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COMPARATIVE EFFECTS OF SELECTIVE CYCLOOXYGENASE 1 AND CYCLOOXYGENASE 2 INHIBITORS ON MYELOPEROXIDASE AND 3α-HYDROXYSTEROID DEHYDROGENASE

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The clinical efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) is believed to result from the ability of these compounds to inhibit the inducible isoform of the enzyme cyclooxygenase, COX2. The gastrointestinal and renal side effects of these drugs, in contrast, are thought to relate to their ability to inhibit the constitutive isozyme, COX1. There is structural and pharmacological evidence that suggests that NSAIDs may also inhibit two unrelated enzymes, myeloperoxidase (MP) and 3α-hydroxysteroid dehydrogenase (3α-HSD), potentially with untoward consequences for the patient. Our laboratories have been investigating a new structural class of potential COX inhibitors, the tri-cyclic aromatics. In this study we have examined the inhibitory potency of selected compounds for the enzymes human COX1, human COX2, human MP, and rat liver 3α-HSD. The compounds selected span a range of COX isoform selectivities, from specific for COX2 to selective for COX1 only, and include three representative tri-cyclic aromatics. We find that compounds within the tri-cyclic aromatic class do not act as potent inhibitors of either myeloperoxidase or 3α-HSD. These results demonstrate the unique inhibitor selectivity that can be achieved with the tri-cyclic aromatics. Examples of COX1 selective, and COX2 selective inhibitors within this structural class are presented.

KEY WORDS: Cyclooxygenase 1, cyclooxygenase 2, myeloperoxidase, 3α-hydroxysteroid dehydrogenase

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly prescribed class of compounds for treatment of the pain, fever, swelling and other symptoms associated with inflammation. These beneficial properties are thought to result from the ability of these compounds to inhibit the activity of the enzyme prostaglandin G/H synthase (EC 1.14.99.1; also known as cyclooxygenase, or COX), a key enzyme in the biosynthesis of pro-inflammatory prostaglandins.[1] These drugs also invariably display severe side effects,

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particularly renal damage and gastrointestinal (g.i.) ulcerogenicity. As with their beneficial properties, it is thought that the side effects associated with NSAIDs derive from their ability to inhibit prostaglandin synthesis in the g.i. tract and kidneys.[2]

Recently, it was discovered that two isozymes of COX exist in human; one is a constitutive form that is expressed in a variety of tissues, including g.i. and kidney (COX1), while the other is induced in response to inflammatory stimuli and is mainly expressed in cells associated with inflammation (COX2).[3,4] These observations have led to the hypothesis that the anti-inflammatory properties of NSAIDs are due to their ability to inhibit COX2, while their ulcerogenicity is related to inhibition of COX1. This has sparked significant effort within the pharmaceutical community to identify compounds that would selectively inhibit COX2, as a means of providing anti-inflammatory efficacy without the associated side effects of current NSAIDs. A number of compounds are now known to selectively inhibit COX2.[5–7] In particular, our laboratories have found that compounds within the general class of tri-cyclic aromatics, in which the A and C rings are substituted phenyl rings and the intervening B ring is a five or six member aromatic ring, can exhibit either COX1 or COX2 selective inhibition.[7] Figure 1 displays the structures for three compounds within this general series that were used here, along with the structures for two well known COX inhibitors, indomethacin and ibuprofen. Within the tri-cyclic aromatics, incorporation of a methyl sulfonyl on either the A or C ring imparts COX2 selectivity to these compounds; **III** and **IV** are examples of such inhibitors. Replacement of this methyl sulfonyl with a methoxy group, as in **V**, switches the isozyme specificity of these inhibitors to greatly favor COX1. Both *in vitro* and *in vivo* studies with compounds demonstrating COX2 selective inhibition have provided support for the hypothesis that anti-inflammatory efficacy and g.i. and renal toxicity can be segregated by selective inhibition of the appropriate COX isozyme.[5–9]

Continued efforts towards more effective inhibitors of COX2 would benefit from structural insights into differences in the active site structures between this enzyme and COX1. No crystallographic data has yet been reported for COX2; however, Garavito's group recently reported the crystal structure of ovine COX1 at 3.5 Å resolution.[10] These workers noted a striking similarity between the active site structure of COX1 and that of myeloperoxidase, another heme-dependent peroxidase whose crystal structure has been solved.[11] The similarity in active site structures for these two enzymes raises the question of whether NSAIDs might also display inhibitory potency against myeloperoxidase. NSAIDs have also been suggested to act as inhibitors of another enzyme, 3α-hydroxysteroid dehydrogenase (3α-HSD). The ability of NSAIDs to inhibit this enzyme has been correlated with the recommended daily dose of NSAIDs for anti-inflammatory therapy.[12]

The ability of a COX2 inhibitor to also inhibit COX1, MP, or 3α-HSD could potentially limit the safety of such a compound as a drug. It is thus important to determine the inhibitory selectivity of potential NSAIDs for these enzymes. In the present paper we have investigated the ability of selected compounds to inhibit human COX1 and COX2, human myeloperoxidase, and rat 3α-HSD. Indomethacin and ibuprofen were selected for this study as representative current NSAIDs that display COX1 selectivity or non-selective isozyme inhibition, respectively. We have also investigated three experimental compounds that belong to the common structural class of tri-cyclic aromatic compounds, and display selectivity towards either COX1 or COX2.

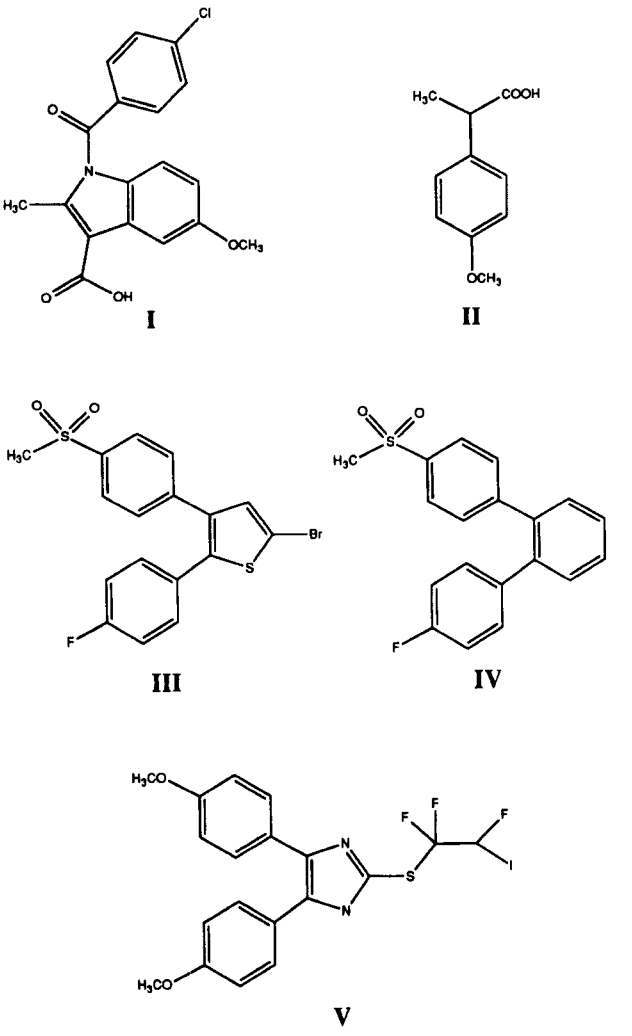

FIGURE 1 Structures of the COX inhibitors used in this study: **(I)** Indomethacin, **(II)** Ibuprofen, **III–V**, experimental tri-cyclic aromatics.

MATERIALS AND METHODS

Ibuprofen, indomethacin, gelatin, hematin, N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), androsterone, β-NAD^+, and 4-aminoantipyrine (4-AAP) were purchased from Sigma. Hydrogen peroxide was purchased from J.T. Baker, and arachidonic acid was

purchased from Nu-Chek Prep., Inc. The experimental compounds **III–V**, were synthesized by the Medicinal Chemistry Department of DuPont Merck Research Laboratories. All other reagents were the highest grades commercially available. All aqueous solutions were prepared with distilled, deionized water.

Human cyclooxygenase 1 and 2 (hCOX1 and hCOX2) were expressed in insect cells and purified as described by George *et al.*[13] The purified enzymes were flash-frozen in a dry ice/ethanol bath and stored at −70°C until use. Human leukocyte myeloperoxidase (MP) was purchased from Sigma as a lyophilized powder with sodium acetate buffer. The powder was rehydrated with distilled water to form a 10 unit/ml solution in 20 mM sodium acetate, pH 6.0. This solution was divided into 50 μl aliquots, flash-frozen in a dry ice/ethanol bath, and stored at −70°C until use. Rat liver 3α-hydroxysteroid dehydrogenase (3α-HSD) was partially purified from rat liver as described by Penning and coworkers.[14] The liver cytosol, containing the enzyme, was concentrated to 68 mg/ml total protein (Biuret assay), divided into 2 ml aliquots, and then frozen and stored as described for the other enzymes (*vide supra*).

Protein concentrations were determined by either the Biuret or Bradford assays as outlined in Copeland.[15] The enzymatic activity of hCOX1 and hCOX2 were assayed in the presence and absence of inhibitors and described by Copeland *et al.*[7] 3α-HSD enzymatic activity was monitored in the presence and absence of inhibitors as described by Penning and coworkers.[12] MP activity, with and without inhibitors, was assayed at 25°C by following the hydrogen peroxide-dependent oxidation of 4-AAP at 490 nm. The assay was performed in 96 well microtiter plates as follows. A stock 4-AAP solution was prepared by dissolving 430 mg of phenol and 25 mg of 4-AAP in 40 ml of distilled water. Each well of the plate receive 90 μl of this stock solution, 65 μl of buffer (40 mM potassium acetate, pH 6.0 containing 0.2% Tween-20), 25 μl of an inhibitor stock solution in dimethyl sulfoxide (DMSO) to yield the desired final drug concentration or, in the case of control wells, 25 μl of neat DMSO, and 10 μl of the stock MP solution (10 units/ml). The reaction in each well was then initiated by addition of 10 μl of an 18 mM hydrogen peroxide solution using a multichannel pipetor. In control experiments we monitor the color formation at 490 nm continuously over a 25 min time period to establish a convenient time window over which linear product formation with time was observed. Subsequently, we routinely performed the assay by allowing the reaction to proceed for 15 min before reading the absorbance at 490 nm as an endpoint assay. The initial velocity of the reaction was then calculated by dividing this absorbance reading by 15 to provide velocity measurements in units of mO.D./min. Blank wells were also run as described above except that the 10 μl of hydrogen peroxide substrate were substituted with 10 μl of distilled water. Absorbance values for the blank wells were subtracted from the measurements for control and experimental wells.

RESULTS

To determine the effects of each inhibitor on the enzymes studied here the individual enzymes were incubated with varying concentrations of the inhibitors for 1–2 min before initiation of the enzymatic reactions with substrate. Dose-response plots were then generated

TABLE 1
Comparison of the inhibitor potency of selected compounds for inhibition of hCOX1, hCOX2, 3α-HSD, and MP.

Compound	K_i Value[a] (μm)			
	hCOX1[b]	hCOX2[b]	3α-HSD	MP
Indomethacin (**I**)	0.03±0.02	20.71±3.80	3.29±0.30	>100[c]
Ibuprofen (**II**)	1.04±0.12	0.92±0.35	76.80±28.2	>100[c]
III	4.37±2.30	0.82±0.62	>100[c]	>100[c]
IV	>1000[c]	1.29±0.29	>100[c]	>100[c]
V	0.02±0.01	45.33±11.50	>100[c]	>100[c]

[a] K_i values represent the mean ± standard deviations of 2 or more replicate studies.

[b] Measured with a fixed preincubation time of 2 min. See Copeland *et al.* for a description of the time dependence of these inhibitors.[6,7]

[c] These compounds demonstrated less than 20% inhibition at concentrations as high as that stated.

with these data, and the inhibitor IC_{50} value was determined by fitting the data to equation (1) by non-linear least squares method:

$$y = \frac{100}{1 + \left(\frac{[I]}{IC_{50}}\right)} \tag{1}$$

Here y is the percent control activity observed at inhibitor concentration $[I]$. The IC_{50} values thus obtained were converted to K_i values by the equation of Cheng and Prusoff, assuming competitive inhibition.[16] These results are summarized in Table 1.

It is clear from Table 1 that none of the COX inhibitors studied here display potent inhibition of human MP. For 3α-HSD we observed K_i values for indomethacin and ibuprofen that are similar to those previously reported by Penning and coworkers.[12,14] This enzyme was not, however, inhibited by any of the experimental tri-cyclic aromatic compounds tested here, despite the potent inhibition of COX isozymes displayed by these compounds. In previous studies, our laboratory has shown that **III** and **IV** are time dependent selective inhibitors of hCOX2,[6,7] while **V** is a time dependent selective inhibitor of hCOX1.[7] The assay conditions used for MP activity provided for prolonged contact between the enzyme and inhibitors, so that any time dependent inhibition of the enzyme by these compounds would have been manifested in the standard assay. For 3α-HSD we performed separate experiments in which the enzyme and inhibitor were preincubated for 5 min prior to initiation of the enzymatic reaction with substrate. This extended preincubation time had no effect on the enzymatic activity displayed in the presence of varying concentrations of the experimental compounds (data not shown). We thus conclude that none of these compounds are capable of inhibiting 3α-HSD in a time dependent fashion.

DISCUSSION

We were motivated to perform the current study by two previous reports in the literature. In the first, Garavito and coworkers pointed out a significant degree of active site structural homology (ca. 70%) between ovine COX1 and MP.[10] Although the two enzymes display quite distinct substrate specificities, they both catalyze peroxidase activities during normal turnover. We were concerned, based on the putative structural similarities and the reaction similarities of these two enzymes, that compounds designed to specifically inhibit hCOX2 activity might also display inhibition of MP. The second report that stimulated our current interest was the demonstration by Penning and coworkers that many non-steroidal anti-inflammatory drugs showed inhibitor potency towards 3α-HSD that appeared to correlate with their therapeutic anti-inflammatory efficacy.[12] Since it is widely believed that the clinical efficacy of NSAIDs results from the ability of these drugs to inhibit COX2 activity, we wondered if the selective hCOX2 inhibitors that we had developed would proved effective inhibitors of 3α-HSD as well. Despite the reported structural and pharmacological similarities between hCOX and MP and 3α-HSD, however, no correlation exists between the inhibitory potency of our experimental compounds for these varied enzymes. We had speculated that inhibition of MP and/or 3α-HSD might correlate specifically with the inhibition for one of the two human isozymes of COX. For this reason we tested compounds within the tri-cyclic aromatic class that span a broad range of hCOX isozyme specificity: **III** and **IV** are hCOX2 selective compounds, while **V** is selective for hCOX1. The inhibitory patterns displayed by this set of compounds for MP and 3α-HSD did not resemble those seen for either hCOX1 or hCOX2. We conclude, therefore that these compounds do not represent potent inhibitors of either MP or 3α-HSD.

The present data provide further evidence of the unique selectivity for hCOX isozyme inhibition displayed by compounds of the tri-cyclic aromatic family. Compounds within this class can be optimized for selective inhibition of either hCOX1 or hCOX2. Unlike currently prescribed NSAIDs, these compounds further do not display any ability to inhibit the unrelated enzyme 3α-HSD. The unique selectivity of this general class of compounds may therefore prove useful in the design of new therapeutic agents for the safe treatment of inflammatory diseases.

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References

1. Vane, J.R. (1971) *Nature New Biology*, **231**, 232.
2. Whittle, B.J.R., Higgs, G.A., Eakins, K.E., Moncada, S. and Vane, J.R. (1980) *Nature (Lond.)*, **284**, 271.
3. Maier, J.A., Hla, T. and Maciag, T. (1990) *J. Biol. Chem.*, **265**, 10805.
4. O'Banion, M.K., Winn, V.D. and Young, D.A. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 4888.
5. Futaki, N., Takahashi, S., Yokoyama, M., Arai, I., Higuchi, S. and Otomo, S. (1994) *Prostaglandins*, **47**, 55.
6. Copeland, R.A., Williams, J.M., Giannaras, J., Nurnberg, S., Covington, M., Pinto, D., Pick, S. and Trzaskos, J.M. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 11202.

7. Copeland, R.A., Williams, J.M., Rider, N.L., Van Dyk, D.E., Giannaras, J., Nurnberg, S., Covington. M., Pinto, D., Magolda, R.L. and Trzaskos, J.M. (1995) *Med. Chem. Res.*, **5**, 384.
8. Gans, K.R., Galbraith, W., Roman, R.J., Haber, S.B., Kerr, J.S., Schmidt, W.K., Smith, C., Hewes, W.E. and Ackerman, N.R. (1990) *J. Pharmacol. Exp. Ther.*, **254**, 180.
9. Masferrer, J.L., Zweifel, B.S., Manning, P.T., Hauser, S.D., Leahy, K.M., Smith, W.G., Isakson, P.C. and Siebert, K. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 3228.
10. Picot, D., Loll, P.J. and Garavito, M.R. (1994) *Nature (Lond.)*, **367**, 243.
11. Fenna, R.E. and Zeng, J. (1992) *J. Mol. Biol.*, **226**, 185.
12. Penning, T.M. and Talalay, P. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4504.
13. George, H., Van Dyk, D.E., Straney, R.A., Trzaskos, J.M. and Copeland, R.A. (1995) *Protein Express. Purif.* in press.
14. Penning, T.M. (1985) *J. Pharm. Sci.*, **74**, 651.
15. Copeland, R.A. (1994) *Methods for Protein Analysis: A Practical Guide to Laboratory Protocols*. New York: Chapman and Hall.
16. Cheng, Y.-C. and Prusoff, W.H. (1973) *Biochem. Pharmacol.*, **22**, 3099.