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Manipulation of kinetic profiles in 2-aryl propionic acid cyclooxygenase inhibitors

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Abstract—The nonsteroidal anti-inflammatory drugs flurbiprofen and ibuprofen were modified in an attempt to alter the kinetics of inhibitor binding by COX-1. Contrary to prior predictions, a halogen substituent is not sufficient to confer slow tight-binding behavior. Conversion of the carboxylate moiety of flurbiprofen to an ester or amide abolishes slow tight-binding behavior, regardless of halogenation state.

1. Introduction

The cyclooxygenase enzymes (COX-1/2, EC 1.14.99.1) catalyze important early steps in the biosynthesis of the prostanoid hormones. These hormones mediate a variety of physiological processes, including inflammation and platelet aggregation, and have been implicated in pathologies such as cardiovascular disease, Alzheimer's disease, and colon cancer.[1–3] Both isoforms of this enzyme possess two distinct activities, catalyzing both cyclooxygenase and peroxidase reactions. The cyclooxygenase active site is the target of the nonsteroidal antiinflammatory drugs (NSAIDs).[4] Considerable effort has been invested in the identification of isoform-selective cyclooxygenase inhibitors; such molecules offer the promise of modulating clinically significant physiological processes such as inflammation (COX-2) or platelet aggregation (COX-1), while minimizing unwanted side effects.

Most NSAIDs can be classified as either slow tight-binding inhibitors (time-dependent binders) or reversible competitive inhibitors (time-independent binders).[5–7] Slow tight-binding behavior is clinically significant, as such behavior can convert a micromolar inhibitor into a psuedo-irreversible nanomolar inhibitor.[8] The slow tight-binding phenomenon appears to underlie the isoform specificity of many COX-2 selective inhibitors; selective compounds are slow tight-binding inhibitors of the target isoform and reversible competitive inhibitors of the other isoform.[9–11] While a wealth of structure–activity data has identified structural modifications of NSAIDS that confer isoform selectivity, there is little structural understanding of the phenomenon of time-dependent inhibition.

Early work by Rome and Lands drew attention to the carboxylic acid moiety possessed by many NSAIDs as a potential determinant of time-dependent inhibition.[7] The free carboxylic acid group found in NSAIDs such as flurbiprofen and ibuprofen forms critical interactions with residues Arg-120, Glu-524, and Tyr-355 within the cyclooxygenase active site,[12–14] and esterification of this group converts such compounds from time-dependent to time-independent inhibitors. Alteration of the carboxylic acid moiety has recently been exploited to convert nonselective inhibitors into COX-2 selective inhibitors.[15–17] Rome and Lands also hypothesized, based on a comparison of the chemical structures of related inhibitors, that a halogen substituent should confer time-dependent inhibition upon an NSAID. However, little evidence has been available to test this hypothesis.

Keywords: NSAIDS; Slow tight-binding inhibitors; Flurbiprofen; Ibuprofen.
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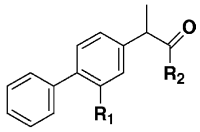
Two members of the 2-aryl propionic acid class of NSAIDs were used to probe structure–activity relationships relevant to inhibitor binding kinetics. Flurbiprofen, a halogenated, slow tight-binding inhibitor, potently inhibits cyclooxygenase activity in both isoforms. Ibuprofen, while similar in structure to flurbiprofen, lacks any halogen substituent and is a reversible competitive inhibitor of both isoforms.[10,18] Crystal structures are available for flurbiprofen in complex with both COX-1[14] and COX-2,[13] and for ibuprofen in complex with COX-1.[14] The structure–activity findings are reported here and discussed in the context of available structural information.

COX-1 enzyme was purified from ovine seminal vesicles as described previously;[19] purity, as estimated from Coomassie-stained SDS PAGE, was greater than 95%. Cyclooxygenase activity was measured at 22 °C using a coupled cyclooxygenase-peroxidase assay, in which the oxidation of the reducing peroxidase cosubstrate *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine (TMPD) was monitored after the addition of arachidonic acid.[20] Assay conditions were as follows: 0.1 M Tris–HCl pH 8.0, 120 μM TMPD, and 80 nM hematin-reconstituted enzyme; reactions were initiated with 60 μM arachidonic acid. Because COX-1 auto-inactivates, initial rate measurements were used in all experiments. IC_{50} measurements were carried out by pre-warming aliquots of enzyme to 37 °C, adding inhibitor, and incubating at 37 °C for an additional 5 min. Samples were then cooled on ice and activity measurements taken. In order to classify inhibitors as time-dependent or time-independent, time-course experiments were carried out in which enzyme was pre-warmed to 37 °C, inhibitor was added at time zero, and aliquots were withdrawn for activity measurements at different time points. K_i and k_{inact} values for **1** and **2** were estimated by incubating enzyme (100 nM) with 120 μM TMPD and different concentrations of inhibitor at 22 °C for varying times, after which the reaction was initiated by the addition of 60 μM arachidonic acid. Control experiments using hydrogen peroxide as an initiator and 250 μM inhibitor showed no peroxidase inhibition by any of the compounds studied.

To examine the contribution of the fluorine atom to the kinetic properties of the inhibitor flurbiprofen [**1,** Sigma Chemical (St. Louis, MO)], the defluorinated analogue, 2-(1,1′-biphenyl-4-yl) propanoic acid (**2**), was synthesized as described previously.[21] Compound **2** has previously been shown to be a potent antispasmotic agent;[22] however, no COX inhibition data were available for this compound. Racemic preparations of **1** and **2** were used for kinetic analysis, but only the *S*-enantiomer is expected to bind.[12] Contrary to the prediction of Rome and Lands, removal of the halogen does not convert flurbiprofen to a reversible competitive inhibitor. However, the fluorine atom does contribute to potency, **1** being almost three-fold more potent than its defluorinated analogue **2** (Table 1). A similar increase in potency is associated with the iodination of the time-dependent 2-aryl propionic acid NSAID suprofen.[23] Recently, high-resolution crystal structures of COX-1 in complex with **1** and **2** have become available[21] (Gupta et al., in preparation). The inhibitors adopt identical positions and orientations in the cyclooxygenase active site, and no changes in the active site geometry are apparent which could account for the difference in potency (Fig. 1). Two alternate positions have been identified for the fluorine atom of flurbiprofen, corresponding to 180° rotations of the phenyl ring; the two positions have approximately equal occupancies. It is unclear if one or both of these alternate binding modes contributes to the greater potency of **1** versus **2**.

Table 1. Inhibition of cyclooxygenase-1 by flurbiprofen derivatives



Compd	R_1	R_2	IC_{50} (μM)[a]	Time Dependent?
1 (flurbiprofen)	F	OH	0.012±0.004	Yes
2	H	OH	0.032±0.002	Yes
3	F	OCH_3	210±28	No
4	F	NH_2	8.3±2	No
5	F	$NH(CH_3)$	24±6	No
6	F	$N(CH_3)_2$	215±7	No
7	H	OCH_3	28.5±1	No
8	H	NH_2	64.5±5	No
9	H	$NH(CH_3)$	19.5±2	No
10	H	$N(CH_3)_2$	4.8±1	No

[a] Standard errors were estimated from two independent trials, each performed in triplicate.

Since **2**, like **1**, proved to be a time-dependent inhibitor of COX-1, experiments were undertaken to determine if removal of the fluorine atom significantly alters the kinetics of binding. The most general model commonly used for time-dependent inhibition of COX-1 involves two reversible steps: first a rapid reversible binding of the inhibitor, followed by a second, much slower inactivation step (Scheme 1A).[24] However, since reversal of the inactivation step is generally quite slow ($k_{-2} << k_2$), a considerably simpler model in which $k_{-2} \approx 0$ has also been used (Scheme 1B).[7]

To estimate the parameters associated with the kinetic model, enzyme can be preincubated with inhibitor, and then assayed after a time t by addition of a large excess of substrate ($[S] >> K_m$). The excess of substrate ensures that any enzyme–inhibitor complexes in rapid equilibrium with free enzyme will bind substrate and catalyze its transformation to product. For such a preincubation experiment, the simple model shown in Scheme 1B predicts that the fractional remaining enzyme activity will vary with time as follows:[7]

$$\frac{E_{active}}{E_{total}} \approx \frac{E_{total} - EI^*}{E_{total}} = \exp\left(\frac{-k_{inact}[I]t}{K_i + [I]}\right) \quad (1)$$

K_i and k_{inact} values for compounds **1** and **2** were determined by fitting to eq 1 above and are summarized in Table 2. The kinetic constants obtained for flurbiprofen (**1**) are consistent with previous determinations.[6,7,24] The K_i value for the defluorinated analogue **2** is seven-fold

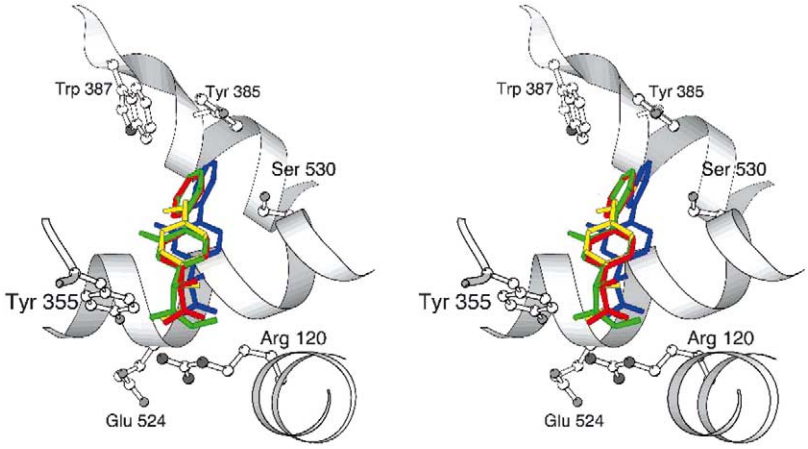
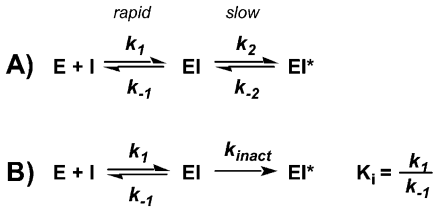


Figure 1.



Scheme 1. Kinetic models for the inactivation of COX by NSAIDs: (A) General two-step mechanism. Free inhibitor is in rapid equilibrium with the enzyme-bound form (EI); EI slowly undergoes a transition to the inactive EI* form. k_{-2} Is typically $<<k_2$, making the EI to EI* transition pseudo-irreversible; (B) Simplified two-step mechanism assuming $k_{-2}=0$, making $k_2=k_{inact}$.

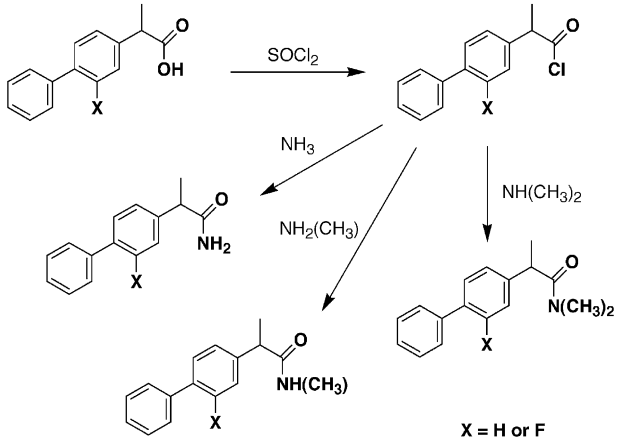
Table 2. Kinetic constants for the inhibition of COX-1 by flurbiprofen (**1**) and its defluorinated analogue (**2**)

Compd	K_i (μM)[a]	k_{inact} (min^{-1})
1	1.0±0.5	1.0±0.4
2	0.13±0.03	0.19±0.02

[a] Time–velocity curves were obtained at 5 different inhibitor concentrations ranging from 5 to 5000 nM. Data from three independent determinations were combined and simultaneously fit to eq 1 using GraphPad Prism 4 nonlinear regression software. Asymptotic standard errors were estimated by the program.

lower than that determined for **1**; however, the rate of inactivation seen with **2** is only about 20% of the rate seen with **1**. Hence, while the fluorine atom might slightly hinder initial binding within the cyclooxygenase active site, it appears to enhance the rate of formation of the EI* complex.

To further probe the structural determinants of slow tight-binding inhibition in flurbiprofen, derivatives of **1** and **2** in which the carboxylic acid moiety was altered were synthesized and characterized for their ability to inhibit cycloxygenase activity in a time-dependent fashion. Methyl esters of **1** and **2** were synthesized as described previously.[14] Amide and amide-substituted derivatives of **1** and **2** were produced by using thionyl chloride to generate the acid chlorides, which were then treated with the appropriate amines to generate the amides **4**–**6** and **8**–**10** (Scheme 2). All preparations used



Scheme 2. Synthesis of amide derivatives of flurbiprofen.

for kinetic analysis were racemic mixtures.

The methyl ester of flurbiprofen (**3**) is a weak inhibitor that displays no time-dependence (Table 1). Interestingly, the methyl ester **7** is over seven-fold more potent than its halogenated analogue **3**. The structural basis for this is not readily apparent from the crystal structures of COX-1 bound to **1**, **2**, and **3**. It has recently been shown that certain carboxylic acid-containing NSAIDs can bind to COX-2 in an 'upside-down' orientation, with the acidic group interacting with Ser-530, rather than Arg-120;[25] perhaps, in a similar manner, the loss of the fluorine atom allows **7** to flip from the expected orientation and find a more favorable binding site with its ester moiety in the upper part of the cyclooxygenase channel.

All of the amide and substituted amide derivatives examined proved to be reversible competitive inhibitors of COX-1 (Table 1), with no slow tight-binding character. Increasing substitution on the amide moiety decreased potency in the series derived from compound **1**. However, the same pattern of substitution had the opposite effect in the dehalogenated series derived from **2**: potency increased with increasing steric bulk at the amide moiety. This result was unexpected, and the structural basis for this trend is not understood, as no crystal structure is currently available for a 2-aryl propionamide NSAID

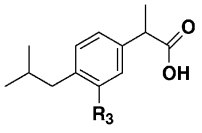
complexed with COX. Similar conflicting trends have been seen with COX-2 inhibitors; for example, increasing the bulk of the substituents on indomethacin amides increases COX-2 inhibitory potency,[26] whereas increasing substituent size on meclofenac amides reduces affinity for the enzyme.[17]

Removal of the halogen from the slow tight-binding inhibitor **1** does not alter its time-dependent character. A converse approach was also attempted, to determine if the addition of a halogen to a time-independent inhibitor could convert it into a slow tight-binding inhibitor. The reversible competitive inhibitor ibuprofen (**11**) was nitrated or brominated at the 3-position on the aromatic ring (analogous to the position of the fluorine substituent in **1**), and the products kinetically characterized. The synthetic Scheme is described in Scheme 2. Again, racemic mixtures were used.

The characteristics of the ibuprofen series are summarized in Table 3. Like the parent compound **11**, both the 3-nitro- and 3-bromo-substituents are reversible competitive inhibitors of cyclooxygenase activity. Nitration reduces potency substantially, increasing the IC_{50} approximately 60-fold. However, bromination improved the potency 10-fold. This result is surprising, given the large atomic radius of bromine and the small size of the constriction (formed by Arg-120, Glu-524, and Tyr-355) through which the ligand must pass in order to reach the cyclooxygenase active site. A small hydrophobic cleft exists within the COX-1 cyclooxygenase active site, and is lined by the side chains of Leu-352, Phe-518, and Ile-523. Based on the crystal structure of the COX-1-ibuprofen complex, it is possible to model **13** into the cyclooxygenase site with its bromine substituent pointing into this hydrophobic cleft. The crystal structure of **11** bound to COX-1 shows that the phenyl and isobutyl groups of the inhibitor make relatively few close contacts within the active site, and that the ligand is somewhat smaller than the actual volume of the cyclooxygenase active site.[14] Therefore, the higher potency of **13** may stem from its improved complementarity with the COX-1 active site (Scheme 3).

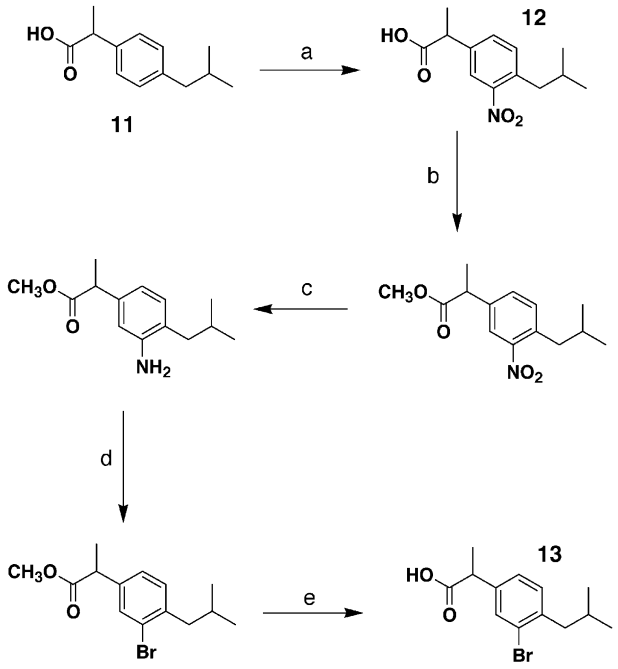
The results presented in this paper show that halogenation is not a determinant of time-dependent inhibition in the 2-aryl propionic acid class of NSAIDs. Neither the removal of fluorine from flurbiprofen nor the addition of bromine to ibuprofen was sufficient to alter these inhibitors' kinetic profiles. Halogenation does contribute significantly to inhibitor potency for compounds such as **1** and **13**; however, this is not generally true, as the bromo-, chloro-, and iodo- derivatives of the related NSAID ketoprofen are ineffective towards COX.[27] Halogenation can also exert more subtle effects on NSAID recognition by COX, as is illustrated by the interplay between the substituents at the 3-position and the amide group in compounds **4**–**6** and **8**–**10**.

Table 3. Inhibition of cyclooxygenase-1 by ibuprofen derivatives



Compd	R_3	IC_{50} (μM)[a]	Time dependent?
11 (ibuprofen)	H	1.06±0.13	No
12	NO_2	59±2.8	No
13	Br	0.11±0.06	No

[a] Standard errors were estimated from two independent trials, each performed in triplicate.



Scheme 3. Bromination of ibuprofen. Reaction conditions: (a) HNO_3, H_2SO_4, 0 °C, 0.5 h; (b) CH_3OH, H_2SO_4, rt, overnight; (c) NH_4Cl, Fe powder, CH_3OH, reflux, 14 h; (d) $NaNO_2$, CuBr, 0 °C, 1 h; (e) NaOH, CH_3OH, reflux, 5 h.

In contrast, alteration of the carboxylic acid moiety of 2-aryl propionic acid NSAIDs appears to be a dependable route to abolishing slow tight-binding binding behavior. These results recapitulate findings in the fenamate and indole acetic acid classes of cyclooxygenase inhibitors, where neutralization of the carboxylic group also leads to reversible competitive inhibitors of COX-1.[15,16,26] The apparent generality of this phenomenon is noteworthy, given that at least one member of the fenamate class of NSAIDs, diclofenac, does not form an ion pair with Arg-120.[25]

Acknowledgements

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