Turning down the heat: new routes to inhibition of inflammatory signaling by prostaglandin H_2 synthases

Many nonsteroidal anti-inflammatory drugs act by inhibiting the cyclooxygenase activity of prostaglandin H_2 synthase (PGHS), a key enzyme in the biosynthesis of prostaglandins. Gastric toxicity remains a serious problem with the current drugs, however. Recent advances in the understanding of PGHS now suggest two possible approaches to producing drugs with fewer side effects.

Chemistry & Biology June 1995, **2**:343–350

Since the discovery in 1971 that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) block prostaglandin synthesis [1], inhibition of this pathway has dominated NSAID research (see [2] for an excellent review). Many well known NSAIDs, including ibuprofen, naproxen and indomethacin, potently inhibit the enzyme prostaglandin H_2 synthase-1 (PGHS1), which catalyzes the first two committed steps in the formation of prostaglandins, thromboxanes and prostacyclin. These prostanoids serve as autocrine or paracrine hormones, signaling a variety of cellular responses such as vasodilation, smooth muscle contraction and platelet aggregation via cell surface receptors of the seven-transmembrane G-protein-coupled superfamily.

As might be expected from the fact that PGHS1 inhibitors have anti-inflammatory effects, prostaglandins initiate inflammatory responses in peripheral tissues, inducing redness, edema, pain and heat sensations. There are also indications that prostaglandin synthesis in the nervous system modulates fever, pain and sleep functions. On the other hand, prostaglandins have functions unrelated to inflammation in kidney, bone and reproductive tissues. It is very likely that prostaglandins have a protective function in the gut, since stomach ulceration is a common side effect of PGHS1 inhibition by NSAIDs. Through the efforts of several groups [3–7], the cDNAs of PGHS1 and a second isoform, PGHS2, were cloned in the late eighties and early nineties. Studies of their expression quickly provided convincing evidence of a clear division of labor between the two isoforms. Although PGHS1 is constitutively expressed in most cell types, PGHS2 expression is usually low or undetectable. Many proinflammatory ligands induce the rapid transcription and translation of PGHS2, resulting in a dramatic increase in prostaglandin production [5–7]. In this article, we examine recent attempts to obtain selective inhibitors of PGHS2, and discuss an alternative mechanism of inflammatory signaling by PGHS which may offer additional targets for drug discovery.

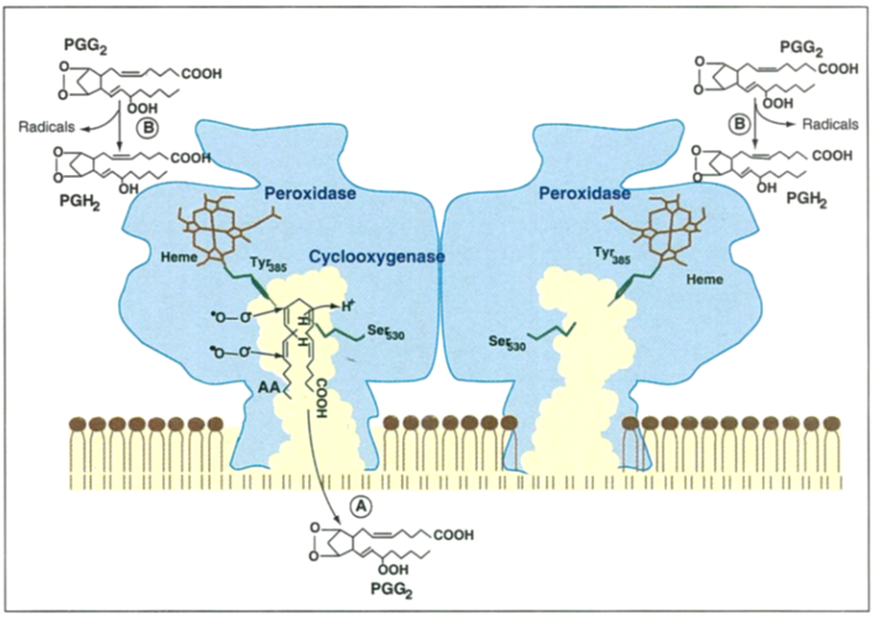


Fig. 1. The PGHS1 cyclooxygenase and peroxidase activities are found in distinct parts of the enzyme. The cyclooxygenase site, where arachidonic acid (AA) is oxidized to PGG_2 (reaction A), is hydrophobic, and buried within a channel that extends inward from the lipid bilayer. A tyrosine residue at position 385 separates the channel from the heme in the peroxidase active site. The peroxidase activity resides within a broad cleft on the surface of the protein, and is solvent-accessible. PGG_2 and other lipid peroxides are reduced here (reaction B), producing radical species. It is not known whether transfer between the cyclooxygenase and peroxidase active sites is passive or facilitated.

Structure of PGHS1

The PGHS enzymes carry out two distinct catalytic steps (Fig. 1). In the first, arachidonic acid is *bis*-oxygenated to form the short-lived cyclic lipid hydroperoxide, PGG_2. The activity responsible for this step in PGHS1 is referred to as PGHS1 cyclooxygenase. In the second step, the hydroperoxide group is reduced to an alcohol by PGHS1 peroxidase, yielding the more stable PGH_2. The recent elucidation of the crystallographic structure of ovine PGHS1 [8] has yielded unexpected insights into the interrelationship of these activities, as well as providing invaluable information for drug design.

PGHS1 is structurally similar to myeloperoxidase, a secreted enzyme with microbicidal functions. Like other members of this conserved gene family, PGHS1 displays peroxidase activity, has an essential heme cofactor, and releases free radicals. Unlike myeloperoxidase, however, PGHS1 is localized to the endoplasmic reticulum and nuclear envelope. PGHS1 is inserted into a single leaf of the lipid bilayer, facing the lumen, as a homodimer of 70 kD subunits (Fig. 1). The PGHS1 peroxidase active site lies within a broad cleft on the exterior surface of the protein, which is freely accessible to solvent and also contains the heme cofactor. In contrast, the cyclooxygenase active site is found in the interior of the protein, at the apex of a hydrophobic channel which extends inward from the membrane bilayer. A tyrosine residue, Tyr385, is also found at the apex of the channel, separating the hydrophobic channel from the heme prosthetic group in the peroxidase cleft on the surface of the protein. This tyrosine participates in a radical-mediated activation of the arachidonic acid substrate [9]. The hydrophobic channel contains the binding sites for competitive inhibitors of PGHS1 such as flurbiprofen, and it is now clear that the irreversible inhibition of PGHS1 by aspirin acetylation of Ser530 results from steric hindrance of substrate access to the channel [10]. There have been no reports yet of inhibitors designed using information from the crystallographic structure of PGHS1, but such inhibitors may be expected soon.

Prostaglandin synthesis during proinflammatory signaling

Prostaglandin production can be induced by inflammatory peptides, growth factors, cytokines or tumor promoters. Cell injury caused by ultraviolet radiation or ischemia–reperfusion also results in prostaglandin release. The released prostaglandins must be synthesized *de novo*, as no intracellular storage of prostaglandins has been observed. Before the PGHS enzymes can synthesize prostaglandins, arachidonic acid or other polyunsaturated fatty acids must be liberated from cellular phospholipids by the action of phospholipase A_2, C or D (Fig. 2). The activation of phospholipases is tightly regulated by intracellular signaling pathways which are normally initiated by ligand binding to cell-surface receptors. Once arachidonic acid has been released, PGHS1 or PGHS2 can use it to synthesize PGH_2. Subsequently, other enzymes further metabolize PGH_2 into various prostanoids specific to each cell type. Some of the inflammatory effects of these compounds are shown in Figure 2.

Several lines of evidence have indicated that PGHS2 mediates the production of prostaglandins in inflammatory responses. First, PGHS2 expression is strongly induced by proinflammatory stimuli *in vitro* and *in vivo*, unlike PGHS1 [5–7,11,12]; second, expression of PGHS2 can be attenuated by anti-inflammatory steroids (the corticosteroids), and this attenuation is paralleled by a reduction in inducible prostaglandin synthesis [6,13].

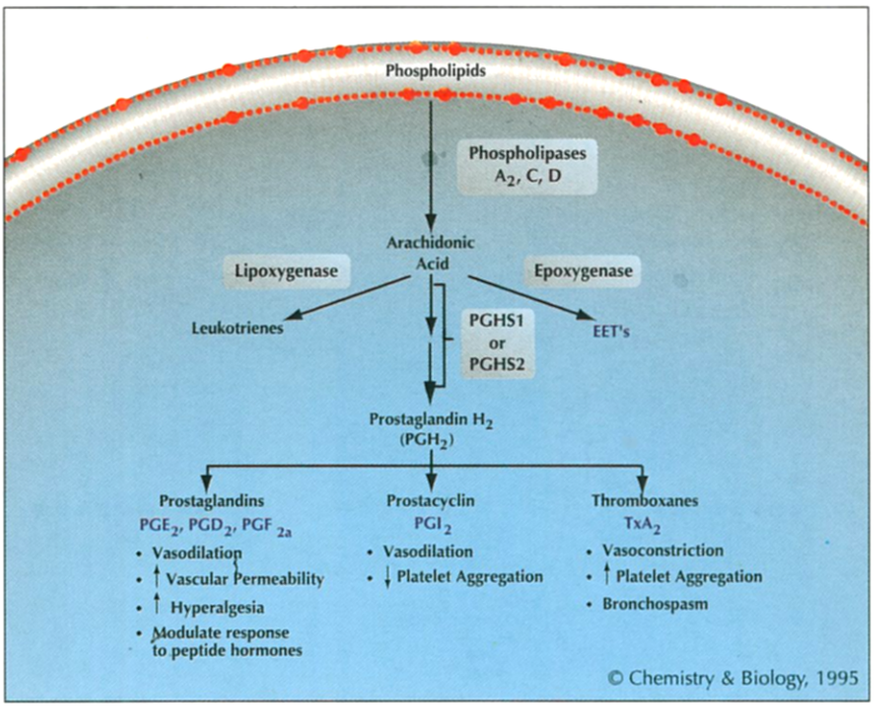


Fig. 2. Prostaglandin biosynthesis and inflammatory effects. Phospholipase A_2 directly releases arachidonic acid from phospholipids, but for the phospholipase C and D pathways di- and monoacylglyceride lipases are also required. Once arachidonic acid has been released, one of the PGHS isoforms converts it to PGH_2. The subsequent conversion of PGH_2 to prostaglandins, prostacyclin or thromboxanes depends on the cell-specific expression of appropriate enzymes. The inflammatory responses to the products of these enzymes vary in different tissues.

Third, both PGHS2 protein synthesis and inducible prostaglandin production can be abolished by cycloheximide [13]; and, finally, an antisense PGHS2 oligonucleotide blocks expression of both PGHS2 and ligand-inducible prostaglandin synthesis [14].

PGHS2-selective inhibitors: a second generation of NSAIDs

The finding that indomethacin, a reversible cyclooxygenase inhibitor, is a potent anti-inflammatory drug sparked a flurry of efforts to make similar inhibitors. In the late seventies and early eighties, powerful compounds such as naproxen, diclofenac and piroxicam were synthesized and characterized. These compounds effectively controlled the edema and pain associated with inflammation, but the beneficial effects carried a price. Prostaglandins have an essential cytoprotective function in the stomach and intestine. Consequently, stomach ulceration seemed to be an unavoidable side effect of cyclooxygenase inhibition, and when few ideas emerged on how to separate toxicity from efficacy the search for new NSAIDs waned.

With the discovery of the inflammation-related PGHS2 in 1991, however, hope quickly revived that PGHS2-selective cyclooxygenase inhibitors might have fewer side effects. Although the existing inhibitors showed little discrimination between the PGHS isoforms, several pharmaceutical companies rapidly identified a number of selective PGHS2 inhibitors by revisiting old compound libraries. *In vivo* studies [11,12,15,16] showed that PGHS2 inhibitors have potent anti-inflammatory activity with little gastric toxicity, quite unlike their non-selective predecessors. Although there is as yet no proof that the improved gastric profile of these compounds directly results from the sparing of PGHS1 cyclooxygenase, PGHS1 is the major isoform detected in the stomach (Fig. 3). Table 1 summarizes the inhibition of PGHS1 and PGHS2 by selected compounds, along with some relevant biological observations. Three classes of inhibitors can be distinguished from these data, based on the mechanism of suppression:

1) Reversible non-selective inhibitors of PGHS1 and PGHS2. Most compounds in this category are competitive substrate inhibitors, including many pre-1990 NSAIDs.

2) Selective PGHS2 inhibitors. These compounds are time-dependent, irreversible inhibitors of PGHS2, but reversibly inhibit PGHS1 at high concentrations [17–19].

3) Covalent inhibitors of PGHS1 and PGHS2. Aspirin is the best-studied example of this class. More recently, certain amino (carboxyalkyl)maleimides were shown to act via this mechanism [20], though the site of modification has not been conclusively identified.

Will PGHS2-selective inhibitors be the NSAIDs of the future? The answer seems likely to be yes. However, several points should be considered before assuming that all currently used NSAIDs will be obsolete. First,

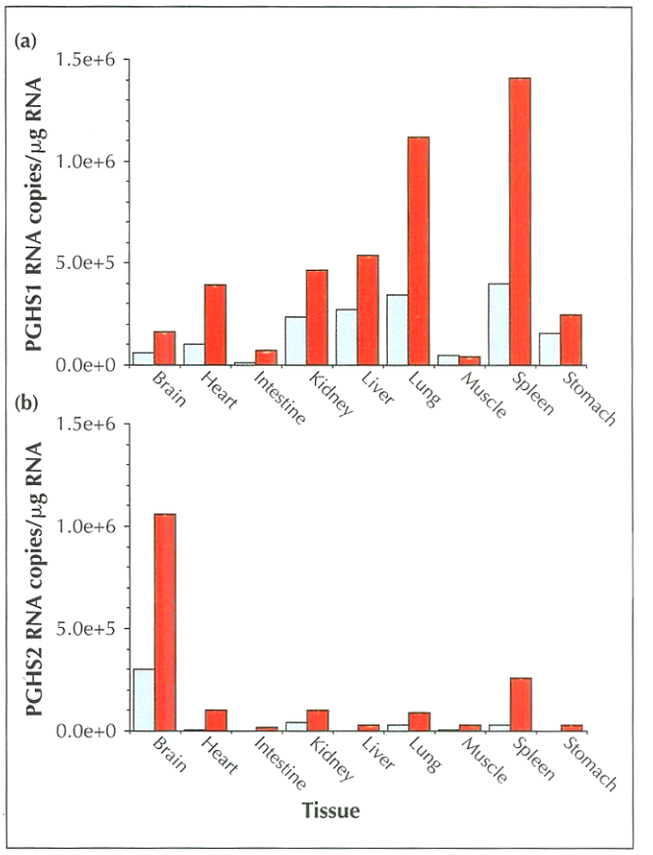
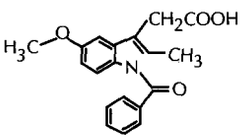
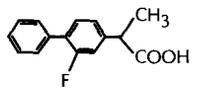
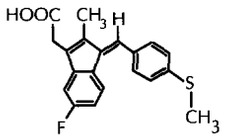
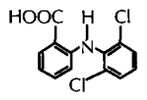
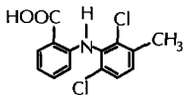
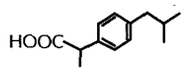
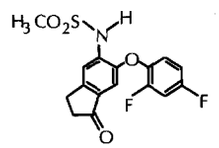
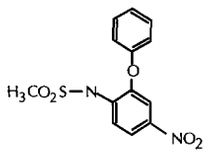
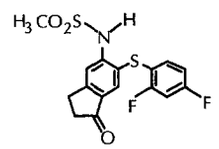


Fig. 3. Distribution of PGHS1 and PGHS2 RNA in normal (blue) and arthritic (red) rat tissues (D.G.M., unpublished data). Total RNA from indicated rat tissues was used for RNase protection with radiolabeled, isoform-specific PGHS1 **(a)** or PGHS2 **(b)** complementary RNA. RNA copy number was determined relative to standards, as quantitated by computer-assisted densitometry of several autoradiographic exposures. Tissues were obtained from control or adjuvant arthritic rats sacrificed 15 days after injection.

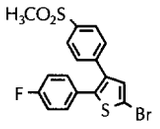
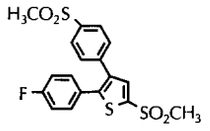
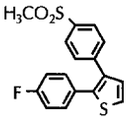
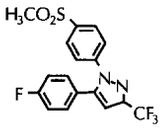
PGHS2 is the predominant isoform in normal rat brain, where it shows a cell-specific expression pattern; furthermore, brain expression can be superinduced by non-inflammatory stimuli ([21,22]; Figs 3,4). Therefore, PGHS2 inhibitors which can pass the blood–brain barrier will have to be assessed for adverse neurological effects. Second, the idea that selective PGHS2 inhibitors will be as effective as the non-selective inhibitors used until now rest in part on the demonstration that PGHS2, but not PGHS1, is upregulated immediately after adjuvant injection [7]. However, a quantitative comparison performed 10–15 days after injection, when swelling is at its peak, shows that PGHS1 levels also rise three- to four-fold (Fig. 3). Because of its high basal level, PGHS1 expression still predominates in most inflamed tissues, particularly the spleen. Therefore it may be too early to discount the possibility that PGHS1 is important in inflammatory responses in at least some tissues. Third, PGHS2 has been implicated in non-inflammatory functions such as reproduction. PGHS2 expression is seen in granulosa cells of ovulatory follicles [23,24], and preliminary results in mice suggest that homozygous PGHS2 gene knockout results in infertility

Table 1. Inhibition of PGHS1 and PGHS2 cyclooxygenase activities by selected NSAIDs.

		IC_{50} values (nM) in PGHS1 and PGHS2 whole cell assays[a]		
		PGHS2	PGHS1	PGHS1/PGHS1
	Indomethacin	50	10	0.2
	Flurbiprofen	1	1	1
	Sulindac sulfide	100	100–1000	1–10
	Diclofenac	1	10	10
	Meclofenamic acid[b]	1–10	10–100	10
	Ibuprofen	1000	10 000	10
	Flosulide	50	>50 000	>1000
	NS-398[c]	1	>10 000	>10 000
	L-745 337	50	>10 000	>200

[a]Reproduced from [32].
[b]Time-dependent inhibitor for both PGHS1 and PGHS2.
[c]Time-dependent inhibitor for PGHS2 but not PGHS1.

Table 1 (continued).

		IC_{50} values (μM) in ovine PGHS1 and human PGHS2 assays[d]		
		PGHS2 (human)	PGHS1 (ovine)	PGHS1/PGHS2
	DuP 697[e]	7	38.5	5.5
	X6882	198	>300	1.5
	X6140	7.8	493	63.2
		IC_{50} values (μM) from PGHS1 and PGHS2 assays expressed in baculovirus[f]		
		PGHS2	PGHS1	PGHS1/PGHS2
	SC58125	0.05	>10	>200

[d]Reproduced from [33].
[e]Time-dependent inhibitor for PGHS2 but not PGHS1.
[f]See [6].

(H. Herschman & D.L. DeWitt, personal communication). Thus, careful evaluation will be necessary before PGHS2 inhibitors can be declared the winners in the NSAIDs sweepstakes.

PGHS peroxidase: a third prong in the NSAID assault?

Although a highly selective PGHS2 inhibitor may well be important in the development of NSAIDs with reduced gastrointestinal toxicity, recent data suggest that another approach is worth exploring. This approach was suggested by the fact that tepoxalin, a compound originally identified as a dual PGHS and 5-lipoxygenase inhibitor, shows potent anti-inflammatory effects without gastric toxicity in rat adjuvant arthritis [25]. The lack of gastric lesions cannot be explained by 5-lipoxygenase inhibition, since pretreatment with tepoxalin did not significantly reduce the damage induced by indomethacin. Intravital microscopy showed that tepoxalin did not cause leukocyte adherence to mesenteric venules, whereas other NSAIDs did (Anderson, D.W., *et al.*, & Capetola, R.J. (1990). *FASEB J.* **4**, A1142, and [26]). Close examination of the differences suggested that tepoxalin might inhibit neutrophil migration into the pin-hole stomach lesions initially caused by cyclooxygenase inhibition. Just such an effect was demonstrated in a mouse skin inflammation model, where tepoxalin effectively inhibited neutrophil infiltration and blocked upregulation of the adhesion molecules E-selectin and MAC-1 (our unpublished data).

The expression of adhesion molecules in inflammation (and also that of chemotactic factors and cytokines) is controlled by the inducible transcription factor NF-κB. The effect of tepoxalin on the expression of several NF-κB-dependent genes was therefore examined. Induction of E-selectin, MAC-1 and VCAM-1 in cultured leukocytes was suppressed by tepoxalin, and activation of NF-κB function was also inhibited ([27] and unpublished data). The drug also inhibited the upregulation of the NF-κB-dependent cytokines IL-2, IL-6, IL-8 and interferon-γ [27,28]. In addition to its anti-inflammatory properties, tepoxalin showed immunosuppressive effects in cultured cells,

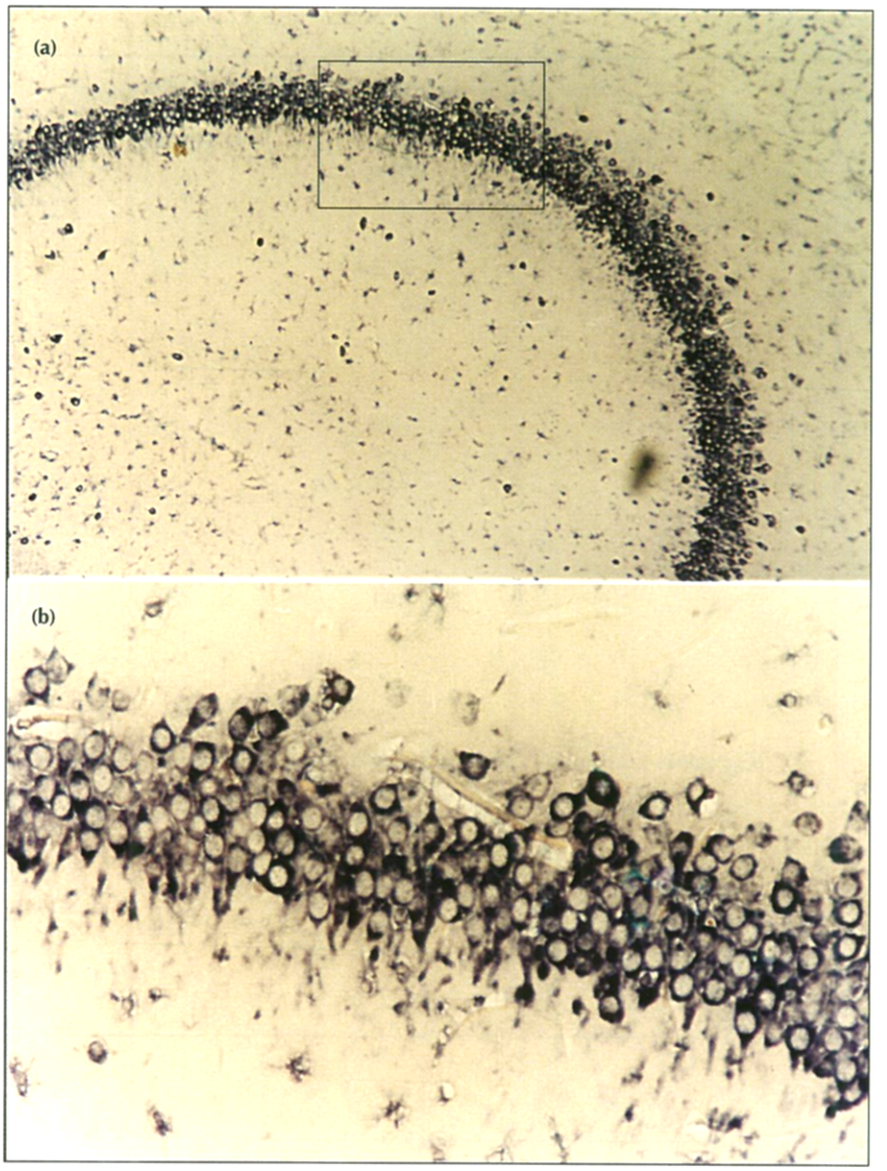


Fig. 4. Cell-specific expression of PGHS2 mRNA in rat brain. *In situ* hybridization was carried out with a digoxigenin-labeled, isoform-specific PGHS2 complementary RNA (unpublished data kindly provided by Dr Lubing Zhou). **(a)** Sagittal section showing a high density of PGHS2-positive hippocampal neurons. **(b)** Boxed area from (a) is shown at higher magnification.

inhibiting T-cell proliferation induced by IL-2 or PMA plus ionophore, and synergizes with cyclosporin A in suppressing mouse skin allograft rejection [29].

How can tepoxalin's actions be explained? NF-κB can be activated by many of the same stimuli that provoke prostaglandin synthesis, apparently using intracellular reactive oxygen intermediates (ROI) as a common second messenger [30]. We have found that PGHS1 can participate in activation of NF-κB by ROI (our unpublished data). Expression of the enzyme in COS cells dramatically enhanced NF-κB activation by PMA, as well as production of ROI. Both functions required intact peroxidase activity, but not cyclooxygenase activity, as shown by site-directed mutagenesis and NSAID inhibition. Therefore, we propose that PGHS1 may be important in activating NF-κB-dependent gene expression during inflammation.

The idea that PGHS1 peroxidase could signal to NF-κB via ROIs makes sense in the light of what is already known about the enzyme. PGHS1 peroxidase can reduce peroxides produced by superoxide dismutase or lipoxygenases, as well as by cyclooxygenase, and produces two radicals for each peroxide reduced. Using purified PGHS1, we found that tepoxalin but not naproxen or indomethacin inhibited PGHS1 peroxidase [31]. Certain analogs of tepoxalin that had previously been shown to inhibit NF-κB activation were also the most potent inhibitors of PGHS1 peroxidase activity. Structure–activity studies demonstrated that tepoxalin has two functionalities (Fig. 5). The substituted pyrazole inhibits PGHS1 cyclooxygenase, whether or not the hydroxamic acid group is present. The hydroxamic acid moiety, however, adds a peroxidase inhibitory functionality to the pyrazole structure, probably by interacting with the hemic iron. Thus, it seems likely that tepoxalin's novel mechanism of action stems from inhibition of PGHS1 peroxidase and subsequent suppression of NF-κB activation during inflammatory challenges.

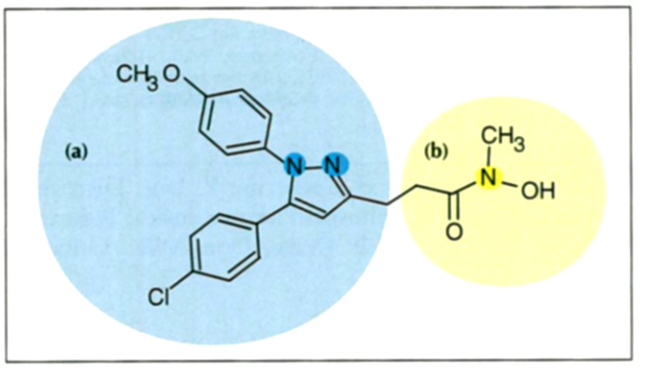


Fig. 5. The structural motifs associated with PGHS1 cyclooxygenase and peroxidase inhibition by tepoxalin. **(a)** The *bis*-substituted pyrazole group is required for high-affinity binding to PGHS1 and cyclooxygenase inhibition, associated with typical NSAID effects. **(b)** The hydroxamic acid group interacts with enzyme-bound heme, inhibiting free-radical release by PGHS1 peroxidase, and thereby blocking subsequent NF-κB activation.

Conclusions

The isolation of the PGHS2 isoform and the identification of PGHS peroxidase as an important contributor to NF-κB activation clearly open new avenues to the discovery of novel NSAIDs. Selective inhibitors of either PGHS1 or PGHS2 and dual inhibitors of PGHS1 cyclooxygenase and peroxidase are available. Interestingly, adding a hydroxamic acid arm to naproxen resulted in a dual cyclooxygenase/peroxidase inhibitor which inhibited NF-κB activation, like tepoxalin (our unpublished data). This may provide a general strategy for the design of other dual inhibitors based on known cyclooxygenase inhibitors, including those which show selectivity for PGHS2. In-depth investigation of these compounds *in vivo* will be necessary before we achieve the ultimate goal of a safe and potent NSAID without gastric toxicity. However, the current generation of NSAIDs provide new tools to dissect the processes controlling inflammation. Equally important, they point the way to future anti-inflammatory compounds with great therapeutic and economic potential.

Acknowledgements: We thank Dr Lubing Zhou for PGHS2 *in situ* hybridization in rat brain, and Dennis Argentieri for tissues from normal and adjuvant arthritic rats. Thanks also to many investigators for sharing results in advance of publication, and to Dr John Booth, Linda Traeger and Anna Bahnesli for help in preparing this manuscript.

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