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Biochemical Pharmacology 70 (2005) 969–986
Review
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Non-COX-2 targets and cancer: Expanding the molecular target repertoire of chemoprevention

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Received 7 January 2005; accepted 4 May 2005

Abstract

Chemoprevention represents a highly promising approach for the control of cancer. That nonsteroidal anti-inflammatory drugs (NSAIDs) prevent colon and other cancers has led to novel approaches to cancer prevention. The known inhibitory effect of NSAIDs on the eicosanoid pathway prompted mechanistic and drug development work focusing on cyclooxygenase (COX), culminating in clinical trials of cyclooxygenase 2 (COX-2) inhibitors for cancer prevention or treatment. However, two COX-2 inhibitors have been withdrawn due to side effects. Here we review several pathways of the eicosanoid cascade that are relevant to cancer; summarize the evidence regarding the role of COX-2 as a target for cancer prevention; and discuss several of the molecular targets that may mediate the chemopreventive effect of NSAIDs. The clinically modest results obtained to date with COX-2 specific inhibitors used in cancer prevention; the multiple COX-2-indpendent targets of both NSAIDs and COX-2 inhibitors; and the limitations of some COX-2 inhibitors indicate that exploiting these (non-COX-2) molecular targets will likely yield effective new approaches for cancer chemoprevention.

Keywords: COX-1; COX-2; COX-2 independent targets; Chemoprevention; Cancer; NSAIDs

Contents

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doi:10.1016/j.bcp.2005.05.004

According to the old adage, ''an ounce of prevention is better than a pound of cure''. This holds true for cancer as well, and it is generally agreed that the best way to control cancer is to find ways of preventing it. Undoubtedly, life style and environmental factors contribute significantly to the number of cancer cases seen each year. During the last two decades cancer prevention has achieved significant prominence amongst the clinical and research communities. This interest has been enhanced by several laudatory results, including those in the prevention of lung cancer (smoking cessation), colon cancer (e.g., screening colonoscopy) and prostate cancer (e.g., prostate specific antigen). The seminal epidemiological observation that nonsteroidal anti-inflammatory drugs (NSAIDs) prevent colon and other cancers has provided the impetus to develop novel chemoprevention approaches against cancer [1]. The recent withdrawal of rofecoxib and valdecoxib, two cyclooxygenase 2 (COX-2) specific inhibitors, on account of their significant cardiovascular toxicity has raised concerns about this class of compounds [2]. In this commentary, we examine cancer chemoprevention against the background of this unfortunate development, assess the concept that the expression of COX-2 is central to carcinogenesis, and propose that shifting our mechanistic focus to targets other than, or in addition to, COX-2 will be a productive approach for cancer prevention.

1. Cancer prevention as a contemporary challenge

Cancer prevention may be categorized as primary, secondary or tertiary [3]. *Primary prevention* modifies genetic, environmental and biological factors etiological in a given tumor to alter their effects on tumorigenesis. For example, elimination of chemicals, radiation, and viruses constitutes primary prevention. Unfortunately, culprit agents, e.g. smoking, are rarely identified. *Secondary prevention* deals with screening for premalignant and early neoplastic lesions and their expeditious treatment. For example, screening for colorectal cancer reduces mortality by 15–33%. *Tertiary prevention* or *chemoprevention* utilizes specific pharmacological agents or nutrients to prevent, delay, or retard the development of cancer. Numerous agents (214 at last count [4]) have been extensively evaluated in the last two decades for their chemopreventive properties against colon cancer. Several clinical trials have been conducted, most notable among them those concerning calcium supplements and vitamin D [5], β-carotene [6], and fiber [7].

Although chemoprevention results have at times been modest, they have, nevertheless, helped the field mature and generated sophisticated expertise and refined methodologies for the evaluation of novel agents. In addition, approaches aimed at shortening the duration of clinical trails are emerging. Promising among them is the use of magnifying endoscopy to detect aberrant crypt foci as an end point in colon cancer prevention trials [8]. Most of the current challenge in cancer chemoprevention is to develop suitable agents and biomarkers both to monitor response to the agents and to identify those subgroups of at risk patients that will benefit the most from the agent.

2. NSAIDs prevent cancer: proof of principle

A new era in cancer prevention was ushered in by the landmark study by Kune et al. [1] showing that subjects using NSAIDs for various indications had a significantly lower incidence of colon cancer. Over thirty epidemiological studies, collectively describing results on >1 million subjects, that followed the original work have established NSAIDs as the prototypical chemopreventive agents against colorectal cancer. The epidemiological findings are in agreement with a considerable body of animal and in vitro data [9]. Three well-designed randomized, double-blind trials of aspirin as a chemopreventive agent

against colorectal adenomas established its chemopreventive effect [10,11]. In the first study, evaluating 81 mg or 325 mg of aspirin daily in patients with a recent history of adenomas, the relative risks of any adenoma (as compared with the placebo group) were 0.81 in the 81 mg group and 0.96 in the 325 mg group. For advanced neoplasms ($\geq$1 cm in diameter or with tubulovillous or villous features, severe dysplasia, or invasive cancer), the respective relative risks were 0.59 and 0.83. In the second study, patients with previous colorectal cancer received either 325 mg of aspirin per day or placebo. The adjusted relative risk of any recurrent adenoma in the aspirin group, as compared with the placebo group, was 0.65. Finally, daily aspirin 160 or 300 mg compared to placebo also reduced the risk of recurrent adenomas at 1 year postcolonoscopy (RR 0.73, 0.52–1.04) [12].

While this work provided a much-needed proof of principle, it also made it clear that aspirin may not be optimal for cancer prevention. This conclusion is inescapable, when these studies are viewed against the fundamental distinction between chemotherapy and chemoprevention. In chemotherapy substantial treatment-related toxicity is accepted because of the imminent risk that cancer poses on the patient's life. In contrast, chemoprevention agents are intended mostly for healthy subjects at risk for a cancer that they may never develop. In this case, criteria for safety and efficacy are stricter than for chemotherapy. It is because of shortcomings in safety and efficacy that aspirin is unlikely to be a practically useful chemoprevention agent. The issue of their safety is clearly illustrated by the report that in 1998 in the US NSAID-induced gastrointestinal complications and AIDS led to virtually equal numbers of deaths (~16,500) [13].

Cancer prevention by NSAIDs is, at least conceptually, largely based on their effects on the eicosanoid pathways. Thus, we review below salient features of these pathways that are relevant to cancer chemoprevention.

3. Linoleic and arachidonic acid metabolism: relevance to cancer

The development of COX-2 inhibitors is a direct outgrowth of extensive work on fatty acid metabolism and what is now known as the eicosanoid field. It is perhaps instructive to note that despite our vast knowledge in this area, there are still details being unraveled (some discussed here) that have direct and at times crucial pharmacological implications.

Polyunsaturated fatty acids such as arachidonic and linoleic acids, when metabolically oxidized by cytochrome P450, COXs and lipoxygenases (LOXs) form an array of biologically active compounds [14] (Fig. 1). Hydrolysis of membrane phospholipids by stimulated lipases produces free arachidonic acid, which serves as a substrate for cytochrome P450, COX and LOX enzymes.

3.1. COX cascade

In the presence of molecular oxygen, the COX (PGH_2 synthase) pathway, through the cyclooxygenase component of PGH_2 synthase, produces the unstable intermediate PGG_2, which is rapidly converted to PGH_2 by the peroxidase activity of PGH_2 synthase. Specific isomerases convert PGH_2 to various PGs and TxA_2. There are three isoforms of COX: (a) The constitutive COX-1 is involved in the maintenance of tissue homeostasis. It is expressed in most tissues and is responsible for platelet aggregation, renal blood flow and maintenance of the gastric mucosa. COX-1 is inhibited by traditional NSAIDs either reversibly or irreversibly, depending on the NSAID. (b) COX-2, identified as an inducible isoform in inflamed and neoplastic tissues, is expressed constitutively in human kidney and brain. Selective COX-2 inhibitors, designated as coxibs, inhibit its enzymatic activity. All NSAIDs are COX-2 inhibitors; however, coxibs are "COX-1 sparing drugs" [15]. (c) More recently, a third isoform, COX-3, has been identified [16]. COX-3 is present mainly in the brain and spinal cord; its product, PGD_2, mediates pain and fever; and its activity is inhibited by acetaminophen. Some consider COX-3 not a distinct entity but a COX-1 variant [17].

The physiological effects of PGs, PGI_2, and TxA_2 are mediated in part by G-protein-coupled prostanoid receptors. There are nine such receptors, eight of which (EP1-4, DP, FP, IP, and TP) are classified according to the prostanoid ligand that each binds with greatest affinity (reviewed in [18,19]). The ninth receptor, CRTH2 or DP2, is expressed on Th2 cells and binds PGD_2 [20]. The diverse effects of PGE_2 may result from its actions on four receptors, EP1, EP2, EP3 and EP4. The EP2 and EP4 (relaxant) receptors are G_s-type leading to cAMP stimulation; the EP1 (constrictor) receptor increases intracellular calcium; and EP3 (inhibitory) receptor is of the G_i-type. Activation of a given receptor may elicit varying responses in different cell types [21] and some of them may be quite important to cancer biology.

PGE_2 stimulation of EP4 (but not of EP2) leads to PI3-kinase-dependent phosphorylation of ERK1/2 [22]; PI 3-kinase/EP4 may also be involved in the β-catenin signaling pathway [23]; EP4 may modulate the PGE_2-stimulated proliferation of colon cancer cells [24]. PPARγ ligands inhibit human lung carcinoma cell growth by decreasing the expression of EP2 receptors through ERK signaling and PPARγ-dependent and -independent pathways [25]. IP, DR and CRTH2 receptors are also coupled with the activation of G_s and linked to increases in cAMP; FP and TP signal by increasing intracellular calcium [26].

3.2. LOX cascades

Leukotrienes (LTs), potent inflammatory mediators, are synthesized in many cells and act through specific receptors. LTB_4 acts by stimulating members of the G-protein-coupled

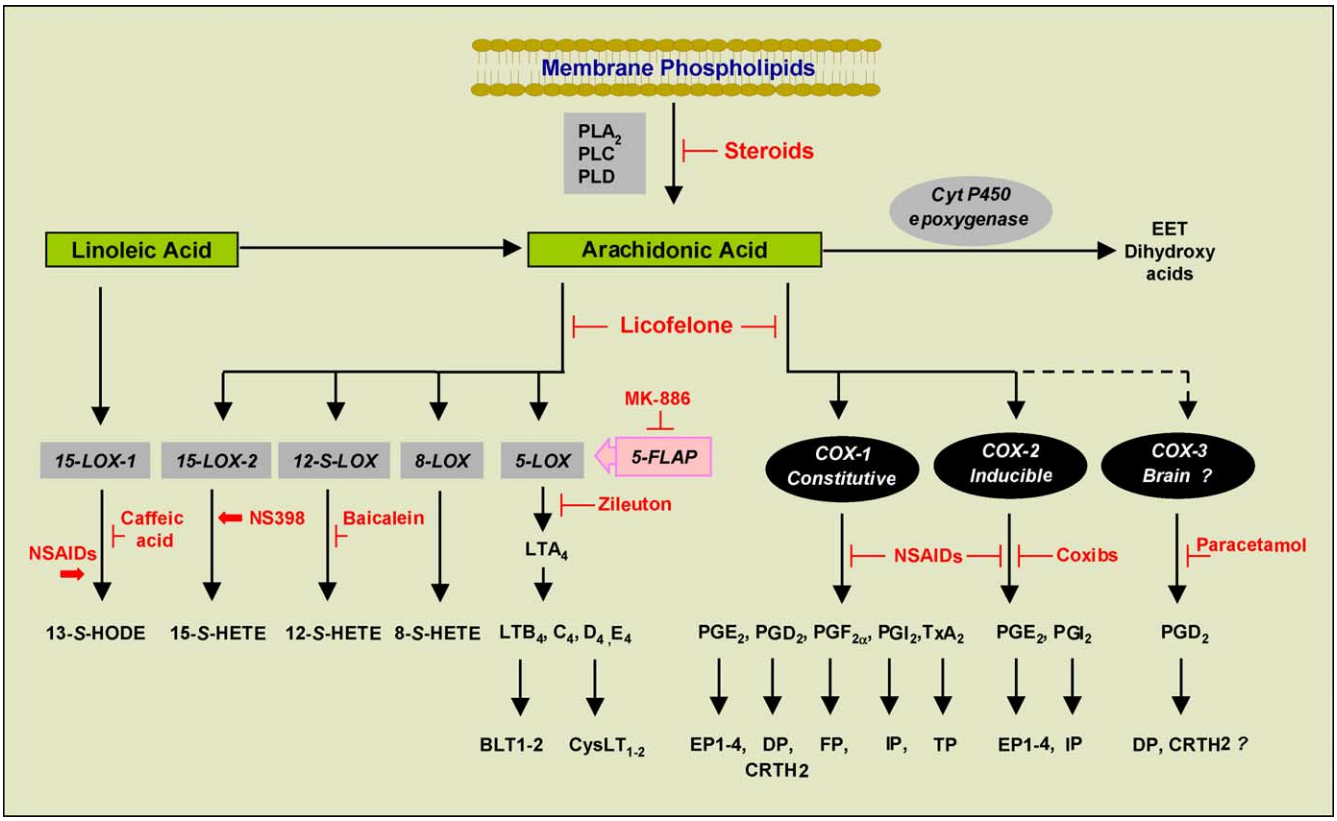

Fig. 1. Overview of the eicosanoid pathway. Arachidonic acid, the substrate of three major biosynthetic pathways, is derived from diet or synthesized from linoleic acid and is released from membrane phospholipids through a series of reactions requiring phospholipases. The COX pathway produces various prostaglandins and thromboxane; the LOX pathways produce leukotrienes and hydroxyeicosatetraenoic acids; and the cytochrome P450 pathways produce EET and dihydroxyacids. *Abbreviations:* phospholipases A_2, C, and D, PLA_2; PLC; PLD; prostaglandins (respective receptors), PGE_2 (EP1-4); $PGF_{2\alpha}$ (FP); PGD_2 (DP, CRTH2); prostacyclin, PGI_2 (IP); thromboxane A_2, TxA_2 (TP). Leukotrienes, LTA_4, LTB_4, LTC_4, LTD_4, LTE_4; LTB_4 receptors, BLT1-2; LTC_4, LTD_4, LTE_4 receptors, $CysLT_{1-2}$. 13-*S*-Hyroxyoctadecadienoic acid, 13-*S*-HODE; hydroxyeicosatetraenoic acid, HETE; Epoxyeicosatrienoic acid, EET. *T-shaped arrows:* inhibition. *Broken arrow*: putative pathway.

BLT receptors [reviewed in [27]. LTD_4, LTE_4 and LTC_4 activate receptors belonging to the CysLT family [28] (Fig. 1).

There are several LOXs; five are shown in Fig. 1. Metabolic products of the 5-, 12-, or 15-LOX biosynthetic pathways modulate the growth of several normal human cells, including T lymphocytes [29], skin fibroblasts [30], epidermal keratinocytes [31] and glomerular epithelial cells [32]. LOX metabolite levels are elevated in various cancers, including colon [33], breast [34,35], prostate [36], lung [37], and skin [38–40]. Some LOX products are procarcinogenic, while others are anticarcinogenic [40].

3.3. Procarcinogenic LOXs

Several LOXs form metabolites that enhance carcinogenesis. These LOXs and metabolites include 5-LOX and its products 5-*S*-HETE and LTB_4; 8-LOX and 8-*S*-HETE; and 12-*S*-LOX and 12-*S*-HETE.

3.3.1. 5-LOX and LTB_4

5-LOX represents an instructive example of both the enormous potential and the challenges that are encountered in trying to exploit pharmacologically this complex system [reviewed in detail in [40]]. As shown in Fig. 1, 5-LOX, which is activated by another enzyme FLAP (five LOX activating protein), converts arachidonic acid to 5-*S*-HETE, which in turn is converted to LTA_4 and then to LTB_4. Several lines of evidence indicate two relevant aspects of its tumor biology: First, 5-LOX, being overexpressed in several cancers (prostate, pancreatic colon, testicular and esophageal), may play a significant role in carcinogenesis, and second, manipulating the metabolic pathway catalyzed by 5-LOX impacts cancer cell kinetics, a finding suggesting a therapeutic or prevention opportunity.

In human prostate cancer, 5-LOX is clearly overexpressed [41]. Furthermore, in prostate cancer cell lines 5-*S*-HETE inhibits apoptosis [42] whereas MK886, a FLAP inhibitor, causes apoptosis of human prostate cancer cells by releasing mitochondrial cytochrome *c* [42]. In lung cancer cells, 5-LOX promotes their growth and 5-LOX inhibitors inhibit cell proliferation and induce apoptosis in many cancer cell lines [43]. In colon cancer, 5-LOX is involved in adenoma formation associated with cigarette smoke exposure [44]. Prompted by our findings of the effect of PGs on colon cancer cell kinetics [45], a decade ago we explored the

effects of LTB_4, LTB_4 methyl ester, LTB_5, 12-*R*-HETE, 12-*S*-HETE, and 15-*S*-HETE on the HT-29 and HCT-15 colon cancer cell lines. Only LTB_4 and 12-*R*-HETE stimulated their proliferation; a competitive antagonist of LTB_4, blocked its effects [46]. There is evidence of 5-LOX overexpression in pancreatic cancer: 5-LOX mRNA is expressed in pancreatic cancer but not in normal pancreatic cells [47]. 5-LOX expression was also high in human testicular cancer but very low in normal testes and 5-LOX inhibitors inhibited the growth of testicular cancer cells [48]. Finally, 5-LOX was expressed in 79% of esophageal cancers but only in 13% of normal esophageal mucosa [49]. 5-LOX was expressed in eight esophageal cancer cell lines, in which 5-LOX inhibitors induced apoptosis.

3.3.2. 8-LOX and 8-S-HETE

The enzymatic activity of 8-LOX increases in the early stages of mouse skin tumorigenesis [50] and its up-regulation promotes skin carcinogenesis [51]. The human 8-*LOX* gene has not been cloned; however, 8-*S*-HETE is genotoxic [50].

3.3.3. 12-S-LOX and 12-S-HETE

Platelet-type 12-*S*-LOX was detected in prostate, melanoma, and other cancer cell lines [52,53]. This LOX isozyme was also found in humans [50] and its expression in prostate cancer correlated with tumor grade and stage [54]. 12-*S*-HETE upregulates adhesion molecules [53] and promotes tumor spread by modulating protein kinase C [55–57]. 12-*S*-HETE also interacts with nuclear receptors such as PPARs and RXR [58]. Finally, 12-*S*-LOX promotes angiogenesis [59].

3.4. Anticarcinogenic LOXs

15-LOX-1 and 15-LOX-2 have anticarcinogenic effects. The preferred substrate for 15-LOX-1 is linoleic acid and for 15-LOX-2 is arachidonic acid [60].

3.4.1. 15-LOX-1 and 13-S-HODE

15-LOX-1 is the main enzyme for metabolizing linoleic acid into 13-*S*-HODE [61,62] and is the only 15-LOX isozyme found in the human colon epithelium [188]. 13-*S*-HODE is linked to cellular differentiation and apoptosis and 15-LOX-1 expression levels are reduced in human colorectal cancers [63]. 13-*S*-HODE induces apoptosis and cell cycle arrest in colorectal cancer cells [63] and, therefore, it is likely to have anticarcinogenic effects. This concept is supported by the observation that linoleic acid inhibits carcinogenesis in a mouse model of skin tumorigenesis [64]. NSAIDs induce 15-LOX-1 expression in colorectal cancer cells [65] and this induction of expression and apoptosis are independent of COX-2 inhibition [66]. 15-LOX-1 is also down-regulated in vitro and in vivo in human esophageal cancers, and NSAIDs induce 15-LOX-1 expression to promote apoptosis in human esophageal cancer cells [40].

3.4.2. 15-LOX-2 and 15-S-HETE

15-LOX-2 is expressed in some but not all normal human tissues (e.g., cornea, prostate, lung, and skin) [60]. 15-LOX-2 expression is reduced in human prostate carcinomas [67]. Reports regarding the role of 15-*S*-HETE in carcinogenesis are conflicting. Some studies suggest that 15-*S*-HETE might have antitumorigenic effects [68–70], while others suggest that it may suppress [71,72] or have no effect on apoptosis [46,73]. However, 15-*S*-HETE inhibits proliferation in PC3 prostate carcinoma cells possibly by activating PPARγ [74]. As recently reported, there is an inverse relationship between 15-LOX-2 and PPARγ gene expression in normal compared to cancer epithelial cells [75].

15-LOX-2 mRNA and protein were expressed in 76% of normal esophageal tissues and only in 46% of esophageal cancers [76]. Study of normal, premalignant, and malignant esophageal cell lines revealed that 15-LOX-2 was essentially non-detectable in the cancer cell lines. Transient transfection of 15-LOX-2 expression constructs into 15-LOX-2-negative esophageal cancer cells significantly inhibited their proliferation. Moreover, the COX-2 inhibitor, NS398, induced 15-LOX-2 expression in esophageal cancer cells [76].

3.5. Pharmacological agents

Modulation of the various LOX isozymes may be of therapeutic value. Such approaches to chemoprevention may include inhibition of 5-LOX, FLAP, and 12-*S*-LOX activities or the use of LT receptor antagonists; several such inhibitors have been developed [reviewed in [77]]. One potentially serious problem with this approach may arise from shuttling of substrate between pathways following inhibition of one of them. For example, once 5-LOX has been inhibited, there may be more substrate available for the COX-2 or LOX pathways that are procarcinogenic. Conversely, inhibition of the COX pathway by NSAIDs could increase LTs. This subtle but potentially critical interplay between the various eicosanoid pathways could decide the final result of interventions directed at single enzymes. Hence, one new approach is to develop agents that inhibit both 5-LOX and COX pathways. Licofelone, currently in clinical development for osteoarthritis, is a substrate analogue of arachidonic acid, which inhibits 5-LOX, COX-1, and COX-2 [78]. Recent data suggest that this approach prevents oral carcinogenesis at the post-initiation stage in a hamster model [79]. At present, there are no clinically useful drugs available to modulate the cytochrome P450 pathway.

4. The targets of NSAIDs: COX-2 dependence and independence

Since our original observation that, even in the absence of any COX enzyme, NSAIDs can modify cancer cell

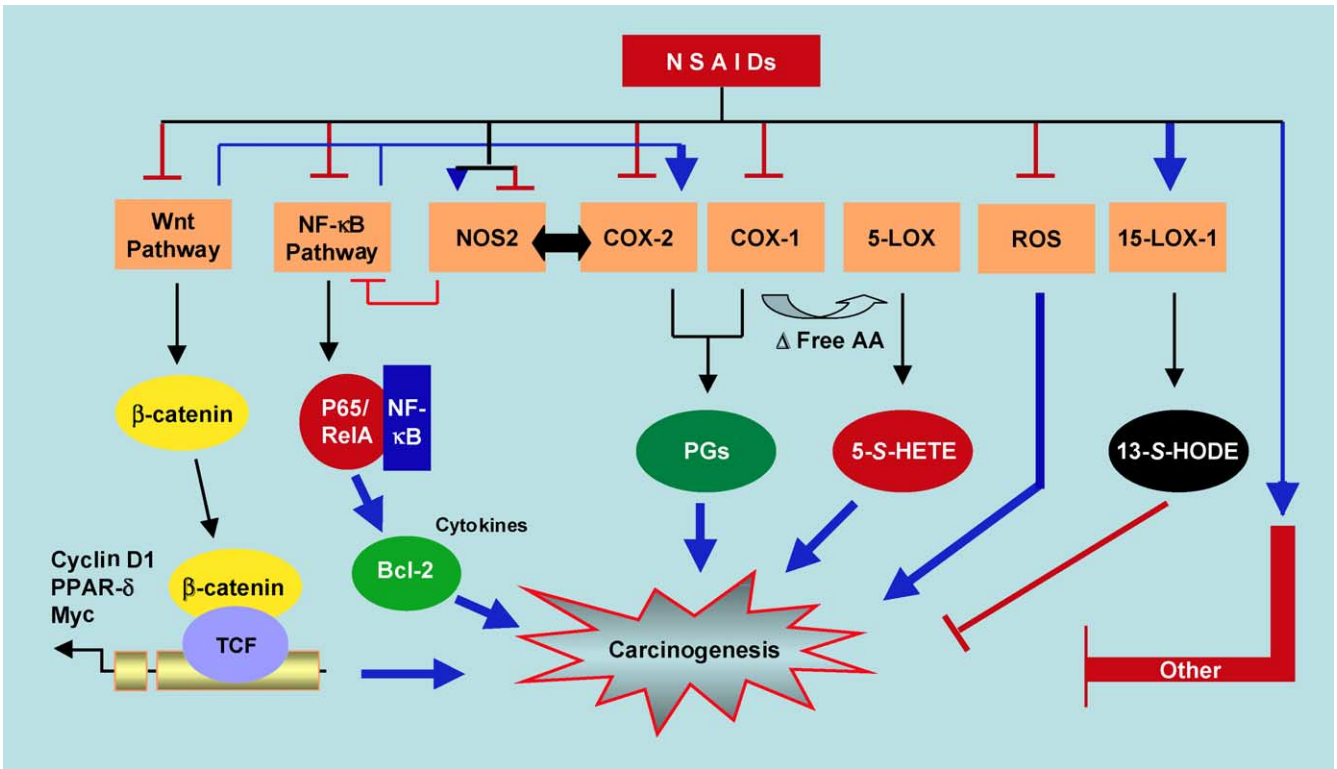

Fig. 2. The pleiotropic effect of NSAIDs on molecular targets. The various molecular targets affected by NSAIDs and their interactions are shown; all of them are potentially relevant to carcinogenesis. *Abbreviations:* NOS2, nitric oxide synthase 2 (inducible isoform); COX, cyclooxygenase; LOX, lipoxygenase; ROS, reactive oxygen species; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; PGs, prostaglandins; 13-*S*-HODE, 13-*S*-hyroxyoctadecadienoic acid; PPARδ peroxisome proliferator-activated receptor δ; TCF, T cell factor. *T-shaped arrows* indicate inhibition; *blue arrows* indicate induction; *all other arrows* indicate pathways.

kinetics in a way consistent with an anticancer effect [80], there has been a growing appreciation of the validity of this concept. The idea that NSAIDs actually have a variety of molecular targets (Fig. 2), not only provides a much-needed explanation of apparently disparate observations, it also underscores the opportunity that these targets represent for cancer prevention and perhaps treatment. Below, we summarize the evolution of current thinking in this area.

4.1. COX-2 inhibition as a dominant mechanism in cancer chemoprevention

That inhibition of COX was the best known biochemical effect of NSAIDs provided a plausible explanation of the epidemiological data on NSAIDs and cancer. Several early observations supported the notion that COX plays a central role in cancer. For example, human colon cancers have elevated PGE_2 levels compared to uninvolved mucosa [81,82]; COX-2 is overexpressed in 45% of colon adenomas and 85% of colon carcinomas [83], a change subsequently demonstrated for several human cancers [84]; and PGE_2 increases colon cancer cell proliferation [45] and suppresses apoptosis [85].

These and other data led to the conclusion that NSAIDs exert their effect by inhibiting COX and that inhibition of COX-2, the isoform overexpressed in cancer, would arrest carcinogenesis. The development of coxibs as selective COX-2 inhibitors, combined with the notion of the centrality of COX-2 in carcinogenesis provided the impetus for their extensive evaluation as chemoprevention agents.

Extensive preclinical data supported this approach. Multiple cell culture studies [reviewed in [86]] indicated the potential efficacy of coxibs against cancer. The many relevant animal studies used either genetically modified animals or COX-2 inhibitors. Oshima et al. showed that deletion of COX-2 decreased significantly the number of intestinal tumors in $Apc^{\Delta 716}$ mice [87]; *COX-1* deletion, however, also attenuated tumor formation in the same mice [88]. Overexpressing the human COX-2 gene in the mammary glands of transgenic mice produced focal mammary gland hyperplasia, dysplasia, and transformation into metastatic tumors [89]. These data indicate that enhanced COX-2 expression is sufficient to induce mammary gland tumorigenesis. Studies addressing whether COX-2 is critical for skin carcinogenesis are equivocal [90,91]. Numerous animal studies using specific COX-2 inhibitors provided support for the concept that COX-2 inhibition both prevents and regresses tumors arising from a variety of tissues, including colon, lung, breast, pancreas and skin [86]. This work and clinical trials [92] culminated in celecoxib receiving FDA approval for its use in patients with familial adenomatous polyposis (FAP).

4.2. The evidence for COX-2 independence

The COX-independent effects of NSAIDs come from several lines of evidence. For example, NSAIDs have antiproliferative and/or proapoptotic effects in cell lines that do not express either COX-1 or COX-2. Both sulindac sulfide and piroxicam induced apoptosis in the COX-2 expressing HT-29 human colon cancer cells and the COX-2 deficient HCT 15 cells. Treatment of HCT 15 cells with various PGs did not reverse the apoptotic effects in HCT 15 cells, further suggesting a COX-independent effect [80]. Similar results were also reported with a COX-2-selective inhibitor, NS-398, in HT-29 (COX-2 positive) and S/KS (COX-2-negative) human colon cancer cell lines [93]. Sulindac sulfide and sulindac sulfone also induced apoptosis in malignant melanoma cell lines independently of COX-2 expression [94]. Sulindac sulfone, which is an active metabolite of sulindac, lacks any COX-1 or COX-2 inhibitory activity. However, this compound inhibits chemical-induced colon carcinogenesis in rats without affecting PG levels [189]. It also induces apoptosis and inhibits the growth of human prostate cancers in a nude mouse xenograft model [95]. The selective COX-2 inhibitor, celecoxib, showed antiproliferative effects on both hematopoietic (BALL-1, COX-2-negative B-leukemia line) and epithelial (A549, COX-2-positive non-small cell lung carcinoma line) cancer cell lines. In addition, the COX-2-negative epithelial lines were found to have IC_{50}s for celecoxib that were very similar to their COX-2-positive counterparts [96]. It is also noteworthy that cells which were manipulated so that they would not express either COX-1 or COX-2 still were responsive to the antiproliferative effects of NSAIDs. Using tetracycline-inducible COX-2 antisense clones, the effect of COX-2 expression on cell viability and sensitivity to apoptosis induced by COX-2 inhibitors was evaluated [97]. COX-2 depletion did not induce apoptosis, whereas COX-2 inhibitors did induce apoptosis in the same cell line. Also, several celecoxib derivatives lacking any COX-2 inhibitory activity caused apoptosis in the androgen-independent prostate cancer cell line PC-3 [97].

Another line of evidence supporting the notion of COX-independent mechanisms comes from studies using both the *R*- and the *S*-enantiomers of ibuprofen. Both enantiomers are equally potent in inhibiting PDGF-induced mitogenesis of smooth-muscle cells; however, *R*-ibuprofen does not have any COX inhibitory activity [98]. Also, *R*-flurbiprofen, which does not inhibit COX, was shown to inhibit colon cancer development in the Min/+ mouse model [99] and it also inhibited prostate cancer progression in the TRAMP mouse [100].

4.3. Targets of NSAIDs other than COX

Over 10 targets (Table 1), in addition to COX, have been recognized as potentially mediating the effect of NSAIDs [101]. Both the strength of the evidence and their relevance to cancer prevention vary among them. Below, we review the most important of them, providing also a brief mechanistic background.

Table 1
Selected COX-independent targets of NSAIDs discussed in the text

Target	References
Cell proliferation	[187]
Induction of apoptosis	[186]
Cell cycle block	[146]
Cytochrome *c*	[163,165,167]
NAG-1	[134]
PDE2/5	[113,116]
NF-κB	[105–107]
PDK1	[121]
Akt	[120]
ERK1/2	[123]
RSK2/MAPKs	[122,124]
PPARγ	[130]
PPARδ	[132]
15-LOX-1	[40,65,66]
15-LOX-2	[76]
Wnt	[136]
Carbonic anhydrase	[172,174]
Cyclin D1	[113,148]
$p21^{cip1}$	[145]

4.3.1. NF-κB

NF-κB controls many genes and is apparently involved in many diseases, including cancer [reviewed in [102,103]]. When bound to IκB proteins, the NF-κB heterodimer is trapped in the cytoplasm. Phosphorylation of IκB in response to inducers such as cytokines, results in its degradation and activation of NF-κB. As a result, NF-κB translocates to the nucleus and binds to the NF-κB binding site in the regulatory region of target genes, thereby promoting the transcription of several genes including *COX-2, c-myc* and *cyclin D1*. Many reports demonstrate that members of the NF-κB and IκB families are involved in the development of cancer [104].

NF-κB is upregulated through chromosomal changes or constitutive activation in various hematological malignancies and in solid tumors, including pancreatic, breast, ovarian, colon and prostate cancer. NSAIDs modulate the NF-κB pathway. For example, aspirin inhibits the activation of NF-κB without interfering with gene transcription [105]. Prolonged treatment of colorectal cancer cells with aspirin decreases cytoplasmic IκB and thus increases translocation of NF-κB to the nucleus; such activation of the NF-κB pathway induced apoptosis in these cells [106]. Sulindac also inhibits activation of NF-κB [107,108].

4.3.2. Phosphodiesterases (PDs)

Phosphodiesterases regulate the levels of cAMP. Their importance to cancer pharmacology stems from findings

that high intracellular cAMP levels arrest the growth, induce apoptosis and attenuate cancer cell migration [109,110]. Agents like theophylline or cholera toxin, which increase intracellular cAMP trigger apoptosis in human cancer cells such as lung and ovarian cells alone or synergizing with chemotherapeutic agents [111,112].

As already mentioned, sulindac sulfone induces apoptosis by a mechanism that does not involve COX inhibition. Sulindac sulfone inhibits PDE2 and PDE5, increasing cellular concentrations of cGMP, leading to activation of cGMP-dependent protein kinase which in turn down regulates β-catenin, suggesting a mechanism for its apoptotic actions [113]. It is interesting that conventional selective PDE5 inhibitors such as sildenafil, which do increase intracellular cGMP levels and lead to inhibition of vascular smooth-muscle cell proliferation, do not induce apoptosis in cancer cells [114]. However, sildenafil induces apoptosis in chronic lymphocytic leukemia cells through a caspase-dependent mechanism [115]. More recently, it has been shown that suppression of PDE5 gene expression by antisense pZeoSV2/ASP5 plasmid transfection results in an increase in $[cGMP]_i$, growth inhibition, and apoptosis in human colon tumor HT-29 cells [116].

Sulindac sulfone also activates JNK1 (c-Jun N-terminal kinase 1) [117], a kinase which can phosphorylate and inactivate the anti-apoptotic proteins Bcl-2 and Bcl-X_L [118,119]. JNK1 can also increase the expression of proapoptotic proteins through activation of the transcription factor AP-1.

4.3.3. 3-Phosphoinositide-dependent kinase-1 (PDK-1)/Akt

The serine/threonine kinase, PDK-1, is activated by PI3K (phosphatidylinositol 3-kinase) which in turn activates Akt, a protein involved in cell proliferation. In human prostate cancer cells (LNCaP, PC-3), celecoxib induces apoptosis by blocking Akt activation independently of Bcl-2 [120], and in the HT-29 human colon cancer cell line, it induces apoptosis by inhibiting PDK-1 activity [121].

4.3.4. Ribosomal S6 kinase-2 (RSK-2)/MAPKs

This serine/threonine kinase which is activated by a MAPK (mitogen-activated protein kinase), can phosphorylate c-Fos and hence may be viewed as a regulator of immediate early gene transcription. NSAIDs inhibit RSK-2 activity by suppressing the phosphorylation of its substrate cAMP response element binding (CREB) protein [122]. The transcription factors c-Fos, c-Jun, Elk-1 and ATF-2 are substrates of MAPKs which comprise three signaling pathways: (a) ERKs, p42/p44 (extracellular); (b) JNKs (c-Jun N-terminal kinases); (c) p38 MAPKs. Some NSAIDs inhibit activation of ERKs [123], hence reducing cell growth; they also induce p38 MAPK [124] and JNK [125], which promote apoptosis.

4.3.5. Peroxisome proliferator-activated receptors (PPAR)

PPARs (α, β, and δ) function as heterodimers with the retinoic acid receptor (RXR), their obligate partner, and regulate transcription of genes involved in apoptosis, differentiation, and inflammation [126]. Higher PPARγ expression is observed in human non-small-cell lung cancer compared to normal tissue [127]. Troglitazone, a PPARγ ligand, increased PPARγ transcriptional activity in lung adenocarcinoma cells (A549) and inhibited their growth predominantly due to inhibition of cell proliferation [127]. Ligands of PPARγ also inhibit carcinogenesis in experimental animal models; for example, treatment of A549 tumor-bearing SCID mice with troglitazone inhibits primary tumor growth [128]. Cell culture and transgenic mice data suggest that PPARγ signaling may serve as a tumor promoter in the mammary gland [129].

Indomethacin binds and activates PPARγ; other NSAIDs, including ibuprofen, and flufenamic acid, are also PPARγ ligands [130]. In matched normal and tumor samples from the colon, PPARδ mRNA was up-regulated in colorectal carcinomas and was shown to be activated by PGI_2 [131]. This elevation of PPARδ in colorectal cancer cells was repressed by APC, an effect mediated by β-catenin/Tcf-4-responsive elements in the PPARδ promotor [132]. Sulindac disrupted the ability of PPARδ to bind its recognition sequences, suggesting that NSAIDs may in part inhibit tumorigenesis through inhibition of PPARδ [132]. Also, 13-S-HODE, a product of 15-LOX-1, down-regulates PPARδ to induce apoptosis in colorectal cancer cells [133].

4.3.6. Products of LOX pathways

They have been discussed earlier.

4.3.7. NSAID activated gene (NAG-1)

NSAID activated gene is a divergent member of the TGF-β family of genes [134]. The TGF-β superfamily of genes plays roles in adult and embryonic growth and development, in inflammation, and in repair including angiogenesis [135]. Multiple lines of evidence suggest that the TGF-β signaling pathway is a potent tumor suppressor of human colorectal carcinogenesis. NAG-1 is of interest because of its characteristics and relation to COX activity: NAG-1 has antitumorigenic and proapoptotic properties [134].

Some NSAIDs (aspirin, indomethacin, sulindac sulfide, and ibuprofen) regulate the expression of NAG-1. NAG-1 expression is up-regulated in human colorectal cancer cells by several NSAIDs that are known to have anti-tumorigenic and pro-apoptotic activities. The increase for NAG-1 mRNA induction was two-fold for ibuprofen and a maximum of 4.6-fold for sulindac sulfide. In addition, the ability of NSAIDs to increase the expression of NAG-1 was not restricted to colorectal carcinoma cells. The pro-apoptotic

NSAIDs that stimulate the expression of NAG-1 are also potent inhibitors of COX enzymes and it was observed that proapoptotic effects reported for COX inhibitors in cell culture were mediated, in part, by NAG-1 expression. Interestingly, in studies using HCT116 cells which were devoid of COX activity there was increased NAG-1 expression by NSAIDs. Furthermore, most COX-2 specific inhibitors were not effective at increasing NAG-1 expression in HCT-116 cells. This suggests a link between apoptosis and NAG-1 expression in such cells and provides a suitable explanation for COX-independent apoptotic effects of NSAIDs in cultured cells.

4.3.8. Wnt pathway

The Wnt pathway, important for normal organ development [reviewed in [136]], has been associated with carcinogenesis, most notably that of colorectal cancer; over 90% of such cancers have an activating mutation. Wnt binds to membrane receptors encoded by the Frizzled genes (FZD1–10). Its canonical pathway involves Wnt binding to FZD receptors, which leads to phosphorylation of the cytoplasmic protein Dishevelled (Dsh), which then binds to axin and causes dissociation of the APC/axin/GSK complex, accumulation of β-catenin and its subsequent translocation to the nucleus. There, β-catenin inactivates gene transcription, some of it (e.g., *c-Myc, cyclin D1*) relevant to cancer. The properties of this pathway and the fact that NSAIDs appear to inhibit the initial stages of the adenoma-carcinoma sequence, prompted studies of the effect of NSAIDs and COX-2 inhibitors on this pathway.

McEntee et al. provided one of the earliest indications that the regression of intestinal tumors in *Min* mice by sulindac was accompanied by changes in β-catenin expression [137]. Ahnen's group determined in human colon cancer cell lines the mechanism of β-catenin protein downregulation by apoptotic concentrations of the COX-inhibitory sulfide and the non-COX-inhibitory sulfone metabolites of sulindac. They demonstrated that sulindac downregulates β-catenin expression by both proteasome- and caspase-dependent mechanisms. Of note, such degradation of β-catenin is COX-independent [138]. A study of nuclear β-catenin expression in five patients with familial adenomatous polyposis treated with sulindac sulphide for 6 months revealed less nuclear β-catenin expression in adenomas compared to pretreatment adenomas of the same patients [139]. Furthermore, an in vitro study by the same authors revealed downregulation of cyclin D1. They considered the inhibition of Wnt signaling by sulindac as an explanation for the COX-2-independent mechanism of chemoprevention by NSAIDs.

Although the interaction of NSAIDs with Wnt signaling is well documented, its precise mechanism of action appears unclear and it may not be common to all of them. For example, aspirin and indomethacin down-regulate β-catenin/TCF signaling in colorectal cancer cells [140]. In this case, however, the reduced signaling activity of β-catenin in response to NSAIDs is a result of its enhanced phosphorylation. Indeed, inactivation of a phosphatase rather than stimulation of a kinase or interference with the ubiquitination machinery may be the cause of the stabilized phosphorylation [141].

Interestingly, the specific COX-2 inhibitor rofecoxib reduced intestinal and colonic polyps in $Apc^{\Delta 716}$ mice, as did sulindac. Polyps from either rofecoxib- or sulindac-treated mice had more membrane-bound β-catenin, but showed unchanged nuclear localization of this transcription factor [142].

We have recently identified an unusual yet powerful mechanism of inhibition of Wnt signaling by the nitric oxide-donating aspirin (NO-ASA). Studying three positional isomers of NO-ASA, a chemopreventive agent against colon cancer, we showed that their cell-growth inhibitory effect was accompanied by significant inhibition of β-catenin signaling. The IC_{50} values for β-catenin signaling were $<1\%$ of the corresponding values for cell growth inhibition, underscoring the potential importance of this effect for cancer prevention. Inhibition of this pathway occurs via steric hindrance of the formation of the β-catenin–Tcf complex in the nucleus and to a much lesser extent through cleavage of β-catenin by caspase 3 [143,144].

4.3.9. Cell cycle effects

Selective COX-1/2 and nonselective COX inhibitors modulate the cell cycle machinery at several sites, which may explain some of their antiproliferative/apoptotic effects. Using three colon cancer cell lines, which differ in their expression of COX-2 (HCT 15, COX-2-deficient; HT-29 and Caco-2, both COX-1 and -2 expressing), it was shown that celecoxib (selective COX-2 inhibitor) and SC560 (selective COX-1 inhibitor) induced a G_0/G_1 phase block and reduced cell survival independent of whether or not the cells expressed COX-2 [145]. In vivo, both celecoxib and SC560 reduced the proliferation of HCT 15 colon cancer xenografts in nude mice, but both agents had no significant effect on HT-29 tumors in this model [145]. Human colon carcinoma cells (Caco-2) transfected with the human COX-2 cDNA, in both sense and antisense orientation, to produce cells which either overexpressed COX-2, or expressed no COX-2, or expressed only a very small amount of COX-2, responded similarly to celecoxib, which reduced cell growth, accompanied by G_0/G_1 phase block and apoptosis [146]. The G_0/G_1 phase block correlated with a decrease in expression levels of cyclin A and cyclin B1 and increased expression of the cell cycle inhibitory proteins $p21^{Waf1}$ and $p27^{Kip1}$ irrespective of the type of cell used [146]. In human umbilical vein endothelial cells (HUVEC) the antiproliferative effects of celecoxib are due to inhibition of PDK-1/Akt signaling and cyclin-dependent kinases. Celecoxib and its COX-2-inactive analogue (4-[5-(2,5-dimethylphenyl)-3(trifluoro-

methyl)-1H-pyrazol-1-yl]benzenesulfonamide, DMC), mediated G_1 arrest in these cells, which was associated with reduced Rb phosphorylation through the inhibition of multiple cyclin-dependent kinases [147].

Sulindac and its sulfide and sulfone derivatives inhibited growth of the normal human mammary epithelial cell line MCF-10F and the human breast cancer cell line MCF-7 [148]. Sulindac sulfide also caused apoptosis and accumulation of cells in the G_1 phase of the cell cycle, which was associated with decreased cyclin D1 levels. Sulindac sulfide induced $p21^{WAF1}$ in MCF-10F cells; however, in the MCF-7 cell line the basal level of $p21^{WAF1}$ was very high, which did not change significantly after drug treatment. Sulindac sulfide, sulindac sulfone and its highly potent analog (CP248) inhibited growth of the human prostate epithelial cell lines BPH-1, LNCaP, and PC3 with induction of apoptosis [149]. Derivatives of LNCaP cells that stably overexpress bcl-2 remained sensitive to growth inhibition and induction of apoptosis by these compounds. Therefore, sulindac derivatives can cause growth inhibition and induce apoptosis in human prostate cancer cells by a COX-1 and -2 independent mechanisms, and this occurs irrespective of androgen sensitivity or increased expression of bcl-2.

Sulindac and sulindac sulfide reduced the proliferation rate of HT-29 colon adenocarcinoma cells, caused them to accumulate in the G_0/G_1 phase of the cell cycle, and reduced the level and activity of several cyclin-dependent kinases (cdks), which regulate cell cycle progression [150,151]. Aspirin caused necrosis of SW620 and HT-29 human colon adenocarcinoma cells and cell cycle arrest in the S and G_2/M phases of the cell cycle independent of prostaglandin production [152,153].

Using the specific COX-2 inhibitor NS-398 and a COX-isoform-specific RNA interference (RNAi), Denkert et al. have shown that in the human ovarian carcinoma cell lines OVCAR-3 (expresses COX-1 constitutively and COX-2 inducibly) and SKOV-3 (COX null) NS398 reduced cell proliferation by induction of G_0/G_1 cell cycle arrest independently of COX-2 inhibition [154]. Using the human prostate cancer cell lines PC3 and LNCaP (both expressing COX-1 but not COX-2), celecoxib at clinically relevant doses inhibited cell growth, which was accompanied by G_1 cell cycle block, reduction in cyclin D1 levels, and reduced DNA synthesis [155]. Interestingly, celecoxib inhibited PC3 xenograft growth without reducing intratumoral PGE_2 levels.

4.3.10. Inhibition of angiogenesis

Angiogenesis, the formation of new capillary blood vessels, is essential for the growth and metastasis of solid tumors. This occurs when endothelial cells respond to the angiogenic growth factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [156]. Both COX-1 and COX-2 are important for the regulation of angiogenesis and both COX-1 and COX-2 selective NSAIDs inhibit angiogenesis. The mechanism for this regulation appears to be through direct effects on endothelial cells involving inhibition of mitogen-activated protein kinase (ERK2) activity, interference with ERK nuclear translocation, is independent of protein kinase C, and has prostaglandin-dependent and prostaglandin-independent components [157,158].

4.3.11. Ca^{2+} mobilization

In the PC-3 human prostate cancer cell line, celecoxib blocks endoplasmic reticulum Ca^{2+}-ATPases causing inhibition of Ca^{2+} reuptake. This results in Ca^{2+} mobilization from endoplasmic reticulum stores followed by capacitative calcium entry, leading to $[Ca^{2+}]_i$ elevation. This Ca^{2+}-ATPase inhibitory activity is highly specific for celecoxib, and is not noted with other COX inhibitors, including aspirin, ibuprofen, naproxen, rofecoxib (Vioxx), DuP697 and NS398. This activity is also observed in other cell lines, including A7r5 smooth muscle cells, NIH 3T3 fibroblast cells and Jurkat T cells [159].

4.3.12. Cytochrome c release

Apoptotic signals can cause the mitochondria to release cytochrome *c*, procaspase 9, and apoptosis inducing factor (AIF), all of which induce cell death [160]. The Bcl-2 family of proteins, Bax, Bak and Bid, can regulate formation of pores in the mitochondrial outer membrane releasing apoptogenic factors [161,162]. Aspirin induces apoptosis through mitochondrial cytochrome *c* release which then interacts with Apaf-1 to activate caspase proteases [163,164]. Ibuprofen also causes apoptosis through a mechanism involving cytochrome *c* release [165]. Indomethacin also caused apoptosis in two non-small cell lung cancer cell lines MOR-P (expresses COX-2) and H-460 (does not express either COX isoforms) by releasing cytochrome *c* and apoptotic inducing factor and caspase activation [166]. The COX-2-selective inhibitor, NS398, induced apoptosis in a number of colon cancer cell lines, including HT-29 (COX positive), HCT 15 (COX negative) and SW480 (COX-2 negative) by releasing cytochrome *c* from mitochondria, leading to the activation of caspase-9 and caspase-3 [167]. Celecoxib also caused apoptosis in Jurkat and BJAB cells involving cytochrome *c* release and activation of caspases-9, -8, and -3 [168].

4.3.13. Inhibition of carbonic anhydrase

Carbonic anhydrases are a group of zinc-containing metalloenzymes that catalyze the hydration of CO_2 to bicarbonate at physiological pH. Virtually ubiquitous in living systems, they facilitate many biosynthetic processes including the synthesis of nucleotides [169]. Carbonic anhydrase expression has been studied most extensively in colorectal tumors and to a lesser extent in hepatobiliary, pancreatic, esophageal, gastric and other tumors. It appears quite likely that some carbonic anhydrase isozymes play a role in carcinogenic processes such

as uncontrolled cell proliferation and malignant cell invasion [reviewed in [170]].

PGE_1, PGE_2 and PGI_2 inhibit carbonic anhydrase (CA) in vitro and in vivo where as $PGF_{2\alpha}$, TXA2, and leukotriens LTB_4 and LTC_4 increase the activity of the enzyme [171,172]. The NSAIDs, aspirin, indomethacin, naproxen, piroxicam and probenecid activated CA I and CA II isozymes in a dose-dependent manner [172].

5-LOX and 12-LOX are upregulated in human pancreatic cancer cells and their inhibition reduces pancreatic cancer cell growth. The general LOX inhibitor NDGA, the 5-LOX inhibitor Rev5901, and the 12-LOX inhibitor baicalein all induced apoptosis in human pancreatic cancer cell lines (PANC-1, MiaPaca2, Capan2, and HPAF), which was accompanied by increased intracellular carbonic anhydrase activity [47]. CA IX expression, which is considered an endogenous marker for tumor hypoxia, was present in 72% of patients with early-stage non-small-cell lung cancer and was associated with tumor necrosis [173]. CA IX-positive tumor areas showed greater cell proliferation as measured by Ki-67 index. Also, the percentage of CA IX-positive tumor cells was significantly related to postoperative recurrence and poor disease-free survival.

Recent studies have also shown celecoxib and valdecoxib to be inhibitors of CA I, II, IV, and IX whereas rofecoxib is not [174]. This may be due to the fact that celecoxib and valdecoxib have an unsubstituted arylsulfonamide moiety which is common to many CA inhibitors and rofecoxib contains a methyl sulfone constituent.

4.4. The issue of drug concentrations and their relevance to targets

A recurring issue in much of biology, including cancer biology, is whether results obtained from cell culture studies using pharmacological agents are directly translatable to humans. A corollary to this is the tendency to ''accept'' only those results that have been obtained with in vitro concentrations that are within the range of blood or serum concentrations obtained in experimental animals or humans. Such reservations have been applied to the study of the molecular targets of NSAIDs as they pertain to cancer. In fact, some investigators have voiced concerns that the non-COX targets affected by NSAIDs are only seen when ''industrial strength'' concentrations of NSAIDs are used [175].

This type of thinking is fraught with the same risks as the uncritical extrapolation to humans of any cell culture results. There are three important (and fundamental) aspects that are often forgotten and thus worth reiterating. First, under no circumstances cell culture or animal data may be extrapolated to the human. We need only inspect the lengthy lists of potential chemopreventive agents that failed when tested in humans [4]. Second, in using cell culture systems, it is often necessary to use high concentrations of an agent because these experiments, by their very nature, are time sensitive. Most studies using cell culture systems have to be terminated by 72–96 h. However, in vivo studies using lower doses can go on for much longer periods of time. So, ''low dose-long duration'' or ''high dose-short duration'' is a balance that needs to be appreciated more. Finally, serum concentrations, an all too common yardstick, do not necessarily reflect actual tissue levels. For example, sulindac sulfide concentrations appear to be 20-fold higher in colonic epithelial cells of guinea pigs compared to those in the serum [176]. Thus, the argument about ''industrial strength'' concentrations represents a misguided form of reasoning and should not be used to accept or reject potentially important findings. It is their ultimate validation in humans that counts—and this is the only (and toughest) criterion we should apply, if progress is to be made.

5. COX-2 specific inhibitors: the limitations of the concept and the agents

The development of COX-2 inhibitors stems from the discovery of COX isoforms. The inducible COX-2 accounts for the generation of PGs at sites of inflammation where COX-2 is overexpressed. The hypothesis that selective inhibition of COX-2 might have therapeutic actions similar to those of NSAIDs, but without their side effects, was the rationale for the development of its selective inhibitors. Selective COX-2 inhibitors include the sulphonamides celecoxib and valdecoxib, the methylsulphones rofecoxib and etoricoxib, and the phenylacetic acid derivative lumiracoxib [177].

Two key observations led to the study of COX-2 inhibitors as chemopreventive agents against colon cancer. First, PGE_2 levels are elevated in colon cancer compared to normal mucosa [82,178]. Second, COX-2 is overexpressed in 45% of colon adenomas and 85% of colon carcinomas [83]; overexpression of COX-2 has been demonstrated for several human cancers [86]. Since NSAIDs protect against cancer and NSAIDs inhibit COX, a convincing case was made to develop COX-2 inhibitors as chemopreventive agents. Abundant preclinical data strongly supported this notion. Implicit in this effort has been the assumption that the overexpression of COX-2 in neoplastic tissues plays a dominant role in carcinogenesis.

To date there are only limited results available on the performance of COX-2 inhibitors either as chemopreventive or as adjuvant chemotherapeutic agents in humans. The most thorough are those on patients with FAP in whom COX-2 inhibitors had a modest effect. When celecoxib (400 mg orally twice a day) was used in FAP patients, the number of colon polyps was reduced by only 28% [92]. A similar effect was obtained on duodenal polyps in FAP patients [179]. In contrast, sulindac (150 mg orally twice a day) was more effective in FAP patients, reducing the

number of polyps by 56% [180]. One of several possible explanations of these findings is that COX-2 overexpression is not a dominant event in colon carcinogenesis and hence its inhibition by celecoxib has only a modest effect. Furthermore, the possibility that celecoxib inhibited targets other than COX-2 may have contributed to this (modest) effect, further detracts from the validity of the notion that COX-2 is a sufficient target for cancer prevention. It is now clear that COX-2 inhibitors act on cancer related pathways through targets other than COX-2 (Table 1).

The combination of COX-2 inhibitors with chemotherapeutic agents has been disappointing. For example, celecoxib plus trastuzumab failed to have an effect in patients with HER2/neu-overexpressing, trastuzumab-refractory metastatic breast cancer [181]. Preclinical studies had linked HER-2/neu overexpression and COX-2 activity. Similarly, rofecoxib combined with chemotherapy showed increased toxicity and no efficacy in metastatic colon cancer [182].

These results also challenge the notion that COX-2 is central to carcinogenesis. It is indeed conceivable that COX-2 expression is *the result* of and not a dominant contributor to carcinogenesis. The strongest argument against this interpretation is the demonstration that disruption of the COX-2 gene reduces the colon tumors in mice [87]. A counterargument to this is the recently reported study in which overexpression of human microsomal PGE synthase-1 in the alveolar type II cells, accompanied by highly elevated PGE_2 production, was not sufficient to induce lung tumors [183]. Of note, *COX-1* disruption in mice has the same effect on colon tumors formation as disruption of *COX-2* [87].

Although there *COX-2* specific inhibitors may be just preferential COX-2 inhibitors or COX-1 sparing inhibitors, the COX-2 effect may account for the cardiovascular toxicity of rofecoxib. While assessing the efficacy of rofecoxib in patients at risk for colon cancer, 3.5% of rofecoxib-treated versus 1.9% of placebo-treated subjects suffered myocardial infarctions or strokes, prompting its withdrawal from the market. Valdecoxib has also been withdrawn. Concerns for the safety of all COX-2 inhibitors have been expressed [2], in view of evidence for increased cardiovascular risk [184]. Since COX-2 is the principal enzyme involved in the synthesis of PGI_2, inhibition by COX-2 inhibitors could tip the balance toward platelet aggregation and vasoconstriction, thus increasing cardiovascular risk [reviewed in [14]].

6. Expanding the repertoire of chemoprevention targets

It is now apparent that in thinking about cancer chemoprevention we should consider two firmly established facts: (a) NSAIDs prevent various human cancers, and (b) NSAIDs act on multiple molecular targets, of which COX-2 is only one. In addition, the notion that COX-2 overexpression is a dominant event in carcinogenesis remains to be proven [14]. Current data suggest that COX-2 may be one of many players in the neoplastic process and, moreover, one that does not participate in its initial stages.

Whereas the efficacy of COX-2 inhibitors in cancer prevention is not yet established, it seems likely that either an alternative or a complementary approach may be needed. This is underscored by the fact that available results with COX-2 inhibitors show only a partial [sometimes marginal [185]] effect in cancer prevention and no effect as adjuvant agents. An additional concern is their safety, especially after their long-term administration, which will be the case in cancer chemoprevention. If no compelling results emerge from the ongoing studies with COX-2 inhibitors, we should reorient our approach to a mechanism-driven drug development that will be centered on the many non-COX-2 molecular targets. Whether COX-2 should be part of exploitable targets will depend on the availability of safe COX-2 inhibitors; on the results of ongoing cancer prevention trials of COX-2 inhibitors; and on the emerging appreciation of the interactions between the various eicosanoid pathways when one of them is inhibited.

On a highly positive note, the work on COX-2 and its relationship to cancer has had a significant impact on the field of cancer prevention. The vigorous pursuit of this mechanism-based approach by academic investigators, the NIH and industry has shown the way to rapidly evaluate chemoprevention agents. Also the wealth of basic science data that have been generated in the process will be most useful in proceeding to the next phase, which will likely include combinations of agents. After all, cancer has been a recalcitrant problem whose solution has eluded medical science for centuries.

7. Future prospects

Despite current difficulties stemming form apparent limitations of coxibs, there is sufficient reason to believe that the future of cancer chemoprevention is indeed very bright. That several cancers can be prevented by the administration of pharmacological agents is essentially certain. Developing the appropriate pharmacological agents and identifying biomarkers that will aid in both monitoring response and selecting the best candidates for chemoprevention should be accomplished in the immediate future.

We believe that what may prove a critical strategic choice in this effort is to expand the scientific inquiry into the several molecular targets that have been identified as potentially mediating the chemopreventive effect of NSAIDs, an effect proven in humans. The methodology to assess the chemopreventive relevance of targets is in

place and innovative approaches should lead to their rapid ''sorting out''. The same applies to pharmacological or natural agents and biomarkers.

The evident ''biochemical promiscuity'' of NSAIDs, if proven relevant, may be of great value to cancer prevention, as it may dictate the rational selection of agents or their most productive combination. Such varied effects on the neoplastic cell may be at the heart of a compound's ability to prevent or even treat cancer. The evolution of the neoplastic cell represents the progressive accumulation of mutations that alter its phenotype. Thus, it is likely that a compound affecting many pathways has a better chance of arresting the carcinogenic process than one that affects a single pathway. It should not be forgotten that in real life subjects achieve less-than-perfect compliance (epidemiological studies likely reflect such imperfection). Therefore, if the chemopreventive agent can block only a single pathway, it would be easier for a resistant phenotype to escape unscathed. Thus, given the low probability of developing a ''magic bullet'', one is forced to predict that a combination of agents will be the most likely winner in the race to prevent cancer.

Acknowledgements

Grant support: NIH R01-CA92423; R01-CA34527; and the Emmanuel Foundation.

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