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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.

Inhibition of cyclooxygenase-2 (COX-2) is a major contributor to the anti-inflammatory, 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 arachidonoylethanolamide (AEA), to prostaglandin-H_2 (PGH_2), PGH_2-glyceryl ester (PGH_2-G) and PGH_2-ethanolamide (PGH_2-EA), respectively (Fig. 1).[1,2] 2-AG and AEA are ligands of the cannabinoid (CB_1 and CB_2) 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 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 (Fig. 2).[7–9] 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 μM) were pre-incubated with 50 nM mCOX-2 protein for

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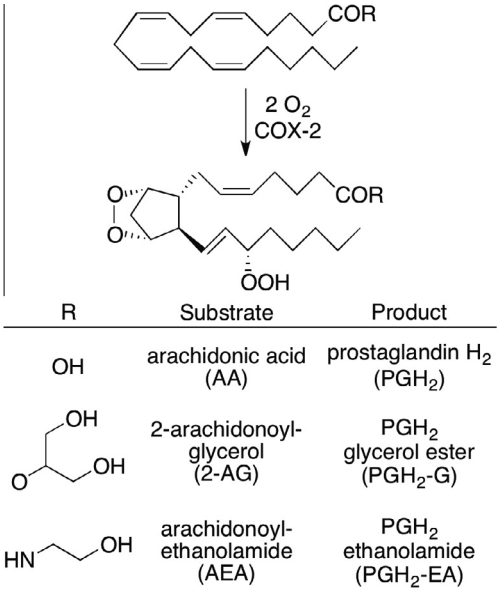


Figure 1. Reaction catalyzed by COX-2. Substrates and products are shown in the table.

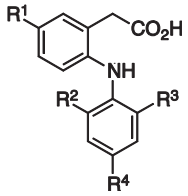
3 min, followed by addition of 5 μM substrate. The reaction was quenched after 30 s, and product formation (PGE_2-G/PGD_2-G and PGE_2/PGD_2 from 2-AG and AA, respectively) was quantified by LC-MS-MS.[6]

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 IC_{50} value of 0.04 μM against 2-AG and inhibited AA oxygenation by 25% at 10 μM 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 IC_{50} value against 2-AG of 0.06 μM, but 0% AA inhibition at 10 μM inhibitor. When the *o*-chlorine of **1** was converted to hydrogen, the resulting molecule, **3**, inhibited only 2-AG oxygenation with an IC_{50} value of 1.8 μM. 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 μM IC_{50}), while maintaining a similar 2-AG IC_{50} value of 0.03 μM. Two *o*-fluorine substituents generated inhibitor **6**, which exhibited moderate substrate-selective behavior (i.e., 1.0 μM 2-AG IC_{50}, 0% AA inhibition at 10 μM 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 IC_{50} value (0.07 μM), while decreasing AA inhibition to 32% at 10 μM 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**,

Table 1
Inhibition 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}[b] (μM)	AA IC_{50}[b] (μM)
1	CH_3	F	Cl	H	0.04 ± 0.01	—(25%)
2	CH_3	H	Cl	H	0.06 ± 0.01	—(0%)
3	CH_3	F	H	H	1.8 ± 0.6	—(0%)
4	CH_3	H	H	H	—(0%)	—(10%)
5	CH_3	Cl	Cl	H	0.03 ± 0.01	0.2 ± 0.1 (60%)
6	CH_3	F	F	H	1.0 ± 0.3	—(0%)
7	CH_3	F	CH_3	H	—(0%)	—(0%)
8	CH_3	Cl	Cl	Cl	0.07 ± 0.01	—(32%)
9	H	F	Cl	H	0.04 ± 0.01	—(15%)
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] IC_{50} values were determined by incubating five concentrations of inhibitor and a solvent control in DMSO with purified murine COX-2 (50 nM) for 3 min, followed by addition of 2-AG or AA (5 nM) 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.

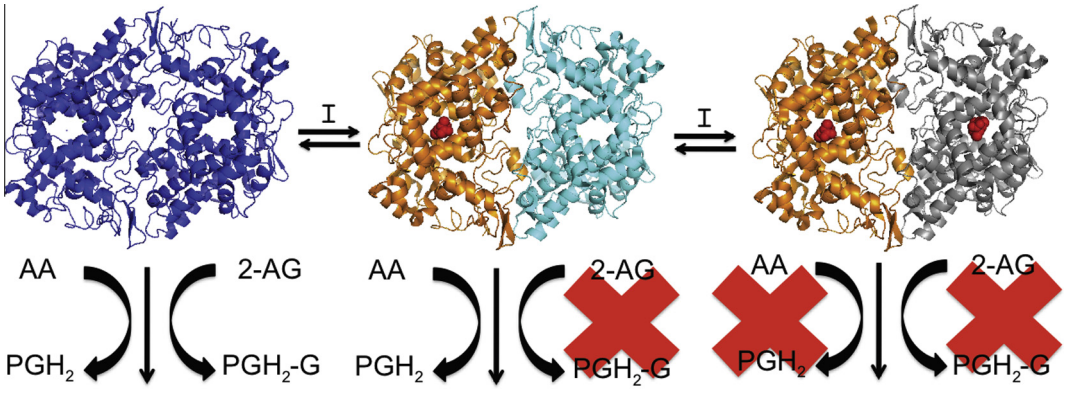


Figure 2. When 1 equivalent of a substrate-selective inhibitor (I, red spheres) binds in an active site of the COX-2 homodimer, a conformational change is induced that prevents oxygenation of 2-AG but allows for AA oxygenation in the remaining active site. Binding of a second equivalent of inhibitor (at higher inhibitor concentration) in the remaining active site is required to block AA oxygenation. Adapted from Ref. 5.