Kinetic basis for selective inhibition of cyclo-oxygenases

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Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the formation of prostaglandins by cyclo-oxygenases (COX). The discovery of a second COX isoform (COX-2) associated with inflammation led to agents that selectively inhibit COX-2, e.g. celecoxib. We evaluated the kinetics of inhibition of celecoxib and several NSAIDs. Celecoxib displays classic competitive kinetics on COX-1 ($K_i = 10\text{--}16\ \mu\text{M}$). An initial competitive interaction with COX-2 can also be discerned with celecoxib ($K_i = 11\text{--}15\ \mu\text{M}$), followed by a time-dependent interaction leading to potent inhibition, characterized as inactivation ($K_{\text{inact}} = 0.03\text{--}0.5\ \text{s}^{-1}$). Half-maximal inhibition (IC_{50}) using end-point assays reflects the competitive component on COX-1 ($\text{IC}_{50} = 4\text{--}19\ \mu\text{M}$) and the inactivation component on COX-2 ($\text{IC}_{50} = 0.003\text{--}0.006\ \mu\text{M}$). NSAIDs exhibit four distinct modes of COX inhibition based on kinetic behaviour: (1) competitive, e.g.

ibuprofen; (2) weak binding, time-dependent, e.g. naproxen, oxicams; (3) tight binding, time-dependent, e.g. indomethacin; (4) covalent, e.g. aspirin. In addition, most NSAIDs display different kinetic behaviour for each isoform. Weakly binding inhibitors show variable behaviour in enzyme assays, with apparent inhibitory activity being markedly influenced by experimental conditions; determination of kinetic constants with this class is unreliable and IC_{50} values are strongly dependent on assay conditions. Although IC_{50} determinations are useful for structure/activity analyses, the complex and distinct mechanisms of enzyme inhibition of each COX isoform by the NSAIDs renders comparison of inhibitory activity on COX-1 and COX-2 using IC_{50} ratios of questionable validity.

Keywords: celecoxib, inhibitors, prostaglandin synthase.

INTRODUCTION

Cyclo-oxygenase (COX), also known as prostaglandin synthase, catalyses two separate reactions, the first being the addition of 2 moles of molecular oxygen to arachidonic acid to form the unstable endoperoxide, prostaglandin G_2 (PGG_2). The second step is a further conversion of PGG_2 into PGH_2 by a peroxidase function. Hence, this enzyme performs the critical initial reaction in the arachidonic metabolic cascade leading to the formation of the pro-inflammatory prostaglandins, thromboxanes and prostacyclins [1,2]. The anti-inflammatory effects of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) are mainly due to their ability to inhibit prostaglandin production by COX [3–5].

Two distinct forms of COX have been identified, based on DNA sequence and expression. A constitutive form (COX-1) has been linked to the production of prostaglandins, which are physiologically important for the maintenance of gastric and renal homeostasis [6]. A second form of COX (COX-2) has recently been identified [7–10] whose expression is induced by cytokines and growth factors. Expression of COX-2 is linked to inflammatory cell types and tissues, and it is this form that is believed to be the target enzyme responsible for the anti-inflammatory activity of NSAIDs [3,5]. All of the commonly used NSAIDs are, however, non-selective inhibitors of both COX-1 and COX-2 [11,12]. This lack of selectivity is thought to account for the increased incidence of gastric ulceration and other deleterious side-effects that accompany the chronic use of NSAIDs. In support of this conclusion, a new generation of compounds has recently been developed that has a high degree of selectivity for COX-2 and has been proven to be both anti-inflammatory and non-ulcerogenic [13]. Examples include NS-398 [14], DFU [15] and SC-58635 (celecoxib, Figure 1) [16].

The discovery of selective COX-2 inhibitors has relied on the development of appropriate *in vitro* assays and a source of COX-

1 and COX-2. *In vitro* assays amenable to large-scale screening efforts have also been developed to assess the potency and selectivity of COX-2 inhibitors. Assay methodologies have varied among groups developing COX-2-selective compounds. Methods of determining IC_{50} s *in vitro* vary widely, with one variable being the source of enzyme activity, where there is species variability [14,17,18], use of recombinant enzymes [12,19–21] and use of cells versus purified enzymes [15]. Most assays rely on measurement of the final product, PGE_2 , either by RIA [14,19,22,23], ELISA [12] or HPLC [15]. Each method typically provides for pre-incubation of enzyme with inhibitor, ranging from 2 min [14] to 15 min [12]. As a result of these differences in methodology and sources of enzyme activity, comparisons of potency and selectivity become difficult. As an example, published selectivity ratios for one of the earliest COX-2 selective inhibitors, NS-398, have ranged from 11 to > 1000-fold [12,14,15,19,22,23].

Figure 1 Structure of celecoxib (SC-58635)

Abbreviations used: C10M, n-decyl β -D-maltopyranoside; COX, cyclo-oxygenase; DEDTC, diethyldithiocarbamic acid; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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Previous work by Rome and Lands [24] demonstrated that certain NSAIDs, exemplified by indomethacin, display an unusual form of enzyme inhibition, termed time-dependent, since the potency of inhibition was markedly increased by time of pre-incubation of enzyme with inhibitor. This type of inhibition is characterized by extremely tight non-covalent binding that is only slowly reversible. Kinetic analysis of this inhibitory profile allows calculation of an inactivation constant, K_{inact} [25]. The discovery of a second COX isoform led to the identification of selective COX-2 inhibitors, where selectivity was based on IC_{50} determinations [11,12,19]. Kinetic analysis demonstrated that isoform selectivity was attained via elimination of the time-dependent component of inhibition on COX-1; i.e. selective COX-2 inhibitors are weak competitive inhibitors of COX-1, but time-dependent inhibitors of COX-2 [22]. Subsequently, a number of publications have used IC_{50} ratios to evaluate the COX isoform selectivity of NSAIDs as well as that of newer agents. Since the mechanism of enzyme inhibition by these drugs can be complex, the present study was performed to evaluate carefully conditions that may influence inhibition by the COX-2-selective compound celecoxib and to determine the kinetic mechanism of inhibition of several NSAIDs.

MATERIALS AND METHODS

Acetyl thiocholine iodide, affinity purified goat anti-rabbit IgG, CHAPS, diethyldithiocarbamic acid (DEDTC), adrenaline, etodolac, Ellman's reagent, hematin, ibuprofen, indomethacin, Tween-20, meclofenamic acid, mefenamic acid, naproxen, phenol, *N,N,N,N'*-tetramethyl-*p*-phenylenediamine (TMPD), Tris and all standard buffer reagents were obtained from Sigma, Chemical Co. (St. Louis, MO, U.S.A.). Arachidonic acid, supplied as the sodium salt, was obtained from NuChek Prep (Elysian, MN, U.S.A.). PGE_2 -acetylcholinesterase and the active metabolite of nabumetone (6-MNA) were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). *n*-Decyl β -D-maltopyranoside (C10M) and *n*-octyl β -D-glucopyranoside were obtained from Calbiochem (La Jolla, CA, U.S.A.).

Preparation of enzyme

Sheep seminal vesicle COX-2

COX-1 was purified from sheep seminal vesicles based on the protocol of Picot and Garavito [26] with slight modification, as summarized here. Sheep seminal vesicles (500 g) were homogenized into 1 litre of 50 mM Tris/HCl, pH 8, containing 1 mM DEDTC, 1 mM EDTA and 0.01 % (w/v) sodium azide. The resulting homogenate was centrifuged at 10000 *g* to remove cell debris. The microsomal fraction was collected by centrifugation at 200000 *g* and then washed once with homogenization buffer containing 115 mM sodium perchlorate. The washed pellet was extracted with 1.5 % (v/v) C10M and the extract was recentrifuged. The supernatant was concentrated to 70 ml by ultrafiltration using a 30 kDa cut-off membrane and was applied to a 1.8 L Sephacryl S-300 (Pharmacia, Piscataway, NJ) gel-filtration column equilibrated with 50 mM Tris/HCl, pH 8, containing 0.1 mM DEDTC, 0.1 mM EDTA and 0.15 % C10M. Protein fractions eluting at dimer molecular mass (145 kDa) were pooled, diluted 2-fold with 0.15 % C10M and applied to a DEAE Trisacryl (Biosepra, Marlborough, MA, U.S.A.) anion-exchange column (150 ml bed vol.) equilibrated with 20 mM Tris/HCl, pH 8, containing 0.1 mM DEDTC, 0.1 mM EDTA and 0.15 % C10M. The column was developed with the same buffer, adjusted to pH 5.3. Active fractions were concentrated to a final volume of 10 ml by ultrafiltration and applied to a 188 ml Ultragel ACA

54 (Biosepra, Marlborough, MA, U.S.A.) gel-filtration column equilibrated with 20 mM potassium phosphate, pH 7.4, containing 0.1 mM DEDTC, 0.1 mM EDTA and 0.3 % (v/v) *n*-octyl β -D-glucopyranoside. The final material was pooled and stored at -80°C until use. The specific activity of this preparation as measured by oxygen consumption is $64 \mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

Recombinant COX-1 and COX-2

Recombinant human and murine COX-1 and -2 were expressed in insect cells infected with baculovirus containing the gene for COX. The cloning and expression of human COX-2 has been described previously [12]. Insect cells were extracted with 1 % CHAPS and the supernatant, after centrifugation at 28000 *g* was used for assay. Detailed kinetic analysis utilized highly purified enzymes, whose purification, with slight modification, is summarized here. Cells from a 40 litre fermentation (60 g, wet weight) were suspended in 600 ml of 25 mM Tris/HCl, pH 8.0, containing 0.25 M sucrose, 1 mM DEDTC with 1 mM EDTA, and washed once. The washed cells were extracted into 600 ml of the above buffer containing 1.5 % C10M. After centrifugation at 28000 *g* for 30 min, the resulting extract was applied to a 150 ml Macro-Prep High-Q (Bio-Rad, Hercules, CA, U.S.A.) anion-exchange column equilibrated with 25 mM Tris/HCl, pH 8.0, containing 0.1 mM DEDTC, 0.1 mM EDTA and 0.15 % C10M, and eluted with a linear gradient to 0.3 M NaCl. The pooled fractions were concentrated to 40 ml by ultrafiltration using a 30 kDa cut-off membrane and applied to a 1 L S-200 Sephacryl (Pharmacia, Piscataway, NJ, U.S.A.) gel-filtration column equilibrated with 25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1 mM DEDTC, 0.1 mM EDTA and 0.3 % *n*-octyl β -D-glucopyranoside. The active fractions were pooled and stored at -80°C until further use. The specific activity of this preparation as measured by oxygen consumption is $11 \mu\text{mol O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

Preparation of celecoxib

Celecoxib was synthesized by the method of Penning et al. [16] and was supplied as a solid. A 10 mM stock solution was prepared in DMSO and successive dilutions were made into 1:9 DMSO/ethanol. Stocks were diluted approx. 20-fold [5 % (v/v) final DMSO/ethanol] into assay buffer for activity measurements.

Enzyme assays

Oxygen uptake assay

Oxygen consumption was measured directly with a Clark-style polarographic electrode in a 600 μl reaction vessel (Instech, Model 203, Horsham, PA, U.S.A.). The assay mixture contained 100 mM Tris/HCl, pH 8.1, 1 μM haem, 0.5 mM phenol and 5–20 μg of enzyme (120–460 nM, assuming 100 % functionality). Enzyme was incubated for 30 s to equilibrate with haem. For the analysis of time-dependent inhibition, test compounds were incubated with enzyme for various amounts of time before initiating the reaction by the addition of arachidonic acid. For the analysis of competitive (i.e. reversible) inhibition, the reaction was initiated by the addition of enzyme to an assay mixture containing substrate and inhibitor. In these cases, enzyme was reconstituted with haem before addition to the reaction. The voltage output from the amplifier was acquired at a rate of 200 data points/min using a Keithley DAS-8 data acquisition board (Keithley, Taunton, MA, U.S.A.) and manipulated using software provided by Instech. Raw data were subjected to three-

point smoothing in order to improve the signal-to-noise ratio without significant compromise of resolution. Maximal rates are expressed as $\mu\text{mol O}_2$ consumed per minute, determined from the first derivative of the reaction time-course as provided by the software.

Peroxidase assay

The COX activity was indirectly measured by utilizing TMPD as a co-substrate with arachidonic acid. TMPD will not turn over without the presence of a hydroperoxide substrate. TMPD oxidation was monitored spectrophotometrically with a 96-well-plate reader at 590 nm. Enzyme was first reconstituted with a stoichiometric amount of haem. Enzyme ($1 \mu\text{g}/\text{well}$) and inhibitor were mixed for various amounts of time in 100 mM Tris/HCl, pH 8, containing $1 \mu\text{M}$ haem. The reaction was started by addition of a mixture of arachidonic acid and TMPD to give a final concentration of $50 \mu\text{M}$ substrate and $85 \mu\text{M}$ TMPD. The initial velocity of the reaction was measured for the first 10 s of the reaction. For steady-state analysis, substrate concentrations were varied from $10 \mu\text{M}$ to $100 \mu\text{M}$.

PGE₂ ELISA

The standard screening assay for inhibitor potency (IC_{50}) was an ELISA method for detection of PGE₂. IC_{50} values were determined by pre-incubation of the inhibitors with enzyme for 10 min at 25°C in a buffer containing 50 mM potassium phosphate, pH 7.5, $2 \mu\text{M}$ phenol, $1 \mu\text{M}$ haem and $300 \mu\text{M}$ adrenaline. The reaction was allowed to proceed for 10 min. For time-dependent inhibition, the initial rate of the reaction was measured by stopping the reaction at 15 s with the addition of 1 M HCl (one tenth of the volume of the reaction mixture); the amount of prostaglandin formed was measured by ELISA (Cayman Chemical Co., Ann Arbor, MI, U.S.A.).

Statistical calculations

Competitive kinetic constants were calculated graphically from double-reciprocal plots of velocity versus arachidonate concentration. This treatment is based on the linearized form of the Michaelis–Menten equation commonly referred to as the Lineweaver–Burk equation. In this form, the reciprocal of the y -intercept is defined as the limiting rate at infinite substrate concentration (V_{max}) and the negative reciprocal of the x -intercept is defined as the concentration of substrate required for half-maximal rate (the Michaelis constant, K_m).

Time-dependent inhibition was evaluated based on a two-step model of a second-order reaction followed by an irreversible first-order reaction, i.e.:

where k_{inact} represents the limiting rate for time-dependent inhibition, which is slow relative to catalytic turnover. From this model, the dependence of observed rate on inhibitor concentration is defined as:

$$k_{\text{obs}} = \frac{k_{\text{inact}} * [\text{I}]}{(K_1 + [\text{I}])} \quad (2)$$

where $K_1 = (k_{-1}/k_1)$ and represents the dissociation constant for inhibitor binding to free enzyme. Eqn. (2) can be transformed

into the more familiar linearized form by taking the reciprocal of both sides of the equation, i.e.:

$$1/(k_{\text{obs}}) = (K_1/k_{\text{inact}}) * 1/[\text{I}] + 1/(k_{\text{inact}}) \quad (3)$$

Kinetic constants for k_{inact} and K_1 were obtained from a linear least-squares fit of $(1/k_{\text{obs}})$ versus $1/[\text{I}]$, where the reciprocal of the y -intercept represents k_{inact} and the negative reciprocal of the x -intercept represents K_1 [25].

RESULTS

Initial assessment of the potency and selectivity of celecoxib with respect to COX-1 and COX-2 was determined from IC_{50} values, expressed as the concentration of compound required to inhibit 50% of the initial rate of (i) oxygen uptake or (ii) TMPD oxidation, or (iii) the amount of PGE₂ detected by ELISA after 10 min (Figure 2). The IC_{50} values obtained for COX-1 were approximately the same for all three methods: $31 \mu\text{M}$ for the oxygraph assay, $20 \mu\text{M}$ for the TMPD assay and $19 \mu\text{M}$ for the PGE₂ ELISA. The IC_{50} values obtained with COX-2, however, varied considerably between the different methods: $0.006 \mu\text{M}$ for the ELISA assay, $0.04 \mu\text{M}$ for the TMPD assay and $0.2 \mu\text{M}$ for the oxygraph assay. From this initial assessment, celecoxib was estimated to be 155–3200-fold more selective for COX-2 than for COX-1, depending on assay conditions. It is interesting to note that the IC_{50} values obtained using the various assays approximate the concentration of enzyme present in each, suggesting that celecoxib is functioning as an active-site titrant with high affinity for COX-2.

In an effort to characterize more fully the mechanism of inhibition of COX-1 and COX-2 by celecoxib, kinetic parameters for competitive (i.e. reversible) and time-dependent inhibition were determined. To minimize any contribution from turnover-dependent auto-inactivation, continuously monitored assays for O₂ consumption and TMPD oxidation were utilized. In these assays, maximal velocity in the absence of inhibitor was achieved within 5–10 s and was constant for several seconds. The maximal rate observed at saturating arachidonate was demonstrated to be

Figure 2 IC_{50} of celecoxib with human COX-1 (open symbols) and -2 (closed symbols) by three different assays

○, ●, TMPD oxidation: maximal rate of TMPD oxidation was measured upon addition of $50 \mu\text{M}$ arachidonic acid following a 1 min preincubation of inhibitor with enzyme. ■, □, Oxygen uptake: maximal rate of oxygen consumption was measured upon addition of $100 \mu\text{M}$ arachidonic acid, after a 1 min preincubation of inhibitor with enzyme. ▲, △, PGE₂ ELISA: PGE₂ was measured after a 10 min incubation with $10 \mu\text{M}$ arachidonic acid following a 10 min preincubation with inhibitor. $\mu\text{M} = \mu\text{M}$.

Table 1 Steady-state and time-dependent kinetic parameters for celecoxib

*Not applicable; ND, not determined.

Assay...	COX-1			COX-2		
	O ₂	TMPD	PGE ₂	O ₂	TMPD	PGE ₂
Time dependence	—	—	—	+	+	+
K _i (μM)	10	16	ND	15	11	ND
K _{inact} (s ⁻¹)	*	*	*	0.5	0.03	0.06
K _i (μM)	*	*	*	4.0	0.4	0.1
Efficiency (K _{inact} /K _i)	*	*	*	0.12	0.08	0.6

Figure 3 Time-dependent inhibition of purified murine COX-1 and sheep COX-2 by three different assays

(1) PGE₂ ELISA: total PGE₂ produced was measured at 15 s after addition of arachidonic acid at 2–120 s preincubation. (2) TMPD oxidation: the maximal rate of TMPD oxidation was measured upon addition of 50 μM arachidonic acid after 2–120 s preincubation of inhibitor with enzyme. (3) Oxygen uptake: maximal rate of oxygen consumption was measured upon addition of 100 μM arachidonic acid, after 2–120 s preincubation of inhibitor with enzyme. μM = μM.

dependent on enzyme concentration. In the case of COX-2, direct plots and double-reciprocal plots of reaction velocity versus substrate concentration demonstrated classical steady-state kinetic behaviour. In the case of COX-1, a slight indication of sigmoidal behaviour was observed in the absence of inhibitor,

consistent with previous observations [17]. This has been attributed to positive co-operativity with COX-1 and was manifested as a slight upward curvature in double-reciprocal plots.

Competitive inhibition by celecoxib was evaluated using the oxygraph and TMPD assays in the absence of any pre-incubation

Figure 4 Time-dependent inhibition of purified murine COX-2 and sheep COX-1 by indomethacin, ibuprofen and naproxen

Maximal rate of TMPD oxidation was measured upon addition of 50 μ M arachidonic acid and 85 μ M TMPD after 2–120 s preincubation of inhibitor with enzyme. uM = μ M.

of enzyme with inhibitor. Initial rates obtained after the addition of enzyme to a reaction mixture containing inhibitor and various concentrations of substrate describe a family of lines which intersect at the y -axis when plotted in double-reciprocal form. This pattern is consistent with reversible competitive inhibition and demonstrates that little or no contribution from auto-inactivation or time-dependent inhibition was observed under these assay conditions. Inhibition constants (K_i) were determined from the negative x -intercept from secondary plots of line slope versus the concentration of inhibitor present. Celecoxib inhibits sheep COX-1, in the oxygen consumption assay, competitively, with a K_i of 16 μ M, and murine COX-2 with a K_i of 11 μ M, suggesting similar initial binding to both isoforms. Similar results were obtained with the TMPD assay (Table 1).

The potency of celecoxib with respect to COX-2 demonstrated a time dependence, as evidenced by decreases in enzyme activity

as a function of increased pre-incubation of inhibitor with enzyme. Kinetic parameters for the time-dependent inhibition of COX-1 and COX-2 by celecoxib were evaluated using each of the three assay methods. Initial rates were determined after pre-incubation of enzyme with celecoxib for various amounts of time (2–120 s) and expressed relative to control rates obtained in the absence of any pre-incubation. Results are presented graphically in semi-log plots of activity remaining as a function of pre-incubation time (Figure 3). Primary plots of results obtained from all three assays indicated no time-dependent inhibition of COX-1 and time-dependent inhibition of COX-2. Time-dependent parameters of K_i and k_{inact} obtained with COX-2 are 4 μ M and 0.5 s^{-1} for the oxygen uptake assay; 0.4 μ M and 0.03 s^{-1} for the TMPD oxidation assay; and 0.1 μ M and 0.06 s^{-1} for the PGE₂ ELISA assay (Table 1). The efficiency of celecoxib with respect to COX-2 can be more conveniently expressed as a function of the k_{inact}/K_i ($s^{-1} \cdot \mu$ M). When expressed in this

ditions differ slightly between the various assays used, potency was not affected by modest (10-fold) changes in substrate concentration.

In contrast to the high degree of selectivity for COX-2 predicted by IC_{50} values, closer examination of COX inhibition by celecoxib demonstrates that inhibitor binding is initially competitive with respect to substrate, and furthermore, is characterized by similar affinity for COX-1 and COX-2. These determinations were performed in the absence of any pre-incubation of inhibitor with enzyme. Consequently, the binding constants obtained reflect the rapid equilibrium between free enzyme and the initial binary complex with inhibitor, which includes little or no contribution from slower, time-dependent processes. Slow, time-dependent processes which occur subsequent to the formation of the initial binary complex with COX-2, but not with COX-1, appear to be the largest contributing factor to the potency and selectivity of celecoxib.

It is of interest to note that K_i values obtained from competitive and time-dependent inhibition of COX-2 by celecoxib are different, indicating tighter binding during time-dependent inhibition. This difference suggests that there is an additional equilibrium, which contributes to the concentration dependence observed during slow inactivation, but which does not contribute to potency during steady-state inhibition by celecoxib. Evidence for multiple, reversible binding steps has been reported previously [27,28]. This behaviour is interpreted as conversion of the initial EI binary complex into a second, tighter EI* complex. (This subject will be discussed further in subsequent publications.)

The basis of selectivity for this new class of highly selective compounds is related to their ability to inhibit COX-2 in a time-dependent manner, while demonstrating no time-dependent inhibition of COX-1 [12,22]. Recently the X-ray crystal structures of COX-1 [29] and COX-2 [30] have been reported. Initial indications are that diaryl heterocycles can be made selective for COX-2 by taking advantage of a side-pocket in the active site of COX-2 that is not found in COX-1. Mutations of this region indicate that time dependence of diaryl heterocycles is mainly due to interaction with this side-pocket [31,32]. However, not all time-dependent inhibitors are affected equally by the enlargement of the side-pocket in COX-2. Compounds that do not have a structure that interacts with the side-pocket can still display time dependence. This time dependence is demonstrated by carboxylic acid compounds that interact with Arg-120. Rome and Lands [24] demonstrated that some NSAIDs with carboxylic acid moieties and halogens at certain positions displayed time-dependent inhibition of COX. The time dependence could be reversed in carboxylic acid classes of compounds with the conversion of the acid into the methyl ester, indicating that the carboxylic acid was contributing to the time dependence. Examples of this observation are mefenamic acid (not time dependent) and meclofenamic acid (time dependent). We now know from structural studies (R. G. Kurumbail, personal communication) that the carboxylic acid of meclofenamic acid forms an ion-pair with Arg-120 (of COX-1) and the halogen serves to place the carboxylic acid in the proper orientation. Mefenamic acid, on the other hand, is not situated in the active site within ion-pairing distance from Arg-120. Hence, there appear to be two types of time-dependent inhibition: one that involves an earlier interaction with the Arg-120 and another which involves the insertion of a portion of the compound into the side-pocket.

Several conclusions can be drawn based on the data reported herein. First, tight-binding, time-dependent and selective inhibitors of COX-2, such as celecoxib, exhibit clearly distinct mechanisms of action with respect to COX-1 compared with COX-2, such that IC_{50} determinations accurately reflect in-

hibitory mechanism; furthermore, incubation conditions and assay type have little effect on relative activity with this class of inhibitor. Secondly, there are at least four categories of inhibition of COX: (i) competitive; (ii) tight binding, time dependent; (iii) weak binding, mixed; and (iv) covalent binding. Some NSAIDs exhibit similar mechanisms of inhibition of both enzymes (e.g. ibuprofen and indomethacin), whereas others show distinct mechanisms of inhibition of COX-1 and COX-2. Thirdly, with the latter category of inhibitors, IC_{50} determinations are strongly influenced by experimental variables and thus are not directly comparable for each isoform. Finally, for inhibitors that display distinct mechanisms of inhibition of COX-1 and COX-2, calculation of a selectivity ratio based on IC_{50} determinations is not supportable, since there are no underlying kinetic constants that are common to each isoform. This points to the need for analysis of selectivity *in vivo* under physiologically relevant conditions.

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