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Effect of inhibitor time-dependency on selectivity towards cyclooxygenase isoforms

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Cyclooxygenase (Cox) is a key enzyme in the biosynthesis of prostaglandins and, as such, is the target of non-steroidal anti-inflammatory drugs (NSAIDs). Two isoforms exist, being expressed consitutively (Cox-1), or inducibly in response to inflammatory mediators (Cox-2). Currently available NSAIDs inhibit both isoforms somewhat equipotently but selective Cox-2 inhibition may eliminate unwanted side effects. We have characterized the kinetic mechanisms of the interactions of purified recombinant human cyclooxygenase-1 and -2 (hCox-1, hCox-2) with the selective Cox-2 inhibitor *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide (NS-398) and some classical non-selective NSAIDs. NS-398, flurbiprofen, meclofenamic acid and indomethacin are time-dependent, irreversible inhibitors of hCox-2. The inhibition is consistent with a two-step process, involving an initial rapid equilibrium binding of enzyme and inhibitor, characterized by K_i, followed by the slow formation of a tightly bound enzyme–inhibitor complex, characterized by a first-order rate constant k_{on}. NS-398 is a time-independent inhibitor of hCox-1, consistent with the formation of a reversible enzyme–inhibitor complex. Flurbiprofen, meclofenamic acid and indomethacin are also time-dependent inhibitors of hCox-1 and hence show little selectivity for one isoform over the other. Flufenamic acid is time independent towards both isoforms and is also non-selective. The high degree of selectivity of NS-398 towards Cox-2 results therefore from the difference in the nature of the time-dependency of inhibition of the two isoforms.

INTRODUCTION

Cyclooxygenase (Cox; prostaglandin G/H synthase, EC 1.14.99.1) catalyses the first two steps in the biosynthesis of prostanoids from arachidonic acid, namely the formation of prostaglandin G_2 and its subsequent reduction to prostaglandin H_2 [1,2]. Cox exists as at least two distinct isoforms. Cox-1 is expressed constitutively in most cell types, whereas the recently discovered Cox-2 is inducibly expressed in response to a variety of proinflammatory agents [3–6]. The current belief is that prostanoids produced by Cox-1 are involved in the maintenance of regular cellular physiology whereas those produced by Cox-2 are involved in inflammatory processes [7–9].

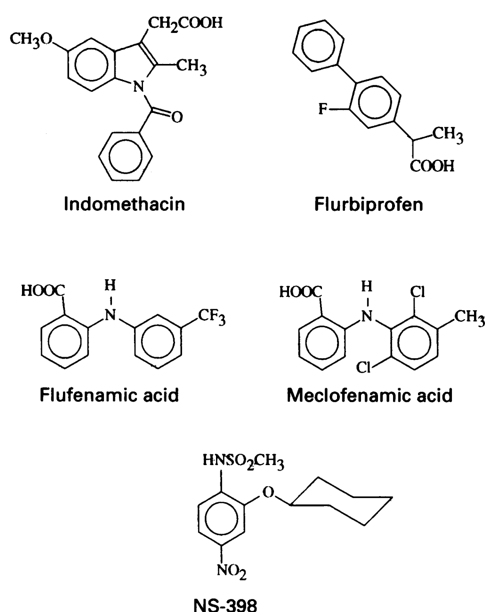

Figure 1 Cyclooxygenase inhibitors used in this study

The cyclooxygenase activity of Cox is the target of a large structurally diverse group of therapeutic agents known as non-steroidal anti-inflammatory drugs (NSAIDs). Two distinct classes of NSAIDs have been identified, differing in the kinetic mechanism by which they inhibit Cox [10,11]. One class operates in a time-independent manner, consistent with the formation of a reversible enzyme–inhibitor complex. The other class demonstrates time-dependent behaviour involving a two-step mechanism of inhibition. Time-dependent NSAIDs are generally more potent since the second, time-dependent step, involves the formation of an essentially irreversibly inhibited non-covalent enzyme–inhibitor complex [10,11]. Members of both classes appear to act competitively with the substrate arachidonic acid [10].

The cyclooxygenase activity of both Cox-1 and Cox-2 are inhibited equipotently, or even somewhat Cox-1-specifically, by currently marketed NSAIDs [8,9,12]. This could probably explain their propensity to cause unwanted side-effects such as gastric and renal damage. Recently, a novel agent, N-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide (NS-398), was described as being anti-inflammatory, analgesic and antipyretic in a number of animal models [13]. NS-398 is, however, much less potent than some other classical NSAIDs as an inducer of stomach lesions [13,14]. NS-398 inhibits purified ovine Cox-2 (oCox-2) with an IC_{50} of 3.8 μM but has no effect on oCox-1 at a 100 μM concentration [15]. Thus, NS-398 appears to be a highly selective inhibitor of Cox-2, as would be predicted on the basis of the pharmacological studies [13,14].

Here we have investigated the kinetic mechanism of inhibition of purified human Cox-1 and Cox-2 (hCox-1, hCox-2) by NS-

Abbreviations used: Cox, cyclooxygenase; h, human; o, ovine; NSAID, non-steroidal anti-inflammatory drug; NS-398, *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide.

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398 and four classical NSAIDs (Figure 1). The results show that the selectivity of NS-398 is due to its behaviour as a time-dependent inhibitor of hCox-2 but time-independent inhibitor of hCox-1. The other non-selective classical NSAIDs show no differences in the time-dependency of their inhibition with Cox-1 and Cox-2.

EXPERIMENTAL

Indomethacin, *R*/*S*-flurbiprofen and arachidonic acid (peroxide free) were from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). Meclofenamic acid and flufenamic acid were from Sigma. NS-398 [13] was synthesized at Merck Frosst Canada.

Recombinant hCox-2 was obtained from vaccinia virus [12] and baculovirus expression systems [16]. The protein was purified by extraction of the membrane fraction by β-octyl glucoside followed by ion-exchange chromatography [17]. A small quantity of recombinant vaccinia virus hCox-1 [12] was purified to enable the comparison of inhibition by NS-398 and other NSAIDs with that of purified hCox-2. The protein was purified as described for hCox-2 except that solubilization was performed with Tween-20, and a second chromatography step involving gel filtration (SW300 Protein Pak, Waters) was required to obtain homogeneous enzyme.

Cyclooxygenase activity was measured at 37 °C by oxygen uptake [18] in reaction buffer (0.6 ml) containing 100 mM Tris (pH 8.0)/5 mM EDTA/1.0 μM hematin/2 mM homovanillic acid/100 μM arachidonic acid. The activity of purified Cox in the presence of inhibitors was determined using a more sensitive fluorimetric assay [19]. The reaction buffer (1.0 ml) contained 100 mM Tris, pH 8.0/5 mM EDTA/1.0 μM hematin/1 mM homovanillic acid/20 μM arachidonic acid. For the K_i determination of NS-398 by instantaneous inhibition, the reaction was initiated by addition of hCox-1 or hCox-2 (0.2 μg) to the above reaction mixture containing 0–35 μM NS-398 and 0.1–10 μM arachidonic acid. The peroxidase activity of Cox was determined using the same fluorimetric assay with 100 μM H_2O_2 as substrate [19]. For both cyclooxygenase and peroxidase reactions, the activity noted represents the highest (initial) velocity attained. IC_{50} titrations were performed using purified vaccinia virus hCox-2. Further kinetic characterizations were performed using purified baculovirus hCox-2. Control experiments showed that there are no differences in inhibitor sensitivities between hCox-2 from the two sources. Residual cyclooxygenase activities of drug-inhibited hCox-2 were determined by oxygen uptake. hCox-2 (5.2 μg) was preincubated for 30 min in the presence of 20 μM NS-398, flurbiprofen and meclofenamic acid and 80 μM indomethacin before dilution (30-fold) in the reaction chamber containing substrate.

Data-fitting was performed by multiple regression using GraFit computer software [20].

RESULTS

Dose-dependent inhibition of purified hCox-1 and hCox-2 by NS-398

The cyclooxygenase activities of purified recombinant hCox-1 and hCox-2 were determined after a 15 min preincubation with NS-398 before initiation of the reaction with substrate arachidonic acid. Cyclooxygenase activity was monitored using a coupled fluorimetric assay which follows the appearance of the oxidized form of the peroxidase cosubstrate homovanillic acid. This assay was employed because of its high sensitivity [19] and the small amount of purified recombinant enzymes available. The resulting titrations (Figure 2) show that hCox-1 and hCox-2 are inhibited with IC_{50} values of 26 μM and 0.1 μM respectively. NS-398 therefore shows a selectivity of approximately 260-fold for hCox-2 over hCox-1.

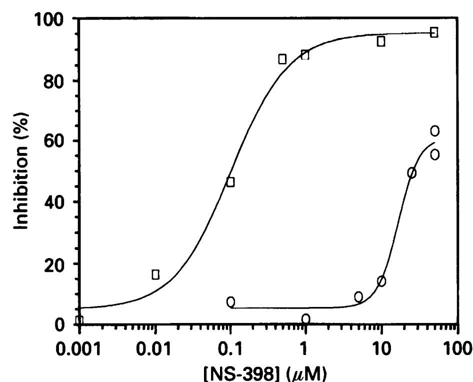

Figure 2 Inhibition of purified hCox-1 and hCox-2 with NS-398

hCox-1 (○) and hCox-2 (□) (0.04 μg of each) were preincubated with NS-398 for 15 min (1.0 ml reaction volume) prior to initiation of the reaction with arachidonic acid (final concentration 20 μM). Inhibition was calculated by comparison of the maximal (initial) cyclooxygenase velocities with those of vehicle-treated controls.

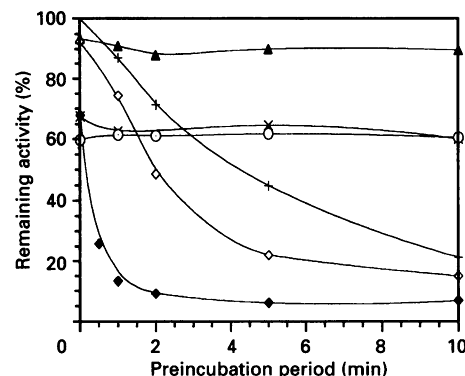

Figure 3 Effect of inhibitor preincubation period on inhibition of hCox-1 and hCox-2

hCox-1 and hCox-2 (0.04 μg of each) were preincubated for the indicated times with inhibitor before initiation of the reaction with arachidonic acid as in Figure 1. The percentage remaining activity was calculated by comparison of the maximal cyclooxygenase velocities with those of vehical-treated controls preincubated for the same period. No loss of activity was observed for the controls (results not shown). ○, hCox-1, 20 μM NS-398; ▲, hCox-1, 1 μM NS-398; ◆, hCox-2, 20 μM NS-398; ◇, hCox-2, 1 μM NS-398; ■, hCox-1, 20 μM flufenamic acid; ×, hCox-2, 20 μM flufenamic acid; +, hCox-2, 10 μM indomethacin.

Time-dependency of inhibition of hCox-1 and hCox-2

The time-dependency of inhibition of hCox-1 and hCox-2 by NS-398, flurbiprofen, indomethacin, meclofenamic acid and flufenamic acid was investigated by preincubation of the enzyme in the presence of drug for increasing periods of time before initiation of the reaction by addition of substrate arachidonic acid. The results (Figure 3) show that NS-398 and indomethacin act in a time-dependent manner with hCox-2. In the absence of a preincubation ($t = 0$) relatively little inhibition is observed, but as the preincubation time is increased a rapid decline in cyclooxygenase activity occurs. Flurbiprofen, meclofenamic acid and indomethacin also show a similar time-dependency of inhibition

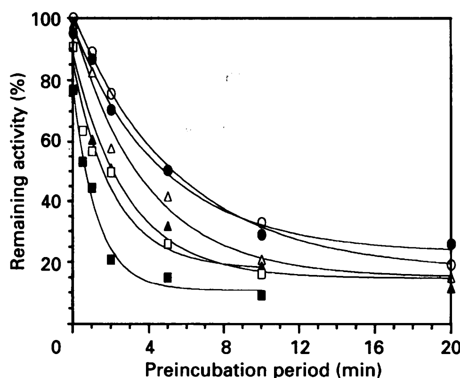

Figure 4 Evaluation of observed first-order rate constants for on-set of inhibition of purified hCox-2 by NS-398

hCox-2 (0.06 μg) was preincubated for the indicated times with NS-398 before initiation of the reaction with arachidonic acid. The percentage remaining activity was calculated as in Figure 2. For each inhibitor concentration the data were fitted to the equation $y = a + b \cdot \exp(-k_{obs.} \cdot t)$. The concentrations of NS-398 and the first-order rate constants ($k_{obs.}$) calculated are: (○) 1.0 μM, 0.176 min^{-1}; (●) 1.33 μM, 0.209 min^{-1}; (△) 2.0 μM, 0.259 min^{-1}; (▲) 2.66 μM, 0.352 min^{-1}; (□) 4.0 μM, 0.467 min^{-1}; (■) 8.0 μM, 0.80 min^{-1}.

with both hCox-1 and hCox-2 (results not shown). In contrast, inhibition of hCox-1 by NS-398 and of both hCox-1 and hCox-2 by flufenamic acid are clearly not dependent on the period of preincubation with drug (Figure 3).

Control experiments were performed to determine whether the inhibition of cyclooxygenase activity noted above could be due to an effect on the coupling peroxidase activity of the enzyme. No significant inhibition of the peroxidase activity was observed, using H_2O_2 as substrate, at a concentration of each inhibitor that produced $< 85\%$ inhibition of the cyclooxygenase activity (results not shown). Similar results have been obtained with a series of NSAIDs using bovine Cox-1 [21], thereby demonstrating that NSAIDs specifically inhibit the cyclooxygenase activity.

To investigate inhibitor reversibility, hCox-1 and hCox-2 were pretreated for 30 min with 20 μM of each inhibitor and an aliquot was diluted 100-fold in substrate-containing reaction buffer. With the previously identified time-independent inhibitors, flufenamic acid and NS-398 for hCox-1 and flufenamic acid for hCox-2, the resulting activity was the same as that of vehicle-treated control. In contrast, no recovery of activity was observed for enzyme treated with time-dependent inhibitors.

Evaluation of kinetic parameters of inhibition

The time-dependent hCox-2 inhibitors identified above were further characterized by preincubating the enzyme with a range of inhibitor concentrations for different periods of time before initiation of the reaction with substrate. An example of the results obtained for NS-398 is shown in Figure 4. The rate of onset of inhibition is increased at higher inhibitor concentrations and the decay in activity at each inhibitor concentration has the characteristics of a first-order process ($k_{obs.}$). The time-dependent inhibition of oCox-1 by some classical NSAIDs has been studied in detail [10,11] and has been shown to be consistent with a two-step mechanism (Scheme 1) in which the initial rapid reversible binding of a drug is followed by a slower irreversible inactivation. This model predicts that the apparent rate constant for the inactivation process ($k_{obs.}$) is related to the concentration of inhibitor (I) by the equation:

$$k_{obs.} = k_{on}/[1 + (K_i/[I])]$$

The values of K_i and k_{on} for each time-dependent inhibitor with hCox-2 were determined by computer-fitting the values of $k_{obs.}$ for each inhibitor concentration to the above equation (Table 1).

The K_i values of NS-398 for hCox-1 and hCox-2 were also determined by measurement of instantaneous inhibition (no preincubation) in the presence of a range of substrate concentrations. Linear double-reciprocal plots of the data were obtained (results not shown). For hCox-2, purely non-competitive inhibition by NS-398 was observed, whereas for hCox-1 the data was consistent with linear mixed-type inhibition [22]. The data were computer-fitted to their respective velocity expressions [22] giving K_i values of 24 ± 6 μM for hCox-2 and 5 ± 1 μM ($\alpha = 5.3 \pm 1.5$) for hCox-1. The K_m values measured for arachidonic acid were 0.60 ± 0.06 μM and 0.43 ± 0.04 μM for hCox-1 and hCox-2 respectively, which are close to those determined under slightly different assay conditions [17].

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \xrightarrow{k_{on}} EI^*$$

Scheme 1

Table 1 Kinetic constants for the inhibition of purified hCox-2 by NS-398, indomethacin, meclofenamic acid and flurbiprofen

	K_i (μM)	k_{on} (min^{-1})	k_{on}/K_i ($min^{-1} \cdot \mu M^{-1}$)	Residual initial velocity (%)
NS-398	14 ± 3	2.1 ± 0.3	0.15 ± 0.03	6
Indomethacin	114 ± 28	2.7 ± 0.5	0.02 ± 0.01	0.6
Meclofenamic acid	5.5 ± 1.4	1.6 ± 0.3	0.3 ± 0.1	2
Flurbiprofen	0.17 ± 0.01	1.05 ± 0.04	6 ± 0.6	13

Residual optimal activity of enzyme–inhibitor complexes

The results in Figure 3 indicate that the time-dependent inhibition of hCox-2 by NS-398 does not result in a complete loss of cyclooxygenase activity. To quantify this residual optimal activity, hCox-2 was preincubated for 30 min with each inhibitor and the cyclooxygenase activity measured by oxygen uptake (Table 1). Indomethacin and meclofenamic acid-treated hCox-2 have relatively little residual activity (0.6 and 2 % respectively), whereas NS-398 and flurbiprofen-treated enzyme retain considerable (6 and 13 % respectively) activity. Although the initial velocity of the cyclooxygenase reaction was inhibited $> 87\%$ in each case, the overall extent of the reaction was between 75 and 125 % of that of the enzyme control preincubated for the same period. Comparable residual optimal cyclooxygenase activities to those noted in Table 1 were obtained when the inhibited hCox-2 was assayed by the fluorimetric method.

DISCUSSION

NS-398 is a selective Cox-2 inhibitor that has anti-inflammatory properties *in vivo* with little gastrointestinal toxicity [13–15]. Under our assay conditions, using purified hCox-1 and hCox-2 and a 15 min preincubation period, NS-398 shows a selectivity of approximately 260-fold for the inducible type-2 isoform. Inhibition of hCox-2 by NS-398 is time dependent, consistent with its operating by a two-step mechanism resulting in the formation

of an irreversibly inhibited complex EI* (Scheme 1). The kinetic parameters for this process, K_i and k_{on}, were evaluated by determination of the rate of onset of inhibition as 14 μM and 2.1 min^{-1} respectively. In contrast, inhibition of hCox-1 by NS-398 is not time dependent, a result consistent with the formation of a rapidly reversible inhibited complex. The difference in the mechanisms by which NS-398 operates on hCox-1 and hCox-2 has important implications, as the degree of selectivity of NS-398 is therefore not limited to a factor of 260. A longer preincubation period would lead to the increased formation of EI*, and hence a lower IC_{50} value for hCox-2, but would have no effect on the IC_{50}value of hCox-1. This was confirmed by titrations with a 5 min preincubation period that gave IC_{50} values of 19 μM and 0.5 μM for hCox-1 and hCox-2 respectively (M. Ouellet, unpublished work). Ultimately, a time-dependent inhibitor, such as NS-398, should inactivate hCox-2 on a stoichiometric basis as has been established for oCox-1 with other NSAIDs [11]. Flurbiprofen, indomethacin and meclofenamic acid inhibit both hCox-1 and hCox-2 in a time-dependent manner. The lack of any high degree of selectivity shown by these NSAIDs [8,9,12] is therefore consistent with their time-dependent, irreversible inactivation of both isoforms. The non-selective NSAID flufenamic acid also inhibits both isoforms similarly, in this case in a time-independent manner, consistent with the formation of a rapidly reversible enzyme–inhibitor complex.

The inhibition of oCox-1 and oCox-2 by NS-398 has been described previously [15]. It is difficult to make a comparison with the present results since these assays were performed at a single drug–enzyme preincubation period. Consequently no information can be gained on the time-dependency of inhibition of NS-398 against the ovine isoforms.

The K_i value of NS-398 for hCox-2 (24 ± 6 μM), determined from the instantaneous inhibition of the cyclooxygenase reaction, is in fair agreement with that obtained from analysis of the time-dependency of inhibition (14 ± 3 μM). The instantaneous inhibition of hCox-2 by NS-398 is of a purely non-competitive nature, the degree of inhibition being independent of the substrate concentration. The same determination of the K_i of NS-398 for hCox-1 gave a value of 5 ± 1 μM. In this case the data fit a linear mixed-type of inhibition ($\alpha = 5.3 \pm 1.5$) [22]. This system can be considered a mixture of partial competitive and pure non-competitive inhibition. Thus, NS-398 acts to increase the apparent K_m of hCox-1 for arachidonic acid and reduce $V_{max.}$. This results in similar levels of instantaneous inhibition of hCox-1 and hCox-2 by NS-398 at high substrate concentrations (see Figure 3) but greater instantaneous inhibition of hCox-1 than hCox-2 at substrate concentrations around and below the K_m value.

The hCox isoforms share 61% identity at the amino acid level [3]. The closeness of the K_i values of NS-398 for hCox-1 and hCox-2 (5 and 24 μM respectively) may indicate that NS-398 interacts initially with the same, or similar, binding sites of both isoforms. However, the second step, involving the isomerization of the initial reversible complex to the irreversibly inhibited complex, can only occur in the case of hCox-2.

The instantaneous inhibition of oCox-1 by the classical NSAIDs used in this study has previously been described as being competitive in nature [10]. The fact that NS-398 represents a new structural class probably explains the difference in the type of instantaneous inhibition.

The K_i values estimated for the four time-dependent inhibitors with hCox-2 vary considerably from 0.17 μM for flurbiprofen to 114 μM for indomethacin (Table 1), although the values of k_{on}, the first-order rate constants for the isomerization of EI to EI*, are fairly constant. The second-order rate constants for the onset of inhibition (k_{on}/K_i) reflect the efficiency with which each inhibitor inactivates hCox-2 (Table 1). These rate constants show that flurbiprofen is the most efficient inactivator of hCox-2, being 40-fold better than NS-398 and 300-fold better than indomethacin. These kinetic constants correctly predict the rank order of potencies obtained for these drugs against microsomal hCox-2 [12,16]. Since the efficiency of inhibition is a function of k_{on}/K_i, an increase in inhibitor potency could be achieved by a novel compound having a decreased K_i and/or increased k_{on}. The highest degree of inhibitor selectivity will be obtained with a compound having a high K_i value and a k_{on} value of 0 for one isoform and a high value of k_{on}/K_i for the other.

Although the inactivation by classical NSAIDs and NS-398 is apparently irreversible, the process is probably not due to a covalent modification of the enzyme. Studies of oCox-1 and indomethacin have shown that the inhibitor could be recovered intact from the EI* complex after enzyme denaturation [11]. By analogy, this is probably the case with other time-dependent NSAIDs and both Cox isoforms.

Apart from the case of aspirin, it appears to be somewhat controversial whether or not the time-dependent, irreversible inhibition of Cox actually occurs *in vivo*. A study *in vitro* [23] of the effects of six NSAIDs on platelets (hCox-1) showed that indomethacin, aspirin and naproxen all acted in a time-dependent, irreversible manner. A later *in vivo* study of platelet thromboxane production [24] found that the degree of inhibition by indomethacin was greater than would be expected from drug plasma concentrations and could not be explained by simple, rapidly reversible inhibition. However, recovery of Cox activity ($t_{\frac{1}{2}} = 24$ h) was observed, suggesting that the formation of the EI* complex may in fact be slowly reversible *in vivo*. In contrast, other workers have reported that indomethacin plasma levels correlate well with the inhibition of platelet Cox activity [25]. They found no evidence for the irreversible inhibition of Cox by indomethacin. In view of the recent discovery of Cox-2 and the renewed interest in Cox inhibitors, it may be timely for a thorough re-evaluation to be made of the reversibility of NSAIDs *in vivo*. The present work favours the position that the time-dependent, irreversible (or slowly reversible) complex formation does in fact occur *in vivo*. NS-398 is believed to be a non-ulcerogenic, anti-inflammatory agent because it selectively inhibits Cox-2 over Cox-1 [13,14]. If the time-dependent inactivation of Cox-2 were not to occur *in vivo*, then NS-398 would completely lose its Cox-2 selectivity, becoming instead somewhat Cox-1 selective. It should be noted that since Cox-2 protein is rapidly turned over *in vivo* ($t_{\frac{1}{2}} \approx 4$ h) [26], the formation of a slowly reversible EI* complex, such as that observed with indomethacin and platelet Cox-1 [24], could effectively inhibit the enzyme for its entire lifetime.

Drug-treated hCox-2 has a greatly reduced optimal velocity of reaction (Table 1) but is relatively unimpaired with regard to the total extent of the reaction. This would appear to argue that inhibitors of this type should not be effective since the total amount of product formed is not reduced. However, studies with oCox-1 have shown that cyclooxygenase activity is dependent upon the maintenance of a sufficient peroxide concentration and that cyclooxygenase activity can be reduced by the presence of other peroxidase enzymes [27,28]. Consequently, when the rate of production of the peroxide prostaglandin G_2 is reduced *in vivo*, by for instance drug inhibition, the balance is tipped in favour of the peroxidases, and cyclooxygenase activity is further inhibited.

In summary, two distinct kinetic mechanisms for the inhibition of hCox-2 have been identified. Inhibitors such as NS-398, flurbiprofen, indomethacin and meclofenamic acid act in a time-dependent manner resulting in the formation of an irreversibly

inhibited enzyme species having a small residual activity. Time-independent inhibition of hCox-2 is observed with flufenamic acid, consistent with reversible complex formation. NS-398 acts in a time-independent manner towards hCox-1. It appears that the high degree of selectivity exhibited by this compound is the result of the two different mechanisms by which it interacts with each isoform. These results establish that a kinetic basis exists for the identification of novel compounds that show a high degree of selectivity for one isoform of Cox over the other.

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