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Short communication

# Etodolac selectively inhibits human prostaglandin G/H synthase 2 (PGHS-2) versus human PGHS-1

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**Abstract**

The isozymes of prostaglandin G/H synthase (PGHS) are shown to be differentially inhibited in vitro by currently marketed nonsteroidal anti-inflammatory drugs (NSAIDs) using microsomal rhPGHS-1 and rhPGHS-2. Comparison of selectivity ratios ($IC_{50}$ rhPGHS-1/$IC_{50}$ rhPGHS-2) demonstrated a 10-fold selectivity of etodolac (Lodine) for rhPGHS-2, whereas the other NSAIDs evaluated demonstrated no preference or a slight preference for inhibition of rhPGHS-1. In vitro enzyme results were supported by a human whole blood assay where etodolac also demonstrated a 10-fold selectivity for inhibition of PGHS-2 mediated $TxB_2$ production. Taken together, these data may be key to explaining the clinically observed gastrointestinal safety of etodolac versus other marketed NSAIDs.

*Keywords:* Prostaglandin G/H synthase (cyclooxygenase); Whole blood, human; Etodolac; Naproxen; Diclofenac; Tenidap

## 1. Introduction

The enzyme cyclooxygenase (prostaglandin G/H synthase, E.C. 1:14.99.1, PGHS) is the rate-limiting enzyme in the production of proinflammatory prostaglandins. Upregulation of PGHS activity and protein by inflammatory stimuli (e.g., IL-1$\beta$ stimulation of fibroblasts (Raz et al., 1988)), was demonstrated to result from induction of a new form of PGHS encoded by a 4.5 kb mRNA (Hla and Neilson, 1992; Kujubu and Herschman, 1992; Xie et al., 1992). This second form of PGHS (PGHS-2) was induced in inflammatory sites and cells, whereas the initially identified isoform of PGHS (PGHS-1) was constitutively expressed in most cells and encoded by a 2.8 kb mRNA. Therefore, PGHS-2 has been postulated to be responsible for proinflammatory prostaglandin production (e.g., at sites of inflammation by macrophages and synoviocytes). In contrast, PGHS-1, the constitutive form, is believed responsible for the production of physiological or homeostatic prostaglandin production (e.g., in the stomach and kidney) (Seibert and Masferrer, 1994).

The ability of NSAIDs (nonsteroidal anti-inflammatory drugs) to inhibit PG production has been well-documented (Smith et al., 1994); however, the relative effects of these drugs on the two PGHS isoforms have not been extensively investigated (Meade et al., 1993; Laneuville et al., 1994). In the present investigation, baculovirus/Sf9 cell expressed rhPGHS-1 and rhPGHS-2 and a human whole blood assay were employed to evaluate the activity of four NSAIDs against the two PGHS isoforms. The results demonstrate that etodolac (Lodine) possessed a 10-fold selectivity for inhibition of PGHS-2, whereas the others were either non-selective (e.g., diclofenac) or had a preference for PGHS-1 (e.g., naproxen and tenidap).

## 2. Material and methods

### 2.1. Cloning and expression of rhPGHS-1 and rhPGHS-2

The human PGHS-1 and PGHS-2 cDNA were cloned from human monocytes, untreated and LPS-treated, respectively, by RT-PCR using oligonucleotide primers based on the published hPGHS-1 and hPGHS-

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2 sequences (Jones et al., 1993). The monocytic PGHS-1 and PGHS-2 cDNAs are identical to those from human umbilical vein endothelial cells (Jones et al., 1993). Both cDNAs were inserted into BamHI to XbaI sites on pcDNA3 for sequencing, and then the BamHI-XbaI fragments were recloned into the baculovirus expression vector pVLI392 at the BglII to XbaI site for expression in Sf9 cells (Invitrogen, San Diego, CA, USA).

Sf9 cells ($1.8–2.2 \times 10^6$ cells/ml, viability > 98%) were infected at an MOI (multiplicity of infection) of 5 with high titer baculovirus stock for rhPGHS-1 or rhPGHS-2. The cells were cultured in spinner flasks for 72 h postinoculation (maximal expression time for PGHS-1 and -2) in Graces Insect media (10% FBS, 0.1% gentamycin) and harvested by centrifugation at $5000 \times g$ for 10 min. Cell pellets were frozen at −70° C until microsomes were prepared (Invitrogen, San Diego, CA, USA).

### 2.2. Microsomal preparation of transfected Sf9 cells

The frozen Sf9 cell pellet was resuspended in Tris buffer (pH 7.8) containing 1 mM PMSF, 100 μM leupeptin, 15 μM pepstatin, 1% FAF-BSA, and sonicated $2 \times 30$ s on 50% duty with a Branson Sonifier microprobe. The homogenate was centrifuged at 8000 $\times g$ for 15 min at 4° C, and the supernatant was then centrifuged at $100\,000 \times g$ for 60 min at 4° C. The microsomal pellet was washed once with Tris buffer (pH 7.8), 1 mM EGTA ($100\,000 \times g$ for 1 h) without protease inhibitors, solubilized in 50 mM Tris buffer (pH 8.0), 10 mM EDTA, 1 mM diethyldithiocarbamic acid with 1% Tween 20 by sonication, and centrifuged at $100\,000 \times g$ for 30 min. The supernatant was aliquoted and frozen at −70° C until utilized in the enzyme assay. Protein was quantitated using the Bradford assay with bovine serum albumin as a standard. Microsomal preparations had specific activities of 0.59 μg $PGF_{2\alpha}$/min/mg protein for rhPGHS-1 and 6.0 μg $PGF_{2\alpha}$/min/mg protein for rhPGHS-2.

### 2.3. In vitro rhPGHS-1 and rhPGHS-2 assays

Recombinant enzymes were reconstituted in buffer (100 mM Tris, pH 7.8 at 37° C) containing 0.5 mM phenol (964 μl total volume). The enzyme preparations were preincubated with vehicle (DMSO) or compounds in DMSO (1% DMSO in final assay) for 30 min at 37° C. Excess hematin was added 1 min prior to initiation of reaction (1.25 μM final hematin) with 30 μM arachidonic acid (sodium salt). The final assay volume was 1.0 ml (100 mM Tris (pH 7.8), 0.5 mM phenol, 1.25 μM hematin and 30 μM arachidonic acid at 37° C).

The reaction was incubated for 35 s (maximum level of $PGH_2$ accumulation as determined from time course studies), and terminated by addition of 50–60 μl of $SnCl_2$ (1 mg/ml) in 0.1 N HCl. $PGH_2$ is quantitatively converted to $PGF_{2\alpha}$ by this reaction (50% efficiency of total conversion). The pH in each tube was adjusted to pH 3.0–3.5 with 1.0 N HCl and extracted twice with 1.5 ml of ethyl acetate (75–90% efficiency per extraction). Combined ethyl acetate layers were dried under $N_2$ (g) and redissolved in EIA buffer (2.0 ml), and $PGF_{2\alpha}$ quantitated by EIA.

### 2.4. Human whole blood assay for PGHS-1 and PGHS-2 mediated $TxB_2$ production

Human whole blood (heparinized) was obtained from Biological Specialties (Landsdale, PA, USA). The blood was aliquoted (2.0 ml) and pre-incubated with compound or vehicle (DMSO, 0.5% final) for 15 min with gentle shaking in a 37° C 5% $CO_2$ incubator. For the PGHS-1 assay, the blood was incubated with gentle shaking in a 5% $CO_2$ incubator for an additional 4.5 h and then stimulated with 20 μM A23187 for 30 min. For the PGHS-2 assay, after the preincubation, LPS (5 μg/ml) was added for an additional 5 h. The incubations were terminated with EGTA (10 mM) on ice; methanol was added (70% final); and proteins were precipitated at −70° C overnight. The samples were centrifuged ($200 \times g$ for 10 min at 4° C); the methanol was dried; the residue redissolved in EIA buffer; and $TxB_2$ measured by specific EIA.

### 2.5. Statistical analysis

Percent inhibition was calculated relative to control (DMSO vehicle) for each experiment with each data point performed in duplicate (whole blood assay) or in triplicate (enzyme assay). A minimum of three ($n = 3$) experiments/donors were combined and dose-response data analyzed by the non-linear logistic model using SAS-JMP.

### 2.6. Materials

Phenol and hematin stock solutions were obtained from Oxford Biochemical (Oxford, MI, USA). Arachidonic acid, Tris buffer, diethyldithiocarbamic acid, EDTA, EGTA, DTT, PMSF, HCl, $SnCl_2$, leupeptin, and pepstatin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). EIA kits for $TxB_2$ and $PGF_{2\alpha}$ were obtained from Cayman Chemical (Ann Arbor, MI, USA). Etodolac, pemedolac, oxaprozin, indomethacin, diclofenac, naproxen, tenidap and 6-MNA (6-methoxynaphthylacetic acid) were synthesized by the Global Chemical Sciences Division, Wyeth-Ayerst Research, using published synthetic protocols.

## 3. Results

### 3.1. Microsomal assay of rhPGHS-1 and rhPGHS-2

rhPGHS activity was measured following conversion of $PGH_2$, the major baculovirus/Sf9 cells-expressed rhPGHS product (Barnett et al., 1994), to $PGF_{2\alpha}$ using $SnCl_2$/HCl. Maximal $PGH_2$ (quantitated by specific EIA for $PGF_{2\alpha}$) formation was observed at 35 S (pH 7.8, 37° C) in the presence of 30 $\mu$M arachidonic acid (6–10 times reported $K_m$ for these isozymes (Laneuville et al., 1994)). A decline in $PGH_2$ levels after 35 S was observed due to non-enzymatic formation of oxygenated arachidonic acid species other than $PGF_{2\alpha}$ (e.g. $PGE_2$, data not shown). The reaction was linear with respect to the concentration of enzyme (6–38 $\mu$g/ml for both rhPGHS-1 and rhPGHS-2) and arachidonic acid (1 to 100 $\mu$M) (data not shown).

### 3.2. Evaluation of NSAIDs using the microsomal assay

In order to ascertain whether etodolac or several reference NSAIDs selectively inhibited one of the PGHS isotypes, the in vitro microsomal assay was employed (Table 1). Etodolac inhibited both the rh-PGHS-1 and rhPGHS-2 in a concentration-dependent manner; the $IC_{50}$ values (Table 1) indicate etodolac has a 10-fold selectivity for inhibiting rhPGHS-2. In contrast, indomethacin, diclofenac, naproxen, (+)-pemedolac (Mobilio et al., 1988) and tenidap inhibited both PGHS isozymes and demonstrated no selectivity for PGHS-2. Diclofenac and (+)-pemedolac were equipotent against rh-PGHS-1 and -2 whereas naproxen, indomethacin, oxaprozin and tenidap were PGHS-1 selective inhibitors (Table 1). The active metabolite of nabumetone (6-MNA) was inactive in our assays up to 1000 $\mu$M. The (−) enantiomer of etodolac was essentially inactive at concentration up to 100 $\mu$M, whereas the (+) enantiomer of etodolac produced an $IC_{50}$ of 1.7 $\mu$M (1.5–1.9, 95% C.L.) for rhPGHS-2. This was also demonstrated with the (−) enantiomer of pemedolac which was 1500–2000 fold less potent than (+) pemedolac (Table 1). Oxaprozin was a weak NSAID using this 30 min preincubation protocol and demonstrated the greatest PGHS-1 selectivity (< 0.01) (Table 1).

### 3.3. Human whole blood assay for PGHS-1 and -2 mediated $TxB_2$ production

Calcium ionophore stimulation of human whole blood produces PGHS-1 derived $TxB_2$, predominantly originating from platelets. Aspirin potently inhibited $TxB_2$ with an $IC_{50}$ of 3.9 $\mu$M. Stimulation of blood with LPS results in a PGHS-2 mediated $TxB_2$ response, which is weakly inhibited by aspirin ($IC_{50}$ = 81 $\mu$M). At concentrations of aspirin which completely abolish PGHS-1 derived $TxB_2$ production (e.g., 10 $\mu$M), no reduction in LPS-generated $TxB_2$ was noted, consistent with the expectation that LPS stimulation reflects predominantly PGHS-2 derived products. Under this paradigm, as shown in Fig. 1, etodolac inhibited PGHS-2 derived $TxB_2$ production ($IC_{50}$ value of 3.4 $\mu$M) at a 10-fold lower concentration than PGHS-1-derived $TxB_2$ ($IC_{50}$ of 34 $\mu$M). Naproxen, in contrast, equally inhibited PGHS-1 and PGHS-2-derived $TxB_2$

Table 1
Comparison of NSAID $IC_{50}$ values for microsomal rh-PGHS-1 [a] and rh-PGHS-2 [a] using a 30 min pre-incubation time with the enzyme prior to initiation of the reaction [95% confidence intervals are given in brackets]

| NSAID | $IC_{50}$ ($\mu$M) [b] | | Ratio (PGHS-1/PGHS-2) |
|---|---|---|---|
| | PGHS-1 | PGHS-2 | |
| Etodolac | 15 [10–26] | 1.4 [0.9–2.0] | 10.7 |
| (+)-Pemedolac | 0.15 [0.12–0.20] | 0.21 [0.05–0.40] | 0.71 |
| (−)-Pemedolac | 233 | 451 | 0.52 |
| Naproxen | 1.6 [0.8–3.3] | 21 [8.9–49] | 0.08 |
| Diclofenac | 0.015 [0.012–0.019] | 0.022 [0.017–0.029] | 0.68 |
| Indomethacin | 0.013 [0.011–0.015] [d] | 0.074 [0.056–0.099] | 0.18 |
| Tenidap | 1.6 [1.1–2.4] | 3.1 [2.2–4.2] | 0.52 |
| 6-MNA [c] | N.A. (100–1000) | N.A. (100–1000) | – |
| Oxaprozin | 97% (1000) | (1000) | ≪ 1.0 |
| | 14.9 [13.5–16.5] [d] | > 1000 | < 0.01 |

[a] rhPGHS-1 and rhPGHS-2 were expressed in the baculovirus/Sf9 cell system. Enzyme assays were performed using solubilized microsomes from transfected Sf9 cells. Compounds were pre-incubated with the enzyme source for 30 min prior to initiation of the reaction with arachidonic acid (30 $\mu$M). After 35 s, PGHS-2 was converted to $PGF_{2\alpha}$ with $SnCl_2$/HCl (1 mg/ml $SnCl_2$ in 0.1 N HCl), extracted twice with ethylacetate, and $PGF_{2\alpha}$ was quantitated by specific EIA. [b] Dose-response studies were performed in at least three separate experiments with each data point performed in triplicate. $IC_{50}$ values and 95% confidence intervals [given in brackets] were determined by non-linear regression analysis of the log dose-response curves (SAS-JMP). [c] 6-MNA is 6-methoxynaphthylacetic acid, the active metabolite of nabumetone. [d] $IC_{50}$ value determined with solubilized microsomal human platelet PGHS-1 prepared from human platelets provided by platelet phoresis (Biological Specialty; Lansdale, PA, USA) as described for Sf9 cell microsome preparation (Materials and methods).

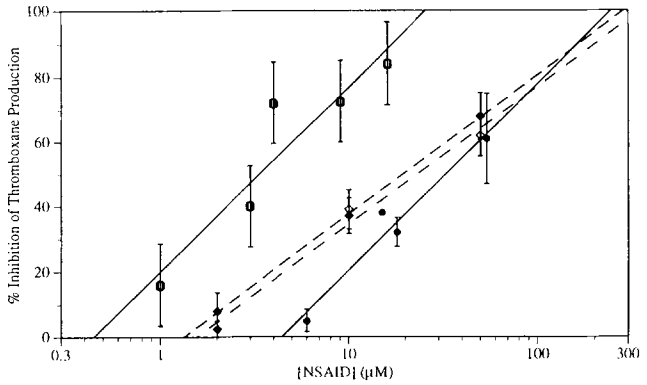


Fig. 1. Effect of etodolac and naproxen on human whole blood $TxB_2$ production. Etodolac (●,○) and naproxen (◆,◇) were pre-incubated for 15 min prior to buffer with DMSO (0.5%) or compound in DMSO (0.5%). For PGHS-1 activity (●,◇), after an additional 4.5 h the blood was stimulated with calcium ionophore A23187 (20 μM) for 30 min (control $TxB_2$ levels were 168–416 ng/ml). For PGHS-2 activity (○,◇) LPS (5 μg/ml) was added for an additional 5 h and the blood was assayed for $TxB_2$ production (control $TxB_2$ levels were 23–41 ng/ml). Each data point is the mean of three donors each performed in duplicate ($n = 3$). Log dose-response curves were analyzed using a linear regression model. $IC_{50}$ values [95% confidence intervals] for etodolac were 3.4 μM [1.2, 9.8] for PGHS-2 and 34 μM [8.0, 221] for PGHS-1; and for naproxen were 23 μM [5.5, 121] for PGHS-2 and 20 μM [5.0, 91] for PGHS-1.

with $IC_{50}$ values of 20 μM and 23 μM, respectively. Oxaprozin demonstrated PGHS-1 selectivity in this assay at 100 μM showing 80% inhibition of PGHS-1 derived $TXB_2$ and no inhibition of PGHS-2 derived $TxB_2$.

## 4. Discussion

The discovery of a second inducible form of PGHS provides an avenue to develop NSAIDs with improved safety profiles. The hypothesis has been proposed that the PGHS-1 enzyme, being constitutive in nature, is the likely candidate to produce homeostatic or physiological prostaglandins which are known to be cytoprotective in the gastric mucosa and to regulate kidney function; and PGHS-2, the form induced by inflammatory stimuli, is responsible for proinflammatory prostaglandin production (Seibert and Masferrer, 1994). Therefore, selective inhibitors of the PGHS-2 isoform may have much improved gastrointestinal and renal safety profiles as compared to non-selective inhibitors of PGHS-1 and -2 (Meade et al., 1993; Seibert and Masferrer, 1994). Since most NSAIDs were developed before the existence of the PGHS-2 isoform had been identified, we evaluated several NSAIDs to test their effectiveness (potency) and selectivity for human PGHS-2.

Meade et al. (1993) were the first to publish data on the effects of a panel of NSAIDs on recombinant murine PGHS-1 and PGHS-2. Instantaneous competitive inhibition was determined by oxygen uptake with microsomal PGHS preparations from COS-1 transfected cells. These studies demonstrated a general selectivity for PGHS-1 inhibition by NSAIDs (> 70-fold preference by indomethacin). 6-MNA, the active metabolite of nabumetone, demonstrated a selectivity for murine PGHS-2; however, upon evaluation with recombinant human enzymes, 6-MNA demonstrated a selectivity for rhPGHS-1 (Laneuville et al., 1994). Using instantaneous-competitive inhibition, etodolac demonstrated a slight selectivity for rhPGHS-2 ($IC_{50}$ = 60 μM for PGHS-2 and 74 μM for PGHS-1). Etodolac was the only NSAID evaluated, out of 11, which demonstrated the slightest selectivity for inhibition of rhPGHS-2 (Laneuville et al., 1994). The data from Laneuville et al. (1994) reflect the affinity (reversible binding phenomenon) of these inhibitors for the PGHS enzymes whereas our assay reflects the time-dependent inhibition observed with these NSAIDs. This difference between assay methods explains the order(s) of magnitude differences between the NSAID $IC_{50}$ values. The time-dependent inhibition observed in vitro may more closely reflect the clinical behavior of these NSAIDs, as aspirin and some other NSAIDs are unable to inhibit PGHS enzymes using instantaneous competitive inhibition measurements as per Laneuville et al. (1994).

The present investigation was undertaken as a result of cellular and clinical data with etodolac which demonstrated selective inhibition of inflammatory prostaglandin production and a safe side effect profile (e.g. from GI microbleeding studies) relative to other NSAIDs. In the assay utilized, a 30 min preincubation of inhibitor with enzyme was chosen to mimic clinical situations where cells/enzymes would be in contact with the inhibitor for extended periods of time (Barnett et al., 1994). Under these experimental conditions, etodolac had a 10-fold selectivity for inhibition of rhPGHS-2. These results are consistent with previous cellular data using human gastric mucosal cells and IL-1 stimulated human synoviocytes ($PGE_2$ generation via predominantly PGHS-1 and PGHS-2, respectively) where etodolac demonstrated a 7.9-fold selectivity for inhibition of $PGE_2$ production in the synoviocytes (Adams et al., 1990). Inoue et al. (1994) also demonstrated an increased selectivity of etodolac for inhibition of $PGE_2$ production in IL-1β stimulated rabbit articular chondrocytes versus rabbit gastric epithelial cells or MDCK cells. To further support these observations, a human whole blood assay was developed where calcium ionophore treatment represents a PGHS-1 response (sensitive to aspirin inactivation) and LPS stimulation (5 h) represents predominantly a PGHS-2 re-

sponse. Etodolac also demonstrated a 10-fold selectivity for inhibition of PGHS-2, whereas naproxen equally inhibited PGHS-1 and -2, aspirin and oxaprozin more selectively inhibited PGHS-1.

Etodolac's selectivity for PGHS-2 inhibition may also be inferred from in vivo models where PGHS-2 would be the dominant isoform (Seibert and Masferrer, 1994). Etodolac is more potent in chronic models of inflammation (established adjuvant arthritis) as opposed to the acute models (e.g., carrageenan paw edema). Furthermore, etodolac is a much more potent inhibitor of inflammatory pain (Randall-Selitto) than chemically induced acute pain (phenylbenzoquinone writhing) (Humber, 1993). Taken together, these observations suggest that the observed selectivity of etodolac for PGHS-2 in vitro may be reflected in its in vivo pre-clinical profile.

Clinical data with etodolac also suggests improved gastrointestinal safety when compared with other NSAIDs (Arnold, 1991). Recent studies in man demonstrate a much weaker effect of etodolac on inhibition of gastric mucosal prostaglandin production (Laine et al., 1995). Thus etodolac's 10-fold selectivity for inhibition of PGHS-2 is consistent with in vivo animal models and clinical findings that show an improved gastrointestinal safety profile.

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