective COX-II inhibitors The substitution of Ile 523 in COX-I by the smaller Val in COX-II (Val 509) permits access to a "side pocket" adjacent to the central COX-channel (Fig. 5). Additionally, the amino acid exchange of His 513 in COX-I for Arg 499 in COX-II allows hydrogen bonding with the sulfone part of COX-II inhibitors[88].

A second difference at the top of the channel results from a second shell amino acid difference. The amino acid Phe 503 in COX-I is replaced by leucine in COX-II (Leu 489). The smaller residue in COX-II allows the first shell amino acid Leu 384 to create an "extra space" at the top of the binding site in COX-II, thus allowing larger inhibitors to bind[84,85,89].

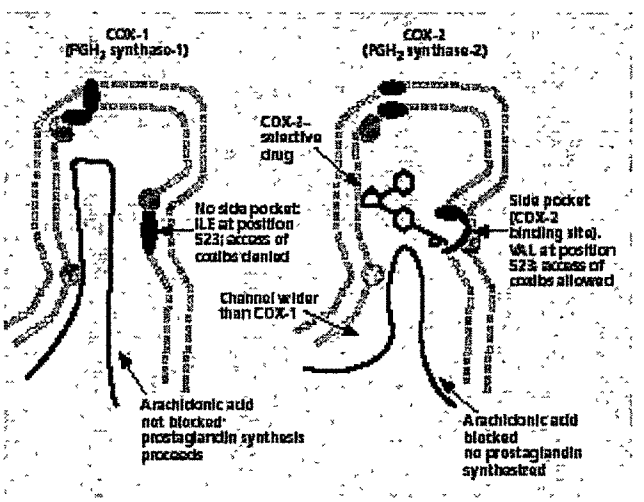


Fig. 5. Substitution of isoleucine of COX-I to valine in COX-II at 523 provides an extra side pocket at COX-II active site.

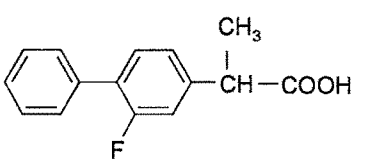
Ionic Binding

Arginine 120 (Arg 106 in COX-II numbering) is one of the very few charged amino acids within the active site. Arachidonic acid as well as acidic COX inhibitors bind via their carboxylate anion to the guanidinium cation of Arg 120. This has been shown by X-ray analysis for COX-I[44] and COX-II[80] for arachidonic acid, indomethacin **(2),** flurbiprofen **(5)** and their derivatives.

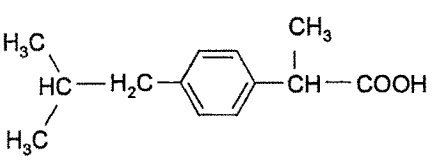
H-Bonding Dynamics

In addition to the functions already mentioned above, Arg 120 and His 513 in COX-I (Arg 106 and Arg 499 in COX-II) are involved together with Tyr 355 (Tyr 341 in COX-II) and Glu 524 (Glu 510 in COX-II) in a

hydrogen bonding network. This has been postulated from X-ray analysis[90], from a COX-II Tyr 355 Phe mutant and detailed kinetic analyses[91]. Tyr 355 lies on the opposite site of the channel from Arg 120 and sterically hinders the mouth of the COX active site, which accounts for the preferential inhibition exhibited by S-stereo isomers of methyl-substituted arylalkanoic inhibitors such as the profens **(5, 6)**. The hydrogen network of Glu 524, Tyr 355 and His 513 (Arg 499) dominates during the binding of the substrate and inhibitors. The hydrogen bonding interactions of Arg 120, Glu 524 and Tyr 355 are proposed to play a dominant role for the irreversible substrate binding status. The two H-bonding networks are also proposed to be responsible for the allosteric activation of the COX enzyme[91]. To date, this is the best structural explanation for the time-dependency of COX-II inhibition and of the loss of COX-I activity due to the need of allosteric enzyme activation of COX-II selective NSAIDs.



Flurbiprofen **(5)**



Ibuprofen **(6)**

1.4.5 STRUCTURAL BASIS FOR SELECTIVE COX-II INHIBITION BY ANTI-INFLAMMATORY AGENTS

Crystal structures of ovine COX-I, murine COX-II and human COX-II have been solved at 3-3.5 A° resolution[44]. The overall folding patterns are very similar, which is not surprising considering that COX isoforms are 65 % identical in amino acid sequence. All COX inhibitors, regardless of their selectivity, bind in the arachidonic acid binding site, which is located in the upper half of a long channel leading from the membrane interface to the interior of the protein. A constriction formed by

the residues Arg 120, Tyr 355 and Glu 524 marks the bottom of the arachidonic acid binding site and restricts access to it. Opening and closing of this constriction might contribute to the time dependence of inhibition exhibited by some NSAIDs[44].

Aspirin's irreversible acetylation of hydroxyl group of Ser 530 of COX-I completely abolishes its cyclooxygenase activity[78] (Fig. **6**), whereas acetylation of COX-II shifts the product profile from primarily PGH_2 to exclusively 15-(*R*)-hydroxyeicosatetraenoic acid (HETE; a mono-oxygenated metabolite of AA)[92,93]. This suggests that the larger

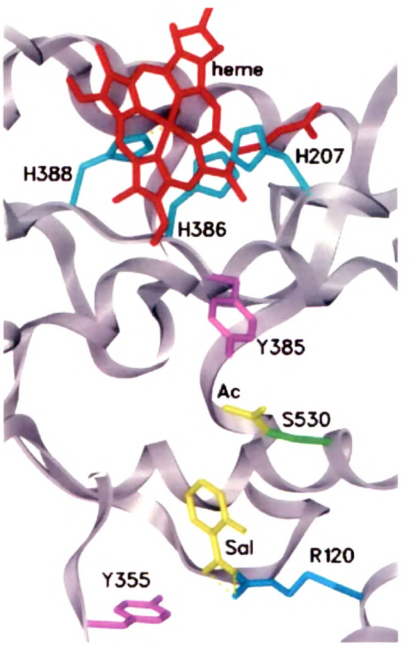
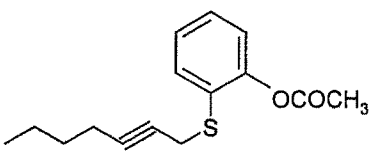


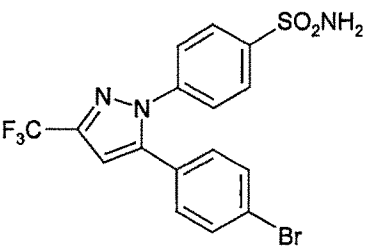
Fig. 6. Crystal structure of the active site of aspirin-acetylated ovine COX-I. The hydroxyl group of Ser 530 (green) is acetylated by aspirin (yellow). The carboxylate group of salicylic acid is found to interact with Arg 120(blue).

cyclooxygenase active site of COX-II is able to bind and metabolize arachidonic acid even after the covalent addition of an acetyl group to the enzyme active site. Replacement of aspirin's carboxylic acid with alkylsulphide or alkynylsulphide functionalities generates COX-II

selective inhibitor that acetylates the same serine residue as aspirin. e.g. APHS [o-(acetoxyphenyl)hept-2-ynyl-sulphide] (**7**). APHS (**7**) is 15-20 fold more active than aspirin[94,95].



APHS (**7**)



SC-558 (**8**)

Flurbiprofen (**5**), a slow-binding competitive inhibitor of both COX-I and COX-II, binds in the long hydrophobic channel and excludes substrate from the cyclooxygenase active site. The binding site of flurbiprofen in COX-II is identical to that observed in COX-I (Fig. **7a**)[44].The carboxylate of the drug forms a salt bridge with guanidinium group of Arg 120 and a hydrogen bond to Tyr 355. The distal aryl ring forms close van der Waals interactions with the main-chain atoms of Gly 526 and Ala 527, and stacks tightly against Tyr 385, which is active in the catalysis or suicide-inactivation of the enzyme[46,47] The distal phenyl ring of flurbiprofen (**5**) also interacts with Ser 530, the residue that is selectively acetylated by aspirin (**1**)[97]. The fluorophenyl ring of the inhibitor makes fewer contacts with protein atoms in COX-II and is involved in van der Waals interactions with Val 349 and the main chain of Ala 527. In COX-I, the fluorine interacts with the side chain of Ile 523, but no such interaction is observed in COX-II, which has a valine at the corresponding position[44].

Indomethacin (**2**), a classic nonselective cyclooxygenase inhibitor, causes slow, time-dependent inhibition of COX-I and COX-II. Indomethacin (**2**) seems to penetrate very deeply into the channel without

a strong effect on COX-II. Its benzoyl group occupies an environment similar to that of the distal phenyl ring of flurbiprofen and is stabilized by hydrophobic interactions. The time dependence may also result from the formation of a salt bridge between the acidic function of indomethacin **(2)** and Arg 120 of the enzyme within the long hydrophobic cyclooxygenase channel followed by conformational changes[80].

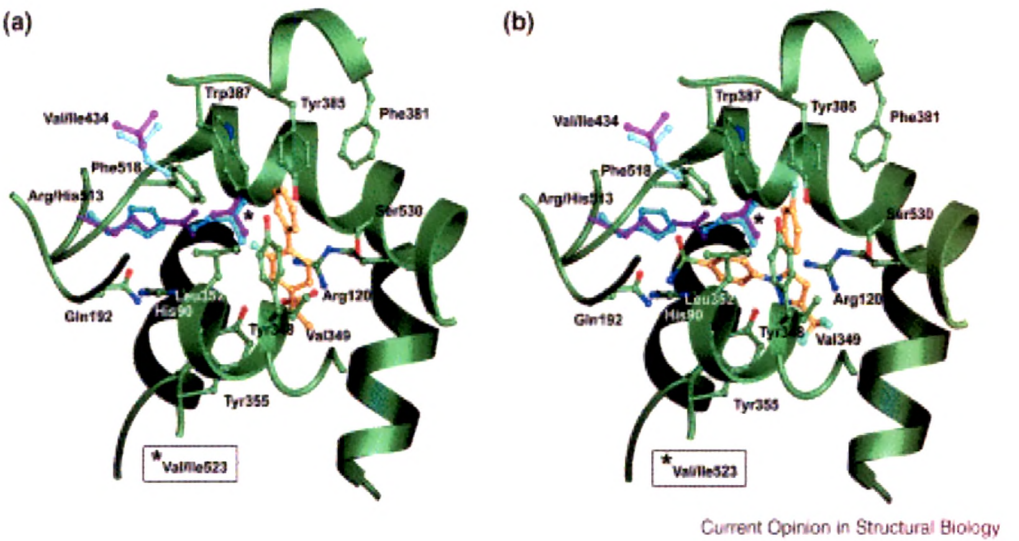


Fig. 7. Binding of a nonselective NSAID (flurbiprofen) and a COX-II specific inhibitor (SC-558) in the cyclooxygenase active site of COX-II.

(a) Structure of the NSAID flurbiprofen (gold) bound to COX-II. The three side pocket residues of COX-I (cyan) differ from those of COX-II (pink).

(b) Structure of the COX-II-specific inhibitor SC-558 bound to wild-type murine COX-II.

SC-558 **(8)**, a diarylheterocyclic inhibitor with 1,900-fold selectivity for COX-II over COX-I, has a central pyrazole ring and a sulphonamide substituent attached to one of the aryl rings. Like flurbiprofen **(5)** and indomethacin **(2)**, SC-558 **(8)** binds to the cyclooxygenase active site (Fig. **7b**). The bromophenyl ring is bound in a hydrophobic cavity formed by Phe 381, Leu 384, Tyr 385, Trp 387, Phe

513 and Ser 530, with contributions from the backbone atoms of Gly 526 and Ala 527. The trifluoromethyl group is bound in an adjacent pocket formed by Met 113, Val 116, Val 349, Tyr 355, Leu 359 and Leu 531. These two features of SC-558 **(8)** binding have rough equivalents in the binding of flurbiprofen **(5)** and indomethacin **(2)** to COX-II[80].

In COX-II, the channel that leads from membrane to the cyclooxygenase active site forks at the SC-558 **(8)** binding site. One branch forms a cavity that accepts the bromophenyl ring of SC-558 **(8)**, whereas the other represents a cavity not observed in the COX-I structure, and accommodates the entire phenylsulphonamide moiety. The phenyl ring is surrounded by hydrophobic residues Leu 352, Tyr 355, Phe 518, Val 523 and the backbone of Ser 353. Beyond this hydrophobic pocket, the sulphonamide group extends into a region near the surface of COX-II that is relatively polar. The sulphonamide interacts with His 90, Gln 192 and Arg 513. One of the oxygen atoms forms a hydrogen bond to His 90; the other oxygen is linked by a hydrogen bond of Arg 513. The amide nitrogen forms a hydrogen bond to the carbonyl oxygen of Phe 518[80,88].

The selectivity of SC-558 **(8)** seems to result from the phenylsulphonamide moiety, which binds in a pocket that is more restricted in COX-I and is unoccupied in complexes of COX-II with non-selective inhibitors. This pocket branches off from the main channel that leads to the cyclooxygenase active site, and is more accessible in COX-II, primarily because of a substitution of isoleucine to valine at position 523 (Fig. 7). In COX-II, the smaller size of the valine side chain coupled with the conformational changes at Tyr 355 opens up the hydrophobic segment of the new pocket that comprises Leu 352, Ser 353, Tyr 355, Phe 518 and Val 523. A similar pocket exists in COX-I, but is inaccessible because of the bulkier isoleucine at 523[80,85,89].

Access of the phenylsulphonamide group to the new pocket in COX-II is facilitated by another isoleucine to valine substitution at position 434. The side chain of the hydrophobic residue at 434 packs against Phe 518, which forms a molecular gate, that extends to the new hydrophilic pocket in COX-II. In COX-I, this gate is closed because of the larger isoleucine side chain. In COX-II, with the smaller side chain at 434, the gate has room to swing open, allowing the entry of the sulphonamide group. Thus the identity of the amino acid at position 434 also appears to make a significant contribution to selectivity in inhibitor binding[80,97].

Another amino acid, Arg 513 in COX-II is replaced by histidine in COX-I; superposition of the two enzymes suggests that an imidazole ring at this position would not extend sufficiently for direct interactions with a sulphonamide group. Therefore, the identity of the residue at position 513 is a third contributor to COX-II specificity. Another residue in this pocket that might contribute to specificity is located at 516; it is alanine in COX-II, but serine in COX-I[80].

Arg 120, the guanidinium group of which stabilizes the carboxylate of classical NSAIDs, is one of the few charged residues in the hydrophobic cyclooxygenase channel. However, in the structure with SC-558 **(8)**, which has no carboxylate group, there is no charge-charge interaction between the inhibitor and Arg 120. The lack of a carboxylate group in SC-558 **(8)** could also be a significant component of its selectivity towards COX-II. Attempts to improve the potency against COX-II by incorporating an acidic group on the pyrazole of the diaryl heterocyclic series results in poor selectivity[80].

1.5 NEED OF SELECTIVE COX-II INHIBITORS

Except few, most currently available NSAIDs inhibit both cyclooxygenase isoforms, i.e. COX-I and COX-II. The notion that blockade of COX-I is responsible for many of the side effects of

nonselective NSAIDs while blockade of COX-II mediates the therapeutically required action, has spurred efforts to develop selective COX-II inhibitors. The main side effects of nonselective NSAIDs are:

1.5.1 GASTROINTESTINAL DISTURBANCES

Common gastrointestinal side effects are dyspepsia, diarrhea (but sometimes constipation), nausea, vomiting and gastric or intestinal ulceration. Chronic use of NSAIDs can lead to gastric damage, hemorrhage and/or perforation[11,12]. In some of those who have bleeding ulcers, the bleeding is sufficiently severe to result in hospital admission, and may cause death. NSAIDs induce gastric irritation and gastric mucosal damage by following mechanism:

When administered orally, NSAIDs allow local irritation of gastric mucosa, which is because of acidic carboxyl group of NSAIDs and by local inhibition of gastroprotective COX-I enzyme, which is responsible for the biosynthesis of gastric PGs, PGE_2 and PGI_2. PGE_2 and PGI_2 inhibit acid secretion by stomach, enhance mucosal blood flow and promote the secretion of cytoprotective mucus in the intestine. Thus, they serve as cytoprotective agents in the gastric mucosa. Inhibition of their synthesis may render the stomach more susceptible to damage. An indirect method to cause local irritation in stomach by these drugs is back diffusion of acid into gastric mucosa from the lumen to induce tissue damage. Parenteral administration of NSAIDs can also cause mucosal damage and bleeding due to inhibition of COX-I[98-101].

1.5.2 INHIBITION OF PG-MEDIATED RENAL FUNCTION

NSAIDs have little effect on renal function in normal human subjects presumably because the productions of vasodilatory PGs have only a minor role in sodium-replete individuals. However, these drugs decrease renal blood flow and the rate of glomerular filtration in patients with congestive heart failure, hepatic cirrhosis with ascitis, chronic renal

disease or in those who are hypovolemic. Acute renal failure may be precipitated under these circumstances. Nephropathy is associated with the long-term use of NSAIDs[102,103].

1.5.2 BLOCKADE OF PLATELET AGGREGATION (INHIBITION OF THROMBOXANE SYNTHESIS)

Function of platelets is disturbed because NSAIDs prevent the formation of thromboxane A_2, a potent aggregating agent by inhibition of cyclooxygenase. Thus NSAIDs increase the bleeding time[27].

1.5.3 MISCELLANEOUS SIDE EFFECTS

Some other side effects like, prolongation of gestation by inhibiting the biosynthesis of PGs, hypersensitivity reactions like skin rashes, urticaria, bronchial asthma, and hypotension, cardiovascular side effects such as congestive heart failure, angina and CNS side effects, which include headache, depression, tremors, drowsiness, vertigo, tinnitus, neuropathy etc are also seen with the usage of NSAIDs[104].

1.6 DEVELOPMENT OF SELECTIVE COX-II INHIBITORS

Prior to the identification of the COX-II enzyme, researchers identified a potent anti-inflammatory compound, Dup-697, which was a relatively weak inhibitor of bovine seminal vesicle PC synthesis, but potent in a variety of anti-inflammatory assays[105]. At first, these results could not be explained, but after identification of COX-II it became evident that this compound possessed a selective inhibitory activity against COX-II. This was the beginning of the search for new anti-inflammatory compounds focusing on COX-II as the target enzyme.

The large number of newly developed COX-II inhibitors demonstrates how promising this field of anti-inflammatory agents is expected to be[106]. More than 500 COX-II inhibitors have been described over the past few years. Until now, rofecoxib, celecoxib, valdecoxib and

etoricoxib have been launched for the treatment of inflammatory processes.

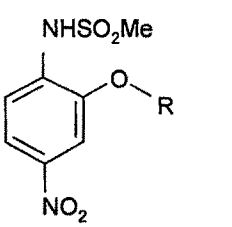
The chemical structures of COX-II inhibitors are heterogenic and can be segmented as follows:

1.6.1 VICINAL DIARYL HETEROCYCLES

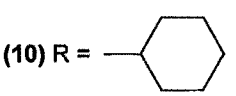
Since the work pertaining to this thesis belongs to this class, these compounds would be discussed at length.

1.6.2 DIARYL– OR ARYL–HETEROARYL–ETHERS (SULFONANILIDE INHIBITORS)

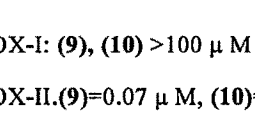
Nimesulide **(9)** was an innovative NSAID, which is chemically different from nonselective NSAIDs **(1-6)** because it is devoid of carboxylic acid group and has an acidic functional group, the sulfonanilide moiety. It is marketed since 1976 as an effective NSAID[107,108]. This

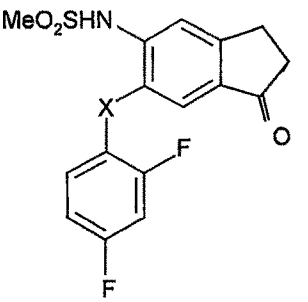


(9) R =



(10) R =





(11) X = O

(12) X = S

IC_{50} COX-I: **(9)**, **(10)** >100 μ M IC_{50} COX-I: **(11)**, **(12)** >100 μ M

IC_{50} COX-II.**(9)**=0.07 μ M, **(10)**=0.05 μ M IC_{50} COX-II: **(11)**=0.015 μ M, **(12)**=0.023 μ M

compound was assayed against COX isoforms and was found to be 1,400 times more selective for COX-II enzyme[109]. The identification of nimesulide **(9)** as a selective COX-II inhibitor has led to extensive

efforts to develop improved analogs such as NS-398 **(10)**[110], and the indanone derivatives, flosulide **(11)**[111,112] and L-745337 **(12)**[113].

VICINAL DIARYL HETEROCYCLES

The greatest amount of research on COX-II has been performed in the preparation and evaluation of this class of compounds. The structural characteristic of these types of compounds is a central ring system (heterocyclic, carbocyclic or aromatic) bearing two vicinal aryl moieties as shown in (Fig. **8**). A wide variety of heterocycles were reported as central ring system e.g. pyrrole, thiazole, oxazole, imidazole, thiophene, pyrimidine, furanone, pyrazole, cyclopentenone, pyran and fused ring systems like, benzofuran and many more.

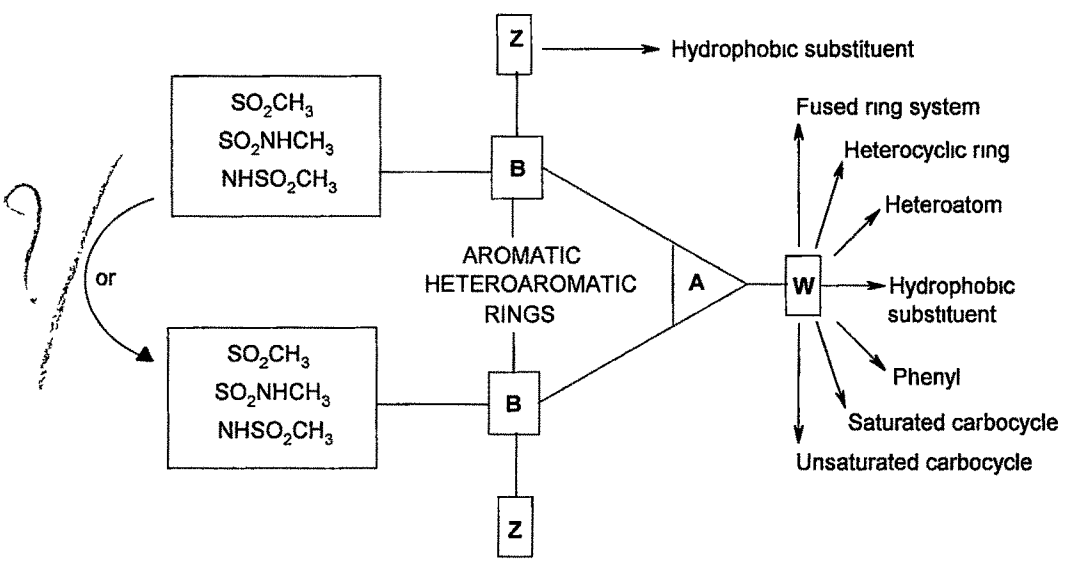


Fig. 8. Structural features of selective COX-II inhibitors.

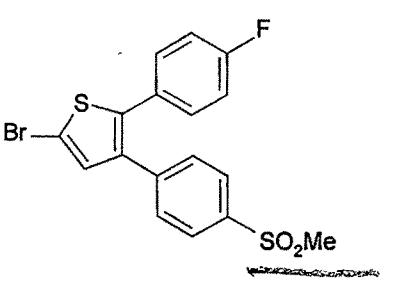
Nonselective NSAIDs **(1-6)** have a carboxylic moiety located in a favorable position for interaction with the guanidium group of Arg-120 in both COX isoforms[114]. Since, many of the different classes of NSAIDs carry carboxylic acid groups, these interactions may prove to be a general binding feature for these groups[77]. However, selective COX-II inhibitors

have this important pharmacophore changed by a methyl sulfone or sulfonamide group[105,106].

It was reported that substitution at position 4- of one of the aromatic systems with a sulfonamide or a methyl sulfonyl group is essential for COX inhibition. Replacement of the methyl sulfonyl group by a sulfonamide group reduces COX-II selectivity but improves oral bioavailability[115].

A brief survey of literature is described here, which shows the chronological development of vicinal diaryl heterocycles as COX-II inhibitors.

In 1990, Gans and co-workers[105] described DuP-697 **(13)** as a new non-acidic NSAID devoid of gastrointestinal toxicity. To gain insights into the features of DuP-697 **(13)** that determine its activity and selectivity, isosteric nuclei of thiophene rings were proposed and the structural activity relationship (SAR) around the thiophene ring was explored[97,116-124].

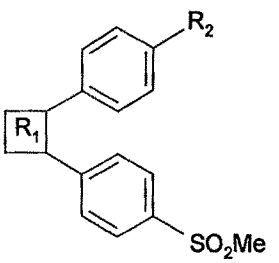


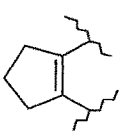
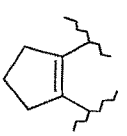
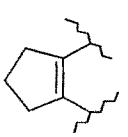
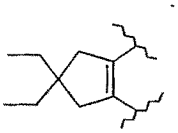
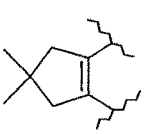
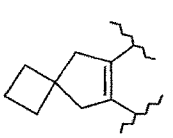
DuP-697 **(13)**

IC_{50} COX-I = 0.60 μ M, IC_{50} COX-II = 0.005 μ M

Previously, it was believed that the spatial disposition of the 2,3-diaryl rings of DuP-697 **(13)** plays an important role in COX inhibition. This geometry would be provided by carbon-carbon double bond of the thiophene ring, which revealed that this heterocycle *in toto* was not essential for activity. To test this hypothesis, Reitz and co-workers[116]

Table 1. Isosteric substitutions of thiophene ring.

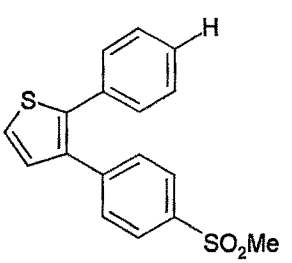


Compound	R_1	R_2	COX-I	COX-II
			IC_{50} (μ M)	
14		F	> 100	0.026
15		Cl	> 100	0.003
16		CH_3	> 100	0.003
17		F	> 100	65
18		F	18.3	0.015
19		F	> 100	0.004

synthesized 1,2-diaryl cyclopentene derivatives. The first compound of this series (SC-57666) **(14)** was found to be a more selective COX-II inhibitor devoid of COX-I activity. However, compound **(14)** was less potent than DuP-697 **(13).** Replacing fluorine at the R_2-position with chlorine **(15)** or a methyl group **(16)** afforded almost 100 times more potency than **(14)** and inactivity towards COX-I (**Table 1**).

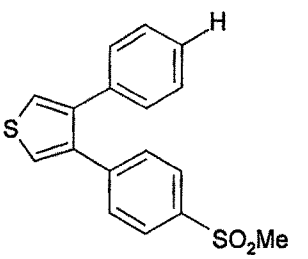
The presence of the ethyl group **(17)** decreased COX-II activity suggesting that these areas of the enzyme are very sensitive to steric bulk substituents[116]. Although methyl group in the same position SC-58231 **(18)** is more potent than **(17)** in enzyme inhibition, the rat adjuvant-induced arthritis model showed that **(17)** is twice more active (ED_{50} = 1.7 mpk) than **(18)** (ED_{50} = 3.2 mpk)[116]. Further modifications at the 4-position of **(17)** resulted in 4-spiro derivatives **(19)**[117].

Leblane and co-workers[118] reported that the substitution of bromine atoms to hydrogen at the 5-position of thiophene ring of DuP-697 **(13)** enhanced selectivity **(20).** However, the COX-II activity decreased.



(20)

IC_{50} COX-I > 100 μ M,
IC_{50} COX-II = 0.25 μ M



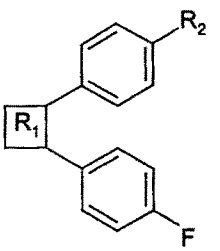
(21)

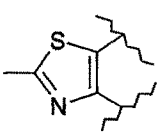
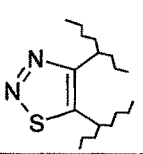
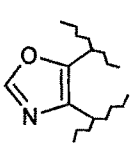
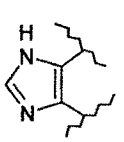
IC_{50} COX-I > 1000 μ M,
IC_{50} COX-II = 0.08 μ M

The synthesis of 3,4–diaryl thiophenes was investigated by Bertenshaw and co-workers[97] and derivative **(21)** showed similar potency in acute animal models of inflammation and was 12,500 times more selective than DuP-697 **(13).**

Thiophene central ring was replaced by Gauthier and co-workers[119] with simple and preferably unsubstituted 5-member heterocycles such as

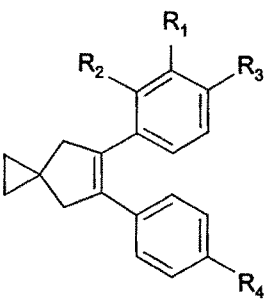
Table 2. Effect of different central ring systems on selectivity of COX-II enzyme.



Compound	R_1	R_2	COX-I	COX-II
			IC_{50} (μ M)	
22		SO_2Me	> 100	0.31
23		SO_2Me	> 100	6.5
24		SO_2Me	> 100	> 30
25		SO_2NH_2	> 100	> 30

isothiazole **(22),** 1,2,3-thiadiazole **(23),** isoxazole **(24)** and imidazole **(25).** None of these derivatives showed significant activity against COX-I and COX- II enzymes (**Table 2**).

In 1996, Huang and co-workers[120] prepared a series of 5,6-diaryl spiro[2.4]hept-5-enes as highly potent and selective COX-II inhibitors. A study of structure-activity relationships in this series suggests that 3,4-disubstituted phenyl analogs are generally more selective than 4-substituted phenyl analogs and that replacement of the methyl sulfone



(26) R_1 = OMe, R_2 = R_3 = Cl, R_4 = SO_2Me

(IC_{50} COX-I > 100 μ M, IC50 COX-II = 0.0221 μ M)

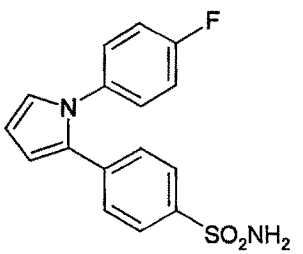
(27) R_1 = Cl, R_2 = OMe, R_3 = H, R_4 = SO_2NH_2

(IC_{50} COX-I > 100 μ M, IC_{50} COX-II = 0.002 μ M)

group on the 6-phenyl ring with a sulfonamide moiety results in compounds with superior *in vivo* pharmacological properties, although lower COX-II selectivity. Compound **(26)** and **(27)** were shown to have superior *in vivo* pharmacological profiles, low GI toxicity and good oral bioavailability and duration of action.

The substituted 1,2-diaryl pyrroles and 1,2- diaryl imidazoles series have been reported by Khanna and co-workers[121,122] as other variants of the central thiophene ring of DuP-697 **(13)**. In both ring systems, the sulfonamide revealed to be a biosteric group of sulfone found in DuP-697 **(13).** The synthesis of derivatives of both series yielded highly selective compounds (**28** and **29** respectively) for COX-II enzyme.

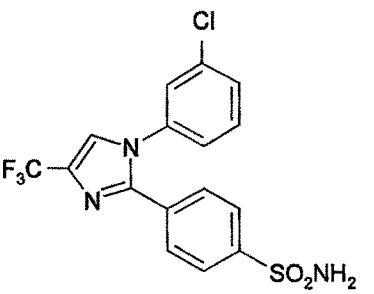
More recently, Khanna and co-workers[123] have explored the replacement of the phenyl ring in the lead template **(29)** by other aromatic



(28)

IC_{50} COX-I > 10.5 μ M,

IC_{50} COX-II = 0.014 μ M



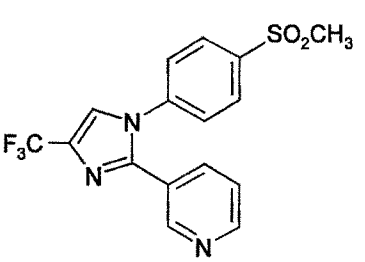
(29)

IC_{50} COX-I > 6.2 μ M,

IC_{50} COX-II = 0.008 μ M

heterocycles as 3-pyridyl derivative **(30)** and 3-thiazole derivative **(31).** Despite these derivatives having improved selectivity for COX-II, they were less potent.

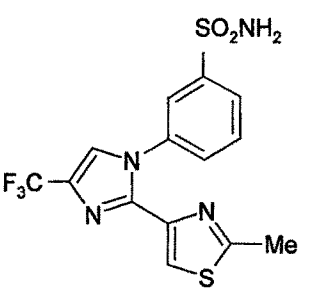
A series of sulfonamide containing 1,5-diaryl pyrazole derivatives were prepared by Penning and co-workers[124]. This work led to the



(30)

IC_{50} COX-I > 10000 μ M,

IC_{50} COX-II = 0.20 μ M



(31)

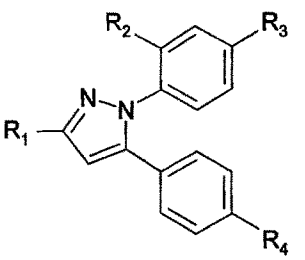
IC_{50} COX-I > 1000 μ M,

IC_{50} COX-II = 0.052 μ M

identification of SC-58635 (celecoxib) **(32),** which was the first selective drug for COX-II enzyme approved by FDA for the treatment of

rheumatoid arthritis and osteoarthritis. This compound showed excellent anti-inflammatory activity and was devoid of gastrointestinal toxicity.

The structural modifications in the diaryl rings and in the central ring system resulted in derivative FR-140423 **(33)**[125,126]. The sulfonamide group of celecoxib **(32)** was replaced with a methylsulfoxide group and the trifluoromethyl group by a difluoromethyl. This compound was ~140



Celecoxib (32) $R_1 = CF_3$, $R_2 = H$, $R_3 = SO_2NH_2$, $R_4 = Me$

IC_{50} COX-I > 15 μ M, IC50 COX-II = 0.04 μ M

FR-140423 (33) $R_1 = CHF_2$, $R_2 = H$, $R_3 = SO_2NH_2$, $R_4 = OMe$

IC_{50} COX-I > 19 μ M, IC_{50} COX-II = 0.013 μ M

(34) $R_1 = CF_3$, $R_2 = F$, $R_3 = SO_2NH_2$, $R_4 = OMe$

% COX-II inhibition; *in vitro*, 10 μ M = 75; *in vivo*, 30 mg/kg = 26

(35) $R_1 = CF_3$, $R_2 = F$, $R_3 = SO_2NNaCOCH_2CH_3$, $R_4 = OMe$

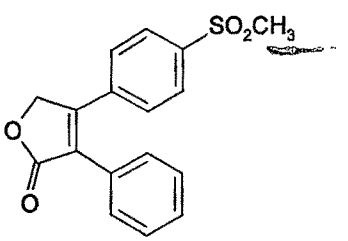
% COX-II inhibition; *in vitro*, 10 μ M = 38; *in vivo*, 30 mg/kg = 26

times more selective to COX-II enzyme. FR-140423 **(31),** unlike indomethacin **(2)** produced an analgesic effect in the tail-flick test. This test is an *in vivo* model to evaluate the central action of analgesic drugs like morphine. Compound **(33)** seemed to be a unique compound having morphine like analgesic activity.

Pal and co-workers[127] modified the benzenesulfonamide moiety of celecoxib **(32).** It was found that the fluorine substitution on the benzenesulfonamide moiety along with an electron-donating group at the

4-position of the 5-aryl ring yielded selectivity as well as potency for COX-II inhibition *in vitro*. The compound (34) displayed interesting pharmacokinetic properties along with anti-inflammatory activity *in vivo*. Among the sodium salts tested *in vivo* (35), the propionyl analogue of (34), showed excellent anti-inflammatory activity and therefore represents a new lead structure for the development of injectable COX-II specific inhibitors.

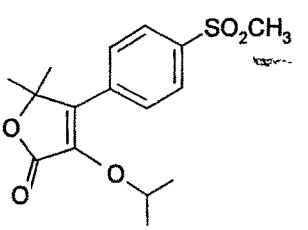
In 1999, Prasit and co-workers[128] synthesized a series of compounds by choosing a lactone moiety as the heterocycle. This study resulted in derivative MK-0966 (rofecoxib) **(36),** which is essentially



Rofecoxib (36)

IC_{50} COX-I = 19 μ M,

IC_{50} COX-II = 0.5 μ M



(37)

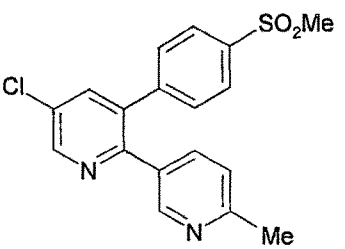
IC_{50} COX-I > 100 μ M,

IC_{50} COX-II = 0.3 μ M

equipotent to indomethacin **(2)** in both COX-II whole cell and COX-II whole blood assays. Rofecoxib **(36)** is the second drug approved by FDA as a selective COX-II inhibitor.

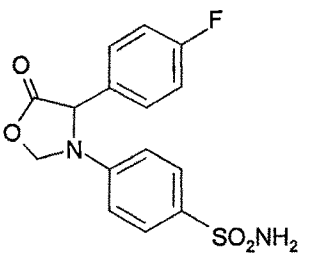
More recently, the insertion of an oxygen atom between the phenyl group and the furanone moiety, resulted in the derivative 5,5-dimethyl-3(2-propoxy)-4-(4-methanesulfonylphenyl)-2(5H)-furanone (DFP) **(37)**[129]. This compound showed to be more selective for COX-II enzyme but during phase-I of the clinical studies, this compound showed poor

pharmacokinetic characteristics in humans having a very long half life ($t^1/_2$= 64 h) These results led to the investigation of a new class of COX-II



Etoricoxib (38)

IC_{50} COX-I > 100 μ M,
IC_{50} COX-II = 1.1 μ M



(39)

IC_{50} COX-I = 13.5 μ M,
IC_{50} COX-II = 1.5 μ M

inhibitors, and MK-0663 (etoricoxib) **(38)** has shown to be a potent and selective COX-II inhibitor[130,131].

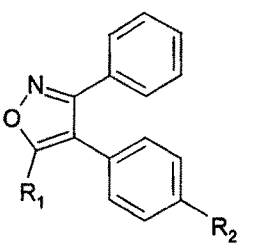
Crespo and co-workers[132] synthesized a series of 3,4-diaryl oxazolone derivatives. The compound **(39)** was selected for further preclinical and clinical assays on the basis of its *in vivo* activity profiles and lack of gastrointestinal toxicity even though it has not shown high selectivity for COX-II enzyme.

Talley and co-worker[133] prepared diaryl isoxazole derivatives. This study culminated in the synthesis of SC-65875 (valdecoxib) **(40),** which showed to be a potent and a selective COX-II inhibitor. This compound is currently in clinical evaluation for the management of pain and inflammation. The compound **(41),** an active metabolite of valdecoxib **(40)** was synthesized. It was more potent in the carrageenan paw edema assay than **(40)** but was less potent against the isolated enzyme and in the air pouch or adjuvant arthritis assays.

Nunno and co-workers[134] synthesized 3,4-diaryl isoxazole analog of valdecoxib **(40).** The compound **(42)** not bearing the sulfonamide group

present in valdecoxib **(40)** was a selective COX-I inhibitor. This result confirms that within the 3,4-diaryl isoxazole class of COX-II inhibitors, the *p*-sulfamoylphenyl group is essential for good COX-II inhibitory potency and lacking the sulfonamide moiety reverses the COX-II selectivity.

In general, COX-II inhibitors of the diaryl heterocycle class such as celecoxib **(32)** possess modest aqueous solubility. In considering the



Valdecoxib(40) R_1=CH_3, R_2=SO_2NH_2, **(41)** R_1=CH_2OH, R_2= SO_2NH_2

IC_{50} COX-I > 140 μ M,
IC50 COX-II = 0.005 μ M

IC_{50} COX-I = 1120 μ M,
IC50 COX-II = 0.18 μ M

(42) R_1 = H, R_2 = CH_3

IC_{50} COX-I = 0.09 μ M,
IC50 COX-II = 2.49 μ M

(43) R_1 = CH_3, R_2 = $SO_2NHCOCH_2Na$

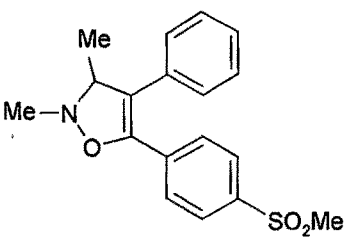
IC_{50} COX-I = 100 μ M,
IC50 COX-II = 20 μ M

development of a COX-II inhibitor for parenteral administration, Talley and co-workers[135] synthesized parecoxib sodium **(43),** water-soluble prodrug of valdecoxib **(40).**

Knaus and co-workers[136] reported the design and synthesis of 4,5- diphenyl-4-isoxazolines for analgesic and COX-II inhibiting activity. After performing molecular modeling studies, they found the compound **(44)** to be a selective COX-II inhibitor out of a series of compounds.

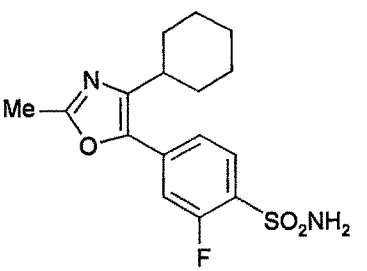
Hashimoto and co-workers[137] synthesized a series of 4-(4-cycloalkyl/aryl-oxazole-5-yl)benzenesulfonamide derivatives. This

study led to the identification of a potent, highly selective, orally active COX-II inhibitor JTE-522 **(45)** that is currently in phase-II clinical trials for the treatment of rheumatoid arthritis, osteoarthritis and acute pain.



(44)

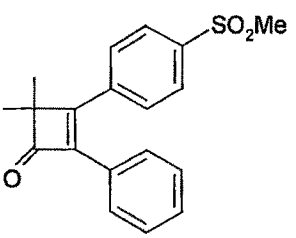
IC_{50} COX-I = 258 11 μ M,
IC_{50} COX-II = 0.0042 μ M



(45)

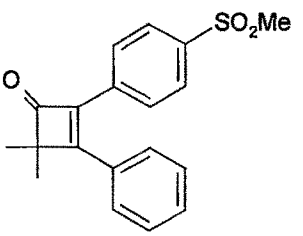
IC_{50} COX-I > 100 μ M,
IC_{50} COX-II = 0.085 μ M

Variations of the central five membered rings are tolerated as well. Hence, ring contraction to smaller carbocycles such as cyclobutenones or an insertion of a six membered carbocyclic or heterocyclic group also lead to potent COX-II inhibitors. 2,3-Diaryl cyclobutenones have been reported to be selective COX-II inhibitors[138]. The compound **(46)** was



(46)

IC_{50} COX-I = 0.12 μ M,
IC_{50} COX-II = 0.002 μ M



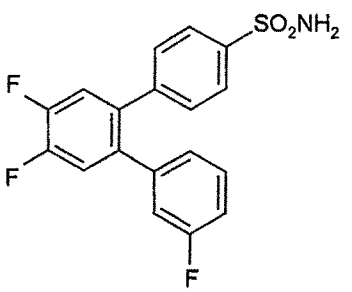
(47)

IC_{50} COX-I > 5 μ M,
IC_{50} COX-II = 0.11 μ M

highly potent on COX-II and displayed a selectivity ratio of approximately 700. Interestingly the regioisomer **(47)** was equally active but with a lowered selectivity ratio. A central six-membered ring with a vicinal

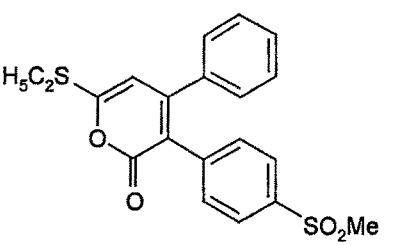
substitution pattern as shown in the compound (48) seems to be accepted as well[139]. Etoricoxib **(38)** that has been described earlier was a compound that belongs to a novel series of bis-pyridinyl-(4-methanesulfonylphenyl) compounds.

Knaus and co-workers[140] synthesized a series of 6-substituted-3-(4-methanesulfonyl)-4-phenylpyran-2-one derivatives as selective COX-II inhibitors. They concluded that a six-membered lactone (pyran-2-one) ring serves as a suitable central ring template to design selective COX-II



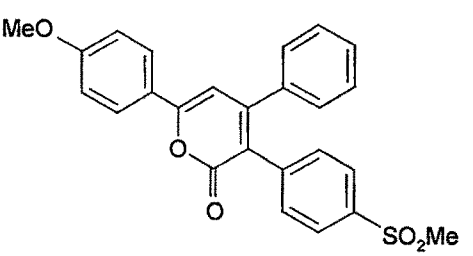
(48)

IC_{50} COX-I = 5.5 μ M,
IC_{50} COX-II = 0.002 μ M



(49)

IC_{50} COX-I = 386.2 μ M,
IC_{50} COX-II = 0.0032 μ M



(50)

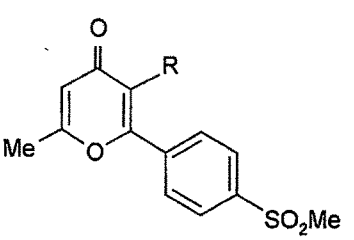
IC_{50} COX-I > 100 μ M,
IC_{50} COX-II = 0.02 μ M

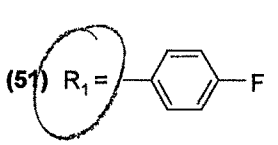
inhibitors when the C=O oxygen atom is suitably positioned to undergo hydrogen bonding to Tyr 355 in the COX-II binding site. The compound

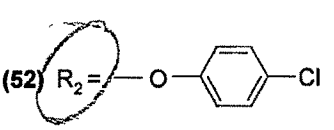
(49) exhibited a very high *in vitro* COX-II inhibitory potency and COX-II selectivity but moderate anti-inflammatory activity compared to celecoxib **(32)** in a carrageenan-induced rat paw edema assay.

The same researchers[141] have prepared 3,4,6-triphenylpyran-2-ones, derivatives with a SO_2Me pharmacophore at the *para* position of either a C-3 phenyl or a C-4 phenyl substituent on the central six-membered pyran-2-one ring. The compound **(50)** obtained proved to be the most potent and selective COX-II inhibitor.

Caturla and co-workers[142] reported a series of 2-phenylpyran-4-ones derivatives as selective COX-II inhibitors. The compound **(51)** having *p*-methylsulphone group at 2-phenyl ring showed the best COX-II inhibitory activity. The introduction of a substituted phenoxy ring at position-3 of the central six-membered pyran-2-one ring **(52)** enhanced both the *in vitro* and *in vivo* activity within the series.





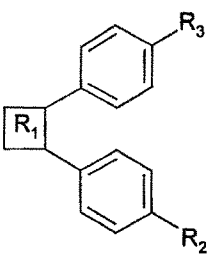


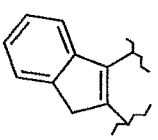
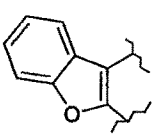
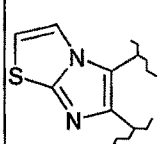
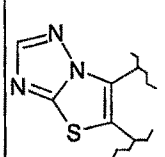
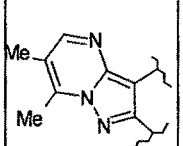
IC_{50} COX-I > 100 μ M,
IC_{50} COX-II = 1.07 μ M

IC_{50} COX-I = 207 μ M,
IC_{50} COX-II = 0.32 μ M

Huang and co-workers[143] synthesized a novel series of diaryl indene. The compound **(53)** showed an IC_{50} value of 0.011 μm for COX-II. Later on, the indene moiety was replaced by benzofuran **(54)** in order to enzymatically prevent the generation of inactive metabolites. This derivative was found to be a very selective COX-II inhibitor **(Table 3).**

Table 3. Effect of different central fused-ring systems on COX-II selectivity.



Compound	R_1	R_2	R_3	COX-I	COX-II
				IC_{50} (μ M)	
53		SO_2Me	F	> 100	0.011
54		SO_2Me	F	> 100	0.02
55		H	SO_2Me	> 50	0.016
56		SO_2Me	H	43	0.01
57		SO_2Me	F	> 10	0.012

Compound L-766112 **(55),** containing the imidazo[2,1-b]thiazole system, was reported to have the selectivity for COX-II enzyme. In carrageenan induced rat paw edema assay, the compound **(55)** showed to be superior to DuP-697 **(13)** [144].

A series of thiazolo[3,2-b] [1,2,4]triazole derivatives was also prepared[145]. This study yielded derivative L-768277 **(56),** which was 4,300 times more selective PGHS-II enzyme **(Table 3)**. This compound was potent in the rat paw edema model (ED_{50} = 1.7 mpk).

The compound **(57)** having the pyrazolo[1,5-a]pyrimidine as a central heterocycle provided the highest potency and selectivity towards the COX-II enzyme in this series[146].

1.7 DIARYL HETEROCYCLES AS SELECTIVE COX-II INHIBITORS FOR ANTI-INFLAMMATORY ACTION: RECENT SETBACKS

Selective COX-II inhibitors, a new generation of NSAIDs designed to overcome the side effects associated with traditional NSAIDs, are devoid of gastrointestinal toxicity. The clinical results for selective COX-II inhibitors such as, celecoxib **(32),** rofecoxib **(36)** and valdecoxib **(40)** are promising. However, the tendency to search for more specific inhibitors has also provoked critical reactions. Certainly selective COX-II inhibitors reduce the risk of GI side effects, but COX-II is not only a proinflammatory inducible enzyme, it also has a number of physiological functions which means that it is constitutively expressed to a high extent in the human body[71,72,147]. COX-II seems to be involved in the regulation of the renin-angiotensin system[148,149], and to possess vasoactive and anti-atherogenic properties[150]. Moreover, the normal induction of COX-II is important for ovulation and, at the end of pregnancy, high uterine levels of COX-II are necessary for the onset of labor[148,151]. The role of elevated

COX-II enzyme levels in ulcerative tissues for wound healing and in new bone growth have already been mentioned.

The inhibition of COX-II may result in unwanted effects. The major side effects observed due to COX-II inhibition are, blood clots, myocardial infarction, heart attack, strokes and congestive cardiac failure[152,153]. On September 30, 2004, Merck and Co. withdrew rofecoxib **(36)** from the international market because of an excess risk of myocardial infarctions and strokes[154]. Pfizer is to withdraw its drugs celecoxib **(32)** and valdecoxib **(40)** due to the risk of heart disease and life-threatening skin reactions[152,155]. Other serious but uncommon problems with COX-II inhibitors include kidney problems (occur rarely), including acute kidney failure[156] and worsening of chronic kidney failure and liver damage[157]. Less serious side effects include heartburn, stomach-pain and upset, nausea and/or vomiting, diarrhea, upper and/or respiratory tract infection and/or inflammation, hypertension, headache, dizziness, swelling of the lower extremities and back-pain[158,159].

The role of COX-II inhibition in producing the side effects is complex and is not fully understood. While nonselective NSAIDs inhibit the formation of platelets-derived thromboxane and endothelial prostacyclin, COX-II inhibitors preferentially suppress the vasodilator and platelet inhibitory prostaglandins without blocking the vasoconstrictive and platelet activating prostaglandins, which could result in a prothrombotic effect. Accelerated atherogenesis of COX-II inhibitors might be further modulated by renovascular hypertension, inhibition of vascular inflammation, improvement of endothelial function and changes in atherosclerotic plaque stability[160-162].

The above mentioned side effects may differ among structurally distinct COX-II inhibitors with different levels of COX-I or COX-II selectivity[162-164]. It was found by different clinical studies that toxicities

related to COX-II inhibition among drugs in a particular class differ. Kimmel and colleagues[165] offered evidence for a differential effect on cardiotoxicity among COX-II inhibitors. They found that the use of rofecoxib **(36)** was associated with statistically significant 2.72-increased odds of myocardial infarction as compared with celecoxib **(32)**. The most reliable evidence of cardiovascular toxicity came from large randomized, double-blind trials. Results of the Vioxx Gastrointestinal Outcomes Research (VIGOR) study[166] revealed an increased risk of myocardial infarction for patients treated with 50 mg of rofecoxib **(36)**, whereas similar large trials with celecoxib **(32)** demonstrated very low risk of cardiovascular side effects that was comparable with nonselective NSAIDs. Several large observational studies found an increased rate of coronary heart disease with high-dose rofecoxib **(36)** but not with celecoxib **(32)**[167,168]. Mamdani and colleagues[169] found an increased risk for congestive heart failure with rofecoxib **(36)** and not with celecoxib **(32)**. It is not clear whether these side effects are really because of unopposed COX-II inhibition or other drug specific mechanisms.

1.8 SELECTIVE COX-II INHIBITORS: NEWER THERAPEUTIC TARGETS

During the past few years, selective inhibitors of COX-II enzyme have emerged as important pharmacological tools for the treatment of pain and inflammation along with some side effects due to its physiological role in body as mentioned previously. But, recently it was found that higher concentrations of COX-II was also observed in several other disease states other than in inflammation, such as colonic polyposis, various forms of cancer, Alzheimer's disease and vascular restenosis following angioplasty[73-76,170]. Prevalence of higher concentrations of COX-II enzyme than the normal in these disease states has opened avenues for the possible therapeutic mitigation of these diseases. Involvement of COX-II expression in these diseases and the role COX-II

inhibitors could play as potential therapeutic agents in future is briefed below.

1.8.1 ROLE OF COX-II IN ALZHEIMER'S DISEASE

A progressive and irreversible brain disorder, Alzheimer's disease (AD) is characterized by three major pathogenic episodes[171] involving (i) an aberrant processing and deposition of β amyloid precursor (β-APP) to form neurotoxic β-amyloid (β-A) peptides and an aggregated insoluble polymer of β-A peptides and aggregated insoluble polymer of β-A that forms the senile plaque (ii) the establishment of intraneuronal neuritic face pathology yielding widespread deposits of agyrophilic neurofibrillary tangles(NFT) and (iii) the initiation and proliferation of a main specific inflammatory response. These three seemingly diverse attributes of AD etiopathogenesis are linked by the fact that proinflammatory microglia, reactive astrocytes and their associated cytokines are associated with the biology of the microtubule- associated protein tue, β-A speciation and aggregation. Specific β-A fragment such as $\beta\text{-A}^{42}$ can further potentiate the proinflammatory mechanism. Expression of the inducible COX-II and cytosolic phospholipase A^2 (CPLA 2) are strongly activated during cerebral ischemia and trauma, epilepsy and AD, indicating the induction of proinflammatory gene pathways as a response to brain injury[172,173]. Studies have identified a reduced risk for AD in patients previously treated with NSAIDs for non-CNS afflictions that include arthritis[174]. In a memory test on rats using specific COX-I and COX-II inhibitors, it was established that COX-II is a required biochemical component indicating the consolidation of hippocampal-dependent memory[175].

1.8.2 IN VIRUS REPLICATION

PGE2, the product of COX-II activity, increased transiently by a factor of more than 50 in cultures of human fibroblasts infected with human cytomegalovirus[176]. Both specific and non-specific COX-II

inhibitors could abrogate the virus-mediated induction of PGE2 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 fibroblasts 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[177] argued that elevated levels of PGE_2 were required for efficient replication of human cytomegalovirus in fibroblasts. Another finding[178] 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 HCC (hepatocellular carcinoma) patients.

1.8.3 COX-II IN CANCER

Several lines of evidence suggest that COX-II enzyme plays an important role in carcinogenesis. Increased amounts of COX-II are commonly found in both premalignant tissues and malignant cancers of the head and neck, oesophagus and lung[179-181] COX-II expression is induced in a variety of cells leading to high levels of prostaglandin production. Such aberrant expressions of COX-II have been reported in murine and human breast cancer, human colon cancer, lung cancer, head and neck cancer and pancreatic cancer[77,182-187]. 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.

Cancer of GIT

Both COX-I and COX-II have been detected in apparently normal GI epithelium, raising the possibility that both may contribute to the

generation of cytoprotective prostaglandins[187]. Over expression of COX-II has been observed in the colon tumors therefore, specific inhibitors of COX-II activity could potentially act as chemoprotective agents[188] Two separate studies[189,190] have indicated protection offered by celecoxib against colorectal carcinoma.

Pancreatic carcinomas

COX-II mRNA and protein expression were found to be frequently elevated in human pancreatic adenocarcinomas and cell lines derived from such tumors. Immunohistochemistry demonstrated COX-II expression in 67 % of pancreatic carcinomas. Sulindac and NS-398 produced a dose-dependent inhibition of cell proliferation in all pancreatic cell lines tested[191]. The findings indicated that COX-II up-regulation is a frequent event is pancreatic cancers and suggested that NSAIDS may be useful in the chemoprevention and therapy of pancreatic carcinoma[192].

Cancer of gall bladder

In a study of a human gall bladder cancer cell line, it was specifically the COX-II inhibitor and not the COX-I inhibitor that decreased mitogenesis and increased gall bladder cell apoptosis[193]. The inhibition of replication of gall bladder cancer cells and the increase in apoptosis produced by the selective COX-II inhibitor suggests that COX enzymes and the prostanoids may play a role in the development of gall bladder cancer and that the COX-II inhibitors may have a therapeutic role in the prevention of gall bladder neoplasms[194].

Cholangiocarcinoma

In normal liver, constitutive expression of COX-II protein was a characteristic feature of hepatocytes whereas no COX-II immunosignal was detectable in bile duct epithelium, Kupffer and endothelial cells. In cholangiocarcinoma cells, COX-II was strongly expressed at high

frequency. It was concluded in the study[195] that aberrant COX-II expression seemed to be related to later stages of cholangiocarcinogenesis.

Malignant pheochromocytoma

In a study[196] on patients with malignant and benign pheochromocytomas and normal persons it was found that normal adrenal medulla does not express COX-II immunohistochemically; however, strong COX-II protein expression was found in malignant pheochromocytomas, whereas most benign tumors expressed COX-II only weakly. These findings suggest that negative or weak COX-II expression in pheochromocytomas favors benign diagnosis.

Malignant thyroid nodules

A study[197] was undertaken to evaluate whether COX-II was up-regulated in human thyroid neoplasia. The data indicated that COX-II was up-regulated in human thyroid cancer, but not in benign thyroid nodules and suggested that COX-II expression might serve as a marker of malignancy in thyroid nodules.

Lung Cancer

Recent studies have demonstrated that increased expression of COX-II was observed frequently in human non-small cell lung carcinoma (NSCLC) and elevated biosynthesis of PG's was found in NSCLC cell lines[198]. NS-398, a specific COX-II inhibitor inhibited PGE_2 synthesis and induced G_1 growth arrest in A549 lung cancer cells[199].

Breast Cancer

Using a highly metastatic mammary tumor cell line that expresses both COX isoforms, it was shown[200] that oral administration of either a selective COX-II inhibitor celecoxib or a selective COX-I inhibitor SC-560 to mice with established tumors results in significant inhibition of

tumor growth. Administration of the dual inhibitor indomethacin leads to even better growth control. Pretreatment of tumor cells with COX inhibitors also reduces metastatic success, indicating that tumor cells may be a direct target of action by COX inhibitors. Results of a study[201] indicated that elevated COX-II expression is more common in breast cancers with poor prognostic characteristics and is associated with an unfavorable outcome. The present finding supports efforts to initiate clinical trials on the efficacy of COX-II inhibitors in adjuvant treatment of breast cancers.