Molecular Determinants for the Selective Inhibition of Cyclooxygenase-2 by Lumiracoxib

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The cyclooxygenase (COX) enzymes COX-1 and COX-2 share high sequence homology (60%), have very similar three-dimensional structures, and catalyze the conversion of arachidonic acid (AA) to prostaglandin H₂. Lumiracoxib is a highly selective COX-2 inhibitor that is weakly acidic and displays a unique pharmacological profile that includes rapid absorption and a relatively short plasma half-life (1). Lumiracoxib displays a 500-fold greater selectivity for COX-2 than COX-1 in vivo (2) and in clinical studies has shown a 3–4-fold reduction in ulcer complications versus classical NSAIDs (3, 4). Lumiracoxib lacks the tricyclic structure of the diarylheterecycle class of COX-2-selective inhibitors (e.g. celecoxib and rofecoxib) and does not contain a sulfonamide or sulfone group. Instead, lumiracoxib is a close structural analog of diclofenac (Fig. 1). Although lumiracoxib and diclofenac share structural similarities, they exhibit large differences in the selectivity of COX-2 inhibition. The molecular basis for this difference in the selectivity of COX inhibition is not entirely understood.

Previous crystal structures of COX enzymes with carboxylic acid-containing NSAIDs reveal that the inhibitors are typically positioned with their carboxylates coordinated to Arg-120 and their aromatic functional groups projecting up into the cyclooxygenase active site (5, 6). However, a crystal structure of diclofenac bound in the active site of COX-2 (Fig. 2B) revealed an inverted binding mode of the molecule with its carboxylic acid moiety coordinated to Ser-530 and Tyr-385 (7). Structure-activity studies have found that another distinctive binding mode is exhibited by indomethacin, a nonselective NSAID that is a time-dependent, functionally irreversible inhibitor of COX enzymes (8). Indomethacin binds in the cyclooxygenase active site with its carboxylic acid group ion-pairing and hydrogen-bonding with Arg-120 and Tyr-355 (Fig. 2A). In addition, its 2′-methyl group binds in a hydrophobic pocket composed of Ala-527, Val-349, Ser-530, and Leu-531 (6). Mutations of Val-349 in this methyl-binding pocket to alanine or leucine alter the size of the pocket and lead to an increase or decrease in the potency of indomethacin, respectively. The 2′-des-methyl analog of indomethacin does not inhibit COX-1 and is a very weak, rapidly reversible inhibitor of COX-2 (8).

Recently, the crystal structure of murine COX-2 with lumiracoxib was solved and revealed that lumiracoxib binds in an inverted orientation similar to that of diclofenac (9). The carboxylate of lumiracoxib forms hydrogen-bonding interactions with Ser-530 and Tyr-385 at the top of the active site. From this crystal structure, it was proposed that the COX-2 selectivity of lumiracoxib arises from the insertion of the methyl group on the phenylacetic acid ring into a small groove provided by the movement of Leu-384 in the COX-2 active site. The movement
of this leucine residue is restricted in the COX-1 active site due to the presence of bulky secondary shell residues behind Leu-384 (Ile-525 and Phe-503) that prevent the movement of this residue with inhibitor bound. The corresponding secondary shell residues are Leu-525 in mCOX-2 and Val-525 in hCOX-2 and Leu-503 in both m/hCOX-2 enzymes. The crystal structure failed to elucidate the precise binding sites of the halogens on the lower aniline ring of lumiracoxib, although it appears that either atom could have accessibility to the small hydrophobic pocket utilized by the 2'-methyl group of indomethacin (Ala-527, Val-349, Ser-530, and Leu-531).

Although the selectivity of lumiracoxib for COX-2 has been determined in vivo and the crystal structure of lumiracoxib-bound mCOX-2 has been solved, structure-activity relationship studies (SARs) have not been performed, and the chemical and structural basis for the balance that exists between potency and COX-2 selectivity remains unknown for this inhibitor. Therefore, we undertook the synthesis and characterization of a number of lumiracoxib analogs in an attempt to uncover the chemical determinants for selective COX-2 inhibition. In addition, the molecular basis for the selectivity of lumiracoxib was investigated by probing these chemical derivatives against Val-349 and Ser-530 mutants in the COX-2 active site.

**EXPERIMENTAL PROCEDURES**

**Materials and Enzymes**—The starting materials, ligands, and catalysts for all chemical reactions were commercially available and were purchased from Sigma and Alfa Aesar (Ward Hill, MA). The synthesis and purification of both methylated and des-methyl lumiracoxib analogs was carried out as described in the supplemental materials with only minor modifications to the reaction conditions being necessary for individual compounds. The expression and purification of murine COX-2 and human COX-2 from insect cells and of ovine COX-1 from ram seminal vesicles was performed according to published methods (10). Site-directed mutagenesis on murine COX-2 to generate V349A, V349I, V349L, and S530A mutants was performed as described by Prusakiewicz et al. (8). [1-14C]AA was purchased from PerkinElmer Life Sciences.

**Synthesis and Structural Characterization of Lumiracoxib Analogs**—Various 5'-methylated and des-methyl lumiracoxib analogs were synthesized as described in the supplemental materials (sections A and B) according to supplemental Schemes 1 and 2. Following synthesis, the mass of the purified product was confirmed by electrospray ionization mass spectra, and the structure was confirmed by one-dimensional 1H NMR. The purity of the product was assessed by high performance liquid chromatography (HPLC) using a light scattering detector. The chemical characterization of each lumiracoxib analog is summarized in the supplemental materials (section C).

**Enzymes**—All activity or inhibition studies were performed in 100 mM Tris-HCl buffer containing 500 μM phenol with hematin-reconstituted protein. All inhibitors were dissolved in Me2SO. Reactions were run with hematin-reconstituted proteins at final enzyme concentrations adjusted to give ~30–35% substrate consumption (hCOX-2 = 145 nM, mCOX-2 = 63 nM, oCOX-1 = 22.5 nM, S530A = 165 nM, V349A = 250 nM, V349I = 268 nM, and V349L = 113 nM).

**Competitive Inhibition Assays for COX**—Several assays were used to determine whether the various lumiracoxib analogs were competitive inhibitors of COX. In one assay, the dual administration of inhibitor and different concentrations of [1-14C]AA (6–50 μM) was performed following a 3-min equilibration of enzyme at 37 °C. In another method, inhibitor was prebound to enzyme for 3 min at 37 °C, followed by the addition of [1-14C]AA at different concentrations for 30 s. In an additional assay that examined the reversibility of COX inhibition, [1-14C]AA was added at a range of concentrations and incubated with inhibitor-bound enzyme for different times (30 s to 5 min) to assess the ability of the substrate to compete off the inhibitor. All assays were termi-
nated and analyzed for substrate consumption by TLC as previously described (11). The values reported were the average of two or more independent determinations.

**COX Inhibition Screening Assay**—Concentration-dependent inhibition reactions were performed by preincubating the inhibitor and enzyme for 17 min at 25 °C, followed by 3 min at 37 °C prior to the addition of 50 μM [1-14C]AA for 30 s at 37 °C. Assays were terminated and analyzed for substrate consumption by TLC as described above. All inhibitor concentrations for 50% enzyme activity (IC50) were determined graphically using Prism and were the average of at least two independent determinations.

**Time-dependent COX Inhibition Assays**—Time-dependent inhibition assays were conducted by preincubating increasing concentrations of the inhibitor with m/hCOX-2, oCOX-1, or the Val-349 mutants (V349A, V349I, and V349I) for various times (0, 0.125, 0.25, 0.5, 1, 3, 5, 15, 30, and 60 min) at 37 °C prior to the addition of 50 μM [1-14C]AA for 30 s at 37 °C. Reactions were terminated and analyzed by TLC as described above. The values of the kinetic parameters were the average of three independent determinations.

**RESULTS**

**Synthesis of Lumiracoxib and a Series of Lumiracoxib Analogs**—A series of lumiracoxib analogs that varied in the substituents on the phenylacetamide ring and on the lower aniline ring were synthesized (Table 1). Lumiracoxib itself (compound 1) was synthesized according to a previously published multistep synthetic method that is described in Scheme 1 of the supplemental materials (12). The various structural analogs of lumiracoxib were also synthesized according to Scheme 1 (methyl derivatives; illustrated for compound 1) or Scheme 2 (des-methyl derivatives; illustrated for compound 11). Mass spectral analysis, one-dimensional proton NMR spectroscopic,
Determinants of Cyclooxygenase-2 Inhibition by Lumiracoxib

and HPLC analyses were performed on each of the lumiracoxib analogs to confirm both the structure and purity of the compounds. The \(^{3}\)H NMR spectrum of each lumiracoxib derivative was compared with previously published spectra where available (12, 13), and each of the analogs was shown by HPLC analysis with evaporative light scattering detection to elute as a single peak. The spectral characterization of each lumiracoxib analog is provided in the supplemental materials.

**Evaluation of COX Inhibition by Lumiracoxib**—The kinetic basis for lumiracoxib inhibition was determined. Following a 3-min equilibration of purified, hematin-reconstituted m/hCOX-2 or oCOX-1 at 37 °C, lumiracoxib and [1-\(^{14}\)C]AA were added simultaneously and incubated for 30 s. No significant inhibition was observed over a wide range of inhibitor and substrate concentrations, indicating that lumiracoxib is not a pure competitive inhibitor (supplemental materials, section D, Fig. 1).

We next tested lumiracoxib against purified m/hCOX-2 and oCOX-1 using a standard time-dependent protocol designed to determine IC\(_{50}\) values for enzyme inhibition. Fig. 3A shows the inhibition curve for lumiracoxib (compound 1). Lumiracoxib did not inhibit oCOX-1 to any appreciable extent, consistent with prior studies (14). Unexpectedly, however, lumiracoxib proved to be a poor inhibitor of both mCOX-2 (~15% inhibition) and hCOX-2 (~50% inhibition). Prior in vitro and in vivo experiments with lumiracoxib have demonstrated that the inhibitor exhibits slow, time-dependent inhibition of purified hCOX-2 with a \(K_i\) of 60 nM and IC\(_{50}\) values of 130 and 140 nM, respectively, for the inhibition of COX-2 in cell-based assays and human whole blood assays (14). In our study, the inhibition of hCOX-2 activity plateaus at ~50% inhibition and displays characteristics of a reversible inhibitor. Although the extent of inhibition of the human isoform is only 50%, our results do support the fact that lumiracoxib exhibits selectivity for hCOX-2 over COX-1, since no appreciable inhibition of oCOX-1 was observed even with very high concentrations of inhibitor (100 \(\mu\)M).

The standard IC\(_{50}\) assay described above uses levels of arachidonic acid (50 \(\mu\)M) that are well above saturation. Thus, if inhibition of m/hCOX-2 by lumiracoxib is readily reversible, lumiracoxib would not be expected to strongly inhibit the COX activity at high substrate concentrations. To probe the effect of substrate concentration on enzyme inhibition, we incubated lumiracoxib with different concentrations of murine or human COX-2 for 3 min at 37 °C prior to the addition of [1-\(^{14}\)C]AA (6-50 \(\mu\)M) for 30 s. Fig. 3B shows that lumiracoxib competes moderately with substrate at 50 \(\mu\)M AA but inhibits hCOX-2 to nearly 100% at high inhibitor concentrations and low arachidionate concentrations. Fig. 3C shows a similar profile for lumiracoxib and mCOX-2. Lumiracoxib is a weaker inhibitor of the murine enzyme, as demonstrated by the low extent of inhibition at 25 and 50 \(\mu\)M AA. In addition, lumiracoxib was not able to fully inhibit mCOX-2 even at the lowest substrate concentrations and highest inhibitor concentrations.

Lumiracoxib, at a concentration of 1 \(\mu\)M, was prebound to purified enzymes for 3 min at 37 °C, followed by the addition of arachidonate at 50 \(\mu\)M for increasing times (30 s to 5 min). The time course for oxidation of arachidonate is a reflection of the dissociation of lumiracoxib; it exhibited rate constants of 0.0098 s\(^{-1}\) and 0.020 s\(^{-1}\) for hCOX-2 and mCOX-2, respectively (Fig. 4). These results corroborate the inhibition plateaus (15% for mCOX-2 and 50% for hCOX-2) that were initially observed with the IC\(_{50}\) screen and indicate a more rapid reversibility for the murine enzyme. Diclofenac was tested in the same assay and showed off-rates from murine and human COX-2 in the range of \(2 \times 10^{-5}\) s\(^{-1}\), suggesting a much more tightly bound inhibitor.

The Kinetics of COX Inhibition by Lumiracoxib—The dependence of COX inhibition by lumiracoxib on time and inhibitor concentration was determined by adding arachidonic acid to wild-type COX preparations following preincubation with inhibitor for various times. The decrease in substrate conversion at different inhibitor concentrations was plotted against the preincubation times and fit to a single-exponential decay with a plateau to determine a value for \(k_{obs}\). The dependence of \(k_{obs}\) on inhibitor concentration is represented by Equation 1, where \(K_i\) corresponds to the inhibitor concentration that yields a rate equal to half the limiting rate, and \(k_{2}\) represents the limiting forward rate constant for inhibition (8). The reverse
Determination of Cyclooxygenase-2 Inhibition by Lumiracoxib

Determinants of Cyclooxygenase-2 Inhibition by Lumiracoxib

FIGURE 4. Arachidonic acid oxygenation time course by inhibited murine and human COX-2. Lumiracoxib or diclofenac (1 μM) were preincubated with m/hCOX-2 for 3 min at 37 °C prior to the addition of substrate (50 μM) for increasing times (30 s to 5 min). Reactions were terminated and analyzed by TLC as described under "Experimental Procedures." Each data point represents at least two independent experiments. Off-rates of lumiracoxib or diclofenac from the enzymes are reported under "Results."

TABLE 2
Kinetic parameters of time-dependent inhibition of COX enzymes and mutants by lumiracoxib (1) and compound 2

|           | Kinetic Parameter | Value          |
|-----------|-------------------|----------------|
| Lumiracoxib, mCOX-2 | k₁ | 0.32 ± 0.11 M/s⁻¹ |
| Lumiracoxib, hCOX-2 | k₁ | 0.087 ± 0.007 M/s⁻¹ |
| Lumiracoxib, mCOX-2 | k₂ | 0.0017 ± 0.0006 M/s⁻¹ |
| Lumiracoxib, hCOX-2 | k₂ | 0.107 ± 0.010 M/s⁻¹ |
| Lumiracoxib, mV349I | k₆ | 0.0025 ± 0.0003 M/s⁻¹ |
| Lumiracoxib, hV521L | k₆ | 0.0006 M/s⁻¹ |

rate constant, k₋₂, is equal to the y intercept and is zero for compounds that display functionally irreversible inhibition.

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k_{\text{obs}} = ((k_2 \times [1])/(K_i + [1])) + k_{-2}
\]

Table 2 shows the kinetic parameters for the time- and concentration-dependent inhibition of wild-type COX enzymes by lumiracoxib. The values for the inhibition of hCOX-2 were in good agreement with previously reported values of Kᵢ (0.06 μM) and k₋₂ (0.005 s⁻¹) (14). Since different experimental assays were used to determine the kinetic parameters for COX inhibition, the present values of Kᵢ and k₋₂ for lumiracoxib inhibition of hCOX-2 are slightly higher (0.32 μM and 0.087 s⁻¹) than the values noted above. As indicated by a plateau at 50% activity remaining in the IC₅₀ curve, the time-dependent inhibition of hCOX-2 by lumiracoxib resulted in a measurable k₋₂ (0.0017 s⁻¹), which indicated that the second binding step was reversible. This k₋₂ is close to the off-rate for lumiracoxib from hCOX-2 measured from the time course of arachidonic acid oxygenation described above (0.0098 s⁻¹) (Fig. 4). Not surprisingly, the kinetic parameters for the inhibition of murine COX-2 by lumiracoxib could not be determined at either room temperature or 37 °C due to poor inhibition of this enzyme by lumiracoxib at high arachidonate concentrations. This is in agreement with the attempted IC₅₀ determination for lumiracoxib inhibition of mCOX-2 where a plateau was reached at 85% remaining activity.

SAR Requirements for Lumiracoxib Potency and COX-2 Selectivity—The COX-2 selectivity of lumiracoxib has been attributed to the methyl group on the phenylacetic acid ring of the inhibitor and its insertion into a small pocket around Leu-384, which is made accessible by small secondary shell residues that exist only in COX-2 (9). The role of the halogen substituents on the lower aniline ring and the nature of their interactions with COX-1 or COX-2 has not been investigated. Hypothetically, the fluorine or chlorine substituents of lumiracoxib may also contribute to COX-2 selectivity and/or to the potency of inhibition. Purified and hematin-reconstituted hCOX-2, mCOX-2, and oCOX-1 were screened against lumiracoxib and the various synthetic analogs (compounds 1–22; Table 1) in our standard time dependence IC₅₀ assay. Several compounds were additionally screened in separate assays to rule out COX inhibition at low arachidonate concentrations (data not shown).

Fig. 5 shows the inhibition curves for diclofenac (A), lumiracoxib (B), and three lumiracoxib analogs (C–E) against purified COX-1 and COX-2. As expected from previously reported inhibition studies (7), diclofenac (compound 10) was a potent, nonselective inhibitor of all three cyclooxygenase isomers. Replacement of the hydrogen with a methyl group at the 5′-position on the phenylacetic acid ring of diclofenac yields compound 2. Compound 2 appears to reversibly inhibit COX-1 (50% inhibition) but potently inhibits both mouse and human COX-2 in a functionally irreversible manner (nearly 90% inhibition) with IC₅₀ values of 63 and 47 nM, respectively (Fig. 5D and Table 1). From these data, it appears that the addition of the methyl group on the phenylacetic acid ring of compound 2 introduces COX-2 selectivity. In support of this hypothesis, a des-methyl lumiracoxib analog (compound 11) exhibits nonselective inhibition of all COX isomers (Fig. 5C) compared with the selective hCOX-2 inhibition demonstrated by compound 1. A fluorine-to-chlorine substitution (compound 1 to compound 2) on the lower aniline ring significantly changes the potency of COX inhibition. Compound 1 inhibits hCOX-2 to only 50%, whereas inhibition of hCOX-2 by compound 2 results in nearly 90% inhibition (Fig. 5, B and D). Although diclofenac and compound 11 are both nonselective inhibitors, diclofenac demonstrates potent inhibition against all COX isozymes (∼90%) compared with its 6-fluoro counterpart (25–40%), supporting a role for the chemical substituents on the lower aniline ring in the potent inhibition of COX (Fig. 5, A and C). An additional substitution at the para position on the aniline ring (compound 6) abrogates the COX-2 selectivity observed with compound 2, suggesting that the precise positioning of substituents on the lower ring is required for selective inhibition of COX-2 (Fig. 5E).

Replacement of the fluorine atom on lumiracoxib with hydrogen to give the monochlorinated compound 3 negates any of the hCOX-2 selectivity observed with lumiracoxib and yields a compound that is a very poor inhibitor of all three COX enzymes (Table 1). The monofluorinated compound 4 and the nonhalogenated compound 5 were similarly shown to be very poor, nonselective COX-1/2 inhibitors in this assay. The des-methyl versions of these mono- or nonhalogenated derivatives
(compounds 12–14) showed identical results (Table 1), suggesting that a 2,6 substitution on the lower aniline ring is required for COX inhibition. However, derivatives that contained a 2,6-difluoro or 2-fluoro-6-methyl substitution did not inhibit any of the COX isoforms (compounds 7, 8, 15, and 16). Interestingly, a 2-chloro-6-methyl (compounds 9 and 17) or 2,6-dimethyl (compound 20) substitution restored potent and time-dependent COX inhibition. The addition of larger alkyl groups, such as ethyl (compound 21) and isopropyl (compound 22), to the ortho positions on the lower aniline ring resulted in a reduction of inhibitor potency (Table 1). Less potent inhibition of COX also was observed with derivatives that contained a trifluoromethyl substitution. Combined, these data indicate that a 2,6-dichloro, 2,6-dimethyl, or 2-chloro-6-methyl substitution is preferred for potent, time-dependent inhibition of COX. In addition, our SAR analysis corroborates the requirement for a methyl group on the phenylacetic acid ring to generate COX-2 selectivity. Compound 7 does not inhibit COX, whereas lumiracoxib (compound 1) is selective for hCOX-2 over oCOX-1. This potential role for the chlorine atom in selectivity is further supported by a comparison between compounds 8 and 9 (a 2-fluoro-6-methyl substitution versus a 2-chloro-6-methyl substitution). Here again, the replacement of a fluorine atom with a chlorine results in selective inhibition of COX-2.

Inhibition of the S530A Mutant by Lumiracoxib Analogs—The crystal structure of diclofenac bound to mCOX-2 shows the inhibitor bound in an inverted orientation in the active site with its carboxylate hydrogen-bonded to Ser-530 and Tyr-385 at the top of the active site (7). Ser-530 also contributes to binding interactions in the small hydrophobic side pocket utilized by the 2'-methyl group of indomethacin (Ala-527, Val-349, Ser-530, and Leu-531). Based on the assumption that lumiracoxib binds in a similar orientation as diclofenac in the COX-2 active site (9) and that Ser-530 is critical for hydrogen bonding interactions between the inhibitor and protein, several lumiracoxib analogs were screened in our standard inhibition assay against a murine S530A mutant. Fig. 6 shows a COX inhibition assay for...
Determinants of Cyclooxygenase-2 Inhibition by Lumiracoxib

FIGURE 6. Inhibition of a S530A mutant by lumiracoxib analogs. Compounds 2 (open symbols) and 9 (closed symbols) were preincubated with wild-type mCOX-2 or a S530A mutant for 20 min prior to the addition of substrate (50 μM), and each reaction was terminated and analyzed as described under “Experimental Procedures” for COX inhibition assays. Each data point represents at least two independent experiments.

Compounds 2 and 9 (2,6-dichloro- and 2-chloro-6-methyl-substituted lumiracoxib derivatives) against both wild-type mCOX-2 and the S530A mutant. As expected, mutation of Ser-530 to alanine reduces both the potency and extent of inhibition observed with the wild-type enzyme and compounds 2 and 9 (from 85% to 20–30% inhibition). Several additional inhibitors were screened against the S530A mutant (diclofenac, lumiracoxib, and compound 20; data not shown). In each case, the mutation of Ser-530 to Ala abrogated the inhibition observed with wild-type mCOX-2. It is clear from these data that these compounds are interacting with Ser-530 and that this interaction is required for COX inhibition.

Inhibition of Val-349 Mutants by Lumiracoxib and Analogs—A small hydrophobic pocket exists in the active site of both COX-1 and COX-2 that is composed of four residues (Ala-527, Val-349, Ser-530, and Leu-531). The potent, time-dependent inhibition of COX by indomethacin is attributed to the insertion of the 2’-methyl group of the inhibitor into this small side pocket (8). Mutation of Val-349 to alanine increases the size of the pocket and increases the potency of indomethacin 3-fold, whereas mutation to leucine has the opposite effect on pocket size and decreases the potency of the inhibitor by 16-fold. A Val-349 to isoleucine mutation resembles wild-type enzyme (8). The crystal structure of lumiracoxib bound to mouse COX-2 indicates that either the fluorine or chlorine substituent on the lower aniline ring may be making contacts in this same small hydrophobic pocket (9). To investigate the importance of interactions between these chemical moieties with the small hydrophobic pocket, various lumiracoxib analogs were screened against V349A, V349I, and V349L mutants in our standard COX inhibition assay, and kinetic parameters were determined for the time-dependent inhibition of these enzymes by key inhibitors.

Fig. 7 shows the results of a concentration-dependent inhibition assay in which the valine mutants were tested against diclofenac (A), lumiracoxib (B), and several lumiracoxib analogs with key substitutions at the 2,6-position on the aniline ring (C–E). Mutation of valine to alanine at position 349 enlarges the size of the side pocket, but, unlike indomethacin and V349A, a decrease in the potency of diclofenac was observed against this mutant. A Val-349 to leucine mutation provides steric bulk and decreases the size of the hydrophobic pocket, which abrogates any inhibition observed with diclofenac and mCOX-2. Interestingly, mutation of Val-349 to isoleucine results in an enzyme that exhibits equal potency with diclofenac against wild-type COX-2 (Table 3). Similar results were observed with compound 2 (Fig. 7D), a diclofenac analog with a methyl group on the phenylacetic acid ring. Perhaps the most intriguing result was observed with lumiracoxib (Fig. 7B) and compound 11 (Fig. 7C), a des-methyl lumiracoxib derivative. Although both compounds were poor inhibitors of the wild-type mCOX-2 enzyme (less than 20% inhibition), the potency of these inhibitors increased dramatically when screened against the isoleucine mutant (Table 3). As with diclofenac, these compounds did not inhibit V349A or V349L to a significant extent. From these data, it appears that there are interactions between the residues in the small hydrophobic pocket and these particular lumiracoxib analogs. It is possible that the ability of the halogens to insert into this pocket may determine the potency and extent of COX inhibition observed with these inhibitors.

To understand more clearly the possibility of inhibitor-protein interactions in the side pocket, several additional lumiracoxib derivatives were screened against the Val-349 mutants. Compound 15 (Fig. 7E), a 2,6-difluoro-substituted analog, did not demonstrate an increased potency against the V349I mutant. This suggests that, at the minimum, a fluorine-chlorine substitution is required at the 2,6-position on the aniline ring to observe an increased potency with V349I. Likewise, derivatives of diclofenac and lumiracoxib that contained only a monosubstituted aniline ring did not inhibit the V349I mutant (Table 3). Interestingly, 2-fluoro-6-methyl derivatives (compounds 8 and 16) resembled a monohalogenated or difluoro-substituted inhibitory profile against the Val-349 mutants, whereas replacement of the fluorine atom with a chlorine atom to yield compounds 9 and 17 generated 2-chloro-6-methyl derivatives that demonstrated increased potency against the V349I enzyme (Table 3). The V349I mutant was resistant to inhibition by compound 22, a 2-methyl-6-isopropyl inhibitor, suggesting that the steric bulk of the isopropyl group was restricting access to the small hydrophobic side pocket. Compound 21, a 2-methyl-6-ethyl derivative, was the only inhibitor tested that showed activity against the V349A mutant with an IC50 of 167 nM compared with wild-type enzyme (174 nM). These data indicated that an ethyl substitution on the lower aniline ring would fill the small hydrophobic binding pocket and inhibit V349A in a manner similar to wild-type mCOX-2. In agreement with this hypothesis, a decrease in potency was observed with compound 21 and the V349I mutant (891 nM), suggesting a steric clash between the inhibitor and protein that leads to a lower affinity interaction.

Altogether, this SAR analysis of lumiracoxib analogs with the Val-349 mutants suggests that a chlorine substituent is preferred on the lower aniline ring for potent inhibition of COX-2 and that a combination of F-Cl, Cl-Cl, or Cl-CH3 is allowed at the 2,6-position to maintain potency. In addition, these data imply that it is the chlorine atom on lumiracoxib that is making binding interactions (perhaps through a halogen bond to the hydroxyl of Ser-530) in the small hydrophobic pocket that consists of Val-349, Ser-530, Ala-527, and Leu-531 and that this interaction may be responsible for the potency of inhibition observed with the various lumiracoxib analogs against COX,
whereas the methyl group on the phenylacetic acid ring determines selectivity.

The Kinetics of COX Inhibition by Lumiracoxib Analogues—
The dependence of COX inhibition by lumiracoxib analogues on time and inhibitor concentration was determined as described above for lumiracoxib. Supplemental Fig. 2 and Fig. 8 show the kinetics of the time-dependent inhibition of hCOX-2 and oCOX-1 by compound 2. Inhibition of the COX enzymes by compound 2 proceeded rapidly, and plateaus were reached at short time points for the higher inhibitor concentrations. Supplemental Fig. 2 shows inhibition time courses out to 30 min preincubation times from which $k_{obs}$ values were obtained and plotted in secondary graphs to determine kinetic constants. Supplemental Fig. 2 shows inhibition time courses out to 30 min preincubation times from which $k_{obs}$ values were obtained and plotted in secondary graphs to determine kinetic constants. The time-dependent inhibition curve for wild-type hCOX-2 approached 0% remaining activity, indicating functionally irreversible inhibition of the enzyme by compound 2. Similar results were demonstrated for wild-type mCOX-2 (data not shown). Inhibition of oCOX-1 by compound 2 approached a nonzero asymptote of nearly 20% remaining activity at the highest inhibitor concentrations, indicating that the second step of binding was moderately reversible. Fig. 8 shows the rapid inhibition kinetics of hCOX-2 (A) and oCOX-1 (C) by compound 2 at time points less than 2 min. For both wild-type human and mouse COX-2, the $y$ intercept of the secondary plot of $k_{obs}$ versus inhibitor concentration was effectively zero (Fig. 8B; data not shown). In contrast, the secondary plot of data for oCOX-1 showed a nonzero $y$ intercept (Fig. 8D), which generated a measurable reverse rate constant for the second binding step ($k_{-2} = 0.0025 \text{ s}^{-1}$). In comparison with indomethacin ($K_I = 7.9 \mu\text{M}$ and $k_2 = 0.052 \text{ s}^{-1}$), a slow and time-dependent nonselective inhibitor of COX (8), compound 2 exhibits an increased binding affinity and similar forward rate constants for both mouse and human COX-2. The COX-2 selectivity of compound 2 is most likely related to the absence of a reverse rate constant for the second binding step in m/hCOX-2 compared with a measurable $k_{-2}$ for oCOX-1 inhibition.

Table 2 shows the kinetic parameters for the time- and concentration-dependent inhibition of wild-type and mutant COX enzymes by compound 2 and lumiracoxib. The kinetic parameters for mCOX-2 and hCOX-2 inhibition by compound 2 were similar ($K_I = 0.82$ and $1.2 \mu\text{M}$, respectively, and $k_2 = 0.069$ and $0.074 \text{ s}^{-1}$, respectively). Inhibition of oCOX-1 by compound 2 resulted in a slightly higher $K_I$, indicating a lower affinity of binding, and a measurable $k_{-2}$. As
noted in Table 2, mutation of Val-349 to isoleucine did not significantly alter $K_I$ and $k_2$ for compound 2. As mentioned previously, the time-dependent inhibition of hCOX-2 by lumiracoxib resulted in a measurable $k_{-2}$ (0.0017 s$^{-1}$), which indicated that the second binding step was reversible. Interestingly, this contrasts with the inhibition of hCOX-2 by compound 2, where a chemical change from a fluorine to chlorine effectively results in a functionally irreversible inhibitor of hCOX-2. As shown previously in Fig. 7, mutation of Val-349 to isoleucine in mCOX-2 results in a protein that is potently inhibited by lumiracoxib. Table 2 shows that lumiracoxib inhibits the V349I mutant with kinetic parameters similar to its inhibition of hCOX-2 except for the loss of a value for the reverse rate constant $k_{-2}$. Mutation of Val-349 to isoleucine increases the binding affinity of lumiracoxib for the enzyme and creates a functionally irreversible inhibitor.

**DISCUSSION**

Diclofenac is a more potent inhibitor of COX-2 than lumiracoxib, but it is also a much more potent inhibitor of COX-1 (Fig. 5). Neither compound is a pure competitive inhibitor of COX-2, but rather both are time-dependent inhibitors that exhibit a two-step mechanism of inhibition. Diclofenac and lumiracoxib differ significantly in the tightness of the inhibitor-COX-2 complex. Diclofenac exhibits a very slow dissociation rate constant from the enzyme of $-2 \times 10^{-5}$ s$^{-1}$. In contrast, lumiracoxib displays a rate constant for dissociation of 0.0017 s$^{-1}$. This relatively rapid dissociation is manifest by the plateau in the IC$_{50}$ curves at high inhibitor concentrations (Figs. [1][2][3][4]).

**TABLE 3**

| Analog | mCOX-2 | V349A | V349I | V349L |
|--------|--------|-------|-------|-------|
| 1      | -      | -     | 114 nM (75%) | -     |
| 2      | 63 nM (80%) | > 4 μM (25%) | 67 nM (85%) | -     |
| 9      | 94 nM (80%) | -     | 120 nM (100%) | -     |
| 10     | 22 nM (85%) | > 4 μM (40%) | 60 nM (85%) | -     |
| 11     | -      | -     | 54 nM (70%) | -     |
| 17     | 35 nM (100%) | -     | 76 nM (100%) | -     |
| 21     | 174 nM | 167 nM | 891 nM | -     |
Determinants of Cyclooxygenase-2 Inhibition by Lumiracoxib

3 and 5) and by the oxygenation of arachidonic acid by preformed COX-2-lumiracoxib complexes (Fig. 4). The lumiracoxib dissociation rate is greater for mouse COX-2 than human COX-2, which accounts for the apparent inability of lumiracoxib to inhibit the mouse enzyme in an IC50 experiment conducted at a high substrate concentration (Fig. 3). Lumiracoxib inhibits both human and mouse COX-2 at low substrate concentrations, which may be relevant to the situation in intact cells where arachidonic acid concentrations are anticipated to be relatively low and constantly changing because of reincorporation into the phospholipid pools.

Previous structure-activity studies with diclofenac analogs have indicated that methyl or chlorine substituents on the lower aniline ring in the ortho position are required for potent inhibition of COX (13). Analogs possessing higher potencies for COX inhibition have halogen substitutions (fluorine or chlorine) at the 5'-position of the phenylacetic acid ring (13). According to patent literature, modifications to the carboxylic acid group of diclofenac can yield potent and selective COX-2 inhibitors (15) and the incorporation of meta-alkyl substituents on the phenylacetic acid ring can also change the selectivity of the inhibitor to favor inhibition of COX-2 (lumiracoxib) (16).

The SAR studies described herein with lumiracoxib analogs indicate that the 5'-methyl group on the phenylacetic acid ring of the inhibitor is the major determinant for COX-2 selectivity and that the chemical nature of the substituents in the ortho positions on the lower aniline ring exert the major influence on the potency of COX inhibition (2,6-dichloro, 2,6-dimethyl, or 2-chloro-6-methyl substitutions are preferred). Interestingly, our data also suggest a contributory role for the chlorine atom of lumiracoxib in COX-2 selectivity.

Ser-530 was shown to be required for inhibition by lumiracoxib and several key analogs. This is not surprising, since the crystal structures of diclofenac- and lumiracoxib-bound mCOX-2 show the inhibitors making hydrogen bonding interactions with Tyr-385 and Ser-530 at the top of the active site (7, 9). Ser-530 also contributes to a small hydrophobic binding pocket that is composed of Val-349, Ala-527, and Leu-531. Fig. 9 (A and C) shows diclofenac bound to mCOX-2 with one of the chlorine atoms inserted into this small hydrophobic pocket. Fig. 9D is an analogous model of indomethacin in the same hydrophobic pocket, illustrating that the chlorine of diclofenac can substitute for the methyl group of indomethacin in terms of filling the space of the binding pocket. Although not designated in Fig. 9C, a halogen bond can be measured between the diclofenac chlorine and the hydroxyl of Ser-530. Since halogens, such as chlorine, bromine, and iodine (but not fluorine), are known to form halogen bonds with the hydroxyls of amino acid residues, it is possible that the chlorine atoms of key inhibitors in this SAR study are making important binding interactions within this hydrophobic pocket. Lumiracoxib exhibits a similar binding mode to diclofenac in the active site of mCOX-2 (9), suggesting that, akin to diclofenac, a halogen bond would be likely between the chlorine of lumiracoxib and the Ser-530 hydroxyl. Disruption of an interaction with Ser-530 by mutation of this residue to alanine may eliminate this binding association. Although a halogen bond may be an important determinant for COX inhibition by some analogs, it is not an absolute requirement, since the 2,6-dimethyl analog (compound 20) exhibits potent inhibition of both mouse and human COX-2 and theoretically would be making binding interactions in the same hydrophobic pocket.

Results of site-directed mutation of Val-349 to alanine, isoleucine, or leucine in murine COX-2 also support a role for inhibitor binding interactions in this small hydrophobic pocket. Although mutations of valine to alanine or leucine changed the size of the binding pocket (increased or decreased, respectively), both mutations abrogated the potent inhibition observed with diclofenac and key lumiracoxib analogs like compound 2. Interestingly, mutation of valine to isoleucine resulted in a protein with equal potency for inhibition as the wild-type mCOX-2 with these compounds. Poor inhibitors of
Determinants of Cyclooxygenase-2 Inhibition by Lumiracoxxib

mCOX-2 in the IC\text{50} assay, lumiracoxxib and compound 11, demonstrated highly potent inhibition of the V349I mutant. This increase in potency was not observed for all inhibitors, suggesting that the chemical nature of the substruents on the lower aniline ring was important for binding interactions in this pocket. The SAR analysis of lumiracoxxib analogs with the Val-349 mutants indicates a preference for a chlorine substituent on the lower aniline ring for potent inhibition of COX-2 with F-Cl, Cl-Cl, or Cl-CH\text{3} substitutions allowed at the 2,6-position to restrict the \text{IC}_{50}.

The relative potency of lumiracoxxib inhibition of oCOX-1, hCOX-2, and mCOX-2 may be a function of the secondary shell residues that lie behind Leu-384, the residue hypothesized to control the COX-2 selectivity of lumiracoxxib by its movement during insertion of the inhibitor’s methyl group. In oCOX-1, the secondary shell residues behind Leu-384 are bulky and restrictive (Ile-525 and Phe-503). In human and mouse COX-2, these residues are Val-525/Leu-503 and Leu-525/Leu-503, respectively; smaller, less restrictive residues that allow Leu-384 to maneuver and permit the 5’-methyl group of lumiracoxxib to insert into the resulting groove. Fig. 9 (A and B) shows the location of Leu-384, Leu-503, and Leu-525 in diclofenac-bound mCOX-2 and demonstrates a space-filling model that the addition of a 5’-methyl group to the inhibitor (lumiracoxxib) may fill a small pocket provided by these residues. The substitution of Leu-525 for Val-525 may contribute to the lower sensitivity of mCOX-2 to lumiracoxxib inhibition compared with hCOX-2.

An interesting question is posed as to why lumiracoxxib is such a poor inhibitor of wild-type mCOX-2 and yet potently and irreversibly inhibits the V349I mutant. It seems likely that the bulkier isoleucine residue reduces the size of the small hydrophobic binding pocket and increases contacts in the pocket for binding interactions between the inhibitor and protein to occur. Ile-349 also may influence the movement and location of neighboring residues or secondary shell residues and could position the hydroxyl of Ser-530 closer to the chlorine atom of lumiracoxxib and strengthen a halogen bond. As mentioned above, our data suggest that chlorine is a preferred substituent for potent COX inhibition and may also influence COX-2 selectivity.

The kinetics of inhibition of wild-type and mutant COX enzymes by lumiracoxxib and compound 2 were compared. Compound 2 was a potent inhibitor of both mouse and human COX-2 and appeared to be functionally irreversible, since no appreciable k\text{m} was observed. The selectivity of compound 2 for COX-2 may be attributed to the existence of a k\text{m} for the inhibition of oCOX-1. Whereas the inhibition of a V349I mutant mirrored wild-type mCOX-2 for compound 2, a dramatic difference was observed with lumiracoxxib inhibition of mCOX-2 (85% activity remaining) compared with V349I (0% activity remaining, IC\text{50} = 114 nM). Inhibition of murine V349I by lumiracoxxib was slow, time-dependent, and functionally irreversible.

The kinetic studies and structure activity series described here reveal that the COX-2-inhibitory activity of lumiracoxxib results from a fine balance between potency and selectivity. The dihaloarylamine ring is the principal determinant of potency, whereas the meta-methylylacylic acid ring controls selectivity. A fluorine-chlorine substitution in the dihaloarylamine ring of lumiracoxxib increases the tightness of binding (potency of inhibition) but also reduces selectivity for COX-2. Introduction of the meta-methyl group in the arylacetic acid ring enhances COX-2 selectivity but reduces potency. This reduction in potency is manifest by a k\text{m} that allows detectable inhibitor exchange with substrate, especially at elevated concentrations.
of arachidonic acid. This behavior contrasts with that of potent slow, tightly binding NSAIDs, such as indomethacin, sulindac sulfide, and diclofenac, and the COX-2-selective diarylheterocycles, rofecoxib, celecoxib, and etoricoxib. However, despite the reduced potency of its inhibition of COX-2 compared with other NSAIDs and COXIBs, lumiracoxib is the most selective COX-2 inhibitor in vivo, as judged by the whole blood assay. This illustrates that factors in addition to the kinetics of inhibitor association and dissociation determined in vitro control the selectivity and potency of inhibition in vivo.

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