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COX-2: A Target for Colon Cancer Prevention

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■ **Abstract** Disease prevention is one area that both public and governmental agencies strongly support owing to its potential for an improved lifestyle and a reduction in health care costs. In this review, we focus on the clinical development of one target for cancer prevention, the COX-2 enzyme. This provides an excellent example of how basic research in biochemistry and pharmacology can lead to translational studies and eventually to approval of a drug by the FDA for use as a chemopreventive agent in humans. It is hoped that, as the genome sequence is understood more clearly, other targets will emerge that will provide even more effective drugs for future cancer prevention.

INTRODUCTION

Disease prevention has emerged as a viable option for reducing the mortality of cancer. Several factors contribute to this development. First, the identification of carcinogens to which the general population is exposed provides opportunities for reducing exposure. For example, the lowered frequency of cigarette smoking in men over the past 25 years has contributed significantly to the reduced incidence and mortality from lung cancer in men observed for each of the past ten years. The cumulative reduction, which is now approaching 16.5%, provides a proof of the concept that preventive strategies, even ones as simple as reduced exposure, have an impact on the incidence and mortality of cancer. Second, studies of the functional genomics of cancer, particularly of early lesions, are anticipated to provide a windfall of new molecular targets directed at preneoplastic disease. This review describes the background for the development of one such target. Third, many signal transduction inhibitors that were developed as therapeutic agents may be as effective or even more effective at preventing the formation of premalignant lesions that contain fewer genetic mutations than advanced cancers. Since these agents have relatively low toxicity compared to traditional chemotherapeutic agents, it is realistic to consider their chronic use as prophylactic agents. Fourth, advances

in diagnostics and imaging are making it possible to detect cancer at an earlier stage where it is much more effectively treated.

COX-2 inhibitors possess outstanding potential as chemopreventive agents and support the notion that cancer prevention is a viable clinical option. COX-2 inhibitors are effective in the prevention of colon cancer in several animal models and can be administered for long periods with fewer toxic effects than nonselective NSAIDs. They are prescribed mainly for the treatment of arthritis and pain so there is a large clinical experience with their use in the general population. Since COX-2 appears to be expressed at high levels in many different types of human tumors, but not in surrounding normal tissue, it seems likely that the paradigm developed for colon cancer can be expanded to other organ sites. COX-2 inhibitors were not developed de novo but arose from centuries of clinical observation and research into the mechanism of action of anti-inflammatory agents (1). Thus, there is a strong base from which to design human trials.

NSAIDS AND CYCLOOXYGENASE

Plant extracts contain numerous materials used for the treatment of a variety of human maladies. For example, extracts of myrtle and willow bark were used centuries ago for the relief of inflammation and fever (1). The active components of these extracts are salicylates or their precursors (Figure 1). Salicylic acid was first synthesized on an industrial scale at Bayer AG in the late 1800s and was marketed as an anti-inflammatory drug. The acetyl derivative was prepared in an attempt to improve its taste and to minimize gastrointestinal side effects. Acetylsalicylic acid (aspirin) was then marketed independently as an anti-inflammatory

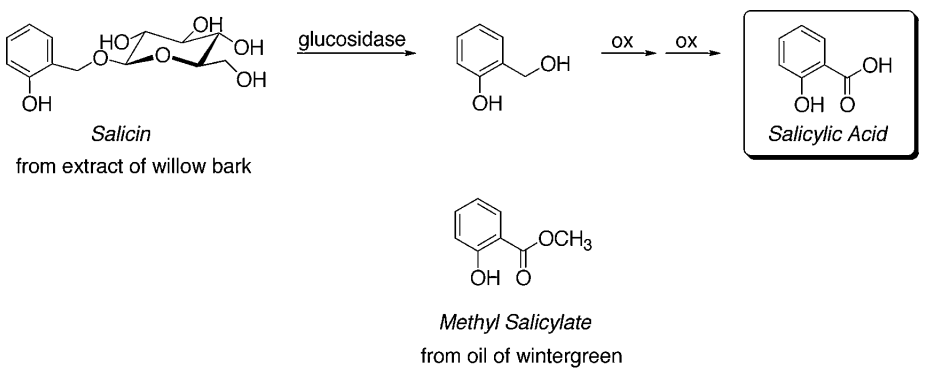

Figure 1 Naturally occurring salicylates. Salicin is the major salicylate precursor in extracts of many plants. On damage to the plant, the glucose residue is hydrolyzed and the salicyl alcohol is oxidized to salicylic acid. The methyl ester of salicylic acid is a component of oil of wintergreen.

drug and for much of the twentieth century was the drug of choice for this indication. Vane and colleagues demonstrated in 1971 that aspirin and other NSAIDs exerted their pharmacological activities by inhibiting the production of prostaglandins; Vane proposed that the cyclooxygenase enzyme was the molecular target (2). This hypothesis has been extensively supported by experimental and clinical data, although it is clear that NSAIDs exert other effects that are not dependent on cyclooxygenase inhibition. These noncyclooxygenase-mediated effects are usually observed at elevated concentrations in cell culture experiments (see below).

Cyclooxygenase catalyzes the double dioxygenation of polyunsaturated fatty acids (Figure 2) (3). Arachidonic acid is the best substrate for the enzyme and is converted to an endoperoxide derivative called PGG_2 that contains a hydroperoxide group at carbon-15. The immediate fate of PGG_2 is reduction of the hydroperoxy group to an alcohol (PGH_2). This reaction is catalyzed by a second enzymatic activity that is a component of the same polypeptide that catalyzes the cyclooxygenase reaction (4). The specificity of the peroxidase component for reducing agents is low, so a number of electron-rich molecules that are likely to be present in cells will serve in this capacity (5). Peroxidase-dependent oxidation has been demonstrated to effect metabolic activation of carcinogens, thereby providing a link between arachidonic acid oxygenation and DNA damage (6, 7). Because the cyclooxygenase protein actually catalyzes two sequential enzymatic reactions of polyunsaturated fatty acid metabolism, it is called prostaglandin endoperoxide synthase, PGH synthase, or PGG/H synthase. The abbreviation COX reflects the first enzymatic reaction and has come into common use to designate the gene and protein.

PGH_2 is converted by a series of metabolizing enzymes into a family of bioactive lipids, which includes prostaglandins or thromboxane (Figure 3) (8). The complete family of metabolizing enzymes is not present in all cells to equal extents, and more typically a single enzyme predominates. For example, PGE_2 is the major product in macrophages, PGD_2 in mast cells, and TxA_2 in platelets. Each of these final metabolites binds to a specific G protein–coupled cell-surface receptor to trigger intracellular responses (9, 10). Each prostanoid has a single receptor with the exception of PGE_2, which has four separate receptors [see however, the CRTH2

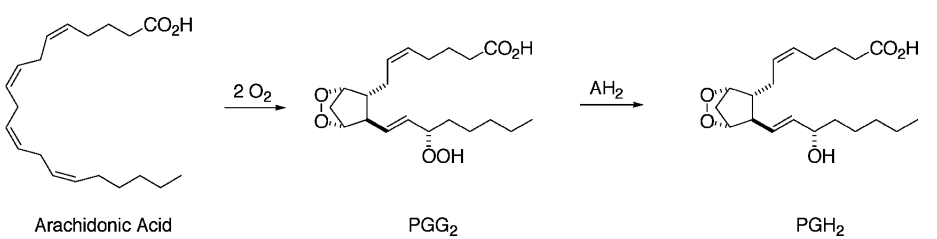

Figure 2 Conversion of arachidonic acid to PGH_2 by the combined action of the cyclooxygenase and peroxidase activities of COX.

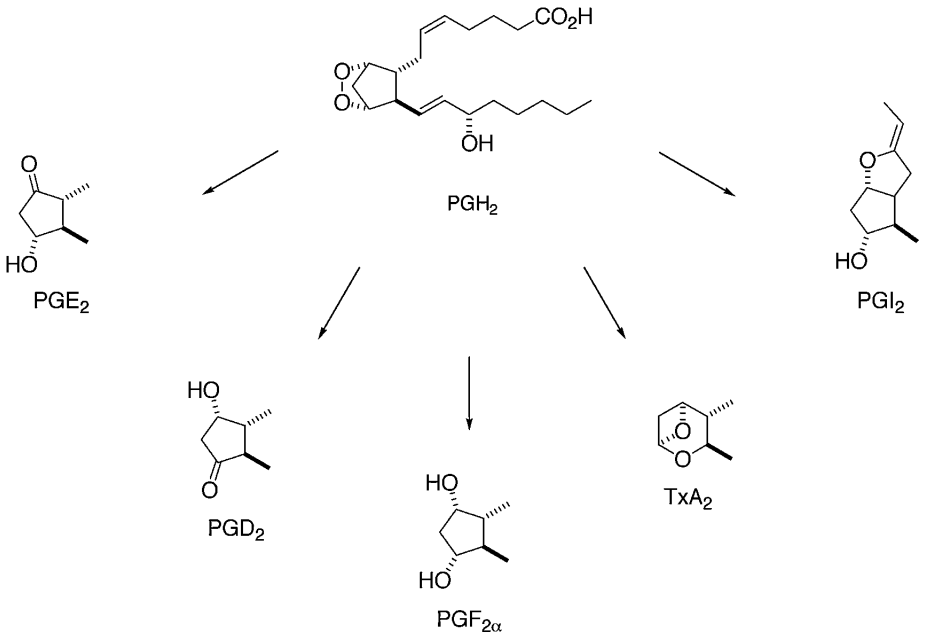

Figure 3 Metabolic transformations of PGH_2 to prostaglandins.

receptor viz the PGD_2 receptor (11)]. Receptor knockout mice have been used to demonstrate a role for PGE_2 in colon carcinogenesis in the *Min* mouse model (EP1 receptor) (12). In addition, the effects of antagonists for the EP4 receptor and the TP receptor implicate PGE_2 and TxA_2 in tumor cell invasiveness and endothelial cell migration, respectively (13, 14).

Recent evidence indicates that prostanoids can also signal via nuclear hormone receptors. The peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors that are members of the nuclear hormone receptor superfamily. Three distinct PPAR isoforms, α, δ, and γ, have been isolated and characterized (15). PPARs bind to sequence-specific DNA response elements as heterodimers with the retinoic acid receptor RXR (16). Although the identity of definitive high-affinity natural ligands for PPARs is lacking, there is evidence that arachidonic acid metabolites can serve as activating ligands. In particular, the PGD_2 metabolite, 15-deoxy$^{\Delta 12,14}$ PGJ_2, is a potent activator of the PPARγ isoform (17, 18), whereas a stable analog of PGI_2, carbaprostacyclin (cPGI), activates PPARδ and to a much lesser extent PPARα (19, 20). The biologic role of these receptors is actively under investigation, but it is interesting to note that He et al. have recently identified PPARδ as a β-Catenin/Tcf regulated gene in APC mutant cells (21). Mutations of the APC gene are thought to occur in over 50% of all colorectal cancers. In their study, Powell et al. (33) found that HT-29 cells induced to express wild-type APC have reduced levels of PPARδ. A Tcf response element

was identified in the 5′ regulatory region of the PPARδ gene, and further studies showed that β-catenin/Tcf upregulates expression of PPARδ in colon carcinoma cells. Since both COX-2 and PPARδ are upregulated in colon tumors, colocalized to similar regions within a given colon carcinoma, and because a cyclooxygenase-generated ligand, PGI_2, activates PPARδ (22), it is possible COX-2 may, in part, modulate cellular processes through activation of this receptor.

NSAIDS AND COLORECTAL CANCER

Extensive literature published mainly in the 1970s and 1980s documented (*a*) stimulation or inhibition of cell proliferation, cell differentiation, or tumor metastasis by prostaglandins; (*b*) generation of a direct-acting mutagen, malondialdehyde, by enzymatic or nonenzymatic breakdown of PGH_2; (*c*) metabolic activation of chemical carcinogens by the peroxidase activity of COX; (*d*) involvement of prostaglandins in tumor promotion; (*e*) immunosuppression by PGE_2; and (*f*) inhibition by NSAIDs of the growth of transplantable or chemically induced tumors (summarized in 23). These data suggested an involvement of arachidonic acid metabolism in all stages of the carcinogenic process. However, the complexity of the results and the occasionally contradictory roles of individual prostaglandins (e.g., stimulation or inhibition of proliferation) made extrapolation of these findings to humans difficult and determination of the importance of arachidonic acid metabolism in human cancer daunting. This situation changed with the publication of the results of multiple epidemiological studies indicating that individuals who consume aspirin have reduced mortality from colon cancer (24, 25).

Colorectal cancer is the second leading cause of death from cancer in the United States. There were over 130,000 new cases of colorectal cancer and about 55,000 deaths from the disease in 2000. Epidemiologic research indicates that there is a 40% to 50% reduction in mortality from colorectal cancer in persons who take aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) on a regular basis (Figure 4). What is the mechanism(s) underlying the antineoplastic effects of NSAIDs? There is strong evidence to suggest that inhibition of the COX enzymes by NSAIDs plays some role in cancer risk reduction (26).

Risk Reduction in Human Sporadic Colorectal Carcinoma

Of the several observational studies examining the effects of chronic NSAID use and the subsequent development of colorectal cancer, all but one has demonstrated a protective effect of NSAIDs. The studies were performed in a variety of settings in the United States and Australia, utilizing both colorectal cancer occurrence and mortality as outcomes (27). Many of these studies were designed so that exposure to NSAIDs was measured by interview or computerized pharmacy records. In the Nurses Health Study, a protective effect was seen only after 10 to 15 years of aspirin use (28). Similar studies have revealed a protective effect of NSAIDs in relation to adenomatous polyp detection (29). Additionally, a small number of observational

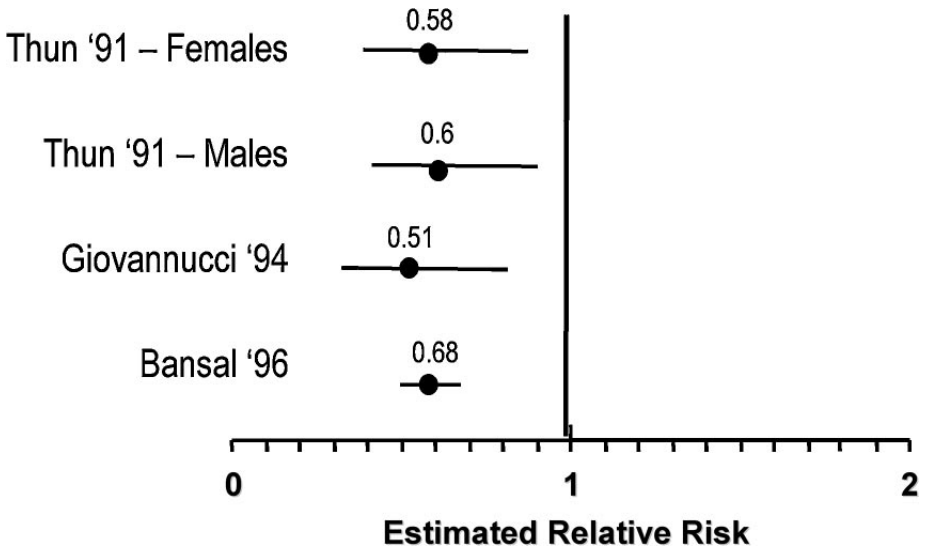

Figure 4 Summary of epidemiological studies relating aspirin intake to reduced mortality from colon cancer.

studies have shown a significant risk reduction with use of non-aspirin NSAIDs (30).

The effect of aspirin use on the development of colorectal cancer has been assessed in a randomized clinical trial that had a principal goal of evaluating aspirin for the prevention of myocardial infarction. A secondary analysis of this study of 22,071 male physicians randomized to placebo or aspirin 325 mg every other day demonstrated no protective effect against the development of colorectal cancer (31). The low frequency of colorectal cancer makes a large-scale randomized clinical trial financially and temporally difficult. More definitive recommendations concerning aspirin use likely will be based on the results of an ongoing randomized clinical trial of aspirin use that utilizes adenomatous polyp incidence as an intermediate endpoint (32). This multicenter study tests the effect of aspirin at one of two doses versus placebo on the development of adenomatous polyps among patients who have undergone prior colonoscopy with polypectomy. Hopefully, data from that study will help determine the degree of benefit and optimal dose of aspirin for cancer prevention.

NSAID USE AND REDUCTION OF ADENOMA SIZE AND NUMBER IN FAP PATIENTS

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease with variable phenotypic expression that is associated with an increased risk of colorectal cancer at a young age (most by age 40). FAP is only responsible for less

than 1% of colorectal carcinomas detected in the general population. The genetic mutation responsible for this disease resides in the adenomatous polyposis coli (APC) gene located on chromosome 5q21. Somatic mutations in the APC gene have also been reported in up to 50% of sporadic colorectal cancers as well (33). Waddell & Loughry first reported that regular use of the NSAID, sulindac, led to regression of rectal adenomas in four patients with FAP, and this phenomenon was confirmed in several other case reports (34, 35). This observation was then tested in randomized, placebo-controlled, double-blinded, crossover studies of sulindac use in FAP patients (36). These studies, collectively, indicate that sulindac has a significant effect on polyp regression in FAP patients. More recently, a selective inhibitor of COX-2 was shown to reduce polyp burden in FAP patients by 30% following a 6-month period of treatment (37)

DISCOVERY OF COX-2

The COX-1 cDNA was cloned in 1988 from sheep, mouse, and human sources (38–40). The gene is 25 kb in size, has 11 exons, and produces a 2.8–3.0 kb mRNA and 68-kDa protein (41). The levels of this mRNA do not vary greatly with cell stimulation, although there are reports of the induction of COX-1 during cell differentiation (e.g., 42). The protein has an N-terminal membrane signal sequence, a C-terminal endoplasmic reticulum retention signal, and four potential glycosylation sites (43). There is no evidence that phosphorylation plays a role in the regulation of COX activity.

Prostaglandin synthesis in cells is known to be enhanced by a variety of growth factors, tumor promoters, etc. (44, 45); this was thought to be the result of activation of phospholipases and the attendant release of stored substrate. Indeed, in most cells, activation of phospholipase A_2 and release of arachidonic acid are rate-limiting events in prostaglandin biosynthesis. Needleman and colleagues demonstrated that cytokines and growth factors enhanced COX activity in fibroblasts and later showed that this was due to the synthesis of new COX protein (46, 47). The synthesis of new COX protein correlated to the increase in COX activity, and both events were inhibited by anti-inflammatory steroids (48). A similar increase in COX activity and protein was observed when cells were treated with the tumor promoter tetradecanoylphorbol acetate (TPA) or when they were transformed with oncogenic viruses (e.g., Rous sarcoma virus or polyoma virus) (49). Stimulation of COX activity by TPA was transient, whereas stimulation by oncogenic viruses was prolonged.

Rosen et al. reported that exposing sheep tracheal epithelial cells in culture to growth factors increased the ability of the cells to synthesize prostaglandins compared to freshly isolated cells (50). This increase in COX activity correlated with the appearance of a 4.0 kb mRNA species that hybridized with COX-1 cDNA under low stringency conditions (50). It is interesting that the 2.8-kb mRNA of COX-1 did not increase when the cells were exposed to growth factors. Rosen et al. proposed that the 4-kb mRNA coded for a COX protein and was the product

of a distinct gene (50). The expression of the inducible form of COX was inhibited by anti-inflammatory steroids, whereas the expression of the basal COX was not, which led Fu et al. to propose the existence of separate pools of COX protein that were regulated independently (49, 51). Simmons et al. cloned a full-length cDNA from src-transformed chicken embryo fibroblasts that produced a 4.1-kb mRNA (52). In vitro, translation of this mRNA produced a protein of ~60 to 70 kDa. The protein sequence was 60% to 65% homologous to the sequence of sheep, mouse, or human COX-1. Similar results were reported by Kujubu et al. for mouse fibroblasts treated with TPA, by O'Banion et al. for src-transformed mouse fibroblasts, and by Hla & Neilson for human endothelial cells or monocytes treated with TPA or lipopolysaccharide, respectively (53–55). Fletcher et al. expressed the cDNA in COS cells and demonstrated that it coded for a functionally active cyclooxygenase (56). Thus, COX-2 was discovered as an immediate early gene inducible by cytokines, growth factors, tumor promoters, and viral transformation.

COX-2 is an 8 kb gene composed of 10 exons; the mRNA of COX-2 is approximately 4.1 kb in size and codes for a protein of 68 kDa (56). The overall exon/intron stucture is similar to that of COX-1, although there are significant differences in the regions corresponding to the N- and C-termini of the proteins (57). The greater size of the COX-2 mRNA is due to a much larger 3′-untranslated region (52–55). COX-2 contains a different membrane signal sequence compared to COX-1 because of the loss of the exon coding for the leader sequence present in COX-1 (exon B). COX-2 has all of the critical active site residues responsible for cyclooxygenase and peroxidase activity, and it has five potential glycosylation sites, four of which appear to be used.

COX INHIBITION

Structural Basis of COX Inhibition

The structure of COX-1 was solved in 1994 and represented a landmark accomplishment because it was one of the first membrane proteins to be crystallized and its structure determined (43). The structure of COX-2 was solved two years later (58, 59). The overall structures of the two proteins are very similar. Each has three structural domains—an epidermal growth factor domain, a membrane-binding domain, and a large catalytic domain that is structurally similar to the mammalian peroxidase, myeloperoxidase (Figure 5). The function of the epidermal growth factor domain is unknown. The membrane-binding domain comprises four helices on the bottom of the protein that present hydrophobic faces to one leaflet of the lipid bilayer. Because each COX enzyme is a homodimer with a C_2 axis of symmetry, the holoenzyme presents two sets of membrane-binding helices. The positioning of the helices in the membrane-binding domain provides a large opening through which arachidonic acid passes to reach the cyclooxygenase active site (43). This opening is termed the lobby of the protein. It is separated from the cyclooxygenase active site by a constriction composed of Arg-120, Tyr-355, and Glu-524. The small size of the opening that is apparent in the crystal structures

of COX-inhibitor complexes suggests that the constriction must open and close to bind and release substrates or inhibitors.

The cyclooxygenase and peroxidase active sites are located on opposite sides of the catalytic domain. The heme prosthetic group is positioned at the base of the peroxidase active site. The proximal ligand to the heme is His-388 and the distal residues—His-207 and Gln-203—are typical of mammalian peroxidases (60). The distal face of the heme is accessible to hydroperoxide substrates that react with the iron to generate higher oxidation states (61–63).

The cyclooxygenase active site is located above the Arg-120/Tyr-355/Glu-524 constriction and penetrates deep into the interior of the protein (Figure 6). The heme group is surrounded by residues that separate it from the cyclooxygenase active site, so it does not make direct contact with fatty acid substrates. Tyr-385 is located near the top of the cyclooxygenase active site at the interface with the heme group. This tyrosine residue has been implicated in the oxidation of arachidonic acid in the first step of the cyclooxygenase catalytic cycle (64). Another noteworthy residue in the cyclooxygenase active site is Ser-530, which is the site for aspirin acetylation (65). The structure of COX-1, which contains arachidonic acid in the active site, was recently solved by substituting cobalt-protoporphyrin-IX for heme (66). The cobalt-substituted enzyme is catalytically inactive so arachidonic acid is not oxidized after binding. The carboxylate of arachidonate ion-pairs and hydrogen-bonds to Arg-120 and extends up into the cyclooxygenase active site. It makes a sharp bend in the vicinity of Tyr-385 and projects into a narrow channel termed the top channel. The 13-*proS*-hydrogen of arachidonate is close to the phenolic hydroxyl of Tyr-385 and the ω-end of arachidonate is located $\sim$3.3 Å from Gly-533. This structure confirms a model proposed on the basis of site-directed mutagenesis experiments (67). Extensive contacts are apparent in the COX-1-arachidonic acid structure between arachidonic acid and active site residues.

NSAIDs (Figure 7) and COX-2 inhibitors bind in the cyclooxygenase active site above the Arg-120/Tyr-355/Glu-524 constriction (43, 59, 68). Most NSAIDs and COX-2-selective inhibitors are slow, tight-binding inhibitors (69–73). The molecular basis for the time-dependence of slow, tight binding inhibitors is unknown, and there are likely to be different mechanisms for different inhibitors. Only aspirin and (2-acetoxyphenyl)heptynylsulfide (APHS) covalently modify the COX protein (74, 75). Aspirin ion-pairs to Arg-120 then moves toward Ser-530 to deliver its acetyl group (76, 77). Acetylation of Ser-530 requires participation of Tyr-385; mutation to Phe reduces acetylation of Ser-530 by greater than 90% (78). Tyr-385 is proposed to H-bond to the carboxyl oxygen of aspirin to stabilize the oxyanion that develops during nucleophilic attack by Ser-530.

DEVELOPMENT OF COX-2 INHIBITORS

The inducibility of COX-2 by cytokines and inhibition by glucocorticoids immediately suggested that this new cyclooxygenase might be responsible for prostaglandin biosynthesis by inflammatory cells (51, 79). If so, selective COX-2 inhibitors

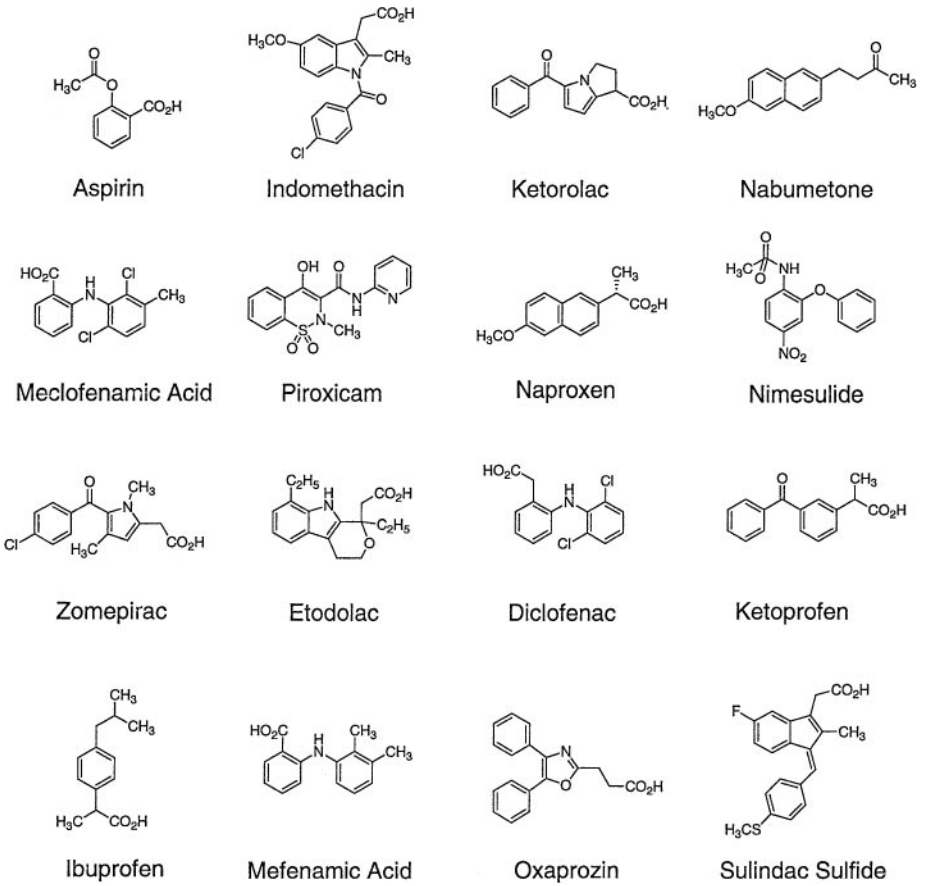

Figure 7 Structures of NSAIDs and related compounds discussed in this review.

might be superior to NSAIDs as anti-inflammatory drugs because they would target the key enzyme of inflammation, pain, and fever. Since COX-2 was not detectable in resting cells in culture or in many tissues, it was also likely that it did not play a role in homeostatic processes in organs such as the gastrointestinal tract or platelets. This was confirmed by extensive analyses of the tissue distribution of COX-1 and COX-2 (80, 81). This increased the excitement surrounding the development of selective COX-2 inhibitors because it was reasonable to hypothesize that such compounds would be anti-inflammatory but would not possess the gastrointestinal side effects that plague traditional NSAIDs. The "COX-2 hypothesis" has been validated in animal models and in human beings (82–84). Evaluation of the ability of a series of NSAIDs to inhibit COX-1 and COX-2 indicate a range of selectivity against the two enzymes (85). Some compounds (e.g., indomethacin, aspirin) are more selective for COX-1, some are equipotent against the two enzymes

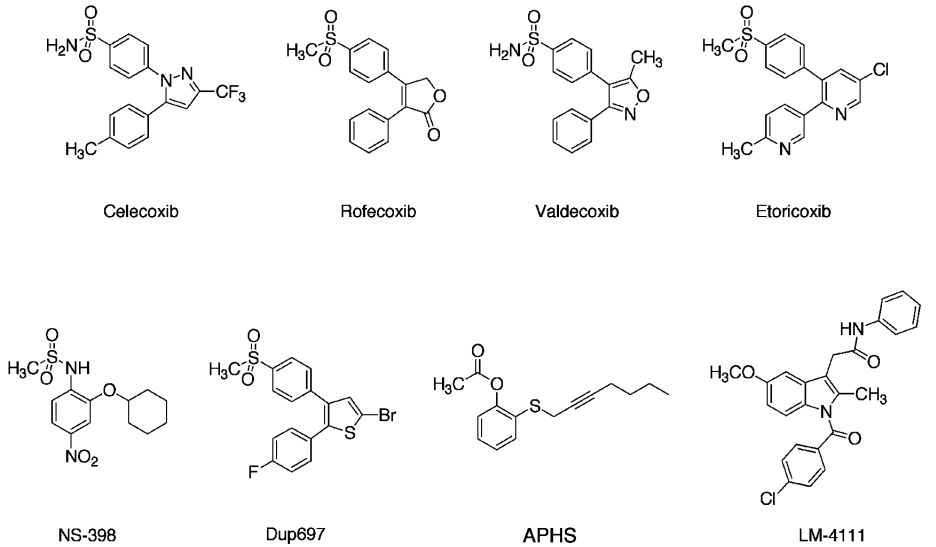

Figure 8 COX-2 inhibitors. The structures highlighted include marketed COX-2 inhibitors (celecoxib and rofecoxib), second generation compounds (valdecoxib and etoricoxib), and compounds commonly used for in vitro studies (NS-398, Dup697, APHS, and LM-4111).

(e.g., diclofenac), and some are more selective for COX-2 (e.g., meloxicam). All of these compounds inhibit both enzymes at pharmacological doses.

Two classes of molecules emerged early on as selective COX-2 inhibitors. These were the acidic sulfonamides (e.g., NS-398, flosulide) and diarylheterocycles (e.g., Dup697) (86, 87) (Figure 8). Diarylheterocycles had been developed years earlier as NSAIDs; oxaprozen (Figure 7) was first patented in 1971 and is still marketed as Daypro. The addition of the methylsulfonyl group on one of the pendant aryl rings of Dup697 represented the critical innovation that introduced COX-2 selectivity into diarylheterocycles (86). Predictably, the diarylheterocycle nucleus was exhaustively manipulated by medicinal chemists to optimize potency, selectivity, and pharmacokinetics (88, 89). Considerable structural variation is tolerated in the heterocyclic ring and even carbocyclic inhibitors have been described (90, 91). Some flexibility is possible in the aryl rings, but the presence of a methylsulfone or sulfonamide on one aryl ring is essential. The oxidation state of the sulfur in diarylheterocycles is a very important determinant of selectivity—sulfones and sulfonamides are selective for COX-2, whereas sulfoxides and sulfides are not.

Structures of complexes of diarylheterocycles with COX-2 reveal the molecular basis for their selectivity (59). The sulfonamido or sulfonyl group inserts into a side pocket off the cyclooxygenase active site and hydrogen-bonds to several side chain and main chain residues (Figure 9). Access to this side pocket is controlled to some extent by the residue at position 523 (92–95). A conserved

valine residue in all COX-2s allows insertion, whereas a conserved isoleucine residue in COX-1 sterically hinders insertion. Other residues that contribute to differences in side pocket binding are Arg-513 and Val-434 in COX-2, which are His and Ile residues in COX-1.

KINETICS OF COX-2 INHIBITION

Detailed kinetic analysis indicates that the binding of NSAIDs and diarylheterocycles to COX-2 is a complex process (69–72). The first step involves the formation of a readily reversible complex, which represents a competitively inhibited enzyme. Some NSAIDs, such as ibuprofen and mefenamic acid, are purely competitive inhibitors of COX-1 and COX-2. Subsequent steps involve the conversion of the reversible complex to a complex, in which the inhibitor is bound more tightly to the enzyme. This conversion occurs in seconds to minutes and may reflect the induction of a protein conformational change. The tightness of binding that develops as a result of the time-dependent step lowers the IC_{50}s for inhibition by 1 to 3 orders of magnitude. COX-2-selective diarylheterocycles are competitive inhibitors of both COX-1 and COX-2 but exert time-dependent inhibition of COX-2 (i.e., COX-2 selectivity is dependent on the time-dependent step) (70). It is conceivable that COX-2 inhibitors, all of which are evaluated initially in time-dependent enzyme inhibition assays in vitro, may exert some COX-1 inhibition in vivo because of their ability to inhibit both enzymes competitively. However, although the COX-2 selectivities of celecoxib and rofecoxib are much lower using the ex vivo human whole blood assay than the selectivities measured using in vitro assays, neither compound inhibits platelet COX-1 in humans following administration of supratherapeutical doses (96, 97). In addition, the low incidence of gastrointestinal side effects observed with celecoxib and rofecoxib (see below) suggests that competitive inhibition of COX-1 does not occur to a significant extent in the gastrointestinal tract.

COX-2 INHIBITORS IN THE CLINIC

The first two COX-2-selective inhibitors to reach the market were diarylheterocycles. Celecoxib (celebrex) is a diarylpyrazole, which contains a sulfonamide group on one phenyl ring and a methyl group on the other (98). Rofecoxib (vioxx) is a diarylfuranone that contains a methylsulfone on one aryl ring; the other ring is unsubstituted (99, 100). The in vitro selectivity of celecoxib for COX-2 is approximately 300, whereas the selectivity of rofecoxib is approximately 1000. Both compounds exhibit anti-inflammatory and analgesic activities in preclinical models and in humans (101–103). They also have superior safety profiles to traditional NSAIDs as judged by endoscopic examination of the gastrointestinal tract (83). Some concern has been expressed that COX-2 inhibitors may exhibit cardiovascular effects based on their ability to lower the levels of the major urinary prostacyclin metabolite (104). Considering the large number of patients who have successfully taken COX-2 inhibitors, it does not appear that cardiovascular complications will

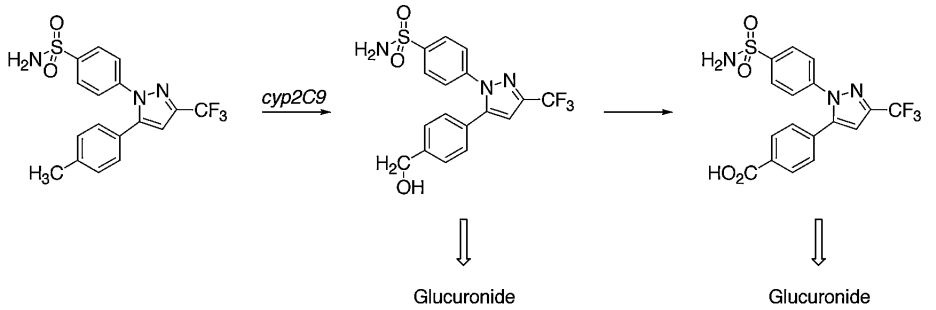

Figure 10 Metabolism of celecoxib in humans.

represent significant safety risks. However, there may be some groups of patients for whom the benefit of a COX-2 inhibitor may not justify the risk of this side effect.

Celecoxib is well-absorbed, and following a single oral dose of 300 mg it reaches a peak plasma concentration of ~4 μM in 1.4 h (105). It is 97% bound to protein and disappears from plasma with a half-life of 11.5 h. The principal metabolic fate is oxidation of the methyl group first to a hydroxymethyl group then to a carboxylic acid (Figure 10) (105). Both metabolites are conjugated to glucuronic acid. The major metabolite is the glucuronide conjugate of the acid, and most of it is excreted in feces (88% to 94%). Cyp2C9 is primarily responsible for celecoxib oxidation, but there may be a minor involvement of cyp3A4 (106).

Rofecoxib reaches a peak plasma concentration of 0.6 μM within 4 h after a single oral dose of 25 mg (97). It is 87% bound to plasma proteins and disappears from plasma with a half-life of 9 h. The principal metabolites are *cis* and *trans* lactones produced by reduction of the 3,4-double bond of rofecoxib (Vioxx package insert) (Figure 11). Some phenol and 3′,4′-dihydrodiol as well as the corresponding glucuronides are also produced. The major route of elimination is via urine (72%).

The economic impact of COX-2 inhibitors is attested to by the statistics in Table 1, which provides a list of the top ten currently available prescription NSAIDs and COX-2 inhibitors with estimates of their annual worldwide sales. It is clear that the COX-2 inhibitors, celebrex and vioxx, have been well-received after only two years. It is interesting that the introduction of COX-2 inhibitors has not substantially depressed the sales of NSAIDs—in other words, COX-2 inhibitors have expanded the market.

MECHANISMS FOR CHEMOPREVENTION OF INTESTINAL TUMORS BY ASPIRIN AND OTHER NSAIDS

Inhibition of Cyclooxygenases

The persistent induction of COX-2 expression by viral transformation of fibroblasts was of considerable interest with regard to previous reports that certain

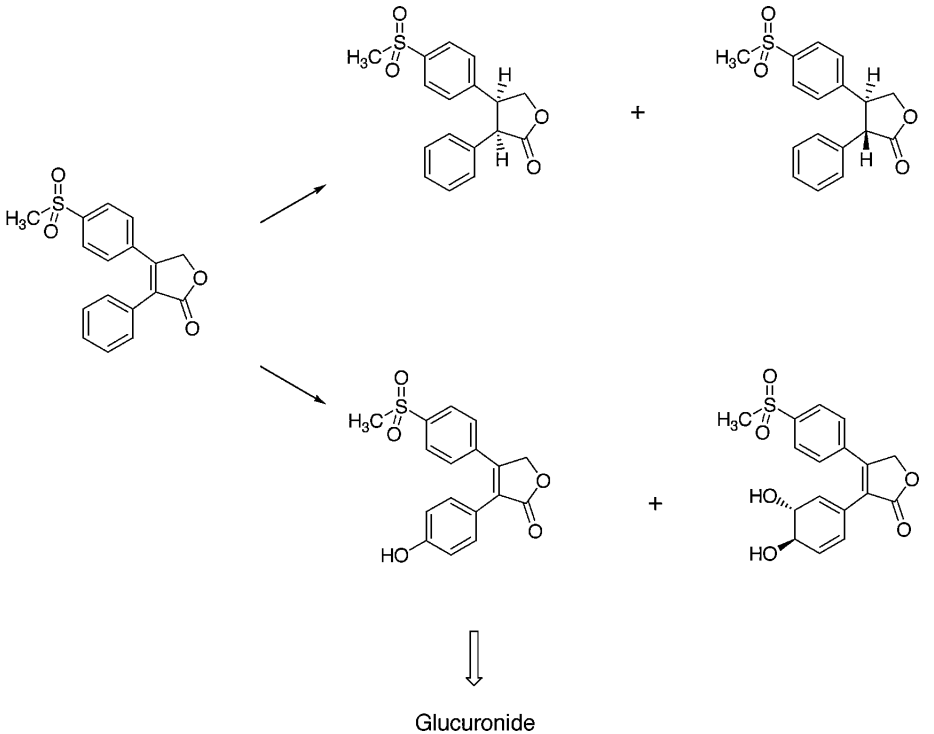

Figure 11 Metabolism of rofecoxib in humans.

human tumors produce substantial amounts of prostaglandins—amounts usually significantly greater than those produced by surrounding normal tissue (107–109). DuBois and colleagues cloned COX-2 as an immediate early gene induced by treatment of rat intestinal epithelial cells with transforming growth factor α (110). Subsequently, they demonstrated that COX-2 is detectable in a range of human colon cancers but not in surrounding normal tissue (111); these findings have been confirmed by other investigators using different techniques and patient populations (112–114). Does dysregulation of COX-2 expression affect the development of gastrointestinal cancer (26)? COX-2 mRNA and protein levels are increased in intestinal tumors that develop in rodents following carcinogen treatment (115) and in adenomas taken from APC mutant mice (116, 117). When intestinal epithelial cells are forced to express COX-2 constitutively, they develop phenotypic changes that include increased adhesion to extracellular matrix and a resistance to butyrate-induced apoptosis (118). Both of these phenotypic changes are consistent with an increased neoplastic potential.

Work by several groups has shown a reduction in tumor multiplicity in *Min* mice treated with sulindac or piroxicam, both potent cyclooxygenase inhibitors (119, 120). More recent studies have demonstrated a significant reduction in

TABLE 1 Worldwide sales of NSAIDs and COX-2 inhibitors

Prescription drug	Annual sales (000's)[a]	Percent total
Celecoxib	2,056,115	24.7
Rofecoxib	1,593,601	19.1
Diclofenac	1,355,194	16.3
Naproxen	398,379	4.8
Nabumetone	340,816	4.1
Nimesulide	325,965	3.9
Ibuprofen	284,551	3.4
Misoprostol	252,487	3.0
Loxoprofen	243,459	2.9
Ketoprofen	196,011	2.4

[a]Figures represent estimates of worldwide sales in 2000.

premalignant and malignant lesions in *Min* mice and carcinogen-treated rats that were given a selective COX-2 inhibitor (121–123). Another study has provided compelling genetic evidence that directly links COX-2 expression to intestinal tumor promotion (117). This report shows that $APC^{\Delta 716}$ mice, which develop numerous adenomas per intestine, bred with COX-2 null mice have an 80% to 90% reduction in tumor multiplicity in the homozygous COX-2 null offspring. These results suggest that COX-2 may act as a tumor promoter in the intestine and that increased levels of COX-2 expression may result directly or indirectly from disruption of the APC gene and/or changes in other signaling pathways.

The precise contribution of increased biosynthesis of prostaglandins by COX-2 to the progression of neoplasia is currently under evaluation. For example, PGE_2 generated in colorectal carcinomas could enhance cell survival and/or affect other aspects of epithelial cell behavior such as cell-cell or cell-substrate adhesion (124). A link between the neoplastic effect of carcinogen treatment and prostaglandin signaling was recently made by the observation that genetic disruption of the EP_1 receptor for PGE_2 results in a reduction in the number of aberrant crypt foci that develop in mice following carcinogen treatment (12). Based on these findings, it would seem important to determine the effects of PGE_2 on the biology of colorectal carcinoma cells. Sheng et al. found that PGE_2 stimulated an increase in the proliferation and invasiveness of colorectal carcinoma cells (13). COX-2 is expressed in both carcinoma and stromal cells (114). Therefore, it is possible that carcinoma cells that do not express COX-2 could receive paracrine signals from PGE_2 produced by neighboring stromal cells. LS-174 cells form crypt-like aggregates when they are cultured in Matrigel® and undergo growth stimulation in response to exogenously added PGE_2. PGE_2 also stimulates a dramatic change in the morphology of the LS-174 colonies. When grown in extracellular

matrix components, LS-174 cells formed well-organized structures consisting of an outside layer of cells and an acellular center. In contrast, the LS-174 cells exposed to PGE_2 form irregular solid clumps of cells with a poorly organized structure (13). In addition, PGE_2 treatment of LS-174 cells results in protruding actin filaments from the cell periphery, in the form of microspikes, and an increase in the number of stress fibers. PGE_2 treatment also increases focal adhesion complexes and results in a significant increase in cell motility, which helps to explain their change in cellular organization when grown as multicellular colonies. LS-174 cells express EP_2, EP_3, and EP_4 receptors, but mRNA for the EP_1 receptor is barely detectable. When LS-174 cells are treated with butaprost (1 μM, a selective EP_2 receptor agonist) and sulprostone (5 μM, a selective EP_3 receptor agonist), there is no significant change in cell morphology. However, when these same cells are treated with the PGE_1 alcohol (10 nM, a selective EP_4 receptor agonist), there is more rapid and significant cell-spreading compared to the effect of PGE_2. Hence, increased LS-174 cell-spreading and migration, stimulated by PGE_2, may be predominantly mediated through the EP_4 signaling pathway. Work is underway to further elucidate the mechanisms involved.

COX-2-selective inhibitors were developed primarily as anti-inflammatory agents that have fewer gastrointestinal side effects than nonselective NSAIDs (83, 125). These selective COX-2 inhibitors have proven effective in inhibiting tumor growth in animal studies, and these agents also exhibit antiangiogenic activity in vitro that may contribute to their antineoplastic effects in vivo (14, 126–129). The antiangiogenic effects of COX-2-selective inhibitors and their ability to reduce hematogenous metastasis of COX-2-expressing tumors (130) has raised the possibility that they may be useful for treatment as well as for prevention of some cancers. Furthermore, the combination of a COX-2 inhibitor with radiation has been demonstrated to provide a significantly enhanced radio response relative to radiation therapy alone (131).

COX-INDEPENDENT MECHANISMS

Epidemiologic data strongly support the chemoprotective effects of NSAIDs for gastrointestinal malignancies, while the data supporting their benefit in other solid tumors is currently being evaluated. The precise mechanism by which NSAIDs prevent and/or cause regression of colorectal tumors is not known. Despite different chemical structures, inhibition profiles, and drug half-lives, all NSAIDs in clinical use possess COX-inhibitory activity. Some investigators have reported effects of NSAIDs that are not likely due to inhibition of COX activity (26). For example, certain NSAIDs induce apoptosis and alter expression of cell cycle regulatory genes in some cell lines when administered at relatively high concentrations (200–1000 μM) (132, 133). By using COX-deficient cell lines or drug metabolites lacking COX-inhibitory activity, these studies rule out the involvement of COX enzymes in the growth-inhibitory effect (129). Certainly, this class of drugs appears to affect biochemical pathways unrelated to COX enzymes, and these effects are

likely to occur in a dose-dependent fashion (some effects occurring only at toxic doses). Work by He et al. has implicated a direct effect of sulindac on inhibition of PPARδ-directed transcription in cell culture models, but only at concentrations of drug above the 100 μM range (21). More recently, this group has shown that sulindac has similar effects on cells that completely lack the PPARδ gene (134), which indicates that other targets are likely responsible. The specific mechanisms of these COX-independent effects and their therapeutic implications are not yet well understood. However, most of the studies demonstrating effects on COX-independent pathways utilize concentrations of NSAID (100–1000 μM) that are difficult to achieve in humans without severe toxic side effects. As noted above, the maximal plasma levels of celecoxib and rofecoxib achieved at therapeutic doses are 4 μM and 0.6 μM, respectively, and the bulk of both drugs is bound to plasma protein.

RISKS OF CHRONIC NSAID THERAPY FOR CANCER PREVENTION

The success of NSAIDs or COX-2 inhibitors for gastrointestinal cancer prevention will depend on the side effects as a result of chronic use of these drugs. There are concerns about the safety of long-term use of aspirin and other NSAIDs in humans. Long term aspirin use can result in serious gastrointestinal and renal adverse effects, even at relatively low doses of drug (135). These side effects tend to increase in older patients. The most significant side effects, in terms of morbidity and mortality, are gastrointestinal, and include dyspepsia, peptic ulcer, and gastrointestinal bleeding (136). It is estimated that regular users of NSAIDs have roughly a threefold greater relative risk of developing serious gastrointestinal complications when compared to non-users of NSAIDs (137). Furthermore, the consequences of NSAID-associated gastrointestinal complications are often dire. A British study of 235 patients with severe peptic ulceration found that the mortality rate from ulceration among regular users of NSAIDs was greater than twice that of non-users (138).

The cost of NSAID-associated complications is also considerable. A retrospective cohort study of 75,350 elderly ($>$65) Tennessee Medicaid recipients by Smalley et al. found that the adjusted mean annual payment for medical care related to gastrointestinal disorders was $134 for non-users of NSAIDs and was $244 among regular users of NSAIDs (139).

As new data become available, we must constantly reassess the risks versus the benefits of chronic NSAID therapy. The aforementioned side effects of aspirin therapy may preclude prophylactic use of that drug in all but the highest risk populations. The deleterious effects of nonselective NSAIDs on the gastrointestinal tract, including gastric erosions, ulcerations, and blood loss, are postulated to result from inhibition of COX-1. COX-2-selective inhibitors appear to have better safety profiles than nonselective NSAIDs (125). The side effects of any chemoprotective agent must be low to ensure ensure compliance and to achieve the desired result, since the absolute risk of colorectal cancer in the general population is quite low.

On the other hand, if high-risk populations can be identified readily by genetic testing or other means (140), the use of these agents in those populations may be more reasonable because of a much more favorable risk-to-benefit ratio.

The risk-benefit ratio for chemoprevention of colorectal cancer would likely improve if accessible techniques were available to identify groups at high risk for the subsequent development of colorectal cancer. It is hoped that advances in the discovery and testing of colorectal cancer genes will make the identification of cohorts at high risk for developing cancer more likely. Additionally, if agents such as the selective COX-2 inhibitors prove to have fewer adverse effects than nonselective NSAIDs, the risk to benefit ratio would improve. Human clinical trials evaluating the antineoplastic effects of selective COX-2 inhibitors are underway to determine if these agents will be effective for cancer prevention in humans.

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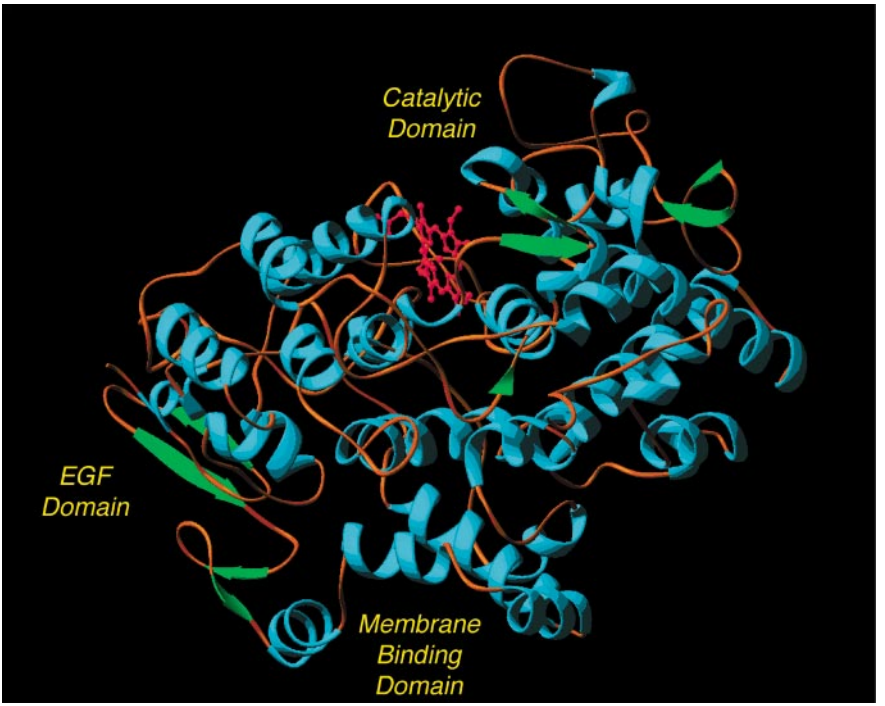

Figure 5 Domain structure of COX enzymes.

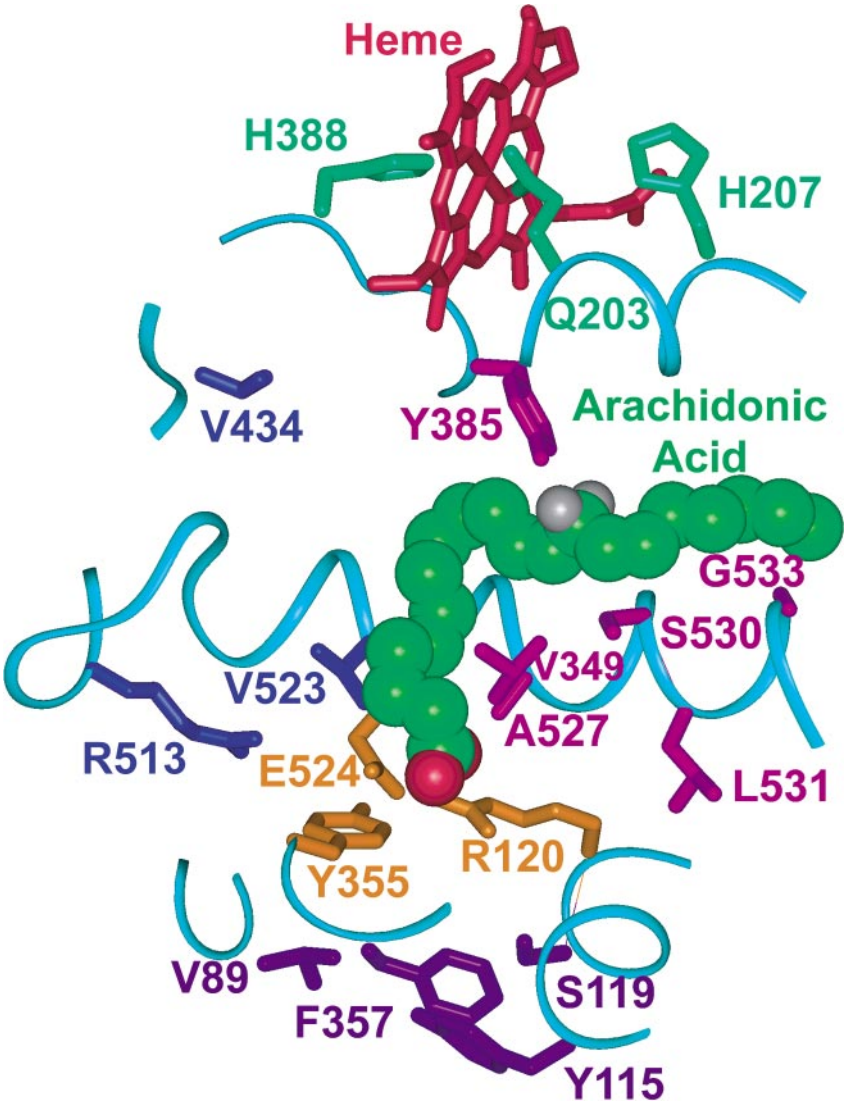

Figure 6 Model of arachidonic acid bound to COX-2. Arachidonate was positioned in the COX-2 active site to approximate its binding to the Co-protoporphyrin-IX derivative of COX-1. The carboxylate of arachidonic acid is ion-paired to Arg-120 and H-bonded to Tyr-355; its 13-*proS*-hydrogen is adjacent to the catalytic oxidizing agent Tyr-385; and its methyl end is placed near Gly-533. Other key residues include Ser-530, which is the site of acetylation by aspirin as well as Val-523 and Arg-513, which are critical differences that give rise to the binding of sulfonamide and sulfone groups of diarylheterocycles in the side pocket of the main active site channel.

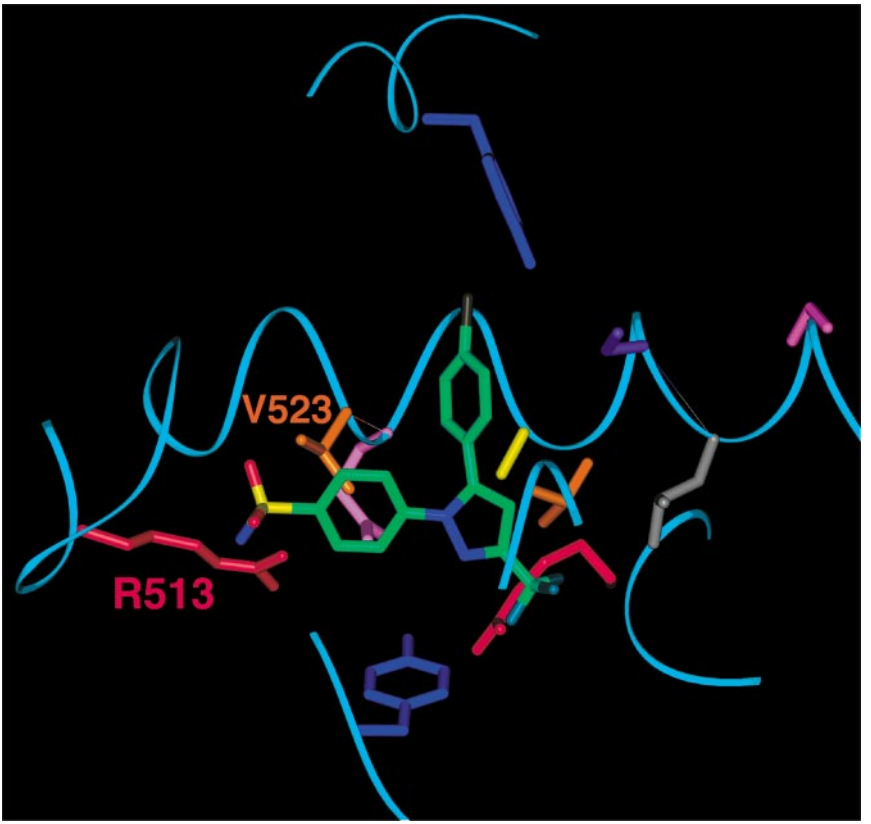

Figure 9 Binding of a diarylheterocycle in the side pocket of COX-2. Compound SC-558 is shown bound in the active site with its sulfonamide group projecting past Val-523 and into the side pocket over Arg-513 and its p-bromophenyl ring projecting up toward Tyr-385.