Cyclooxygenase inhibitors: From pharmacology to clinical read-outs☆

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Highlights

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit prostanoid biosynthesis.

They comprise traditional (t) NSAIDs and selective inhibitors of COX-2 (coxibs).

They are classified by assessing their impact on biomarkers of COX-1 and COX-2 activities.

Use of these biomarkers allows a mechanistic interpretation of their clinical outcomes.

Abstract

Acetylsalicylic acid (aspirin) is a prototypic cyclooxygenase (COX) inhibitor. It was synthesized serendipitously from a natural compound, i.e., salicylic acid, with known analgesic activity. This chemical modification, obtained for the first time in an industrial environment in 1897, endowed aspirin with the unique capacity of acetylating and inactivating permanently COX-isozymes. Traditional nonsteroidal anti-inflammatory drugs (tNSAIDs) were developed to mimic the pharmacological effects of aspirin, using aspirin-sensitive experimental models of pain and inflammation as the template for screening new chemical entities. Among the tNSAIDs, some were endowed with moderate COX- selectivity (e.g., diclofenac), but no studies of sufficient size and duration were performed to show any clinically relevant difference between different members of the class. Similarly, no serious attempts were made to unravel the mechanisms involved in the shared therapeutic and toxic effects of tNSAIDs until the discovery of COX-2. This led to characterizing their main therapeutic effects as being COX-2-dependent and their gastrointestinal (GI) toxicity as being COX-1-dependent, and provided a rationale for developing a new class of selective COX-2 inhibitors, the coxibs. This review will discuss the clinical pharmacology of tNSAIDs and coxibs, and the clinical read-outs of COX-isozyme inhibition. This article is part of a Special Issue entitled "Oxygenated metabolism of PUFA: analysis and biological relevance."

Abbreviations

2-AG, 2-arachidonoyl-glycerol;5′UTR,untranslated region;AP-1, activatorprotein1;AA, arachidonic acid; AEA, arachidonoyl-ethanolamide; ARE,AU-rich element; C/EBP, CCAAT-enhancer-binding protein; CRC, colorectal cancer; CREs, cAMP-response elements; COX, cyclooxygenase; c, cytosolic; PGES, PGE synthase; GI, gastrointestinal; GPCRs, G-protein coupled receptors; HuR, human antigen R; KD, knock-down; KO, knock-out; miRNAs, microRNAs; NF-IL6, nuclear factor for interleukin-6; NF-κB, nuclear factor κB; OA, osteoarthritis; OTC, over-the-counter; PD,

pharmacodynamic; PK, pharmacokinetic; PL, phospholipases; PGI2, prostacyclin; PG, prostaglandin; RCTs, randomized clinical trials; RA, rheumatoid arthritis; TCF4, transcription factor 4; TX, thromboxane; tNSAIDs, traditional nonsteroidal anti-inflammatory drugs; UGIB, upper GI bleeding

Keywords

NSAID

Coxib

COX-1

COX-2

Aspirin

1. Introduction

Acetylsalicylic acid (aspirin) (Fig. 1) is a prototypic cyclooxygenase (COX) inhibitor. It was synthesized serendipitously from a natural compound, i.e., salicylic acid, with known analgesic activity. This chemical modification, obtained for the first time in an industrial environment in 1897, endowed aspirin with the unique capacity of acetylating and inactivating permanently COX-isozymes [1]. Traditional nonsteroidal anti-inflammatory drugs (tNSAIDs) were developed to mimic the pharmacological effects of aspirin, using aspirin-sensitive experimental models of pain and inflammation as the template for screening new chemical entities. The chemical structures of major NSAIDs are shown in Fig. 1. Among the tNSAIDs, some were endowed with moderate COX-2 selectivity (e.g., diclofenac), but no studies of sufficient size and duration were performed to show any clinically relevant difference between different members of the class [2]. Similarly, no serious attempts were made to unravel the mechanisms involved in the shared therapeutic and toxic effects of tNSAIDs until the discovery of COX-2 [3]. This led to characterizing their main therapeutic effects as being COX-2-dependent and their gastrointestinal (GI) toxicity as being COX-1-dependent, and provided a rationale for developing a new class of selective COX-2 inhibitors, the coxibs [3].

Except for a well known effect of NSAIDs (including coxibs) on blood pressure control, no cardiovascular safety issue had been raised until 1999, when the COX-2-dependence of prostacyclin (PGI2) biosynthesis in humans was first reported [4]. Until then, practicing physicians had not been concerned about any potential increase in cardiovascular risk associated with tNSAIDs because it was widely assumed that these drugs would mimic the antithrombotic effect of aspirin [5] through a COX-1-dependent antiplatelet effect.

At the same time, there was some interesting observational evidence that both aspirin and tNSAIDs might protect against some cancers, particularly of the lower GI tract [6].

All we knew about the safety of tNSAIDs was largely derived from observational studies that perhaps overemphasized the burden of GI toxicity of these agents [7]. The real turning point of our understanding of NSAID safety was represented by: i) the large GI outcome trials of coxibs in the post-marketing phase of their development [8], [9]; ii) the long-term, placebo-controlled trials of celecoxib [10] and rofecoxib [11] for chemoprevention of sporadic colorectal adenoma recurrence; and iii) meta-analyses of all the randomized coxib and tNSAID trials [12], [13].

2. Mechanism of action

NSAIDs act by inhibiting the biosynthesis of prostanoids [14], a family of bioactive lipids [i.e. prostaglandin (PG)E2, PGF2α, PGD2, PGI2 and thromboxane (TX)A2], which interact with specific cell-membrane receptors of the superfamily of G-protein coupled receptors (GPCRs), and play important roles in many cellular responses and pathophysiologic processes, such as modulation of the inflammatory reaction and its resolution, erosion of cartilage and juxtaarticular bone, GI cytoprotection and ulceration, angiogenesis and cancer, hemostasis and thrombosis, renal hemodynamics and progression of kidney disease, atheroprotection and progression of atherosclerosis [15], [16].

Prostanoids are autacoids which act in an autocrine and paracrine manner [15]. Their functional responses are orchestrated by the level and type of the specific prostanoid receptor(s) expressed on cells/tissues [17], [18], [19]. The prostanoid receptor family consists of eight rhodopsin-like (class A) GPCRs each being the product of an individual gene: DP1 (for PGD2), EP1, EP2, EP3 and EP4 (for PGE2), FP (for PGF2α), IP (for PGI2) and TP (for TXA2). DP1, EP2, EP4 and IP receptors are classically associated with elevation of intracellular cyclic adenosine monophosphate (cAMP) levels through activation of Gs proteins; EP1, FP and TP receptors induce elevation of intracellular calcium through Gq, and EP3 causes the reduction of intracellular cAMP levels through Gi. Moreover, splice variants have been identified for EP1, EP3 and TP which couple with different G proteins [17], [20]. A ninth prostanoid receptor named CRTH2 or DP2, belonging to the GPCR family A, was also

identified [21]. PGD2 is its natural ligand but the receptor shows higher sequence homology with other leukocyte chemoattractant receptors than prostanoid receptors [22]. It is coupled via pertussis-toxin sensitive Gαi/o to reduction in intracellular cAMP and calcium mobilization presumably via G-βγ subunits [22], [23].

Prostanoids are generated by the enzymes PGG/H synthase-1 and -2 (also known as COX-1 and COX-2) which are homodimers of 576 and 581 amino acids, respectively [24]. Each subunit of the dimer contains the cyclooxygenase (COX) and peroxidase active sites. Thus, the two isozymes share the same activities and catalyze the rate-limiting step of prostanoid biosynthesis, i.e., the production of PGH2 from arachidonic acid (AA; 20:4, n− 6). AA is released from membrane phospholipids by phospholipases (PL), mainly cytosolic (c) PLA2 upon cellular activation [24]. PGH2 is then transformed to prostanoids by the activity of different synthases. The generation of PGE2 is catalyzed by three different synthases: a cytosolic PGE synthase (cPGES) and two membrane-bound PGESs, i.e., mPGES-1 and mPGES-2 [25]. Whereas cPGES and mPGES-2 are constitutive enzymes, mPGES-1 is encoded by an inducible gene. It is thought that the coordinated expression of COX-2 and mPGES-1 is responsible for enhanced biosynthesis of PGE2 which occurs in inflammation and cancer [25]. The biosynthesis of PGD2 is regulated by the activity of two PGD synthases, lipocalin (L-PGDS) and hematopoietic (H-PGDS) [26] that are quite different from each other in terms of biochemical features, tissue distribution and functional relevance. Finally, the biosynthesis of TXA2 and PGI2 (prostacyclin) involves the activity of TX-synthase (TXS) and PGI-synthase (PGIS), respectively [15], [16].

Although COX-1 and COX-2 share the same catalytic activities and generate the same product, each isozyme subserves different biological functions [14]. This is due to several differences in the biology of COX-isozymes, such as the regulation of gene expression, the stability of transcripts and proteins, the requirement of different levels of hydroperoxides and AA to initiate COX catalysis and different coupling with downstream synthases [14], [24], [27], [28].

The role of COX-1 is to maintain a basal rate of prostanoid biosynthesis in the body and to allow a rapid, but short-lasting increase in the generation of prostanoids when the levels of free AA are transiently increased [14]. Two examples of COX-1-dependent AA metabolism are the constitutive synthesis of PGE2 by the GI tract to maintain GI homeostasis, and enhanced generation of TXA2 by activated platelets involved in primary hemostasis and atherothrombosis [14], [29]. In contrast, COX-2 induction plays a role in enhanced generation of prostanoids in the presence of low levels of free AA, such as in response to inflammatory stimuli and growth factors [27]. Constitutively expressed COX-2 contributes to the continuous generation of vasoprotective PGI2 in endothelial cells (characterized by a low hydroperoxide tone) [30] constantly exposed to mechanical forces resulting from steady physiological blood flow [14], [31].

The characterization of the functional roles of COX-2 in vivo was obtained in experimental models using genetically manipulated mice, such as COX-2 knock-out (KO), conditional COX-2 KO causing tissue-specific inactivation of the COX-2 gene, and COX-2 knock-down (KD) associated with partially reduced expression of COX-2 mimicking pharmacological inhibition of COX-2 [32], [33], [34], [35]. The roles of COX-2 inhuman health and disease were substantiated by the clinical findings of coxib trials demonstrating their efficacy in reducing pain and inflammation [3] and in preventing the recurrence of sporadic colorectal adenomas [10], [11], [36]. These therapeutic effects of coxibs were associated with reduced GI toxicity as compared to tNSAIDs which affect COX-1-dependent prostanoid production in the GI tract [29]. However, the reduced biosynthesis of endothelial COX-2-dependent PGI2 by coxibs and some tNSAIDs was associated with a modest but consistent increase in cardiovascular risk [31], [37].

It has been shown that there is a marked variability in the therapeutic and adverse effects of tNSAIDs and coxibs [37], [38]. This is associated with intra- and inter-subject variability in the extent of COX-isozyme inhibition and COX-2 selectivity achieved at therapeutic doses of these drugs [29], [39].

Sources of variability are related not only to pharmacokinetic (PK)/pharmacodynamic (PD) properties of the drugs, but also to factors within an individual that modulate drug response, such as her/his genetic background [39].

More recent work has uncovered novel functions of COX-2. In fact, COX-2 may oxygenate neutral AA derivatives, such as the endocannabinoids 2-arachidonoyl-glycerol (2-AG) and arachidonoyl-ethanolamide (AEA), leading to the formation of metabolites with a unique repertoire of physiological activities mediated by orphan GPCRs, heterodimers of eicosanoid receptors and their splice variants, or nuclear lipid receptors. Moreover, COX-2 may play a role in modulating endocannabinoid tone [40].

Endocannabinoids activate cannabinoid receptors to serve a variety of regulatory functions, some of which are now beginning to be understood. They act near their site of synthesis and their concentrations are rapidly reduced by transport and subsequent enzymatic hydrolysis [40]. It has been reported that members of the profen family of NSAIDs (i.e. propionic acid-derivatives) potently inhibit COX-2-mediated oxygenation and inactivation of endocannabinoids [41]. Ibuprofen and flurbiprofen are constituted of (S) and (R) enantiomers (racemate). (S) enantiomers, but not (R) enantiomers, inhibit AA oxygenation by COX-isozymes via freely reversible binding to the COX active site. In contrast, the (R) enantiomers decrease the levels of endocannabinoid oxygenation by COX-2 and this effect was associated with increased levels of 2-AG and AEA [41]. Whether these effects can be obtained in vivo after dosing in humans at therapeutic doses of NSAIDs requires further investigation. Some findings suggest a limited contribution of the endocannabinoid pathway to the clinical efficacy of racemate ibuprofen in vivo, due to unidirectional metabolic chiral inversion of the R-enantiomer to the S-form [42]. This reaction requires the formation of R-ibuprofenoyl-CoA thioester catalyzed by the acyl-CoA synthetase (an enzyme expressed in several isoforms and showing broad tissue distribution); then, an epimerase catalyzes the epimerization of R-ibuprofenoyl-CoA thioester to S-ibuprofenoyl-CoA thioester which is converted to S-ibuprofen by a CoA thioester

hydrolyse. Differently from R-ibuprofen, the S-enantiomer is not able to form a CoA thioester in vivo.

3. Clinical pharmacology of COX-isozyme inhibition

The kinetics, potency and selectivity of COX-isozyme inhibitors can be assessed in vitro using purified enzyme systems, isolated cellular preparations or whole blood samples. We have developed whole blood assays of COX-1 [43] and COX-2 [44] inhibition, using endogenous thrombin to trigger platelet thromboxane (TX)A2 production and exogenously added lipopolysaccharide (LPS) to induce COX-2 expression in circulating monocytes (Fig. 2). Measurement of serum TXB2 after 1-hour whole blood clotting at 37 °C is an index of platelet COX-1 activity induced by released AA in response to endogenously generated thrombin. The assessment of plasma PGE2 levels generated in heparinized whole blood samples incubated with LPS for 24-hour at 37 °C provides a sensitive and specific index of monocyte COX-2 activity [43], [44]. In fact, COX-2 is not detectable in blood cells but is induced in a time-dependent fashion in monocytes in response to LPS. The possible contribution of platelet COX-1-derived PGE2 to whole blood COX-2-derived PGE2 was minimized by adding aspirin in vitro (50 μM) or by pre-treating the volunteers with a single dose of aspirin 300 mg (given 48 h before blood collection). We exploited the capacity of aspirin to irreversibly acetylate platelet COX-1 as well as its blood instability (t1/2: 39 min) due to enzymatic hydrolysis. At this concentration, aspirin inhibits whole blood platelet COX-1 activity by approximately 90% while not significantly affecting COX-2 activity [44]. Thus, no acetylsalicylic acid was present in blood at the time of COX-2 induction. The possible contribution of salicylic acid to COX-2 inhibition was excluded due to the its low potency in inhibiting COX-isozyme activities [45]. A concentration-dependent reduction in serum TXB2 and LPS-induced PGE2 in response to exogenously added COX-inhibitors in vitro or to their circulating blood levels ex vivo allows characterizing the pattern of COX-isozyme selectivity or monitoring the extent and duration of pharmacological inhibition, respectively [43], [44].

After the discovery of COX-2 and the development of a new class of selective COX-2 inhibitors, the coxibs [3], a dichotomous definition of “selective” and “non-selective” NSAIDs became widely accepted in review articles and Pharmacology textbooks. In fact, all NSAIDs are COX-2 inhibitors with some degree of COX-1 inhibition as a “side-effect”. Moreover, based on in vitro testing of these agents in human whole blood assays and measurement of the IC50s for inhibition of platelet COX-1 and monocyte COX-2, it became apparent that COX-2 selectivity is a continuous variable with overlap between some tNSAIDs (e.g., nimesulide and diclofenac) and some first-generation coxib (i.e., celecoxib) (Fig. 3). From a clinical perspective, COX-2 selectivity can be described as the variable probability of sparing COX-1 activity (e.g., in the GI mucosa and platelets) at therapeutic drug concentrations of the NSAID: low (e.g., paracetamol), intermediate (nimesulide, meloxicam, diclofenac), or high (rofecoxib, etoricoxib, lumiracoxib). High doses (e.g., twice the therapeutic dose) of an NSAID with intermediate COX-2 selectivity will reduce its COX-1 sparing potential, as shown by the main results of the CLASS trial [8], a randomized comparison of high-dose regimens of celecoxib vs diclofenac and ibuprofen in patients with osteoarthritic conditions, that failed to demonstrate a statistically significant difference in ulcer complications between celecoxib and the tNSAID comparators.

Aspirin represents a notable exception to the rule. After the first 80 years of its commercial life, largely spent as an over-the-counter (OTC) analgesic, antipyretic, and anti-inflammatory agent, it underwent a de novo clinical development (entirely driven by the medical/scientific community) as an antiplatelet agent [46]. When tested in vitro on purified enzymes, aspirin inhibits both COX-1 and COX-2 with similar potency [47]. However, in isolated human platelets expressing only COX-1 and in isolated human monocytes expressing COX-2 (due to overnight incubation with LPS), aspirin resulted approx. 60-fold more potent in inhibiting COX-1 than COX-2 [45]. Moreover, its unique PK (i.e., short half-life) and PD (i.e., irreversible inactivation of COX-isozymes) properties allow a selective, cumulative inhibition of platelet COX-1 activity at low doses (e.g., 30 mg) given once daily [48] (Fig. 4). Thus, in vivo, low-dose aspirin (75–100 mg daily) is a relatively selective inhibitor of

platelet COX-1, with transient inhibition of COX-1 and COX-2 in extra-platelet cellular targets. From a current clinical perspective, aspirin is a COX-1 inhibitor with some dose-dependent inhibition of COX-2 as a "side-effect", as exemplified by inhibition of PGI2 biosynthesis at daily doses ≥ 160 mg [49].

It seems important to emphasize that whole blood assays of COX-isozyme activity [43], [44] provide capacity indexes of the virtually maximal prostanoid production by circulating blood cells, but are not informative of the actual rate of prostanoid biosynthesis in vivo. Both PGI2 and TXA2 are produced at a very low rate in healthy subjects, as reflected by the urinary excretion of their major enzymatic metabolites, PGIM and TXM, respectively [50], [51]. The biosynthesis of these important prostanoids can increase by 10- to 20-fold in response to pathophysiologic stimuli [52]. While the relationship between inhibition of COX-2 activity measured ex vivo and reduction in PGI2 biosynthesis measured in vivo is linear [37], the relationship between inhibition of platelet COX-1 activity and reduction in TXA2 biosynthesis is strikingly non-linear [53], [54] (Fig. 5). Thus, greater than 97% inhibition of platelet COX-1 activity (as assessed by measuring TXB2 generation in whole blood after dosing, i.e. ex vivo) is required in order to achieve 70% to 80% reduction in TXA2 biosynthesis in vivo.

Among the studied tNSAIDs, naproxen appears to produce aspirin-like antiplatelet effects throughout the 12-hour dosing interval, when given at high doses (i.e., 500 mg) twice daily [55], [56]. This is due to its profile of COX-isozyme inhibition (Fig. 3) as well as to its longer half-life as compared to other tNSAIDs (e.g., ibuprofen and diclofenac). Lower doses of naproxen and/or less frequent dosing are associated with inadequate inhibition of platelet COX-1 [56].

Therefore, based on the non-linear relationship between inhibition of platelet COX-1 activity and reduction in TXA2-dependent platelet activation (Fig. 5), most tNSAIDs and coxibs behave similarly in not suppressing COX-1 beyond the threshold for inhibition of platelet function, while reducing PGI2 biosynthesis in a dose-dependent fashion [37].

Because acetylation of platelet COX-1 by aspirin requires initial low-affinity anchoring to the Arginine-120 residue of the COX-channel, a common docking site for all NSAIDs, concomitant treatment with ibuprofen [57], [58] or naproxen [59], [60] can interfere with the antiplatelet effect of low-dose aspirin. Novel mass spectrometric assays to measure the extent of COX-1 acetylation in platelets may shed additional light of the mechanistic aspects of this clinically relevant drug–drug interaction [61].

4. Pharmacological effects of NSAID

4.1. Reduced pain and inflammation

The clinical efficacy of structurally distinct NSAIDs, all of which share the capacity to inhibit COX-2-dependent prostanoids, points to the importance of these mediators in promoting pain, fever and inflammation. The finding of a comparable pain relief by coxibs and tNSAIDs both in acute (for example, dental surgery) and chronic pain models (such as osteoarthritis [OA] and rheumatoid arthritis [RA]), supports a major role of the inhibition of COX-2 in their clinical efficacy [36]. However, it should be pointed out that often the randomized clinical trials (RCTs) comparing tNSAIDs and coxibs had inadequate statistical power to detect small differences in efficacy possibly associated with inhibition of COX-1.

NSAIDs exert an antinociceptive action by acting both at peripheral and central sites, mainly through the inhibition of COX-2 [62]. However, there is some suggestion that both COX-isoforms may contribute to the acute inflammatory response. In fact, both COX-1 and COX-2 mRNA as well as COX-1 and COX-2 proteins are co-expressed in circulating inflammatory cells, in inflamed RA synovium [63], [64] and in the spinal cord. COX-1-derived prostanoids may be generated in the initial phase of acute inflammation, while COX-2 upregulation which requires several hours is presumably the dominant pathway in the chronic phase of the inflammatory response [15].

In inflammation, both PGE2 and PGI2, produced in the peripheral terminals of sensory nerve endings, are hyperalgesic and enhance nociception produced by other mediators (e.g., bradykinin). In fact, prostanoids increase neuronal activity in nociceptive nerve fibers by lowering the activation threshold for opening sodium channels in the neuronal membrane [65], [66]. Peripheral inflammation also generates pain hypersensitivity in neighboring uninjured tissue (secondary hyperalgesia), because of increased neuronal excitability in the spinal cord (central sensation) and a syndrome comprising diffuse muscle and joint pain, fever, lethargy and anorexia [67]. These effects are mediated by the upregulation of COX-2 and PGE2 in spinal cord neurons and other regions of the CNS in response to proinflammatory IL-1β [68], [69], [70].

4.2. Gastrointestinal complications

COX-1 is the major COX-isoform expressed in the gastric mucosa of healthy individuals, and is involved in the production of cytoprotective PGE2 and PGI2. Prostanoids have an important role in protecting the GI mucosa by stimulating the synthesis and secretion of mucus and bicarbonate, inhibiting gastric acid secretion, increasing mucosal blood flow and promoting epithelial proliferation [71].

tNSAIDs cause mucosal injury throughout the GI tract [71]. Their use can lead to trivial lesions, such as petechiae, or superficial erosions, and more serious (and also less frequent) lesions such as ulcers that can complicate with bleeding, perforation or obstruction [71]. Their use is associated with around a 4-fold increased risk of serious upper GI complications [72], [73], [74]. The increased risk is dose-dependent [72], [73], [74], and is maintained even after many months of chronic treatment but disappears completely about two months after treatment withdrawal [29]. Individuals with advanced age or a history of complicated peptic ulcer disease have the greatest absolute risk [74]. The increased risk is common to all studied tNSAIDs suggesting a class effect [38].

The development of gastro-duodenal lesions in a large proportion of patients exposed to tNSAIDs is plausibly a consequence of persistent, moderate inhibition of mucosal COX-1 activity [29].

Differently, the increased risk of upper GI bleeding complications detected in tNSAID users – some of which can be serious or even fatal – has been proposed to occur as a result of transient, high-grade inhibition of platelet COX-1 in a very small percentage of exposed patients [29]. The improved GI tolerability demonstrated for two highly selective COX-2 inhibitors, rofecoxib and lumiracoxib, reflects the substantial sparing of COX-1 activity in the gastric mucosa and platelets [9], [75]. The important contribution of the alteration of primary hemostasis in the serious GI events caused by tNSAIDs is supported by the fact that the advantage of selective COX-2 inhibitors – which do not affect platelet TXA2 biosynthesis – is lost when these drugs are coadministered with low-dose aspirin [76].

Experimental results using selective pharmacological inhibition or genetic deletion of COX-1 and COX-2 in mice have shown that COX-2 plays an important role in the healing of preexisting ulcers [77], [78]. In fact, COX-2 is rapidly upregulated in response to growth factors and cytokines, and both COX-2 mRNA and protein are strongly expressed in mouse stomachs in which ulcers had been induced [79], [80]. Clinical data showing that coxibs are associated with a small risk of upper GI bleeding (UGIB), though smaller than that caused by tNSAIDs, support the protective role of COX-2 for the GI tract [13].

Altogether these results suggest that both COX-isozymes are a source of cytoprotective prostanoids; simultaneous inhibition of COX-1 and COX-2 that translates into profound suppression of prostanoid biosynthesis might be a hazard for the GI system [29], [38]. This hypothesis is mechanistically consistent with results obtained in the mouse showing that inhibition of both COX-1 and COX-2 is required for the formation of gastric lesions [81]. Massó González et al. [38] have recently conducted a systematic review of observational studies, published between 2000 and 2008, addressing the risk of upper GI bleeding/perforation among NSAID users (tNSAIDs and coxibs). This analysis allowed characterizing some factors associated with increased risk of serious GI events by NSAIDs. Thus, it was found that drugs with long half-life or slow release formulation and/or associated with profound

and coincident inhibition of both COX-isozymes – which translates into deficiency of prostanoid generation in the GI tract – were associated with a greater risk of UGIB [38].

4.3. Preventing or causing myocardial infarction

Atherothrombosis is a multifactorial process affecting coronary, cerebral and peripheral arterial vessels, in which platelet activation plays important roles both in the initiation and progression of the underlying vascular disease and in its thrombotic complications [52]. The participation of TXA2 (largely derived from platelet COX-1 activity) as an important mechanism amplifying platelet activation in response to vascular injury is supported both directly by urinary TXM measurements in acute coronary [82], [83] and cerebrovascular [84], [85] ischemic syndromes, and indirectly by the results of aspirin trials [5]. The former have demonstrated episodic increases in TXA2 biosynthesis in patients with unstable angina [82], [83] and with ischemic stroke [84], [85]. The latter have shown that low-dose aspirin reduces the risk of atherothrombotic complications in primary [86] and secondary prevention [87], as well as in the acute setting of myocardial infarction and ischemic stroke [87].

The “fingerprints” of the antithrombotic effect of low-dose aspirin [88] are represented by the following findings: i) a reduction in major vascular events was demonstrated with daily doses as low as 50 mg in placebo-controlled, randomized trials [87]; ii) this protective effect is saturable at low doses, i.e., much higher doses (up to 1500 mg) are not more effective [87]; iii) a cardioprotective effect was reported in a trial using a unique controlled release formulation of low-dose aspirin developed to maximize presystemic inhibition of platelet COX-1 and minimize systemic inhibition of endothelial COX-2 [86]; and iv) the antithrombotic effect and bleeding liability of low-dose aspirin were closely matched by a selective TP-antagonist, terutroban, in patients with cerebral ischemic events [89]. These findings are consistent with the mechanism of action being primarily, if not exclusively, related to permanent inactivation of platelet COX-1 and consequent suppression of TXA2-dependent platelet activation [5], [46], [88]. While for secondary prevention the benefits of

antiplatelet therapy substantially exceed the risk of major bleeding complications, for primary prevention the balance between vascular events avoided and major bleeds caused by low-dose aspirin is substantially uncertain [86]. This apparent discrepancy is due to the fact that in primary prevention (i.e., in people without a previous vascular event) the cardiovascular risks without aspirin, and hence the absolute benefit of antiplatelet prophylaxis, are at least an order of magnitude lower than in secondary prevention [86].

While the role of platelet TXA2 in atherothrombosis has been well characterized [52], the role of endothelial PGI2 has remained elusive for at least 25 years after its discovery due to the lack of selective inhibitors or antagonists [31]. Endothelial PGI2 production provides an important mechanism of thromboresistance of the vessel wall, and is largely driven by vascular COX-2 expression in both mice [90] and men [91]. The role of human endothelial PGI2 has been illuminated by the results of coxib and tNSAID trials and their meta-analyses [12], [13]. In particular, a meta-analysis of individual participant data from 639 randomized trials [13] has provided important information on the cardiovascular consequences of COX-2 inhibition by high-dose regimens of agents (both coxibs and some tNSAIDs) that do not inhibit platelet COX-1 activity beyond the threshold for functional inhibition. Compared with placebo, the risk of major vascular events (the combination of non-fatal myocardial infarction, non-fatal stroke or death from vascular causes) was increased by approximately 40% by a coxib (rate ratio [RR] 1.37, 95% CI 1.14–1.66; p = 0.0009) or diclofenac (RR 1.41, 95% CI 1.12–1.78; p = 0.0036), largely due to a doubling in major coronary events (coxibs 1.76, 1.31–2.37; p = 0.0001; diclofenac 1.70, 1.19–2.41; p = 0.0032), with no apparent change in the risk of any stroke [13]. It should be emphasized that relatively few strokes were recorded in these trials, and the absence of any stroke risk for NSAID regimens known to increase blood pressure is implausible [13]. Ibuprofen also significantly increased major coronary events (2.22, 1.10–4.48; p = 0.0253), but the effect on major vascular events was statistically uncertain (1.44, 0.89–2.33). Consistently with the favorable PK/PD features noted above, naproxen (mostly, 500 mg bid) did not increase major vascular events (0.93, 0.69–1.27) [13]. Vascular death was increased

significantly by coxibs (1.58, 1.00–2.49; p = 0.0103) and diclofenac (1.65, 0.95–2.85, p = 0.0187), non-significantly by ibuprofen (1.90, 0.56–6.41; p = 0.17), but not by naproxen (1.08, 0.48–2.47; p = 0.80). Although these analyses suggest that high-dose naproxen may not increase the risk of major vascular events in low-risk individuals (annual rate in the control arm < 1%), this result should not be interpreted as reflecting an intrinsic safety quality of the drug under all circumstances. First of all, in aspirin-treated high-risk patients naproxen would not be expected to produce any additional inhibition of platelet COX-1 (presumably, fully suppressed by low-dose aspirin), and may actually interfere with the antiplatelet effect of aspirin [59], [60]; furthermore, the longer half-life of naproxen versus diclofenac or ibuprofen (14 h vs 2–3 h) may produce longer-lasting suppression of PGI2 biosynthesis [55]. Secondly, the cardiovascular effects of lower, OTC doses of naproxen (e.g., 220 mg bid) are largely unknown because they were rarely employed in the coxib and NSAID trials. Thirdly, the apparent safety advantage of high-dose naproxen over other tNSAID regimens, as recorded in the coxib and tNSAID trials, may be attenuated during long-term treatment because of similar untoward effects on blood pressure control and renal function [92].

Overall, celecoxib and rofecoxib, that differ in COX-2 selectivity by about 20-fold (Fig. 3), increased the risks of major vascular events to the same extent (celecoxib 1.36, 1.00–1.84; rofecoxib 1.38, 1.07–1.80) [13]. This finding is consistent with the mechanistic considerations outlined in Section 3.

In this meta-analysis, the proportional effects of coxibs and tNSAIDs were similar, irrespective of baseline characteristics, including the level of risk of major vascular events (< 5%, 5–10%, > 10% over 5 years) [13]. Thus, the effects of different NSAID regimens in particular patients can be predicted, which could help in personalizing NSAID therapy. These analyses did not provide any convincing evidence that the effect of coxibs on the risks of major vascular events may be attenuated by concomitant use of low-dose aspirin [13]. A mitigating effect of low-dose aspirin is biologically plausible, as COX-1 knock-down attenuates the prothrombotic effect of COX-2 inhibition in mice [34]. Moreover, the neutral cardiovascular phenotype of high-dose naproxen would suggest that

profound and persistent inhibition of platelet COX-1 activity may mask the prothrombotic consequence of high-grade COX-2 inhibition in the vasculature [92]. However, it should be pointed out that low-dose aspirin inhibits primarily TXA2-dependent platelet function while PGI2 counteracts the effects of an intricate network of stimuli, not just TXA2 [31].

All NSAIDs doubled the risk of heart failure causing hospital admission, consistent with this being a COX-2 dependent hazard unrelated to variable platelet inhibition [13]. The finding that coxib and tNSAIDs caused a similar increased risk of heart failure provides indirect evidence that the high-dose regimens used in the randomized trials of these agents produced comparable levels of COX-2 inhibition [92]. It should be emphasized that the absolute excess of hospitalizations due to heart failure is at least as large as the excess in major vascular events produced by the same drugs [13].

4.4. Renal effects

Given the constitutive expression of COX-2 in the human kidney, it is not surprising that all NSAIDs can cause renal adverse events in at-risk individuals [3]. COX-2 is involved in prostanoid production (particularly, PGE2 and PGI2) in both cortical and medullary structures. These prostanoids have vasodilator and natriuretic properties [93]. Thus, renal vasoconstriction and salt and water retention resulting from COX-2 inhibition can contribute to increased blood pressure, particularly in hypertensive patients, and impaired response to antihypertensive drugs. In a randomized, placebo-controlled trial in approximately 19,000 hypertensive patients, aspirin (75 mg daily) did not affect blood pressure control or the need for antihypertensive therapy [94], consistent with the lack of renal COX-2 inhibition by low-dose aspirin [48].

In patients with ineffective circulatory volume, maintenance of renal hemodynamics is critically dependent on intact prostanoid production, and administration of any NSAID can acutely decrease renal blood flow and glomerular filtration rate [3].

4.5. Chemoprevention of colorectal cancer

A large body of evidence has demonstrated that constitutive aberrant COX-2 expression is a contributing factor promoting colorectal cancer (CRC) [95]. Various genetic, epigenetic, and inflammatory pathways have been identified to be involved in the etiology and development of CRC [95]. Alterations in these pathways can influence COX-2 expression at multiple stages of colon carcinogenesis allowing for elevated prostanoid biosynthesis to occur first in the tumor microenvironment [95]. Later, aberrant expression of COX-2 occurs in epithelial cells and may contribute to the different steps of intestinal tumorigenesis from hyperplasia, dysplasia, to carcinoma and metastasis [96], [97], [98].

COX-2-dependent PGE2 production plays a predominant role in CRC [99], [100]. PGE2 is the most abundant prostanoid detected in human CRC [101], [102], [103]. PGE2 modulates a number of signal transduction pathways that may affect proliferation, programmed cell death (apoptosis), angiogenesis, immune responses, cellular adhesion, differentiation, and tumor invasion [99], [104]. Overexpression of COX-2 also increases cell migration and proliferation in intestinal epithelial cells [100], [105]. COX-2 gene expression is up-regulated in most human CRC compared with normal intestinal mucosa [106]. In contrast to COX-2, COX-1 expression seems to be unaltered in CRC [107], [108], [109].

In normal cells, COX-2 expression levels are largely regulated at the post-transcriptional level through various RNA sequence elements present within the mRNA 3′ untranslated region (3′UTR) of COX-2 mRNA (Fig. 6). A conserved AU-rich element (ARE) functions to target COX-2 mRNA for rapid decay and translational inhibition through association with various RNA-binding proteins to influence the fate of COX-2 mRNA. Specific microRNAs (miRNAs) bind regions within the COX-2 3′UTR and control COX-2 expression (Fig. 6). Moreover, altered expression and cytoplasmic accumulation of transacting factors that bind to ARE of COX-2 mRNA and influence its stability, such as the mRNA-stability factor human antigen R (HuR) [110], [111] may play a role in controlling the extent and duration of COX-2 expression (Fig. 6).

In human colon carcinoma cells, the COX-2 ARE does not function properly and enhanced mRNA stability is detected [112], [113], [114], [115]. It has been hypothesized that the ability of HuR to promote mRNA stabilization requires its translocation to the cytoplasm. HuR is expressed at low levels and is localized to the nucleus in normal tissue, whereas HuR overexpression and cytoplasmic localization is observed in colon adenomas, adenocarcinomas, and metastases and it colocalizes with COX-2 [111].

The role of COX-2 in human tumorigenesis is supported by the efficacy of coxibs in reducing the risk of colorectal adenoma recurrence [10], [11], [116]. However, the long-term use of selective COX-2 inhibitors is contraindicated due to the interference with cardiovascular homeostasis by the concomitant inhibition of vascular COX-2-dependent PGI2 biosynthesis [31].

Similarly to coxibs, aspirin, given once daily even at the doses used for the prevention of cardiovascular events [5], can prevent sporadic colorectal adenoma recurrence [105]. These drugs have a different mechanism of action regarding the COX-isozyme which is targeted: low-dose aspirin affects preferentially platelet COX-1 while coxibs are selective inhibitors of COX-2 [29]. Thus, it has been proposed that different cells of the tumor environment play a role in the early phase of tumorigenesis. In particular, platelets, activated in response to mucosal injury/dysfunction, may interact with stromal cells and this cross-talk may lead to the release of several mediators involved in cell growth and angiogenesis [29], [45], [105]. These events may contribute to epithelial cell transformation in the GI tract. In this scenario, a central event is related to the aberrant expression of COX-2 and the synthesis and release of PGE2 that impacts on different molecular signaling pathways which promote tumorigenesis and its progression to cancer (Fig. 7). Platelet-derived mediators (e.g., cytokines and growth factors) may upregulate COX-2 expression in adjacent nucleated cells of the intestinal mucosa [110], [117]. This hypothesis would explain the apparently similar chemopreventive effect of low-dose aspirin acting upstream to suppress platelet activation at sites of

intestinal mucosal injury and coxibs acting downstream to inhibit COX-2 activity in intestinal epithelial and endothelial cells [29], [45], [105], [118].

This hypothesis is supported by the finding of aspirin's beneficial effect in reducing the risk of CRC-related death detected on long-term follow-up of RCTs which were designed to test its antithrombotic efficacy [119], [120]. Aspirin was effective at low-doses and its anti-cancer effect was not increased at higher doses, a finding which is compatible with the antiplatelet action of the drug contributing to the prevention of both atherothrombosis and cancer [45], [105], [118]. Thus, atherothrombosis and cancer may share a common mechanism of disease, i.e., platelet activation in response to vascular and mucosal injury, respectively.

Activated platelets may also enhance the metastatic potential of cancer cells (through a direct interaction and/or the release of soluble mediators or exosomes) [121], at least in part by inducing the overexpression of COX-2 [117]. COX-independent mechanisms of aspirin, such as the inhibition of NF-kB and Wnt/β-catenin signaling and the acetylation of other proteins, have been suggested to play a role in the chemopreventive effects of aspirin [45], [118]. However, their relevance remains to be demonstrated in vivo at low doses.

5. Conclusions

The last 15 years have witnessed a substantial improvement in the assessment of NSAID efficacy and safety, largely due to the development of coxibs and the meta-analyses of their trials. The appreciation of a sizeable cardiovascular risk associated with the use of high-dose regimens of these agents has resulted in the discontinuation of long-term trials for cancer prevention. Moreover, it led to more stringent requirements for new drug approval, and discouraged pharmaceutical companies from developing additional members of the class.

Clearly, novel drugs are required given the size of the unmet medical need in this field, possibly targeting downstream enzymes in AA metabolism, prostanoid receptors, or drug targets unrelated to

this metabolic pathway. mPGES-1 inhibitors are being developed by several companies [122], and hold the promise for improved cardiovascular safety compared to existing NSAIDs [123]. The important lessons learned from the coxib experience will hopefully guide a more rational approach to drug development.

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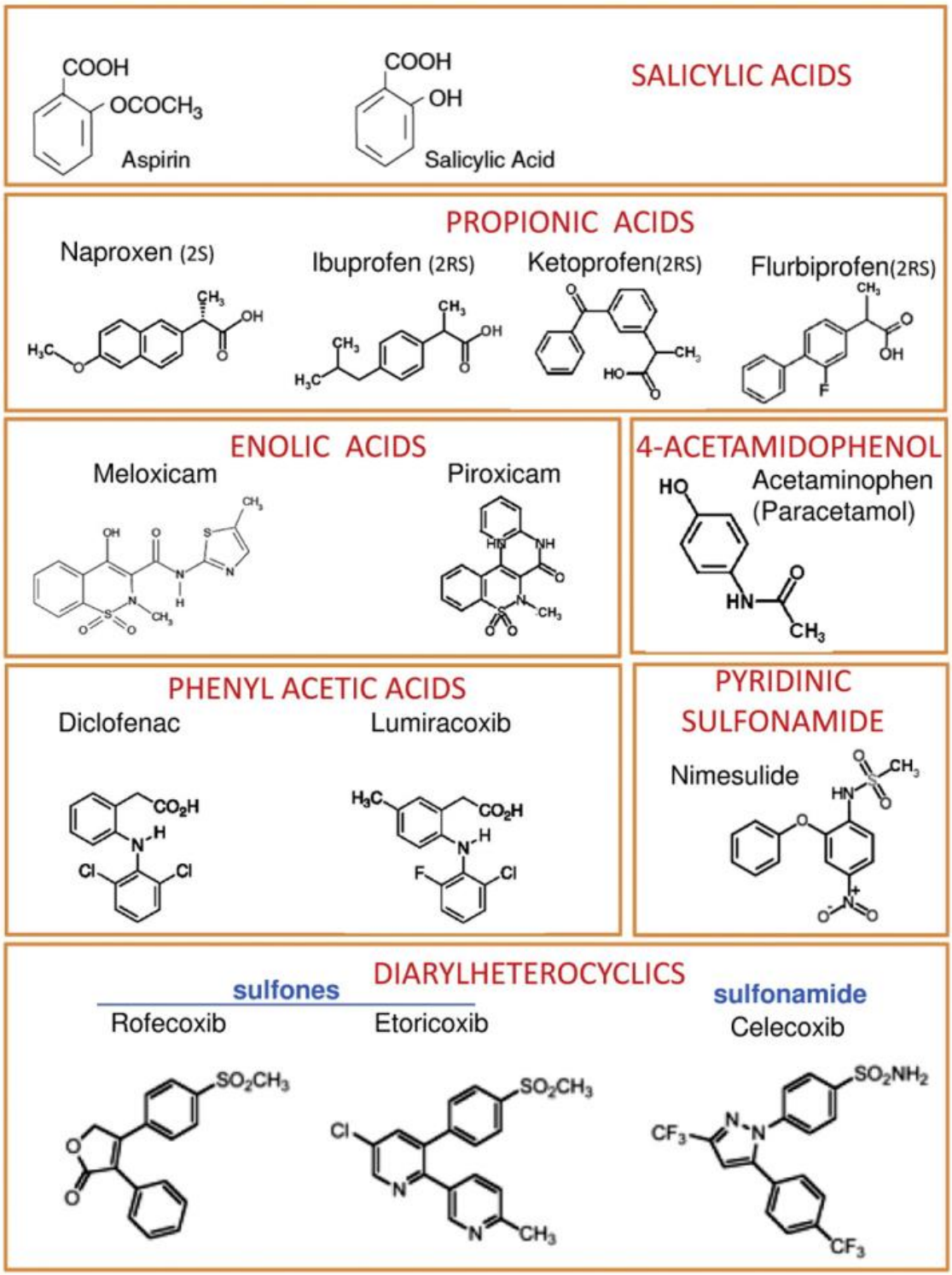

Fig. 1. Chemical structures of NSAIDs. The drugs are grouped on the basis of their chemical features. Ibuprofen, like other 2-arylpropionate derivatives (including ketoprofen, flurbiprofen, naproxen), contains a chiral carbon in the α-position of the propionate moiety. As such, there are two possible enantiomers (R) and (S). Except naproxen clinically used as (S) enantiomer, the other propionic acid-derivatives are used clinically as racemic agents.

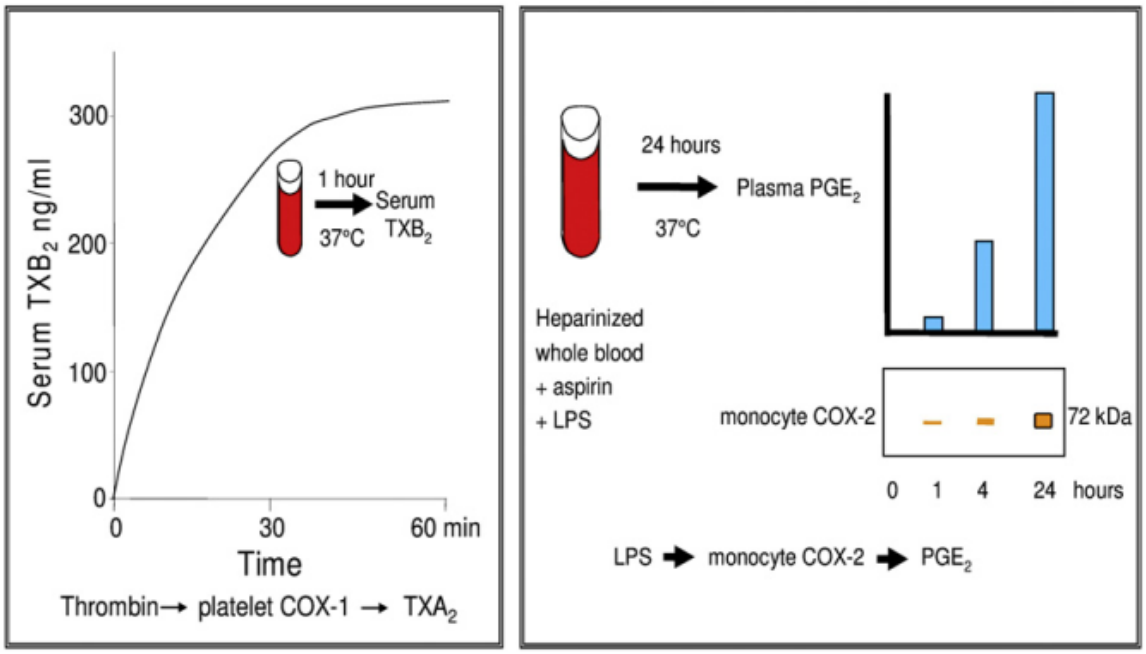

Fig. 2. Schematical representation of whole blood assays for platelet COX-1 activity (left panel) and monocyte COX-2 activity (right panel).

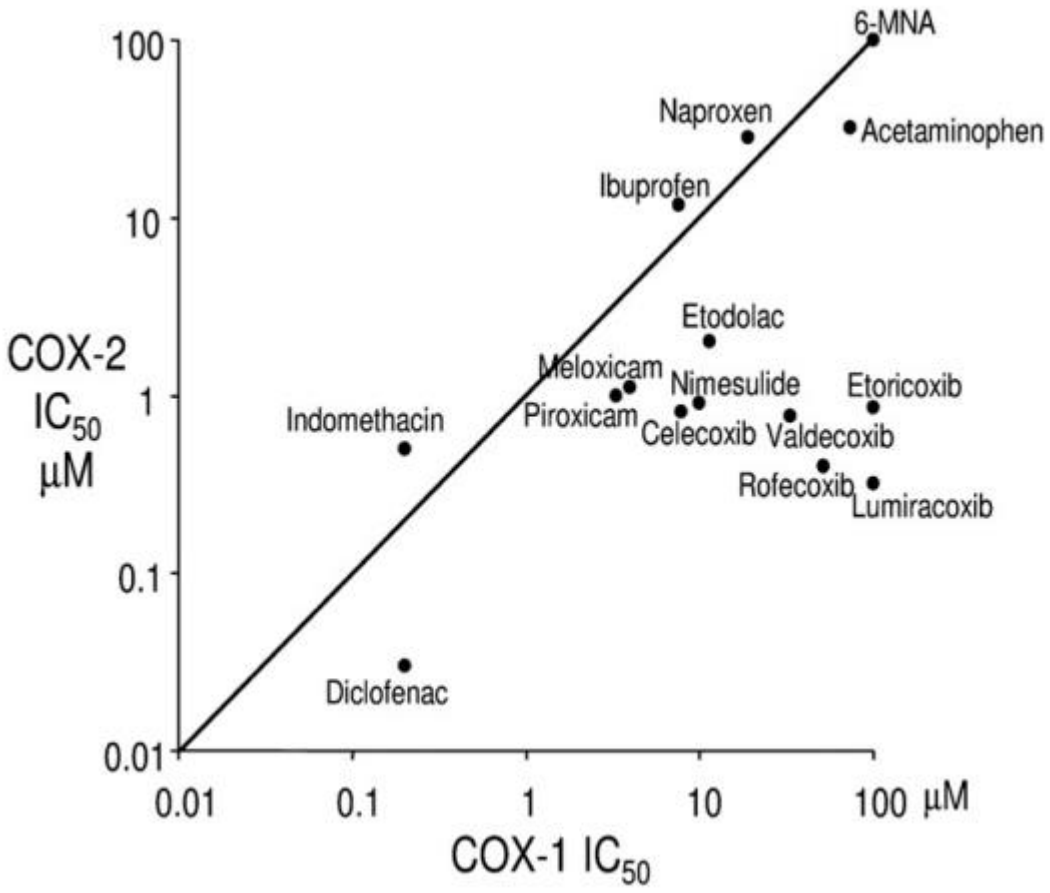

Fig. 3. Concentrations of various drugs required to inhibit the activity of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) by 50 % (IC50) in whole blood assays. Each point is the mean of three or four values. Drugs plotted below the diagonal line indicating equivalence are more potent inhibitors of COX-2 than COX-1. 6-MNA denotes 6-methoxy-2-naphthylacetic acid.

Modified and updated from FitzGerald & Patrono, N Engl J Med, 2001 [3].

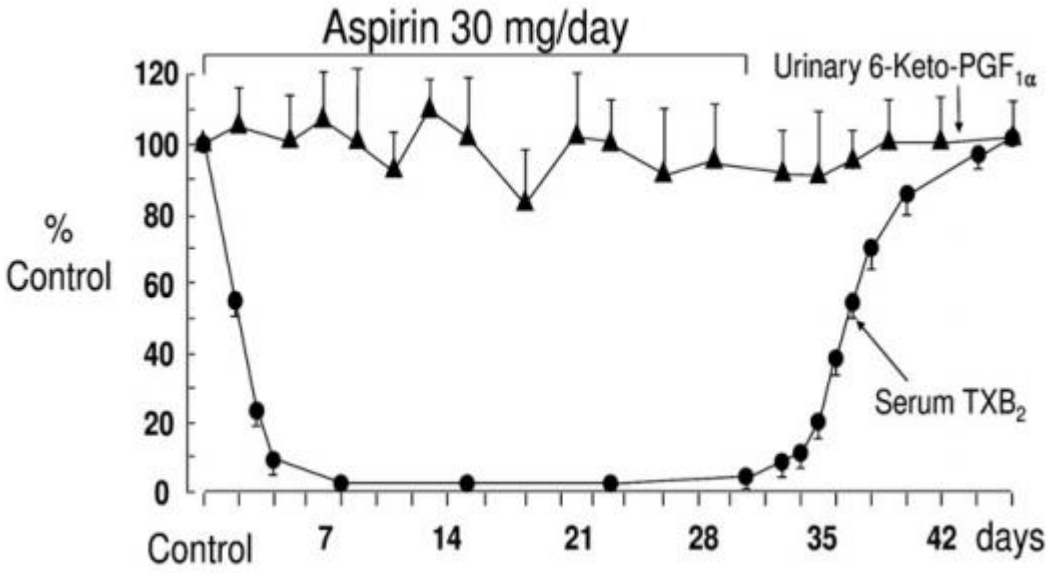

Fig. 4. Long-term effects of low-dose aspirin (0.45 mg/kg per day) on platelet TXB2 and renal PGI2 synthesis. Serum TXB2 concentrations and urinary excretion of 6-keto-PGFIα were measured in three healthy subjects before, during, and after aspirin therapy. Mean values ± SEM are plotted. The arrows indicate duration of daily aspirin therapy. Modified from Patrignani et al., J Clin Invest, 1982 [48].

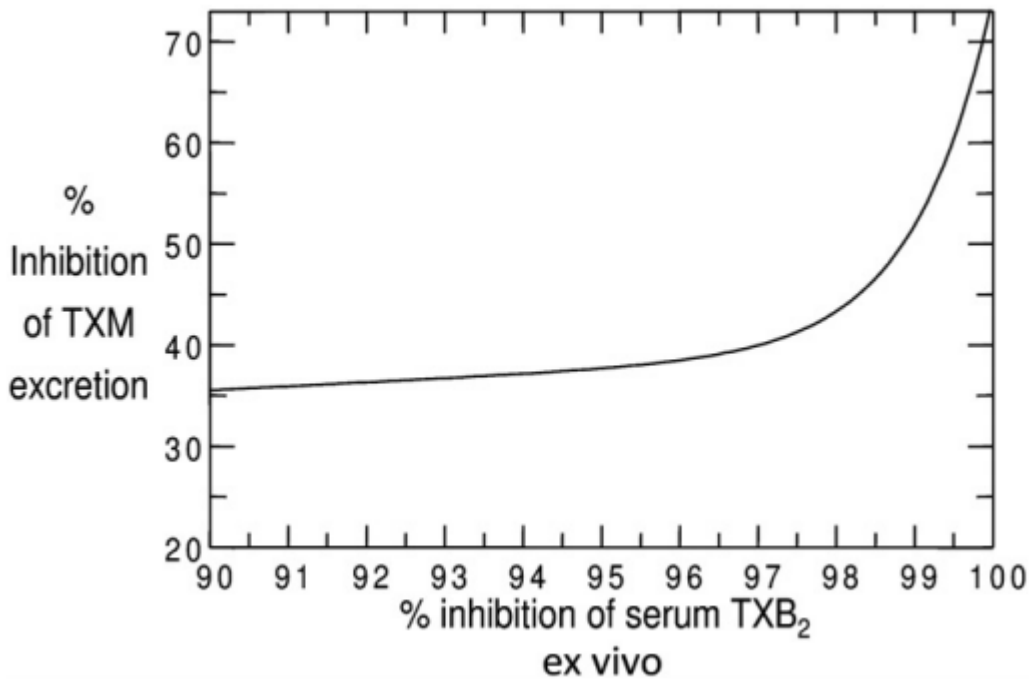

Fig. 5. Non-linear relationship between inhibition of platelet COX-1 activity (x-axis), as reflected by serum TXB2 (the nonenzymatic hydrolysis product of TXA2) measurements ex vivo, and inhibition of TXA2 biosynthesis in vivo (y-axis), as reflected by measurements of the urinary thromboxane metabolite 11-dehydro-TXB2 (TXM). Modified from Santilli, Rocca et al., J Am Coll Cardiol, 2009 [54].

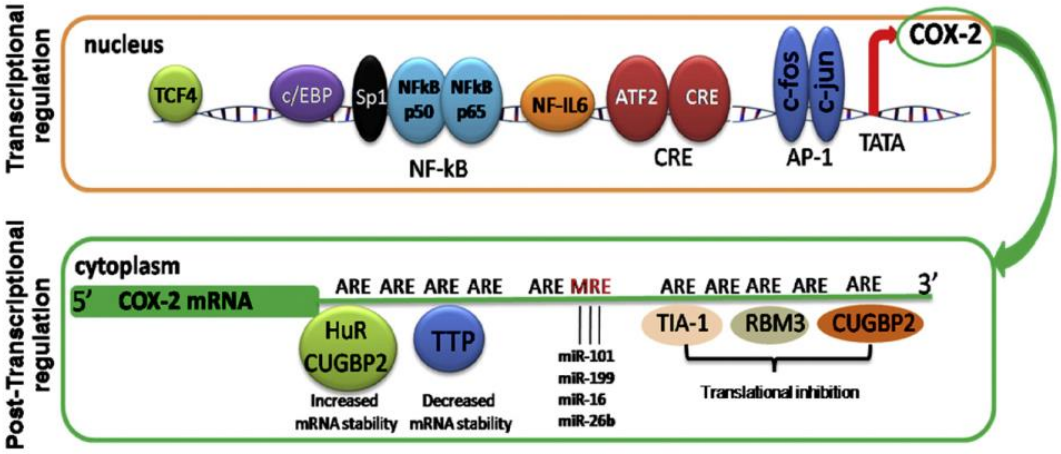

Fig. 6. Transcriptional and post-transcriptional regulation of COX-2 expression. The transcription of COX-2 gene is precisely regulated by the action of numerous transcription factors which are able to recognize and bind specific sequences of the COX-2 gene promoter. In fact, the 5′UTR (untranslated region) of the COX-2 gene contains binding sites for numerous regulatory transcription factors, including TCF4 (transcription factor 4), C/EBP (CCAAT-enhancer-binding protein), Sp1, two NF-κB (nuclear factor κB) motifs, NF-IL6 (nuclear factor for interleukin-6), two CREs (cAMP-response elements), and two AP-1 (activator protein 1) sites. In addition to the transcriptional regulation, COX-2 expression is controlled by several mechanisms at the post-transcriptional level. COX-2 mRNA contains at 3′UTR a region rich in AU-element (ARE sequence). These sequences are recognized by several RNA-binding proteins which alter the COX-2 mRNA stability or its translational efficiency. Among them there are some proteins which promote mRNA stability (HuR and CUGBP2), mRNA decay (TTP), and translational inhibition (TIA-1, CUGBP2, and RMB3). In addition the 3′UTR region of COX-2 mRNA contains some microRNA-binding sequence (MRE) and specific microRNAs (miR-101, miR-199, miR-16 and miR-26b) have been found to bind this sequence thus controlling COX-2 expression.

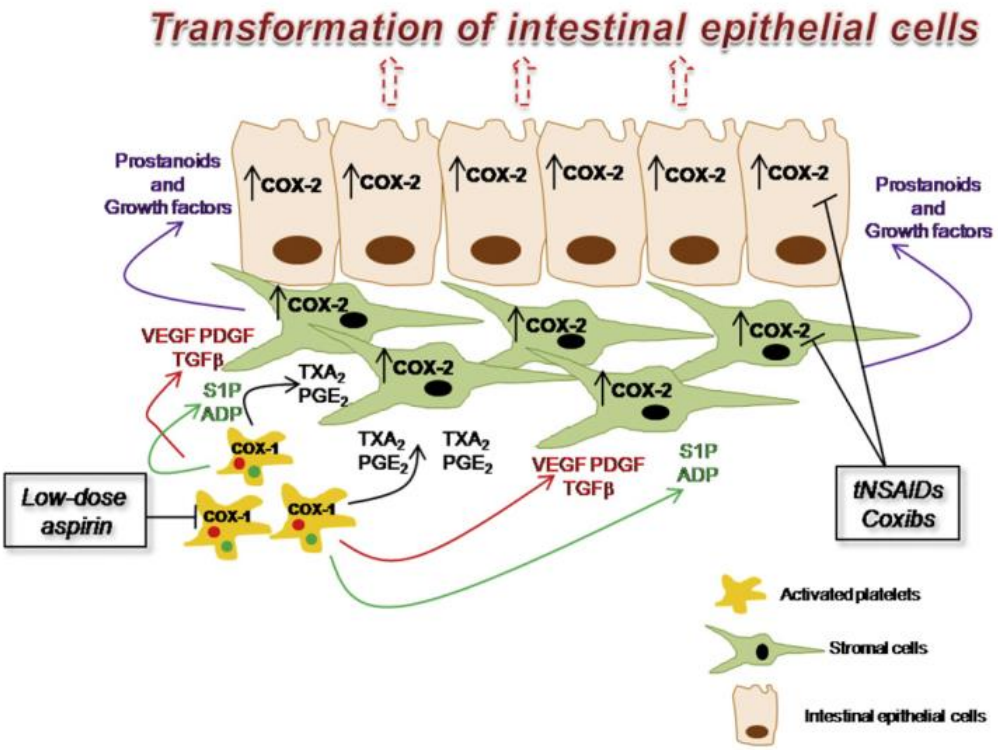

Fig. 7. Activated platelets trigger early events of intestinal malignant transformation. Platelets upon activation release several mediators such as COX-1-derived prostanoids (mainly TXA2 and PGE2), growth and angiogenic factors stored in α-granules (VEGF, PDGF, TGFβ), and other mediators released from dense granules (i.e., ADP and S1P). These factors can activate stromal cells (mainly fibroblasts) which respond with an up-regulation of COX-2. Activated stroma, in turn, releases prostanoids and growth factors which can act on epithelial cells provoking the induction of COX-2 in these cells. The overexpression of COX-2 in intestinal epithelial cells is a key step of intestinal malignant transformation. This cascade of sequential events would explain the similar impact of low-dose aspirin (acting upstream) and NSAIDs (acting downstream) on sporadic colorectal adenoma recurrence.