Structural and Functional Basis of Cyclooxygenase Inhibition

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Received November 13, 2006

I. Introduction

Brief History. The use of medicinal substances for the treatment of pain and fever dates to ancient Egyptian and Grecian civilizations, where dried myrtle leaves or bitter extracts from the bark of poplar trees were used to treat back and abdominal pain. The Ebers papyrus from ancient Egypt (1850 B.C.) is the oldest preserved medical text and contains the first record documenting the use of plant remedies for the treatment of pain and inflammation. Other records show that in 400 B.C., Hippocrates prescribed the bark and leaves of the willow tree to reduce fever and to relieve the pain of childbirth. The first published report documenting the antipyretic and analgesic properties of willow bark appeared in England in 1763 in a presentation to the Royal Society by Reverend Edward Stone.1 The active component of willow bark was later identified as salicin, which is metabolized to salicylate. In 1832, the French chemist Charles Gerhardt experimented with salicin, generating salicylic acid, and in 1860 Kolbe and Lautemann developed a highly efficient method for the synthesis of salicylic acid from phenol, which led to the use of the compound in the general population as an antiseptic and antipyretic.

In 1897, Felix Hoffman from the Bayer Company developed a more palatable form of salicylate by synthesizing acetylsalicylic acid, which was called “aspirin” and distributed by Bayer in tablet and powder form in 1899. In the decades that followed, other compounds that possessed similar antipyretic, analgesic, and antiinflammatory properties (phenylbutazone (4-butyl-1,2-diphenylpyrazolidine-3,5-dione), 1949, and indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid), 1963) were developed, although the mechanism of action was unknown. In 1971, John Vane showed that aspirin, indomethacin, and sodium salicylate all cause a dose-dependent decrease in the synthesis of prostaglandins from cell-free homogenates of lung tissue.2 Vane was awarded the Nobel Prize in physiology and medicine in 1982, in conjunction with Sune Bergstrom and Bengt Samuelson, for “discoveries on prostaglandins and related biologically active substances”.

Vane,2,3 concurrent with Smith and Willis,4 proposed that aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs5) inhibited the enzyme activity that converts polyunsaturated fatty acids to prostaglandins during the inflammatory process. The prostaglandin endoperoxide synthase or fatty acid cyclooxygenase (COX) that catalyzes the dioxygenation of arachidonic acid (AA) to form prostaglandin H2 (PGH2) and the resultant prostaglandins was first characterized in detail in 1967 using preparations from sheep seminal vesicles.5 A purified and enzymatically active COX was isolated in 1976,6 and the existence of two COX isoforms (encoded by distinct genes) with high amino acid sequence homology (60%) but differential expression profiles was reported in 1991.7,8 Both COX isoforms are bifunctional, membrane-bound enzymes located on the luminal surfaces of the endoplasmic reticulum and on the inner and outer membranes of the nuclear envelope.9 Found in most tissues, COX-1 is the constitutively expressed isoform and is involved in the production of prostaglandins that mediate basic housekeeping functions in the body. Although COX-2 is

* Abbreviations: APPROVe, Adenomatous Polyp Prevention on Vioxx trial; APC, Adenoma Prevention with Celecoxib trial; AA, arachidonic acid; COX, cyclooxygenase; hCOX-2, human cyclooxygenase-2; mCOX-2, murine cyclooxygenase-2; NSAID, nonsteroidal antiinflammatory drug; POX, peroxidase; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PGF2α, prostaglandin F2α; PGG2, prostaglandin G2; PGH2, prostaglandin H2; PGI2, prostaglandin I2 or prostacyclin; oCOX-1, ovine cyclooxygenase-1; TxA2, thromboxane A2.

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Although the precise molecular mechanism involved is primarily an inducible enzyme, the expression of which is activated in response to cytokines, mitogens, endotoxin, and it is constitutively expressed in some tissues (e.g., brain and kidney), the COX-2 isoenzyme has proven to play a central role in the chemopreventive action of these inhibitors. However, important questions remain concerning the benefit–risk profiles of traditional NSAIDs and both the diaryl heterocycle class of COX-2 selective inhibitors and new, structurally distinct inhibitors like lumiracoxib (2-[[2-chloro-6-fluorophenyl]amino]-5-methylphenyl)acetic acid) that are selective for COX-2.

There are several excellent reviews of the structure and mechanism of COX enzymes and the structure–function relationships of COX inhibitors. This review will focus on the structural and functional basis of the inhibition of COX enzymes by nonselective and COX-2 selective inhibitors. It will integrate kinetic, mechanistic, and structural information to illustrate not only the range of molecules with COX inhibitory activity but also the diversity of mechanisms by which they act.

II. Cyclooxygenase Enzymes: Structure and Mechanisms

COX-1 and COX-2 are bifunctional enzymes that carry out two sequential reactions in spatially distinct but mechanistically coupled active sites: the double dioxygenation of arachidonic acid to prostaglandin G2 (PGG2) and the reduction of PGG2 to PGH2. Arachidonic acid oxygenation occurs in the cyclooxygenase active site, and PGG2 reduction occurs in the peroxidase active site. PGH2 diffuses from the COX proteins and is transformed by different tissue-specific isomerases to prostaglandins (PGE2, PGD2, PGE2, PGF2α, PGI2) and thromboxane A2 (TXA2) (Figure 1).

COX-1 and COX-2 are homodimers of 70 kDa subunits and dimerization is required for structural integrity and catalytic activity. 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. 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. 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 (APC, celecoxib) and the Adenoma Prevention with Celecoxib trial (APC, celecoxib). 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 APC 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), parecoxib (N-[4-(5-methyl-3-phenylisoxazol-4-yl)phenyl]-sulfonamide), and valdecoxib (4-(5-methyl-3-phenylisoxazol-4-yl)benzenesulfonamide)) and some nonselective classical NSAIDs also might pose a risk for increased cardiovascular events. Nevertheless, COX-2 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 concerning the benefit–risk profiles of traditional NSAIDs and both the diaryl heterocycle class of COX-2 selective inhibitors and new, structurally distinct inhibitors like lumiracoxib (2-[[2-chloro-6-fluorophenyl]amino]-5-methylphenyl)acetic acid) that are selective for COX-2.

Figure 1. COX enzymes catalyze the committed step in prostaglandin synthesis. In the cyclooxygenase reaction, two molecules of oxygen are incorporated into arachidonic acid to yield PGG2. PGG2 diffuses to the peroxidase (POX) active site and undergoes a two-electron reduction to form PGH2. PGH2 is converted by tissue/cell specific enzymes (synthases) to various prostaglandins and TXA2.
Although it has been assumed that both subunits are active simultaneously, recent work suggests that substrate or inhibitor binding in the cyclooxygenase active site at one subunit precludes binding of another molecule at the other subunit.29

Each monomer of COX consists of three structural domains: a short N-terminal epidermal growth factor domain, a membrane binding domain, and a large, globular C-terminal catalytic domain (Figure 2).28,30,31 The COX and peroxidase (POX) active sites are located on opposite sides of the catalytic domain with the heme prosthetic group positioned at the base of the peroxidase site. The epidermal growth factor domain and catalytic domain create the dimer interface and place the two membrane binding domains on the same face of the homodimer about 25 Å apart.25 The membrane binding domain of cyclooxygenase is composed of four amphipathic α-helices, with hydrophobic and aromatic residues that project from the helices to create a surface that interacts with one face of the lipid bilayer.26 Three of the four helices lie in the same plane, whereas the last helix (helix D) projects up into the catalytic domain.25

The catalytic domain constitutes the majority of the COX monomer and is the site of substrate binding and NSAID action. The entrance to the COX active site occurs at the base of the membrane binding domain and leads to a long hydrophobic channel that extends deep into the interior of the catalytic domain (Figure 3).28 The COX channel narrows at the interface between the membrane binding domain and the catalytic domain to form a constriction composed of three residues (Arg-120, Tyr-355, and Glu-524) that separates the “lobby” from the active site.28 The COX-1 and COX-2 active sites are very similar but differ in the presence of a side pocket in COX-2 located above the active site and is surrounded by six aromatic amino acids. Mutation of Gly-533 at the top of this channel seals off the channel and abolishes the oxygenation of arachidonic acid but not that of fatty acids with shorter carbon chains.33

Crystal structures and molecular models of ovine COX-1 (oCOX-1) and murine COX-2 (mCOX-2) complexed with arachidonic acid indicate that the carboxylic acid of the substrate ion-pairs to the guanidinium group of Arg-120 and hydrogen-bonds to Tyr-355. The aliphatic backbone projects up into the top of the cyclooxygenase active site from the hydrophobic channel and then makes a sharp bend in the vicinity of Tyr-385 (Figure 5).32,33 In these structures, the vast majority of contacts between the substrate and various protein residues in the active site involve van der Waals interactions. The C-terminal constriction residues, although some inhibitors have been shown to make interactions with lobby residues. Several residues in the lobby region that are thought to be important for inhibitor interactions (Pro-86, Ile-89, Leu-93, and Val-116) are shown in red. Twenty-four residues line the COX active site with only one difference between COX-2 and COX-1 (Val-523 to Ile-523). Eight COX active site residues are shown in purple. The heme group in the POX active site is designated in red.

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Figure 2. Structural representation of the murine COX-2 dimer.31 The N-terminal epidermal growth factor domain is designated in pink and leads into the four α-helices of the membrane binding domain (yellow). Helix D projects up into the COX active site, which is located at the base of the large, globular catalytic domain (cyan). The heme prosthetic group (red) lies in the POX active site.

Figure 3. Cyclooxygenase active site of murine COX-2. Both COX-1 and COX-2 contain a 25 Å length hydrophobic channel that originates at the membrane binding domain (yellow) and extends into the core of the catalytic domain (cyan). The long hydrophobic channel can be divided into the lobby and the substrate/inhibitor binding site of COX by a constriction of three residues: Arg-120, Glu-524, and Tyr-355 (labeled in white). Most inhibitors bind in the COX active site above the constriction residues, although some inhibitors have been shown to make interactions with lobby residues. Several residues in the lobby region that are thought to be important for inhibitor interactions (Pro-86, Ile-89, Leu-93, and Val-116) are shown in red. Twenty-four residues line the COX active site with only one difference between COX-2 and COX-1 (Val-523 to Ile-523). Eight COX active site residues are shown in purple. The heme group in the POX active site is designated in red.
to PGH₂. Although the POX reaction is generally considered the second step in the formation of product, the COX reaction is absolutely dependent on POX activity for its activation.10,35 In short, one turnover of the POX reaction is required to provide the tyrosyl radical for initiation of the COX reaction, which continues to turn over, in the presence of fatty acid substrate, until radical-induced inactivation occurs (Figure 6). The productive conformation of arachidonic acid observed in crystal structures positions the 13-pro(S) hydrogen of the substrate 2.3 Å from the phenolic oxygen on Tyr-385, ideally located for abstraction by the tyrosyl radical produced during catalysis.37–40

Interestingly, a crystal structure of mCOX-2 is available in which a mixture of structures was observed containing substrate (AA) and product (PGH₂) bound in the cyclooxygenase active site.32 Although the product species is ambiguous (it could be PGG₂ or PGH₂), the carboxylate of the molecule is positioned near Arg-120 and Tyr-355 and the ω-end is bound in the top channel, similar to arachidonic acid.41 This conformation of product, in which the PGG₂/PGH₂ species hydrogen-bonds with the constriction site residues and bends in an L-shaped fashion at Tyr-385, suggests that arachidonic acid was positioned properly for catalysis. The mixed structure cocrystal also yielded a conformer of arachidonic acid that is bound in an inverted configuration with its carboxylate hydrogen-bonded to Tyr-385 and Ser-530. This binding conformation of arachidonic acid in the active site is thought to reflect a noncatalytic or inhibitory binding mode where no viable products can be produced. High-resolution crystal structures of mCOX-2 with arachidonate and eicosapentanoic acid (EPA) also have revealed an inhibitory or nonproductive binding mode of substrate in the active site.41 Mechanistic studies have confirmed that this binding mode is not viable for catalysis.32,42

III. Kinetic and Molecular Basis of Cyclooxygenase Inhibition

III.1. Introduction. A general model for COX inhibition is emerging in which multiple equilibria are established between free enzyme, inhibitor, and two or three enzyme—inhibitor complexes. All COX inhibitors can be discussed on the basis of which steps or series of steps are observed in their inhibitory
mechanisms. Aspirin (2-(acetyloxy)benzoic acid) is the only clinically used inhibitor to irreversibly inactivate COX-1 and COX-2 through time-dependent, covalent modification of the COX active site. All other COX inhibitors, whether nonselective or COX-2 selective, associate with the protein in a noncovalent manner. The most potent COX inhibitors are slow, tight binding inhibitors that form very stable binary complexes. In some cases, the dissociation rates of enzyme–inhibitor complexes are so slow that the inhibitors appear to be functionally irreversible.

The structural requirements for the time-dependent inhibition of prostaglandin biosynthesis by different antiinflammatory drugs were first evaluated by Rome and Lands in 1975. In this study, ibuprofen (2-[4-(2-methylpropyl)phenyl]propanoic acid) and mefenamic acid (2-(2,3-dimethylphenyl)aminobenzoic acid) (Figure 7) display competitive and rapidly reversible inhibition of the COX activity of sheep seminal vesicle preparations that is characterized by a single-step mechanism (one forward and one reverse rate constant to describe enzyme–inhibitor association and dissociation, respectively). On the other hand, flurbiprofen (2-(3-fluoro-4-phenylphenyl)propanoic acid) and meclofenamic acid (2-(2,6-dichloro-3-methylphenyl)aminobenzoic acid) exhibit time-dependent, functionally irreversible inhibition of COX activity that displays a two-step mechanism. Rome and Lands developed a kinetic model to explain their observations in which there is an initial rapid, reversible binding of the inhibitor to the enzyme characterized by a dissociation constant, $K_I$, followed by an essentially irreversible time-dependent change in the enzyme–inhibitor complex characterized by the rate constant $k_{\text{inact}}$:

$$E + I \xrightleftharpoons[k_1]{} [EI] \xrightarrow{k_{\text{inact}}} EI^* \quad K_1 = k_{-1}/k_1$$

(1)

In the decades that followed, many other COX inhibitors were investigated for their kinetic mode(s) of inhibition with most NSAIDs and selective COX-2 inhibitors displaying time-dependent inhibition with either a two-step mechanism (e.g., diclofenac (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid) and indomethacin),

$$E + I \xrightarrow[k_1]{} [EI] \xrightarrow[k_{\text{inact}}]{} EI^*$$

(2)

or a three-step (diaryl heterocycles like celecoxib) mechanism.

$$E + I \xrightarrow[k_1]{} [EI] \xrightarrow[k_{\text{inact}}]{} [EI^*] \xrightarrow[k_3]{} EX$$

(3)

As Rome and Lands observed for flurbiprofen, these inhibitors interact with COX initially through a rapid association that is then followed by one or more slow, time-dependent steps, which leads to a much more tightly bound complex. The magnitudes of the rate constants for the reverse reactions are the major determinants of the potency and selectivity of COX inhibition. The molecular basis for the time dependence of some COX inhibitors has been established recently and will be a major focus of this review.

III.2. Aspirin. Aspirin (Figure 7) inhibits cyclooxygenase activity in a time-dependent fashion, although it is the least potent of the time-dependent COX inhibitors as reflected in its unusually high $K_I$ value for initial association with the enzyme. Reaction of COX with aspirin containing a radiolabeled acetyl group leads to incorporation of radioactivity into the protein. Arachidonic acid inhibits acetylation, suggesting that aspirin acetylates a residue in the COX active site channel. Ser-530 is the only residue in COX that is acetylated by aspirin, and a S530A mutant is resistant to aspirin acetylation and time-dependent inactivation. Interestingly, the $K_{m}$ values for arachidonate binding and $IC_{50}$ values for reversible inhibition by several COX inhibitors (aspirin, flurbiprofen) are the same for both the native (Ser-530) and mutant (Ala-530) enzymes. Only the wild-type enzyme is irreversibly inactivated by aspirin, suggesting that the active site serine is not essential for catalysis or substrate binding but is required for the time-dependent covalent inhibition of COX. Despite aspirin’s low affinity for the active site of COX ($K_I = 20 \text{ mM}$), acetylation of Ser-530 progresses rapidly once aspirin has bound in the active site (as indicated by the high values of $k_{\text{inact}}$) and the acetylated serine side chain is stable to hydrolysis following modification.
In the existing crystal structures of COX with aspirin analogues, a hydrogen bond is formed between the carbonyl oxygen of the acetyl adduct and the phenolic hydrogen of Tyr-385. The presence of Tyr-385 across the active site from Ser-530 appears to be a critical determinant of acetylation. Mutation of Tyr-385 to Phe reduces aspirin acetylation of the serine hydroxyl by 93%. Tyr-385 hydrogen-bonds to the acetyl group of aspirin, which increases its reactivity by stabilizing the negative charge of the tetrahedral intermediate of acetylation. This action of Tyr-385 is analogous to the activation of substrate acylation of serine proteases by the oxyanion hole in the active site. Interestingly, aspirin acetylates heme-reconstituted enzyme 100-fold more quickly than apoenzyme. One possible explanation for this effect is that the heme group restricts protein conformational mobility and facilitates enzyme—aspirin interactions. The inhibition of COX enzymes by aspirin is also dependent on the oxidative state of the enzymes; stimulation of COX with peroxides to form the Tyr-385 tyrosyl radical reduces acetylation by aspirin. The requirement of Tyr-385 and the heme prosthetic group for acetylation of Ser-530 underscores the active roles of the different components of the cyclooxygenase active site in the overall inhibition of its enzymatic activity by aspirin.

The use of low-dose aspirin is recommended for cardiovascular prophylaxis. Aspirin irreversibly inactivates platelet COX-1, leading to a decrease in the production of proaggregatory TXA2. Since platelets are enucleated cells and are not able to generate new enzyme, TXA2 synthesis is inhibited for the entire lifetime of the platelet (8–10 days in circulation). Aspirin also selectively targets platelet COX-1 in the presystemic circulation, reducing the possible inhibitory effects on COX-2 derived from the liver. Therapeutically, this 10- to 100-fold selective inhibition of COX-1 over COX-2 by low-dose aspirin is employed in the prophylactic treatment of thromboembolic disease and myocardial infarction. However, the cardioprotective effect of aspirin may be compromised by coadministration of aspirin with other NSAIDs. Ibuprofen, indomethacin, and the COX-2 selective inhibitors N-(2-(cyclohexyloxy)-4-nitrophenyl)methanesulfonylamide 1 (NS-398) and 5-bromo-3-(4-fluorophenyl)-2-(4-(methylsulfonyl)phenyl)thiophene 2 (DuP-697) block aspirin inactivation of platelet COX-1 in vitro and in human studies. Conversely, studies with celecoxib and rofecoxib in healthy human subjects indicate that these highly COX-2 selective inhibitors show no such interference with the antiplatelet activity of aspirin. The results of these clinical studies can be explained by experiments with purified COX-1 and activated human platelets, which demonstrate that the ability of NSAIDs and COX-2 inhibitors to interfere with the aspirin inhibition of COX-1 directly correlates with their inhibitory potency against COX-1 (ibuprofen > celecoxib > valdecoxib > rofecoxib > etoricoxib). Thus, a low affinity for COX-1 and high COX-2 selectivity confer a low potential to block the inhibition of platelet COX-1 by aspirin.

III.3. Phenylpropionic and Arylacetic Acid Inhibitors. The phenylpropionic acid inhibitors ibuprofen and mefenamic acid (Figure 7) are competitive and rapidly reversible inhibitors of COX that inhibit with a single-step kinetic mechanism, whereas structural analogues of each inhibitor (flurbiprofen and meclofenamic acid) are time-dependent, functionally irreversible inhibitors that follow a two-step mode for COX inhibition. The original kinetic model developed by Rome and Lands explains the kinetic differences between these two classes of inhibitors (eq 1). Ibuprofen and mefenamic acid associate with...
the enzyme in an initial step that is both rapid and reversible and is characterized by a dissociation constant $K_i$. In the case of flurbiprofen and meclofenamic acid, this first step is then followed by an essentially irreversible time-dependent change in the enzyme−inhibitor complex characterized by a rate constant $k_{\text{act}}$. Indomethacin (Figure 7) and other arylacetic acid derivatives are also examples of time- and concentration-dependent inhibitors of prostaglandin synthesis. Treatment of enzyme preparations or purified cyclooxygenase with indomethacin for different times prior to the addition of substrate leads to progressive, time-dependent, functionally irreversible inhibition of COX. Like flurbiprofen, indomethacin conforms to a two-step binding mode for COX inhibition. The tight binding of indomethacin to COX-1 requires the carboxylic acid group because its methyl ester lacks the time-dependent step but retains the fast-reversible (time-independent) step. In contrast, esterification of indomethacin does not abolish time-dependent inhibition of COX-2, although it does reduce its tightness of binding to the enzyme. Indomethacin is recovered intact after prolonged incubation with either enzyme, suggesting that the time-dependent inhibition of COX likely involves a conformational change rather than a covalent interaction with the protein as is seen for aspirin inhibition. Naproxen ((S)-6-methoxy-α-methyl-2-naphthaleneacetic acid) is a phenylpropionic acid inhibitor (Figure 7) that exhibits unique binding kinetics with COX-1 and COX-2. Naproxen is representative of a group of NSAIDs, including some oxicams, that exhibit “mixed” inhibition of COX and display neither classic time-dependent inhibition nor competitive inhibition. Mixed inhibition is characterized by an initial time-dependent loss in enzyme activity, which never reaches a zero end-point, consistent with a slow, reversible, weakly bound inhibitor. Studies probing the differential inhibition of COX-1 and COX-2 by naproxen reveal that the inhibitor displays “mixed” inhibition of COX-2 but lacks a time-dependent component for COX-1 inhibition. Interestingly, the ability of naproxen to inhibit COX slowly and reversibly (albeit weakly), as opposed to NSAIDs that rapidly and reversibly inhibit COX (ibuprofen, mefenamic acid) or inhibit COX in a slow and functionally irreversible manner (indomethacin, diclofenac), may contribute to the potential cardioprotective effects of naproxen noted in clinical trials. This question has received considerable attention in recent years, and studies in humans show that naproxen can mimic the antiplatelet effect of low-dose aspirin.

X-ray crystallography of COX−inhibitor complexes and site-directed mutagenesis studies have helped to elucidate the molecular basis behind the time-dependent inhibition of some arylpropionic and arylacetic acid inhibitors. COX-1 crystal structures of competitive reversible (ibuprofen and methylflurbiprofen) and time-dependent (flurbiprofen and alclofenac (2-(4-(allyloxy)-3-chlorophenyl)ethanoic acid)) inhibitors reveal the same binding conformation of inhibitor in the active site, suggesting that the mechanism of time-dependent inhibition of COX by NSAIDs does not involve global conformational changes in the enzyme structure or the binding of inhibitor to different active sites. In the crystal structure of COX-1 with flurbiprofen, the propionate inhibitor binds in the cyclooxygenase active site with the carboxylate of the inhibitor making hydrogen-bonding contacts with Arg-120 and Tyr-355 at the constriction site (Figure 9A). The structurally similar, but kinetically distinct, ibuprofen binds in a nearly identical manner in the COX-1 active site, making an ion pair with Arg-120 and a hydrogen bond with Tyr-355 (Figure 9B). These crystallographic results suggest that the kinetic differences between competitive, reversible and time-dependent, functionally irreversible inhibitors cannot be entirely explained through the binding modes exhibited by these inhibitors. Site-directed mutation of Arg-120 to alanine in COX-1 reveals that carboxylic acid-containing time-dependent NSAIDs (indomethacin and flurbiprofen) form an ion pair and/or hydrogen bond with Arg-120 and that this interaction is critical for inhibition. The same mutation in COX-2 is not equally effective in eliminating inhibition by indomethacin. Interestingly, a methyl ester derivative of indomethacin exhibits more potent inhibition of the COX-2 R120A mutant than wild-type enzyme, suggesting that interactions with Arg-120 are less important for inhibitor binding and potency with COX-2.

Cocrystals of indomethacin and COX-2 show that indomethacin binds deeply within the cyclooxygenase active site (Figure 10A). The $p$-chlorobenzoyl group projects up into the active site channel, and the chlorine atom interacts with Leu-384 at the top of the active site, while the benzoxy oxygen interacts with Ser-530. The benzoyl group itself is stabilized by hydrophobic interactions with Leu-384, Tyr-385, Phe-381, and Trp-387. The carboxylate of indomethacin forms a salt bridge with Arg-120 and makes additional contacts with Tyr-355 at the constriction site. The $o$-methoxy group protrudes into a large cavity provided by Ser-353, Tyr-355, and Val-523. The indole ring interacts with Val-349 and the 2′-methyl group projects...
increase in activity almost 4-fold lower than wild-type mCOX-2 with a slight decrease in $K_m$. The $k_2' (0.074 \text{ vs } 0.052 \text{ s}^{-1})$, whereas V349L demonstrates a 3-fold increase in $K_1$ that accompanies a $k_2' \geq 0.074 \text{ s}^{-1}$. Indomethacin exhibits slow, time-dependent, and functionally irreversible inhibition of both wild-type mCOX-2 and the V349A mutant with no appreciable reverse rate constant ($k_{-2}$) for the second inhibitory step (eq 2). However, indomethacin demonstrates reversible inhibition with the V349L mutant with a measurable $k_{-2} \geq 0.01 \text{ s}^{-1}$. A 2'-des-methyl analogue of indomethacin is a poor inhibitor of mCOX-2 and the V349 mutants and is readily competed off the enzymes by arachidonic acid. 2-Des-methyl indomethacin does not inhibit COX-1 at all. Therefore, a critical determinant of the time-dependent inhibition of COX by indomethacin is attributed to the binding of the 2'-methyl group of the inhibitor into this small hydrophobic pocket.

![Figure 10](image1.png)

**Figure 10.** Crystal structure of indomethacin in the active site of murine COX-2. Panel A shows key active site residues important for inhibitor binding. Indomethacin is shown in green and is colored by atom. Arg-120, Tyr-355, and Glu-524 at the constriction site are shown in purple. Residues that constitute the small hydrophobic binding pocket (Val-349, Leu-531, Ala-527, and Ser-530), as well as additional active site residues (Tyr-385), are shown in yellow. Panel B is a space-filling model of the 2'-methyl group of indomethacin (green) inserted into the hydrophobic binding pocket (yellow).

Unlike indomethacin, diclofenac inhibition of COX-2 is unaffected by mutation of Arg-120 to alanine or of Tyr-355 to phenylalanine, with $IC_{50}$ values for inhibition that are similar to that of wild-type mCOX-2 (wt, 77 nM; R120A, 257 nM; Y355F, 137 nM). However, a S530A COX-2 mutant is resistant to diclofenac inhibition ($IC_{50} > 50 \mu M$), suggesting that Ser-530 is important for inhibitor binding in the COX-2 active site. In support of this hypothesis, a S530M COX-2 mutant displays a greater than 240-fold increase in $IC_{50}$ for diclofenac over wild-type enzyme. In addition, diclofenac quenches the internal protein fluorescence of apo COX-1 but does not quench the fluorescence of the aspirin-acetylated enzyme, suggesting that diclofenac must interact with Ser-530 in the COX active site for binding and inhibition. Most crystal structures of COX enzymes with carboxylic acid-containing NSAIDs show the inhibitors positioned with their carboxylates coordinated to Arg-120 and their aromatic functional groups projecting up into the cyclooxygenase active site. In contrast, diclofenac binds in the active site of COX-2 in a unique inverted binding mode with its carboxylic acid moiety hydrogen-bonded to Ser-530 and Tyr-385 (Figure 11). The inhibitor also forms extensive van der Waals interactions with several hydrophobic residues within the active site. For example, the phenylacetic acid ring is surrounded by the side chains of Tyr-385, Trp-387, Leu-384, and Leu-352. The dichlorophenyl group forms van der Waals contacts with Val-349, Ala-527, Leu-531 (the indomethacin binding pocket), and Val-523. Unlike most other NSAIDs with carboxylic acid moieties, neither Tyr-355 nor Arg-120 makes contact with the inhibitor. This unique binding mode of diclofenac is analogous to the structure of the nonproductive complex of arachidonic acid and COX-2 in which the substrate binds in an inverted conformation with its carboxylate coordinated to Ser-530 and Tyr-385. This orientation contrasts with most structures solved for fatty acid substrates bound to COX, including arachidonic acid, where the carboxylate of the substrate forms hydrogen bonds or ion pairs with Tyr-355 and Arg-120. The novel, inverted binding of diclofenac and the nonproductive conformation of arachidonic acid in COX-2 highlight the importance of Ser-530 and Tyr-385 in ligand...
association and the chelation of negative charges in COX-2 and may represent a new binding mode exhibited by some classes of NSAIDs.

Recently, a crystal structure of mCOX-2 with lumiracoxib, a COX-2 selective phenylacetic acid derivative of diclofenac (Figure 7), was solved and shows that lumiracoxib also binds in the COX active site in an inverted orientation. The carboxylate of lumiracoxib forms hydrogen-bonding interactions with Ser-530 and Tyr-385 at the top of the active site, similar to diclofenac. The methyl group on the phenylacetic acid ring of lumiracoxib projects into a small groove near Leu-384 in the COX-2 active site. When lumiracoxib is modeled into COX-1, the methyl group of the inhibitor clashes with the side chain of Leu-384 because of changes in its position caused by bulky amino acids in the second shell (Ile-525 and Phe-503 in oCOX-1 and Val-525 and Leu-503 in hCOX-2), suggesting a possible mechanism for the COX-2 selectivity of lumiracoxib. Although the selectivity of lumiracoxib for COX-2 has been determined in vitro and in vivo and the crystal structure of lumiracoxib-bound mCOX-2 has been solved, structure—activity relationship studies (SARs) have not been reported and the chemical and structural determinants for this inhibitor’s COX-2 selectivity remain unknown.

Many attempts have been made to convert nonselective NSAIDs into COX-2 selective inhibitors (Figure 12). Flurbiprofen is the only example of a phenyl propionate inhibitor that has been successfully elaborated into a selective COX-2 inhibitor. Examination of flurbiprofen bound to COX-1 and COX-2 suggests that modification of the 4-phenyl ring to induce steric constraint should result in increased selectivity for COX-2. This hypothesis was validated through introduction of diethoxy substituents in the 4-phenyl ring to generate a potent and selective COX-2 inhibitor. Modification of the N-substituted indole-3-acetic acid framework of indomethacin is also an effective strategy. For example, conversion of the carboxylic acid group to the corresponding ester or amide as well as to the reverse esters/amides results in selective COX-2 inhibition (Figure 12). Interestingly, the 2′-methyl group of indomethacin is critical for inhibitory potency in the neutral amide and ester series, as the des-methyl derivatives are extremely poor inhibitors of the COX enzymes. Structure—activity studies with diclofenac analogues indicate that methyl or chlorine substituents on the lower aniline ring in the ortho position are necessary to achieve potent inhibition of COX. An o,o-difluoro substituted analogue is considerably less active, and compounds that are synthesized to resemble hydroxylated metabolites of diclofenac show inhibitory potencies that are 100 times lower than that of diclofenac. Analogues that possess higher potency than diclofenac itself have halogen substitutions (fluorine or chlorine) at the 5′ position of the phenylacetic acid ring and compounds that are synthesized to resemble hydroxylated metabolites of diclofenac show inhibitory potencies that are 100 times lower than that of diclofenac. Analogues that possess higher potency than diclofenac itself have halogen substitutions (fluorine or chlorine) at the 5′ position of the phenylacetic acid ring.

Patent literature suggests that modifications to the carboxylic acid group of diclofenac have yielded potent and selective COX-2 inhibitors (Figure 12). Incorporation of meta-alkyl substituents on the phenylacetic acid ring of diclofenac changes the selectivity of the inhibitor to favor inhibition of COX-2, whereas the incorporation of halogens at the 2 and 6 positions on the lower aniline ring influences the potency of cyclooxygenase inhibition (Figure 12).
can be traced to an older COX inhibitor, phenylbutazone (Figure 13). Phenylbutazone and other diaryl heterocycles, such as oxypHENylbutazone (4-butyl-1-(4-hydroxyphenyl)-2-phenyl-3,5-pyrazolidinedione) and oxaprozin (3-(4,5-diphenyl-1,3-oxazol-2-yl)propanoic acid), were developed as antiinflammatory and analgesic agents. A multitude of other diaryl heterocycles with variations on the central heterocyclic ring were synthesized and screened as COX inhibitors and antiinflammatory agents.91 Compound 2 (DuP 697, Figure 13) was a Dupont-Merck lead compound that was able to inhibit the COX activity of activated macrophages but not the COX activity present in platelets. This was a puzzling finding at the time but was later explained by the discovery of the second COX isoform (COX-2) and its differential expression profile from COX-1. Compound 2 was able to selectively inhibit the activity of COX-2 protein induced in lipopolysaccharide-activated macrophages but not that of the constitutively expressed COX-1 in platelets. With the discovery that mammalian tissues code for another form of COX whose expression is induced during macrophage activation, compound 2 became an attractive lead for the development of selective inhibitors. Extensive structure–activity studies demonstrated that a 4-sulfonamido or 4-methylsulfonyl substitution on the phenyl ring of the inhibitor provides COX-2 selectivity but that no other substitutions are tolerated.92,93 From additional structure–activity analysis, it became clear that the fundamental factors responsible for the potent and selective inhibition of COX-2 include (1) two aromatic rings on adjacent positions on a central scaffold and (2) the presence of a sulfonamide or methyl sulfone group on one of the phenyl rings.94

The kinetic mechanism behind the selective COX-2 inhibition of compound 2 was probed in recombinantly expressed and purified human COX-1 and COX-2. Compound 2 showed a competitive reversible association with COX-1, indicative of the kinetic profile of noncovalent inhibitors that display a rapid, single-step mechanism for COX inhibition.95 Interestingly, compound 2 also showed an initial competitive interaction with COX-2 that was followed by a slow, time-dependent step, which was responsible for the inhibitor’s selectivity for the inducible enzyme.95 Dialysis of the inhibited enzyme showed no recovery of COX-2 activity but complete recovery of COX-1 activity. Denaturation of COX-2 released compound 2, which could inhibit another sample of enzyme, showing that the inhibition of COX-2 was not due to covalent binding.

Sequence alignments between COX-1 and COX-2 and homology modeling of human COX-2 based on the existing ovine COX-1 crystal structure28 identified Val-523 as the only residue in the main channel of the COX-2 active site that is not conserved in COX-1.96,97 Two groups simultaneously studied the role of Val-523 to investigate the structural features of COX-2 that influenced the mechanism of the time-dependent action of COX-2 selective inhibitors. Guo et al. created a series of Val-523 mutants in human COX-2 (including V523I) and tested the mutants against compounds 1 and 2 and 5-(4-fluorophenyl)-1-(4-(methylsulfonyl)phenyl)-3-(trifluoromethyl)-1H-pyrazole 3 (SC-58125). These COX-2 selective inhibitors suppress wild-type COX-2 activity through an initial step that involves competitive, reversible binding followed by a time-dependent transition to a tightly bound enzyme–inhibitor complex, whereas inhibition against COX-1 is rapid and reversible. Against the V523I mutant, all inhibitors lose the time-dependent component of their inhibition of COX-2, pointing to a role for this residue in the structural transition that underlies the time-dependent inhibition of these COX-2 selective agents. Gierse and co-workers probed the molecular basis of the time-dependent inhibition of purified human COX-2 by diaryl heterocycles by also constructing a V523I mutant of human COX-2.97 The V523I mutation abolishes the selectivity of all the inhibitors for COX-2, whereas classical NSAIDs like indomethacin show no change in their selectivity profiles. In addition, a series of mutations were made at the mouth of the active site of human COX-2 (Y115L, S119V, and F357L) to evaluate these residues for their contribution to the selective inhibition of COX-2 and the molecular basis of the time-dependent inhibition of this enzyme.
inhibitors and the substitution of His-513 in COX-1 for increases the affinity of the enzyme for COX-2 selective and COX-2 is sufficient to confer COX-2 selectivity for these indicating that the single Val-523 difference between COX-1 group of compound COX-2 active site compared to that of COX-1.30 The nearly effect on the selectivity of compounds inhibition of COX-2 by the diaryl heterocycles. Substitution of Val-434 and Arg-513. 31 Compound 4-(5-(4-bromophenyl)-3-pyrazol-1-yl)benzenesulfonamide (SC-299) is a fluorescent diaryl heterocycle that is a potent, selective inhibitor of COX-2 but a weak, competitive inhibitor of COX-1.100 The binding of 5 to both COX-1 and COX-2 proceeds in a time-dependent fashion. Binding to COX-1 occurs in a rapid bimolecular reaction that is followed by a slower unimolecular step. Binding to COX-2 also demonstrates a rapid bimolecular reaction but is followed by two unimolecular steps. The slower of these steps is abolished by mutation of Val-523 to Ile. A dramatic difference exists in the rate of dissociation of compound 5 from the two enzymes, which accounts for the selectivity of inhibition of COX-2. Dissociation of 5 from COX-1 in the presence of the nonfluorescent competitor, flurbiprofen, is rapid and complete in less than 1 min, whereas the dissociation of 5 from COX-2 is extremely slow and occurs over several hours.100 Mutation of Val-523 to Ile drastically reduces the half-life for dissociation from COX-2 to a value that approximates the half-life for dissociation from COX-1. Consequently, it is the time-dependent step in the inhibition of COX (two-step for COX-1, three-step for COX-2) that is responsible for the COX-2 selectivity of compound 5; however, it is the tightness of binding reflected in the off-rate that determines the magnitude of selectivity. The slow step in association and dissociation appears to reflect inhibitor binding in the side pocket because mutation of Val-523 eliminates the third step in binding and drastically accelerates the rate of dissociation.

The kinetic basis for the selective inhibition of COX-2 by celecoxib has also been evaluated. As expected, it exhibits competitive reversible kinetics with COX-1.14 An initial competitive interaction is also observed with COX-2, which is followed by a time-dependent interaction that leads to potent inhibition of the enzyme. A more detailed kinetic study indicates the existence of a three-step kinetic mechanism for the selective inhibition of COX-2 by celecoxib.101 The reversible association of diaryl heterocycle inhibitors with COX, measured by following the inhibition of consumption of molecular oxygen as arachidonic acid is oxygenated by COX-2, comprises two steps that may or may not appear kinetically distinct, depending on the relative magnitudes of the rate constants.101 The selectivity of the inhibitors for COX-2 is derived from the contribution of a third pseudoirreversible step that leads to a tightly bound complex. The difference in the rate constants for association of diaryl heterocycles with COX-1 and COX-2 does not correlate to the differences in selectivity. Rather, selectivity is determined by the rate constants for dissociation. These studies of the kinetics of inhibition correlate closely to the fluorescence quenching studies summarized above and can be interpreted in the context of the crystal structures demonstrating the importance of insertion of the sulfonamide or methylsulfonyl groups into the side pocket as the mechanism of time-dependent inhibition.31

IV. Cardiovascular Toxicity and the Benefit–Risk Calculation

Rofecoxib was withdrawn from the market in 2004 following the detection of an increase in cardiovascular events (myocardial
infarctions and strokes) in individuals taking a 25 mg daily dose as part of a colorectal polyp prevention trial. A prior trial of a 50 mg dose of rofecoxib had revealed a higher incidence of cardiovascular events compared to naproxen, but there was uncertainty about whether rofecoxib induced cardiovascular effects or naproxen protected against them. The polyp prevention trial was conducted for 3 years and compared the anti-inflammatory dose of rofecoxib to placebo. Although rofecoxib doubled the incidence of cardiovascular events compared to placebo, no difference was observed between the treated and placebo groups until after approximately 18 months. The findings in this trial resulted in the voluntary withdrawal of rofecoxib from the market and raised many questions that are only now beginning to be answered. FitzGerald and colleagues have recently reviewed the relevant scientific and clinical literature.

The first and most important question is whether the cardiovascular side effects are unique to rofecoxib or are a class effect due to COX-2 inhibition. Results of a similar placebo-controlled polyp prevention trial with two different doses of celecoxib (200 or 400 mg twice daily) revealed dose-dependent cardiovascular side effects. Analogous to the rofecoxib trial, no difference between treated and placebo groups was observed for 12 months, but ultimately there was a clear statistically significant difference in both dose groups (2.6-fold in the 200 mg twice daily dose group and 3.4-fold in the 400 mg twice daily dose group). Shortly thereafter, the results of two separate trials of valdecoxib (with pretreatment with its injectable produg parecoxib) for postsurgical pain associated with coronary artery bypass grafting revealed a 3-fold increase in drug-induced cardiovascular events compared to placebo. Importantly, both valdecoxib trials revealed cardiovascular side effects after only 10 or 14 days of treatment. The similarity of the results with rofecoxib, celecoxib, and valdecoxib, which are structurally and electronically different members of the diaryl heterocycle group of COX-2 inhibitors, strongly suggests that the cardiovascular side effects are a class effect.

The second question is how COX-2 inhibition leads to cardiovascular toxicity. The anticipated finding that COX-2 inhibitors reduce the urinary excretion of the major metabolite of prostacyclin (PGI₂) provides critical insight into this issue. Prostacyclin is the major COX-2-dependent product of vascular endothelial cells, and it plays an important role in regulating vascular tone and atherosclerosis. Although COX-1 is the major cyclooxygenase enzyme when vascular endothelial cells are cultured in vitro, COX-2 is induced in these cells by laminar flow stress. This appears to explain why COX-2 inhibitors have such a significant effect on prostacyclin levels in vivo. It is noteworthy that COX-2-selective inhibitors also significantly depress the levels of the urinary metabolites of PGE₂, another important mediator of inflammation and vascular tone.

The third question is why the cardiovascular side effects are only observed in a subset of individuals taking COX-2 inhibitors. Different individuals respond differently to a given COX-2 inhibitor, but more than 2% of the individuals in the polyp prevention trials experienced significant reduction in PGI₂ production. Does the low incidence of toxicity have a statistical or biological basis; i.e., would everyone who experiences COX-2 inhibition eventually develop cardiovascular toxicity or do other factors collaborate with COX-2 inhibition to cause the side effects? Neither explanation can be ruled out at this time, but there are data to support a multifactorial basis for cardiovascular toxicity. Recent studies in mice have shown that COX-2 inhibitors and NSAIDs do not induce hypertension in vivo or constriction of aortic rings ex vivo unless the animals are pretreated with a nitric oxide synthase inhibitor. This suggests that nitric oxide is the primary mediator of vascular tone and that prostacyclin plays a subsidiary role. However, PGI₂ plays a critical role in animals (and presumably people) with reduced nitric oxide synthesizing capacity, which leads to the vascular effects of COX-2 inhibition. This suggests that individuals with preexisting vascular disease or individuals who develop it while taking a COX-2 inhibitor may be at increased risk of cardiovascular side effects. Indeed, the majority of cardiovascular events in the APC trial (celecoxib) were in individuals with risk factors for cardiovascular disease. Another important factor appears to be the severity of the cardiovascular challenge; individuals in the coronary artery bypass grafting trials exhibited cardiovascular events within 2 weeks of taking valdecoxib, whereas those in the APC and APPROVe trials exhibited adverse events over much longer periods of time. These observations suggest that individuals with risk factors such as atherosclerosis, diabetes, cigarette smoking, etc. would be more sensitive to the side effects of COX-2 inhibition than healthy individuals. Whether interindividual genetic differences can be identified, e.g., SNPs for genes involved in thrombosis or prostaglandin biology that alter an individual’s risk of cardiovascular complications, remains an important unanswered question. Understanding the potential for predisposing conditions to COX-2-induced cardiovascular side effects may provide approaches for developing predictive biomarkers of risk.

NSAIDs inhibit COX-2 in vascular endothelium as effectively as COX-2-selective inhibitors, so the fourth important question is whether NSAIDs also induce cardiovascular side effects. Extensive data are not available on this point, but some epidemiological and clinical studies suggest certain NSAIDs may induce cardiovascular side effects. Two epidemiological studies indicate that prolonged NSAID use is associated with a small increase in cardiovascular risk. In addition, a recent meta-analysis of short-term and long-term clinical studies that compared COX-2 selective inhibitors to placebo or traditional NSAIDs concludes that cardiovascular events (mainly myocardial infarctions) are comparable between COX-2 selective inhibitors and the traditional NSAIDs ibuprofen and diclofenac. Interestingly, the traditional NSAID naproxen was not associated with an increase in cardiovascular events in either the epidemiological or clinical studies. The basis for the difference between naproxen and all other COX-2 inhibitors (selective or nonselective) is not clear but may be related to naproxen’s much longer half-life compared to ibuprofen or diclofenac. Prolonged inhibition of platelet COX-1 by naproxen would prevent biosynthesis of the prothrombotic TXA₂, which might counterbalance the effect of inhibition of COX-2 driven PGI₂ biosynthesis in the vascular endothelium. Additional studies will be required to test this hypothesis and to further define the magnitude of the differences in cardiovascular effects of naproxen and non-naproxen NSAIDs.

Another key question, for which there are insufficient data, is whether the dose response for inhibition of a pathophysiological event (e.g., inflammation) is the same as the dose response for inhibition of a physiological event (e.g., in the gastrointestinal tract or vascular endothelium). Differences have been noted in the apparent dose responses for inhibition of platelet function and inhibition of endothelial prostacyclin biosynthesis by NSAIDs. In the colon polyp recurrence trial alluded to above, it was noted that twice daily administration of 200 or 400 mg of celecoxib induced both a dose-dependent...
inhibition of polyp recurrence and a dose-dependent increase in cardiovascular risk. A related trial of once-a-day celecoxib (400 mg) for sporadic polyp prevention exhibited clinical benefit but did not exhibit increased cardiovascular risk, which may suggest some digression of the dose responses for pathophysiological and physiological effects at low drug levels. This speculation must be tempered with the fact that the two polyectomy trials had different designs, so comparing results between the two is problematic. Moreover, the studies did not have the statistical power to fully address the issue of cardiovascular toxicity, so it remains possible that celecoxib at a dose of 400 mg once daily increases the risk of cardiovascular complications.

This discussion leads to the fifth and perhaps most important question: Where can COX-2 inhibitors be used clinically? Celecoxib is still marketed for treatment of inflammatory disorders, and lumiracoxib and etoricoxib are on the market in Europe, but sales are well off those exhibited prior to the withdrawal of rofecoxib. As with any drug, their prescription must reflect a balance between potential benefit and risk. COX-2 inhibitors were developed for individuals who are sensitive to the gastrointestinal side effects of traditional NSAIDs, but sales were dramatically expanded to the general population. This altered the benefit-risk calculation significantly because there is not an obvious benefit of COX-2 inhibitors for individuals who can tolerate NSAIDs. However, COX-2 inhibitors offer a clear benefit to individuals with severe gastrointestinal responses to NSAIDs and there may be other niche populations that would benefit from treatment with COX-2-selective inhibitors over NSAIDs.

Cancer patients are an obvious such population. Exhaustive preclinical and clinical data exist demonstrating that COX-2 plays a role in tumor growth and/or metastasis. In experimental animals, selective COX-2 inhibitors including celecoxib block the formation, growth, and metastases of multiple tumor types. Celecoxib demonstrates a dramatic ability to reduce the recurrence of colon polyps. For example, a 400 mg dose twice daily for 3 years reduced the incidence of recurrent adenomas of any type by 45% and of high risk lesions by 66%, suggesting that celecoxib is likely to be an effective colon cancer chemopreventive agent. However, despite this clear efficacy, the benefit-risk calculation does not appear to be high enough to recommend celecoxib for colon cancer prevention because of the magnitude of the cardiovascular side effects.

The primary factor in this recommendation is that the development of colon cancer is a slow process that occurs over many years and the percentage of patients with polyps that convert to cancer is low. So individuals with polyps would need to take celecoxib for a long time and the actual clinical benefit would not justify the enhanced risk of cardiovascular side effects. Obviously, this benefit-risk calculation might change if the individuals at the highest risk of cancer development could be prospectively identified or if biomarkers of cardiovascular toxicity could be developed. In support of this idea, celecoxib was previously found to cause a reduction in the colorectal polypl burden in familial adenomatous polyposis (FAP), a heritable condition that predisposes a person to colorectal cancer. Celecoxib continues to be used as an adjunctive therapy for the management of adenomatous colorectal polyps in patients with FAP.

The situation is likely to be very different for cancer treatment. Trials are underway to explore the utility of celecoxib for cancer treatment, primarily as an adjuvant agent in combination therapy (for example, with an agent designed for a different mechanism of action, such as inhibition of the epidermal growth factor receptor axis). If any of these trials are successful, the cardiovascular risks associated with the use of celecoxib may be acceptable given the mortality associated with many cancers and the limited treatment options. In fact, current treatment options include the use of agents, such as adriamycin, that induce direct cardiovascular damage in a sizable fraction of patients once they reach a certain cumulative dose.

Do the structural, functional, and kinetic data presented in this review illuminate the benefit-risk calculation of the use of COX-2 inhibitors and NSAIDs, or do they provide insights that may improve new drug discovery? This is difficult to answer with certainty because the kinetics of binding of COX-2 inhibitors and NSAIDs represent only a single step in their pharmacological properties. Absorption, distribution, and metabolism would seem to overshadow the effect of enzyme-inhibitor association-dissociation kinetics. However, it should be noted that cardiovascular side effects were first observed in the diaryl heterocycle class of inhibitors, which are compounds that exhibit the highest potency and selectivity. As described above, both the potency and selectivity of COX-2 inhibition are determined by the rate of dissociation of the enzyme-inhibitor complex.

The structural and functional information described above may be useful for altering the properties of existing NSAIDs or COX-2 inhibitors to incorporate new or protective functionalities. For example, the discovery that amide derivatives of indomethacin and several other NSAIDs bind to COX-2 but not COX-1 provides a potential strategy for incorporating additional functionality into COX-2 inhibitors. Our laboratory has had success in tethering fluorophores to indomethacin to generate compounds that retain inhibitory potency against COX-2 in intact cells and selectively image COX-2-expressing cells (but not COX-2-negative cells) (M. J. Uddin and L. J. Marnett, unpublished results). A modification of this strategy would allow the introduction of functionality, such as the inhibition of thromboxane synthesis or antagonism of the thromboxane receptor, induction of Nrf2, or activation of PPARγ, all of which might be anticipated to introduce cardiovascular protective activity. These combined dual function molecules might prevent the cardiovascular toxicity associated with COX-2 inhibition while enabling effective inhibition of inflammation or cancer.

V. Conclusions

Antiinflammatory preparations have been used for millennia, but their mechanism of action was elucidated less than 40 years ago. Our understanding of the inhibition of COX enzymes by NSAIDs has expanded dramatically in the past 10–15 years through a combination of structural, functional, and kinetic investigations. It is clear that the combination of these complementary techniques is required to truly understand enzyme-inhibitor interactions, and in fact, there are multiple examples of accurate predictions of enzyme-inhibitor binding modes based on a kinetic analysis of site-directed enzyme mutants. The diversity of binding mechanisms to COX-1 and COX-2 demonstrated by different compounds is impressive. The ability of COX enzymes to accommodate structurally distinct inhibitors is particularly remarkable because there is no evidence for sizable changes in protein conformations in the interactions of COXs with various NSAIDs. The structures of the proteins are very similar even in complexes with structurally diverse inhibitors bound to different residues in the active site. The catalog of various interactions described above may provide useful insights for efforts to design new NSAIDs with novel properties.
NSAIDs have generally been considered safe drugs; indeed, multiple NSAIDs are marketed as over-the-counter medications. The major side effect to their use has been gastrointestinal toxicity, which affects a subset of individuals taking NSAIDs on a chronic basis. The discovery of COX-2 and the development of COX-2-selective inhibitors provided a strategy to circumvent this toxicity that was hailed as a major therapeutic advance. Yet not long after the first COX-2-selective inhibitors were marketed, evidence for a new side effect appeared. Cardiovascular toxicity appears to affect only a small percentage of individuals taking the drugs but is quantitatively significant because of the large numbers of individuals involved. The controversy surrounding the removal of rofecoxib and valdecoxib shone a bright light on many issues related to the FDA approval process, direct-to-consumer marketing, postmarketing surveillance, and benefit–risk analysis. One suspects this experience will change the way drugs are developed, approved, and marketed. One hopes that more careful analysis of benefit and risk will occur between patient and physician as part of these changes.

COX-2 clearly plays an important role in multiple diseases through its role in the pathogenesis of inflammation. The massive clinical experience with NSAIDs throughout history attests to this. Consequently, COX-2 remains an attractive but now more challenging target than it was 7 years ago. Just as our understanding of COX–inhibitor interactions required a combination of multiple complementary experimental approaches, further exploitation of COX-2 as a pharmaceutical target may require multiple complementary clinical approaches, including not only new drug discovery, but also closer clinical monitoring utilizing novel biomarkers and pharmacogenomics. Inflammation is linked to several major unmet medical needs including osteoarthritis, rheumatoid arthritis, cancer, and neurodegeneration. The efficacy of NSAIDs and COX-2 inhibitors is a testimony to the importance of prostaglandins and thromboxane in these disorders and validates this entire pathway as a target for therapeutic intervention. Successful exploitation of the opportunities presented by the cyclooxygenase pathway may serve as a test for a new model of multidisciplinary approaches to therapeutic development.

Acknowledgment. We are grateful to Carol Rouzer and Andrew Dannenberg for critical readings and helpful discussions and to Jeffery Prusakiewicz, Melissa Turman, and Eric Dawson for assistance with the graphics. Research on NSAIDs and COX-2 inhibitors in the Marnett laboratory is supported by research grants from the National Cancer Institute (Grants CA89450, CA105296, and CA119629).

Biographies

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Lawrence J. Marnett received his undergraduate degree from Wayne State University in 1969 and his Ph.D. in Chemistry from Duke University in 1973 under the direction of Ned Porter. He did postdoctoral work at the Karolinska Institute with Bengt Samuelsson and at Wayne State University with Paul Schaap. He began his academic career in 1975 at Wayne State University where he rose to Professor of Chemistry. In 1989, he moved to Vanderbilt University as the Mary Geddes Stahlman Professor of Cancer Research, Professor of Biochemistry, Chemistry, and Pharmacology. Dr. Marnett’s research program focuses on the role of the enzyme cyclooxygenase-2 in cancer and inflammation and on the contribution of inflammation and oxidative stress to the generation of DNA damage and mutation and alterations in cell signaling. He is the author of over 350 research publications and 11 patents. He is the founding and current Editor-in-Chief of the American Chemical Society journal Chemical Research in Toxicology and is the Director of the Vanderbilt Institute of Chemical Biology.

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JM0613166