COX-2 Inhibition, Apoptosis, and Chemoprevention by Nonsteroidal Anti-inflammatory Drugs

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Abstract: Non-steroidal anti-inflammatory drugs (NSAIDs) have as their common mechanism the inhibition of cyclooxygenase (COX) enzymes, of which two isoforms (COX-1 and COX-2) exist. The effect of NSAIDs on chemoprevention and tumor regression has been shown in animal models, epidemiologic studies, and in treatment of patients. The exact biochemical and cellular mechanisms underlying each of these phenomena is only partially understood. Processes that have been recently implicated as being important include the inhibition of tumor cell growth, prevention of angiogenesis, and induction of apoptosis in neoplastic cells.

Introduction

Chemotherapeutic treatment of neoplasia has historically relied heavily on genotoxic agents which, in addition to inhibiting cell growth and inducing apoptosis are themselves mutagens and are typically toxic. In the future, however, a wider array of efficacious anti-neoplastic agents that lack the toxic/mutagenic properties of genotoxins may be used. Promising mechanisms of action of such drugs include inhibition of angiogenesis in tumors, selective inhibition of cell growth, and/or induction of apoptosis in neoplastic cells. One class of drugs that recently has been shown to exhibit all of these properties, and might be of important use as antineoplastic agents in the future, is, ironically, one of the oldest man-made pharmaceuticals in use today: non-steroidal anti-inflammatory drugs (NSAIDs). Aspirin (acetylsalicylate), the progenitor and still widely used member of the NSAID family, was first made and used in 1897 by Felix Hoffman at Bayer and Co. (See [1] for review of the history of aspirin and NSAIDs) and most NSAIDs currently on the market are at least 20 years old [Fig. (1)]. These drugs all exert their anti-inflammatory, analgesic, antipyretic, and anticoagulatory effects by inhibiting the production of prostaglandins.

Cyclooxygenases and NSAIDs

The rate-limiting step in the synthesis of prostaglandins is catalyzed by the cyclooxygenase (COX) enzymes, also known as prostaglandin synthases. Two isoforms of COX (COX-1 and COX-2) have been identified. Both are inhibited by all commercially-available nonsteroidal anti-inflammatory drugs (NSAIDs) available before the year 1999 [Fig. (2)]. However, very recently two COX-2-selective inhibitors, celecoxib and rofecoxib, have been pharmacologically marketed [Fig. (1)]. These two drugs exert low inhibition of COX-1, which is expressed as a constitutive enzyme in virtually all tissues, including stomach. In contrast, these drugs potently inhibit COX-2, which is expressed at low levels in most tissues unless induced by mitogens or other stimuli [3]. The isoenzyme-selective action of these drugs [Fig. (2)] avoids gastric toxicity, mediated primarily by inhibition of COX-1, and retains high anti-inflammatory activity, which has been proposed to be mediated primarily by COX-2 [4,5].

Competitive and Non-competitive Activities of NSAIDs

COXs are catalytically bifunctional enzymes and contain two separate and distinct active sites [Fig. (3)]. The cyclooxygenase active site catalyzes the oxygenation of arachidonic acid and performs its cyclization to form the pentane ring-containing endoperoxyl intermediate PGG$_2$. A separate peroxidase active site in the enzyme reduces a hydroperoxyl in PGG$_2$ to a hydroxyl to produce PGH$_2$ from which all other prostaglandins are made [3,6-8]. It has long been known that aspirin has a mode of action that is significantly different from other commercially available NSAIDs. Aspirin covalently modifies the
**Evolution of NSAIDs**

(Market Release Date)


Indomethacin (1964) → Ibuprofen (1969)

Aspirin (1899)

**Fig. (1).** Evolution of NSAID development. Beginning with Aspirin (bottom), developed at Bayer in 1897, the sequential development of non-selective, competitively-acting NSAIDs (middle), and, finally, COX-2-selective inhibitors (top) are shown. Only a representative sample of non-selective, competitively active NSAIDs are shown. COX-2 preferential drugs such as etodolac, meloxicam, and nimesulide, which show less selectivity for COX-2 than rofecoxib or celecoxib, constitute an intermediate group between selective and non-selective inhibition and are not shown. Dates in parentheses are dates of market release.
cyclooxygenase active site of COX-1 and COX-2 while other NSAIDs competitively "plug" this site. In a landmark study, Garavito and colleagues achieved a high-resolution X-ray crystallographic structure of sheep seminal vesicle COX-1 [6-8] co-crystallized with flurbiprofen. Subsequently, this laboratory was successful at viewing the binding of other NSAIDs to COX-1 [7-8], and other researchers have applied the same technique to COX-2 and the binding of COX-2 selective inhibitors [9,10]. Together, these studies have allowed precise elucidation of the cyclooxygenase and peroxidase active sites, as well as determination of the binding interactions of NSAIDs with COX. These studies demonstrated that the

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**Fig. (2).** Comparison of NSAIDs for COX-2 selectivity. Determinable log [IC$_{50}$ ratio (COX-2/COX-1)] was measured using William Harvey Human Modified Whole Blood Assay (WHMA). The "0 line" indicates equipotency, i.e., an IC$_{50}$ ratio of 1. Italics indicate compounds with very low potency. Reproduced from reference [2] with author's permission.
cyclooxygenase active site is a long, narrow, hydrophobic channel with only a small number of hydrophilic residues, most, if not all of which are essential for catalysis. Both NSAIDs and arachidonic acid, the primary substrate of the enzyme, enter this active site via a mouth that is found largely buried in the lipid bilayer of the endoplasmic reticulum and nuclear envelope to which COXs are attached. (See [11,12] for recent reviews on the structure of COXs, including their membrane binding, catalytic, and dimerization domains). After diffusing into the active site, however, aspirin is transiently coordinated electrostatically via its carboxyl group to the amine of arginine 120 [Fig. (3)], one of the hydrophilic residues of the active site. This weak interaction places it within 5 angstroms of serine 530, to which aspirin diffuses and transfers its acetyl group. The resulting salicylate that is formed is held poorly in the active site and readily diffuses out. However, aspirinÆEs 2-carbon adduct to serine, which is spacially near tyrosine 385, a residue that directly participates in catalysis, is sufficient to permanently block PGG₂ formation. In contrast, flurbiprofen, indomethacin and other competitively active NSAIDs form more complex interactions within the COX active site. These include the formation of electrostatic interaction with arginine 120 as well as interactions with hydrophobic, neutral and polar residues in the narrow channel. Because of these multiple favorable interactions, these drugs compete for binding to the active site better than arachidonic acid does and thus inhibit PGG₂ synthesis.

Fig. (3). COX-1 and COX-2 active sites. (a) α-tracing of the 3-dimensional structure of COX-2 monomer, with active sites and critical residues designated [Brookhaven databank code 1CX2]. Cut-away representations of COX-2 (b) and COX-1 (c) in the same orientation as in (a) are shown. The membrane binding (blue) and dimerization (red) domains are represented by shading with the remainder of the protein being the catalytic domain. Both the peroxidase and cyclooxygenase active sites are designated, although the shapes of each are approximate. Critical hydrophilic residues in the active site are Arg 120, Ser 530, Tyr 385. Ile 523 in COX-1 is substituted for Val 523 in COX-2, the smaller side chain in this residue in COX-2 provides access to the hydrophobic side channel (shown) that is exploited by COX-2 selective drugs.
COX-2 selective inhibitors additionally exploit the fact that the COX-2 cyclooxygenase active site is slightly larger than that of COX-1 and has a hydrophobic side-pocket [Fig. (3)] that can be accessed by aromatic containing substituents on these drugs [Fig. (1)].

**COX-2 and Cancer**

In addition to its role in inflammation, multiple pieces of evidence suggest that COX-2 plays an important role in cell proliferation. First and foremost was its cloning and characterization as an immediate-early gene product induced by serum, phorbol ester, and the v-src oncogene in fibroblasts [13-16]. Numerous studies have now extended these original observations to a wide variety of cultured cells and mitogenic inducers. At the organismal level, COX-2 is induced physiologically during the mitogenic response occurring in wound healing [17,18] and, very significantly, has been found to be over-expressed in many types of pre-malignant and malignant neoplasms in humans and other organisms. The tumor best characterized for COX-2 overexpression is adenocarcinoma of the colon/rectum with its precursor lesion, adenoma of the colon/rectum. COX-2 is overexpressed at an early stage of adenoma development in approximately 80-90% of tumors and this overexpression extends through the adenoma/adenocarcinoma transition, such that a similar percentage of adenocarcinomas as adenomas overexpress COX-2 [19-23]. In both adenomas and adenocarcinomas in humans the primary cell-type over-expressing COX-2 is the neoplastic cells themselves and not invading stromal cells or leukocytes [19,20,22]. However, in adenocarcinomas COX-2 overexpression has frequently been reported to be more focally expressed within the tumor mass, in contrast to a more homogeneous overexpression of COX-2 seen in adenomas. COX-2 overexpression in carcinomas is also often greater in well- or moderately-differentiated carcinomas than in poorly differentiated tumors.

The paradigm of COX-2 overexpression in both pre-malignant precursors and malignant lesions extends beyond colorectal cancer. COX-2 is highly elevated in 80-90% of epithelial biopsies of Barrett/Es esophagus as well as in squamous cell carcinomas and adenocarcinomas of the esophagus in Barrett/Es and non-Barrett/Es patients [24,25]. Likewise, COX-2 is elevated in atypical alveolar epithelium of the lung which is a proposed precursor to lung adenocarcinomas, a malignancy where COX-2 is also elevated [26,27]. Thus, existing evidence suggests that COX-2 becomes elevated early in tumor progression. Early COX-2 expression is also likely in carcinoma of the stomach [28], breast [29], head and neck [30], pancreas [31], and well-differentiated hepatocellular carcinomas [32,33] where COX-2 has thus far been found to be overexpressed in the majority of tumors in humans.

**COX-2 Expression in 2-Stage Models of Chemical Carcinogenesis**

One of the earliest studies indicating a role of cyclooxygenases in cancer was by Verma and Boutwell [34], who showed in a two-stage model of skin carcinogenesis that prostaglandin synthesis activity was increased in papillomas resulting from repeated phorbol ester treatment of mutagen-initiated mouse skin. Furthermore, they demonstrated that cotreatment during the promotion stage of carcinogenesis with a cyclooxygenase inhibitor, indomethacin, completely inhibited tumor formation [34]. Two-stage rodent models of carcinogenesis in skin [35] and colon (reviewed in [36]) have all shown that tumor formation, in some cases even beginning at formation of dysplastic foci, is accompanied by a marked induction of COX-2. Moreover, the initial observation of Verma and Boutwell regarding the chemopreventive property of NSAIDs in inhibiting tumor formation is also seen in these tissues as well [35,36].

**Chemopreventive and Chemotherapeutic Uses of NSAIDs in Humans**

Numerous studies have epidemiologically associated regular NSAID use with an approximately 50% decrease in the incidence of colorectal cancer (see [36] for a recent review). Additionally, multiple studies suggest that NSAIDs exert a similar level of protection (approximately a 50% reduction) in esophageal and stomach cancer [37-39]. It is unclear at present what optimum dose is needed to obtain NSAID-induced chemoprotection in the colon/rectum. However, the largest study thus far done (>600,000 cohorts [39]) used as its criteria for regular NSAID use a minimum of 16 doses of aspirin per month. The primary obstacle to the direct determination of the chemopreventive activity of NSAIDs and the establishment of an optimum dose in humans is the demonstrated gastric and renal toxicity of this class of drugs, which negatively counterbalance chemopreventive benefits.

Through the early studies of Waddell and Loughry done in 1983 [40,41], the NSAID sulindac was shown to have a chemotherapeutic effect, in that it could cause regression of colorectal adenomas in patients suffering from familial polyposis coli. Since that discovery, many other investigators have shown this drug to be highly effective in causing regression of
both sporadic and familial-inherited forms of colorectal adenomas [42-49]. Piroxicam has experienced limited testing for its ability to cause adenoma regression with mixed results [47,48]. In one patient with familial adenomatous polyposis, complete regression of villous adenomas was observed [48]. Whereas in another small study, partial regression of adenomas was seen after piroxicam treatment [47]. Gastric toxicity problems have prevented long term treatment with piroxicam and resulted in a 50% withdrawal rate [47]. As described below, the high efficacy of sulindac in causing colon adenoma regression likely has to do with the unusual mechanism by which this pro-drug is activated in the body.

**NSAID-induced Chemoprevention vs Tumor Regression**

Are chemoprevention and tumor regression the same biological phenomenon? The answer to this question, as well as to the question of whether non-competitive NSAIDs such as aspirin act in the same way to prevent cancer as competitive-acting NSAIDs, is presently unknown. Indeed the recent explosion in knowledge regarding COX-2 in neoplastic cells suggests the role of COX-2 is multi-factorial. COX-2 overexpression in neoplastic cells is involved in promoting cell division, inhibiting apoptosis, altering cell adhesion, increasing cell motility, and inducing neovascularization. COX-2 expression may also be involved in decreasing immnosurveillance.

The primary distinguishing biological feature of chemoprevention vs. tumor regression is that in the former tumors never arise, whereas in the latter visible masses, that may be greater than 2 cm in size, are caused to shrink and disappear in a matter of days or weeks. In tumor regression, NSAID treatment must result in a massive induction of apoptosis. Apoptosis is a two-stage process that includes programmed cell death and resorption of dead cells. NSAID induction of apoptosis in both sporadically-occurring and experimentally-induced tumors undergoing tumor regression has been histologically confirmed by several studies [42,46,49-51]. In contrast, induction of apoptosis, failure to establish a vasculature, or any of a number of other COX-2 mediated effects may be the mechanism of chemoprevention by NSAIDs.

**Clues from Genetically Altered Mice and Two-stage Chemoprevention Studies**

Dissecting differences in mechanism(s) between NSAID-induced chemoprevention from those of tumor regression in colorectal cancer is presently possible only in rodents, where chemically-induced and genetic models exist for intestinal neoplasia. Two caveats of these models are that murine tumors histologically overexpress COX-2 in stromal cells and invading leukocytes, rather than in the neoplastic cells themselves [52]. As described above, the opposite is true of human tumors. Additionally, in the APC
d or APC
d mutant strains, the adenomatous polyposis coli (APC) gene is altered, giving rise to hundreds of intestinal polyps. However, unlike familial polyposis in humans, where polyps arise primarily in the colon/rectum, polyps in these mice are found throughout the intestine and are most abundant in the small intestine, which is a rare site for tumorigenesis in humans [53].

Aspirin [54,55], piroxicam [56-58], sulindac [53,58,59], or COX-2-selective inhibitors [53] at doses ranging from 200 ppm (roughly 0.5 mg/day) and upward can reduce tumor incidence by 40-50% in the case of aspirin, and to greater than 95% in the cases of the other drugs. In these chemoprevention studies NSAID administration is typically begun immediately after weaning and continues throughout the life of the organism. In contrast to this regimen needed for chemoprevention, tumor regression studies in APC mutant mice can employ aggressive NSAID treatment for relatively short periods of time since evidence for tumor regression is seen within 4 days after the start of NSAID treatment [59]. In most chemoprevention studies and essentially all tumor regression studies, the NSAID doses used to effectively reduce neoplasia are high, at least one order of magnitude higher (on a weight-weight basis) than doses recommended for incidental or anti-inflammatory use in humans or animals. For example, sulindac, piroxicam and aspirin are used at doses ranging from 7.5-200mg/kg. At these high doses, up to 95% percent of animals in one study using piroxicam experienced ulceration of the GI tract [56].

**Tumor Regression: Is the Vasculature or the Neoplastic Cell the Ultimate Anti-neoplastic Target of NSAIDs**

In the case of tumor regression, visible tumors that regress must already have an established vasculature. However, the apoptosis accompanying NSAID-induced tumor regression could be caused by destruction of the existing vasculature by an anti-angiogenic action. In this case NSAIDs would be acting to inhibit COX-2 over-expressed in non-vascular tumor cells (i.e. neoplastic cells, stromal cells, or leukocytes) to prevent PGE synthesis and thereby inhibit production of the angiogenic factors needed for the survival and maintenance of endothelial cells [60]. Without an intact vasculature, the tumor then dies through anoxia. This
mechanism is presumably that by which anti-angiogenic agents such as TNP-470, angiostatin, and endostatin induce tumor regression.

Angiogenesis is essential for tumors to grow beyond 1-2 mm in size [61]. COX-2 has been postulated along with iNOS and p53 to be a central player in angiogenesis in tumors (reviewed in [62]). Dubois and colleagues have shown that the PGE$_2$ synthesized by overexpressed COX-2 in CaCo2 colon carcinoma cells can induce secretion of VEGF, TGF$-_1$, bFGF, and other angiogenic factors that in turn stimulate vascular tube formation [63]. Sulindac and COX-2 selective inhibitors inhibit angiogenesis [49,64], presumably by completely inhibiting the production of these angiogenic factors by the tumor cells [63]. These same angiogenic growth factors not only stimulate endothelial cell growth but also are required for endothelial cell maintenance and survival in vivo [65-67]. Hence, it is assumed that an absence of angiogenic factors due to COX-2 inhibition would result in apoptosis in the tumor vasculature, although this remains to be shown.

Interestingly, Dubois and colleagues also found that COX-1 expression is induced in endothelial cells undergoing tube formation [63], a phenomenon associated with cell differentiation in a variety of cell types [68-70]. Inhibition of COX-1 synthesis using antisense nucleotides resulted in a decrease in expression of Ets-1, a transcription factor involved in angiogenesis [63]. This finding illustrates a possible target for NSAIDs in tumor regression which would be unaffected by COX-2 selective drugs. Further studies will be needed to determine whether inhibition of endothelial COX-1 potentiates the anti-angiogenic effects of NSAIDs.

An anti-angiogenic action should require anti-inflammatory levels of NSAIDs, since NSAID concentrations within tumors, and particularly near blood vessels should be at least as high as in the synovium of joints. However, certain factors may increase the dose level needed to produce an anti-angiogenic response in tumors that would be sufficient to result in vasculature destruction. First, prostaglandin synthesis may need to be repressed below a threshold level in order to be anti-angiogenic. Second, prostaglandin synthesis may need to be suppressed for prolonged periods of time. This is consistent with the fact that relatively high (micromolar rather than nanomolar) concentrations of NSAIDs are needed to evoke apoptosis in both in vitro and in vivo systems [71-73].

Alternatively, COX-2 over-expression in rat intestinal epithelial cells makes them resistant to butyrate-induced apoptosis [71] and NSAID treatment increases the apoptotic index of colonic crypt cells and cells in dysplastic foci too small to be influenced by vasculature. This suggests that an anti-angiogenic action may be just one biochemical mechanism for NSAID-induced tumor regression.

Comparison of Tumor Regression Activity of NSAIDs and Anti-angiogenic Agents

As described above, COX-2 overexpression in murine tumors is localized to stromal cells and invading leukocytes, whereas in humans COX-2 is expressed in the neoplastic cells themselves. If inhibition by NSAIDs of this overexpressed COX-2 is necessary and sufficient to induce apoptosis, this pattern of expression is consistent with COX-2 making a diffusible product acting in a paracrine fashion. Hence, overexpressed COX-2 targeted by NSAIDs need not be located in the neoplastic cells to have an effect on tumor regression [Fig. (4)].

Fig. (4). The effect of NSAIDs on tumor growth. Prostaglandins or other COX-generated downstream mediators may signal in a (1) autocrine or (2) paracrine manner to tumor cells, promoting cell proliferation and survival. Another essential paracrine signaling pathway involves production of PGE$_2$ by tumor cells, which in turn acts in an autocrine/paracrine fashion to promote synthesis by the tumor cells of angiogenic growth factors such as VEGF, TGF$-_1$, bFGF, and PDGF. These factors act in a paracrine fashion (3) to promote vascular tubule formation and survival. Inhibition of all of these signals results in inhibition of cell growth, angiogenesis, and promotes the induction of apoptosis.
An important question is whether angiogenesis is the sole mechanism by which a diffusible product generated by COX-2 results in tumor maintenance and growth [Fig. (4)]. If so, this would predict that tumor regression and potentially chemoprevention by NSAIDs are caused almost exclusively by their anti-angiogenic properties. A comparison of the anti-angiogenic agents and NSAIDs shows some similarities and some differences in effect. Similar to the induction of apoptosis in neoplasms by NSAIDs, classic angiogenesis inhibitors control apoptosis in tumor cells. For example, the potent angiogenesis inhibitor TNP-470, thought to inhibit the intracellular enzyme, methionylaminopeptidase-2, induces apoptosis in vascular endothelial cells [74].

Recently, using a multistage carcinogenesis model (RIP1-Tag2) for pancreatic cancer in mice, Bergers et al. [75] treated pancreatic insulinomas, islet cell carcinomas, and their pre-malignant precursors with angiogenesis inhibitors TNP-470, angiostatin, endostatin, and BB-94. Depending on the stage of carcinogenesis, the four angiogenesis inhibitors had differing efficacies. Tumor burden in highly vascularized small tumors (1-2 mm) was reduced up to 87% by inhibitors, a figure that approximates the high level of tumor regression achieved with NSAIDs, as previously noted. Yet in large tumors and invasive carcinomas, total reduction possible was less than 59%, analogous to the low regression rates of NSAID treatment in carcinomas, as noted previously. Interestingly, they found that treatment increased the number of apoptotic tumor cells 2 to 3 fold and that tumor cells close to, rather than distal to capillaries were frequently apoptotic. This finding suggests an interesting paradox: that apoptosis in tumor cells can be induced by paracrine signaling from apoptotic endothelial cells rather than by hypoxia.

In human and animal colorectal adenomas, NSAID treatment may result in nearly 100% tumor regression [56-59], and multiple studies have shown that regression is independent of tumor size [43,45,59]. Although angiogenesis inhibitors significantly reduce tumor mass, the total tumor prevention and total regression observed with NSAIDs, particularly of large tumors, is not seen [75,76]. This may be because, in contrast to NSAIDs, angiogenesis inhibitors do not inhibit synthesis of vascular endothelial growth factors, but act at individual sites in the vascular cell [74,77]. In this well-controlled study on the efficacy of anti-angiogenic drugs during tumor progression, TNP-470 and other agents became less effective as adenomas grew larger than 3mm. In contrast, NSAIDs appear to affect regression regardless of size. One caveat is that vascularization of tumors in the pancreatic model of tumor progression may differ significantly from that which occurs in adenoma progression. In support of this concept is the fact that pancreatic tumors of 1-3mm are highly vascularized, whereas colonic tumors of this size show the same vasculature as normal crypts, and increased vascularization is seen only in larger (greater than 3mm) adenomas and in carcinomas [78,79]. Since in familial polyposis the majority of polyps are in the 1-3mm range, yet experience NSAID-induced regression, it appears that inhibition of angiogenesis is only one of the mechanisms by which NSAIDs cause tumor regression in the colon.

Other Pathways: Is the Apoptosis-inducing Activity of NSAIDs Dependent on COX-2 Inhibition?

The majority of NSAIDs cause apoptosis; however, in many cell types concentrations > 100 µM are needed to evoke programmed cell death [80]. Lu et al. [72] showed that almost all NSAIDs cause apoptosis in RSV-transformed fibroblasts where COX-2 is induced as part of the process of transformation to the neoplastic state [Table I]. Essentially 100% of cells could be induced to undergo apoptosis and this event was readily detectable at NSAID doses as low as 32 µM [73]. This apoptotic activity is accompanied by generation of nucleosomal ladder DNA and other biochemical events associated with apoptosis. The fact that the majority of NSAIDs cause apoptosis is significant because the history of NSAID pharmacology is replete with unique properties, other than COX inhibition, being associated with individual NSAIDs [81].

There may be more than a single pathway through which individual NSAIDs act to cause apoptosis, because their relative potency in producing the various apoptotic effects is not the same [72]. Furthermore, addition of PGE2 or other prostanooids does not reverse this NSAID-induced apoptosis (NIA), suggesting that inhibition of one or more COX-generated products other than PGE2 is the major mechanism of NIA.

Studies involving the NSAID sulindac have provided contradictory clues about the role of COX-2 in apoptosis signaling pathways. Sulindac [Fig. (1)] is a pro-drug that must be reductively metabolized to its active form, sulindac sulfide, in the colon by bacterial flora and in the liver. Sulindac is also oxidized in the liver to form sulindac sulfone. Lu et al. [72] showed no ability of the pro-drug to induce apoptosis [Table I]. In contrast, sulindac sulfide, but not sulfone, efficiently causes apoptosis in this system. The sulfide is a potent COX inhibitor, but the sulfone is at least 5000-fold less potent for inhibiting COX-1 and -2 [82]. Currently it is thought that sulindac and sulindac sulfide are in equilibrium and can be interconverted into each other in the body. In contrast, oxidation to the sulfone is considered to be largely irreversible.
Table I. Morphological Inhibition of Transformation in Response to NSAID Treatment

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X, complete inhibition of focus formation by RSV; \, partial inhibition that was characterized by cell rounding and formation of small clumps of cells; -, no effect. Inhibition of focus formation was shown to result from induction of apoptosis in all drugs listed [72]. With the exception of aspirin, ineffective drugs were either pro-drugs (e.g. sulindac) or were very weak inhibitors of COX activity (e.g. acetamidophenol). Reproduced from [72].

Importantly, in some systems, the sulfone has shown chemopreventive activity comparable to that of sulindac sulfide [83,84], and that activity has been shown to be apoptosis-inducing [83-86] and anti-angiogenic [87]. However, many other studies have shown that sulfone is much less potent than sulindac sulfide and must be used at high concentrations to achieve similar results [84,88].

The contradictory results obtained with sulindac sulfone have resulted in efforts to identify other non-COX pathways for NSAIDs. Recently it has been reported that sulindac sulfide directly binds p21ras, thereby inhibiting interaction of p21ras with the Raf protein and inhibiting Ras-induced malignant transformation [89]. This may explain why rat enterocytes transformed by the oncogene c-K-ras show marked resistance to sulindac-induced apoptosis [90], which may be due to target upregulation due to antagonist binding. If this finding is correct, sulindac sulfone and sulfide would represent the first c-K-ras antagonists known. Unexplained, however, is whether all other apoptosis-inducing NSAIDs function through c-K-ras binding, which at this point seems unlikely.

In addition to studies on sulindac, other experimental results have suggested a non-COX mechanism for NSAID-induced apoptosis. For example, at high concentrations competitively acting NSAIDs induce apoptosis in HCT-15 cells, which have been reported by PCR analysis to lack COX-1 and COX-2 transcripts [80], suggesting an additional mechanism for NIA besides COX inhibition. However, reevaluation of this cell system by others found that it expressed COX-2, but not COX-1 mRNA [91], although at 80% lower levels than the colorectal-derived HT-29 cells used as a positive control in the original study.

The inability of prostanoids to rescue from NSAID-induced apoptosis in many in vitro cell systems is puzzling and suggests that any COX mediated product being interrupted by NSAIDs may be unstable. Studies by Chan suggest that the product may not be a prostanoid at all, but arachidonic acid that is shunted to neutral sphingomyelinase, with concomitant production of ceramide and activation of apoptosis pathways [30].

Are High NSAID Concentrations Needed for Apoptosis and Tumor Regression in vivo?

It is important to note that in all reported cases, NSAID concentrations needed to cause apoptosis are typically higher than the sub-micromolar levels that are frequently needed for 50% inhibition of COX-1 or COX-2 in most cell types. Instead they approximate, and in
some cases exceed, the micromolar concentrations needed for 80% inhibition of COX-1 and COX-2 recently reported by Warner, Vane and colleagues [2]. Are the high NSAID doses needed for apoptosis in vitro physiologically relevant in the colon? Important clues may come from sulindac, the most effective NSAID in causing tumor regression thus far described. Reduction of sulindac to the active sulfide is actively carried out by aerobic gut flora on the lumenal surface of the colon rectum [92-94]. Because of this mode of activation, systemic sulindac sulfide concentrations are significantly reduced in colonectomized patients [92]. The unusual bioactivation of sulindac on the surface of the colon mucosa may be responsible for the creation of locally high concentrations of the active sulindac sulfide, that may topically affect tumor growth. Topical inhibition of colonic explants has been demonstrated in rodents [49]. This postulate would predict that sulindac will not be as effective in causing tumor regression in other tissues as in the colon. If future study finds that chemoprevention and tumor regression are caused by the same mechanisms (e.g. apoptosis), NSAIDs may also have diminished chemopreventive efficacy in non-colon tissues than in the colon. Alternatively, if chemoprevention does not require high NSAID doses needed to evoke apoptosis, systemic delivery of drug may be sufficient to prove equally effective in tissues outside the GI tract.

A topical effect of sulindac may explain why this drug effectively causes adenoma regression in the colon, but is ineffective in causing adenoma regression in the duodenum in the same patients [95,96]. Such an effect may also explain why NSAID-reduction of tumor incidence is epidemiologically most closely associated with tumors of the GI tract [97]. Similarly, the high, ulcerogenic doses of piroxicam used in rodent tumor regression studies [56] may be effecting tumor regression through topical (i.e. piroxicam in the feces) as well as systemic delivery of the drug.

If tumor regression and/or chemoprevention is mediated by strongly inhibiting COX-2 with high concentrations of NSAIDs, COX-2 selective inhibitors may be extremely valuable in causing anti-neoplastic effects in the absence of gastric toxicity.

**Evidence of Sub-populations of COX-2 or a COX-3**

In their purified states, COX-1 and COX-2 have very similar properties [98], yet only COX-2 generates PGE2 following mitogen-induced substrate release, even when COX-1 is expressed at high levels [99]. Additionally, COXs in different tissues vary in their sensitivities to NSAIDs. For example, NSAID IC50 values are typically $1 \times 10^{-4}$ M or greater in some fibroblasts [72], in contrast to macrophages, in which NSAIDs typically inhibit COX at sub-micromolar concentrations [100]. Are the COX populations the same in all cells, or may differences in COX make some cells more sensitive to the anti-neoplastic effects of NSAIDs? Similarly, can the activities of COX-1 and COX-2 be directly regulated during apoptosis and in tumor proliferation, resulting in sub-populations of COX isoforms that recognize different substrates and have distinct sensitivities to NSAIDs?

A search for proteins that might act as modifiers of COX-1 and COX-2 has been conducted by the yeast 2-hybrid system [101]. Extensive library screening by this method has thus far yielded one protein that associates with both COX-1 and COX-2 in the yeast system. This protein, nucleobindin, was first identified, purified, and molecularly cloned as an autoimmunity- and apoptosis-associated protein (see [101] and refs. therein). Nucleobindin also is a calcium-binding protein containing high-affinity EF hand binding sites [Fig. (5)]. Like COXs, nucleobindin has a hydrophobic signal peptide and is synthesized into the ER where it can associate with COXs. Farquhar and colleagues [102,103] have recently shown that nucleobindin is a resident golgi protein where it may also participate in regulating calcium release to the cytosol. Binding of nucleobindin to COX-1 or COX-2 may alter their enzymatic properties (e.g., kinetics, substrate recognition, product shunting, etc.) or subcellular localization. It is also possible that nucleobindin interaction with COXs may alter their sensitivity to NSAIDs.

![Functional domains of murine nucleobindin](image)

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**Fig. (5).** Functional domains of murine nucleobindin. Nucleobindin (Nuc) is an intra-lumenal resident of the ER and the cis-golgi, and is directed into the lumen of these compartments by a hydrophobic N-terminal signal peptide. COX interaction has been shown to require the N-terminal 40% of the protein. Nuc binds calcium with two functional EF-hands, and other domains (basic region, leucine zipper, nuclear localization signal). Nucleobindin is involved in the initiation of a systemic lupus erythematosus-like disease in mice, where it is found bound to nucleosomal-laddered DNA released from apoptotic cells. Modified from [101].
Preliminary studies show that co-expression of COX-2 and Nuc increased the Vmax of COX-2 in whole cells [unpublished data]. However, interaction of the two purified proteins in solution had a negligible or a slight inhibitory effect [unpublished]. These data suggest that the increase in COX activity seen in whole cells may require other factors of which nucleobindin is only a part. Currently nucleobindinÆs precise role in regulating COX signaling, inhibition by NSAIDs, or in creating enzymatically distinct sub-populations of COX-1 and COX-2 remains to be elucidated.

In addition to protein regulators that create sub-populations of COXs, recent pharmacological data have detected distinct COX enzyme activities that differ significantly with those associated with either COX-1 and COX-2 [105]. This activity is associated with and may be involved in apoptosis. In studies done on a murine monocyte/macrophage transformed cell line, Simmons et al. [105] reported induction of an acetaminophen-sensitive cyclooxygenase with reduced sensitivity to NSAIDs. The enzyme activity identified was induced by treating cells for a prolonged period of time (48 hours) with high (500 µM) concentrations of diclofenac. In the process COX-2 was induced as was a COX enzyme activity that could readily be revealed by rapidly removing the diclofenac from the cells. This NSAID-induced COX differs from COX-1 or COX-2 in that: (1) time-dependent, pseudo-irreversible, competitive inhibitors that normally bind tightly to COX-1 and COX-2 readily wash out of the active site of this COX; (2) acetaminophen, a weak inhibitor of COX-1 and COX-2, inhibits this activity; (3) this activity is not inhibited by aspirin, and (4) this COX activity is less sensitive to inhibition by NSAIDs [104]. In response to chronic diclofenac treatment in a fibroblast cell system, a COX-2-luciferase fusion protein localized preferentially to the cytosol rather than the membrane of the cell, suggesting that the diclofenac induced a subcellular redistribution of the COX-2 reporter [104].

Induction of a similar activity by the COX-2 selective inhibitor NS398 has recently been shown by another laboratory [105]. Moreover, induction of a cytosolic [106] or nuclear COX-2 have also been reported [107]. Clearly different sub-cellular COX localizations is a mechanism that could result in enzymatically distinct COX-2 populations. Efforts to further characterize this enzyme should provide greater clarity regarding the role of COX-2 and other COX isoforms in tumorigenesis and apoptosis.

Conclusions

The effect of NSAIDs on chemoprevention and tumor regression has been clearly shown in numerous studies, although the exact biochemical mechanism underlying each of these phenomena is still poorly understood. COX-generated biomediators such as prostaglandins, or other downstream mediators have an important role in regulating cell proliferation and survival. This can be signaled for either in an autocrine or a paracrine fashion to other tumor cells [Fig. (4)]. Inhibition of these signals results in inhibition of cell growth and the induction of apoptosis. Other COX-2-dependent paracrine signaling involves production of growth factors such as VEGF, TGF-β1, bFGF, and PDGF by the tumor cells that signal to the vasculature. This serves to mediate angiogenesis and vascular tubule survival [Fig. (4)]. Inhibition of these signals results in inhibition of angiogenesis and the possibly the induction of apoptosis in neovessels.

While some data suggest that high concentrations of NSAIDs may induce apoptosis and have chemopreventive action through mechanisms other than COX inhibition, a reasonable conclusion is that the majority of NSAIDs induce apoptosis by inhibiting synthesis of COX-2-generated biomediators. It is becoming increasingly evident that selective COX-2 inhibitors have a growing potential as chemopreventive and tumor regression agents for colorectal, esophageal, and other cancers of the GI tract.

Abbreviations

NSAIDs = Nonsteroidal anti-inflammatory drugs
NIA = NSAID-Induced apoptosis
COX = Cyclooxygenase
APC = Adenomatous polyposis coli
GI = Gastro-intestinal
PGE2 = Prostaglandin E2
iNOS = Inducible nitric oxide synthase
VEGF = Vascular endothelial growth factor
bFGF = Basic fibroblast growth factor
PDGF = Platelet-derived growth factor
TGF-β1 = Transforming growth factor-beta 1
RSV = Rous sarcoma virus
WHMA = William Harvey Human Modified Whole Blood Assay

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References


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