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Exploring the Molecular Determinants of Substrate-Selective Inhibition of Cyclooxygenase-2 by Lumiracoxib

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Abstract

Lumiracoxib is a substrate-selective inhibitor of endocannabinoid oxygenation by cyclooxygenase-2 (COX-2). We assayed a series of lumiracoxib derivatives to identify the structural determinants of substrate-selective inhibition. The hydrogen-bonding potential of the substituents at the *ortho* positions of the aniline ring dictated the potency and substrate selectivity of the inhibitors. The presence of a 5'-methyl group on the phenylacetic acid ring increased the potency of molecules with a single *ortho* substituent. *Des*-fluorolumiracoxib (**2**) was the most potent and selective inhibitor of endocannabinoid oxygenation. The positioning of critical substituents in the binding site was identified from a 2.35 Å crystal structure of lumiracoxib bound to COX-2.

Keywords

COX-2; substrate-selective inhibition; lumiracoxib; endocannabinoids; prostaglandins

Inhibition of cyclooxygenase-2 (COX-2) is a major contributor to the antiinflammatory, analgesic and anti-pyretic effects of non-steroidal anti-inflammatory drugs (NSAIDs). COX-2 oxygenates arachidonic acid (AA) and the endocannabinoids, 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide (AEA), to prostaglandin-H₂ (PGH₂), PGH₂-glyceryl ester (PGH₂-G) and PGH₂-ethanolamide (PGH₂-EA), respectively (Figure 1).^{1, 2} 2-AG and AEA are ligands of the cannabinoid (CB₁ and CB₂) receptors and are involved in regulating locomotion, temperature and pain.³ Currently, the biological properties of endocannabinoid derived prostaglandins are poorly understood, with evidence suggesting an active role in malignant cells, neurons, and macrophages.⁴ Determining the

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biological consequences of endocannabinoid oxygenation by COX-2 has been difficult due to the lack of probes that inhibit COX-2 oxygenation of endocannabinoids without inhibiting the oxygenation of AA.

Our laboratory discovered the existence and mechanism of action of substrate-selective inhibitors of COX-2.^{5, 6} Rapid reversible inhibitors such as ibuprofen, mefenamic acid and lumiracoxib block COX-2-catalyzed oxygenation of endocannabinoids, but not of arachidonic acid. Substrate-selective inhibitors bind in one of the two active sites of the COX-2 homodimer and induce a conformational change in the remaining catalytic active site that inhibits 2-AG or AEA oxygenation, but not AA oxygenation (Figure 2).^{7,8, 9} Binding of a second molecule of inhibitor in the catalytic active site inhibits AA oxygenation.

We are investigating the molecular determinants of substrate-selective inhibition by a series of NSAID scaffolds. To date, only molecules that bind in the canonical orientation in the COX-2 active site (i.e., with a carboxylic acid of the inhibitor interacting with the COX-2 residues Arg-120 and Tyr-355, at the base of the active site) have been investigated in detail.^{10, 11} The substrate-selective COX-2 inhibitor, lumiracoxib, is anticipated to bind in an inverted orientation in the active site similar to diclofenac. The carboxylic acid group of diclofenac projects up into the main channel of the active site forming hydrogen bonds with Ser-530 and Tyr-385; mutation of Ser-530 to Ala abolishes diclofenac inhibition.^{12–14} In the present work, we evaluated a series of lumiracoxib analogs for their ability to inhibit endocannabinoid and AA oxygenation by COX-2. The results of these experiments were integrated with a crystal structure of lumiracoxib complexed to mCOX-2 to explain the structure-activity relationships observed.

Lumiracoxib and its derivatives were synthesized as described previously and tested in an *in vitro* assay.¹² Briefly, inhibitors (up to 10 μ M) were pre-incubated with 50 nM mCOX-2 protein for 3 min, followed by addition of 5 μ M substrate. The reaction was quenched after 30 s, and product formation (PGE₂-G/PGD₂-G and PGE₂/PGD₂ from 2-AG and AA, respectively) was quantified by LC-MS-MS.⁶

The number and size of the *ortho* substituents on the lower aniline ring of the lumiracoxib derivatives had a significant effect on the molecules' selectivity and potency (Table 1). Lumiracoxib, **1**, possessed an IC₅₀ value of 0.04 μ M against 2-AG and inhibited AA oxygenation by 25% at 10 μ M inhibitor. The incomplete inhibition of AA oxygenation is consistent with previous observations from our laboratory.¹² Replacing the *o*-fluorine of **1** with hydrogen produced an inhibitor (**2**) with an IC₅₀ value against 2-AG of 0.06 μ M, but 0% AA inhibition at 10 μ M inhibitor. When the *o*-chlorine of **1** was converted to hydrogen, the resulting molecule, **3**, inhibited only 2-AG oxygenation with an IC₅₀ value of 1.8 μ M. Inhibitor **4**, in which both *ortho* halogens were replaced by hydrogens, did not inhibit 2-AG or AA oxygenation. These results demonstrate that replacing an *o*-halogen of lumiracoxib with hydrogen reduces the ability of lumiracoxib derivatives to inhibit 2-AG and AA oxygenation, although the effect is less dramatic when fluorine is replaced instead of chlorine.

Placement of two chlorine atoms in the *ortho* positions of the lower aniline ring (**5**) led to a potent, but less selective, COX-2 inhibitor. Relative to **1**, compound **5** possessed greater AA inhibition (0.2 μ M IC₅₀), while maintaining a similar 2-AG IC₅₀ value of 0.03 μ M. Two *o*-fluorine substituents generated inhibitor **6**, which exhibited moderate substrate-selective behavior (i.e., 1.0 μ M 2-AG IC₅₀, 0% AA inhibition at 10 μ M inhibitor). Expanding beyond halogen substituents, replacing the *o*-chlorine of **1** with a methyl group generated compound **7**, which was inactive. Introducing a *p*-chlorine substituent to **5** generated inhibitor **8**.

Compared to **5**, **8** maintained a similar 2-AG IC₅₀ value (0.07 μ M), while decreasing AA inhibition to 32% at 10 μ M inhibitor.

The 5'-methyl group of lumiracoxib is believed to be a major determinant of the molecule's COX-2 selectivity over COX-1.¹² To determine its role in substrate-selective inhibition of COX-2, we prepared a series of *des*-methyl derivatives. *Des*-methyl lumiracoxib, **9**, exhibited the same 2-AG IC₅₀ value as lumiracoxib (**1**), but less AA inhibition (15%) at 10 μ M inhibitor. The *des*-methyl analog, **10**, bearing a single *o*-chlorine, exhibited a significantly weaker 2-AG IC₅₀ value of 3.9 μ M compared to its 5'-methylated analogue, **2**, but no AA inhibition. *Des*-methyl inhibitor, **11**, bearing a single *o*-fluorine substituent on the lower aniline ring, only partially inhibited 2-AG conversion (25% at 10 μ M inhibitor), whereas its 5'-methylated analogue, **3**, had a 2-AG IC₅₀ of 1.8 μ M. Finally, diclofenac, **13**, possessed an AA IC₅₀ value of 0.1 μ M (after 15 minutes of incubation with the enzyme due to its time-dependent mechanism of inhibition), lower than the 0.2 μ M AA IC₅₀ value observed from its 5'-methylated analogue, **5**.⁷ *Des*-methyl inhibitors **12** and **14–16** showed little difference in behavior from their methylated derivatives **4** and **6–8**, respectively.

In light of diclofenac's time-dependent mechanism of inhibition, we evaluated our most potent and selective derivatives (**2**, **8**, **9**) for AA inhibition after 15 min pre-incubation with mCOX-2. There was little difference observed between these data and those collected after 3 min pre-incubation. Inhibitor **8** showed 55% AA inhibition at 10 μ M inhibitor after 15 min pre-incubation compared to 32% inhibition at 3 min pre-incubation. Inhibitor **9** increased its inhibition of AA oxygenation from 15% at 3 min incubation to 25% at 15 min incubation with 10 μ M of inhibitor. *Des*-fluoro lumiracoxib, **2**, continued to lack any inhibition of AA oxygenation after 15 min pre-incubation, identifying it as the most potent, substrate-selective inhibitor evaluated.¹²

Consideration of the results of these two structural series indicates that a halogen, preferably chlorine, is required for optimal substrate-selectivity and that a 5'-methyl group can increase the potency of inhibition for analogs with a single halogen in the aniline ring. To identify the structural basis for these observations, we determined the crystal structure of lumiracoxib bound to COX-2. A crystal structure of the lumiracoxib-COX-2 complex has been reported in abstract form but no atomic coordinates are available in the protein data bank.¹⁴ So, we attempted to crystallize complexes of lumiracoxib with apo or holo mCOX-2. The apo-mCOX-2:lumiracoxib complex was successfully crystallized using a previously described protocol.^{6,11} The crystals diffracted to 2.35 Å (Supplemental Table 1). Structural studies demonstrate that the heme group does not significantly alter the binding of molecules in the cyclooxygenase active site.^{15,16} In the structure of apo-mCOX-2:lumiracoxib, no significant electron density was visible in the peroxidase site; that which is present can be accounted for by solvent molecules.

The complex crystallized in the I222 space group and contains a single COX homodimer in the asymmetric unit. Clear electron densities in each monomer led to the unambiguous assignment of lumiracoxib in the active site of COX-2 (Supplemental Figure 1). Lumiracoxib binds in an inverted orientation similar to diclofenac.¹³ The carboxylic acid group forms hydrogen bonds to Tyr-385 and Ser-530 with effective distances of 2.5 and 2.6 Å, respectively. In determining the identity of the *ortho*-halogen substituents on the lower aniline ring, we relied on the electron density from the difference map ($F_o - F_c$). Switching the current positions of the chlorine and fluorine atoms resulted in significant negative electron density around the chlorine and some positive electron density around the fluorine in the difference map. The *o*-chlorine atom is adjacent to Ser-530 in a hydrophobic pocket comprised of Val-349, Ala-527, and Leu-531. Since the chlorine atom is 3.6 Å away from the hydroxyl oxygen of Ser-530, it is possible that it is participating in a weak hydrogen

bond; assigning fluorine to this position would reduce the strength of this interaction. The rest of the molecule interacts with COX-2 in the hydrophobic channel mainly through van der Waals interactions. The constriction site residues Arg-120 and Tyr-355 at the base of the active site, which hydrogen bond to the carboxylic acid of profen COX-2 inhibitors, show little interaction with lumiracoxib (Supplemental Figure 2).

This complex provides the structural basis for lumiracoxib's COX-2 selectivity over COX-1. Unlike diclofenac, lumiracoxib has a 5'-methyl substituent on the phenylacetic acid ring. The methyl group is inserted into the hydrophobic pocket made by Phe-381, Leu-384, Tyr-385, Trp-387, Phe-518 and Met-522 (Figure 3A). The C_α-C_β-C_γ plane of Leu-384 is rotated away from the active site by approximately 150° when compared to the orientation of Leu-384 in the diclofenac crystal structure (Figure 3B). This rotation shifts the γ-carbon of Leu-384 toward the interior of the enzyme by 1.8 Å, generating space to accommodate the 5'-methyl group of lumiracoxib. Leu-384 is able to rotate away from the active site in COX-2 because there is space between it and the residue behind it, Leu-503. In COX-1, Phe-503 fills that space and does not allow Leu-384 to rotate away from the active site. Without space for Leu-384 to move away from the 5'-methyl group, lumiracoxib is unable to bind to COX-1.

To test our hypothesis that Leu-503 is key to lumiracoxib's COX-2 selectivity, we evaluated the molecule in our *in vitro* assay with mCOX-2 L503F. With wild-type mCOX-2, lumiracoxib (**1**) had a 2-AG IC₅₀ value of 40 nM. In the presence of mCOX-2 L503F, **1** only partially inhibited 2-AG conversion (25% at 5 μM inhibitor, Figure 4). Percent inhibition of AA oxygenation was 25% at 5 μM inhibitor for both enzymes. The loss of activity due to substitution of the bulkier phenylalanine at position 503 is evidence that the residue is a critical determinant in lumiracoxib's COX-2 selectivity.

The crystal structure illustrates the origins of the two molecular determinants identified by the enzyme assay. First, effective *ortho* substituents on the lower aniline ring are hydrogen-bond acceptors. Molecules with an *o*-chlorine group were the most potent inhibitors identified, reflecting the atom's ability to donate electrons in a hydrogen bond with Ser-530. Substituents that are hydrophobic (i.e., methyl, hydrogen) result in inactive molecules. Derivatives without an *o*-chlorine but with an *o*-fluorine have moderate potency, illustrating fluorine's limited propensity to participate in a hydrogen bond. Second, the presence of the 5'-methyl group restores inhibition of 2-AG oxygenation to derivatives with a single *ortho* substituent. As comparison of the lumiracoxib and diclofenac crystal structures clearly shows, the presence of a 5'-methyl group forces Leu-384 to rotate away from lumiracoxib and generates a small hydrophobic cavity. The additional hydrophobic contacts between the 5'-methyl substituent and residues Phe-381, Leu-384, Tyr-385, Trp-387, Phe-518, and Met-522 provide additional binding energy to mitigate the loss of binding from the absence of a second *ortho*-halogen. Molecules without the 5'-methyl group may also lose their selectivity for COX-2 (e.g., diclofenac).¹²

The results here and elsewhere suggest that substrate selectivity originates from the affinity of an inhibitor for the COX-2 active site. Molecules that bind very strongly to the binding pocket (e.g., indomethacin, diclofenac, (*S*)-flurbiprofen) are slow, tight-binding inhibitors that cause a significant structural rearrangement of the COX-2 homodimer.^{17–19} Because of this large-scale rearrangement, only one equivalent of inhibitor is required to block substrate oxygenation. When the structure of these slow, tight-binding inhibitors is changed slightly (i.e., *des*-methyl indomethacin, lumiracoxib, (*R*)-flurbiprofen, respectively),^{6, 12, 20} the molecules lose potency and apparently induce a less dramatic conformational change in the catalytic subunit.

Despite multiple crystal structures of substrate-selective inhibitors bound to COX-2, we still do not have a detailed structural understanding of how these molecules preclude the oxygenation of 2-AG, but not AA. This ambiguity stems from each crystal structure having two equivalents of inhibitor bound to the COX-2 homodimer, when only one equivalent is necessary for substrate-selective inhibition (Figure 2). Having both active sites occupied may be required for crystallization. As a result, substrate-selective inhibitors have generated crystal structures that overlay closely (outside of the binding pocket) to those of non-substrate selective molecules, making structural determination of substrate selectivity difficult.

A structure-based hypothesis on the origin of substrate selectivity suggests that the oxygenation of 2-AG is more susceptible to disruption than AA oxygenation. The distance between Tyr-385, the catalytic residue of COX-2, and 2-AG's 13-*pro-S* hydrogen, which is abstracted in the first step of substrate oxygenation, is greater than the distance between Tyr-385 and AA's 13-*pro-S* hydrogen (3.3 Å vs. 2.1 Å, respectively).^{21, 22} Thus, even a weakly bound inhibitor in one active site of the homodimer may cause enough of a long-distance structural change to effect the conformation of 2-AG in the second active site. Disruption of AA oxygenation would require a more significant structural rearrangement, likely resulting from a more potent inhibitor.

The present study has expanded our understanding of COX-2 inhibition by lumiracoxib and its derivatives. We have identified Leu-503 as the key determinant of lumiracoxib's selective binding to COX-2 over COX-1. Lumiracoxib derivatives possess varying degrees of potency and substrate-selective inhibition dependent on the identity of the *ortho* substituents on the aniline ring, and the presence or absence of a 5'-methyl group. In particular, we identified *des*-fluoro lumiracoxib (**2**) as a potent, substrate-selective inhibitor. As illustrated by our crystal structure, compound **2** derives its activity from a hydrogen bond between the *ortho*-chlorine atom and Ser-530, hydrogen bonding between the carboxylic acid and residues Tyr-385 and Ser-530, and hydrophobic interactions involving the 5'-methyl group and neighboring side chains. Work exploring **2** in a biological setting, in addition to identifying new scaffolds of substrate-selective COX-2 inhibition, is ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Reaction catalyzed by COX-2. Substrates and products are shown in the table.

Figure 2.

When one equivalent of a substrate-selective inhibitor (I, red spheres) binds in an active site of the COX-2 homodimer, a conformational change is induced that prevents oxygenation of 2-AG but allows for AA oxygenation in the remaining active site. Binding of a second equivalent of inhibitor (at higher inhibitor concentration) in the remaining active site is required to block AA oxygenation. Adapted from reference 5.

Figure 3. Stereodiagram highlighting the change in the mCOX-2 active site surface (grey) in the presence of lumiracoxib (A, magenta) and diclofenac (B, pink). The 5'-methyl group of lumiracoxib forces Leu-384 (red) to rotate away from the active site, creating a cone-shaped hydrophobic pocket that contributes to lumiracoxib's binding affinity. Leu-384 faces the active site in the presence of diclofenac, resulting in a smaller binding pocket.

Figure 4.

Oxygenation of 2-AG and AA by wild-type and L503F mCOX-2 vs. lumiracoxib. The dotted lines describe the percent conversion of 2-AG (blue) to PGE₂-G/PGD₂-G and AA (red) to PGE₂/PGD₂ by wild-type mCOX-2. The solid lines describe the percent conversion of 2-AG (blue) to PGE₂-G/PGD₂-G and AA (red) to PGE₂/PGD₂ by mCOX-2 L503F. The single point mutation is enough to eliminate lumiracoxib's behavior as a substrate-selective inhibitor.

Table 1Inhibition of mCOX-2 Dependent Oxygenation of 2-AG and AA by Lumiracoxib and Derivatives *In Vitro*^a

Inhibitor	R ¹	R ²	R ³	R ⁴	2-AG IC ₅₀ (μ M) ^b	AA IC ₅₀ (μ M) ^b
1	CH ₃	F	Cl	H	0.04 $\pm$ 0.01	-(25%)
2	CH ₃	H	Cl	H	0.06 $\pm$ 0.01	-(0%)
3	CH ₃	F	H	H	1.8 $\pm$ 0.6	-(0%)
4	CH ₃	H	H	H	-(0%)	-(10%)
5	CH ₃	Cl	Cl	H	0.03 $\pm$ 0.01	0.2 $\pm$ 0.1 (60%)
6	CH ₃	F	F	H	1.0 $\pm$ 0.3	-(0%)
7	CH ₃	F	CH ₃	H	-(0%)	-(0%)
8	CH ₃	Cl	Cl	Cl	0.07 $\pm$ 0.01	-(32%)
9	H	F	Cl	H	0.04 $\pm$ 0.01	-(15%)

Inhibitor	R ¹	R ²	R ³	R ⁴	2-AG IC ₅₀ (μM) ^b	AA IC ₅₀ (μM) ^b
10	H	H	Cl	H	3.9 ± 2.0	—(0%)
11	H	F	H	H	—(25%)	—(0%)
12	H	H	H	H	—(8%)	—(15%)
13^c	H	Cl	Cl	H	0.03 ± 0.01	0.1 ± 0.01 (85%)
14	H	F	F	H	1.2 ± 0.4	—(10%)
15	H	F	CH ₃	H	—(15%)	—(0%)
16	H	Cl	Cl	Br	0.06 ± 0.02	0.14 ± 0.05 (55%)

^a IC₅₀ values were determined by incubating five concentrations of inhibitor and a solvent control in DMSO with purified murine COX-2 (50 nM) for three min, followed by addition of 2-AG or AA (5 μM) at 37 °C for 30 s.

^b Mean ± standard deviation (n = 6); dash (—) indicates < 50% inhibition of 2-AG oxygenation at 10 μM inhibitor. Numbers in parentheses indicate maximum inhibition (when not equal to 100%) at 10 μM inhibitor.

^c Inhibitor incubated with enzyme for 15 min before addition of 2-AG or AA.