**Differential Sensitivity and Mechanism of Inhibition of COX-2 Oxygenation of Arachidonic Acid and 2-Arachidonoylglycerol by Ibuprofen and Mefenamic Acid†**

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**ABSTRACT:** Ibuprofen and mfenamic acid are weak, competitive inhibitors of cyclooxygenase-2 (COX-2) oxygenation of arachidonic acid (AA) but potent, noncompetitive inhibitors of 2-arachidonoylglycerol (2-AG) oxygenation. The slow, tight-binding inhibitor, indomethacin, is a potent inhibitor of 2-AG and AA oxygenation whereas the rapidly reversible inhibitor, 2'-des-methylindomethacin, is a potent inhibitor of 2-AG oxygenation but a poor inhibitor of AA oxygenation. These observations are consistent with a superior inhibitor of 2-AG oxygenation but a poor inhibitor of AA oxygenation. Despite this similarity, we report here that COX-2 oxygenates a range of fatty acyl substrates including fatty acids, esters, and amides. Arachidonic acid (AA) and 2-arachidonoylglycerol (2-AG) are the best acid and ester substrates and display comparable $k_{cat}/K_m$’s for oxygenation (4). Despite this similarity, we report here that COX-2 oxygenation of 2-AG is dramatically more sensitive to inhibition by a series of acidic NSAIDs than is oxygenation of AA. In fact, these compounds, which have been considered relatively weak COX inhibitors (i.e., ibuprofen and mfenamic acid), inhibit 2-AG oxygenation at concentrations that are orders of magnitude lower than the concentrations required for inhibition of AA oxygenation.}

The potencies of individual inhibitors toward AA and 2-AG oxygenation were determined in instantaneous inhibition assays in which the substrate and inhibitor were incubated in an O$_2$ monitor followed by addition of murine COX-2 (mCOX-2) purified as previously described (5). Substrate oxygenation velocities were measured at multiple concentrations of substrate and inhibitor. A plot of $V$ vs $[S]$ for AA oxygenation at increasing ibuprofen concentrations demonstrated increased $K_m^{app}$'s but comparable $V_{max}$’s consistent with competitive inhibition (Figure 1A). Similar results were obtained with mfenamic acid (Supporting Information, Figure S1). The $K_i$'s were calculated from a secondary plot of the dependence of $K_m^{app}$ on inhibitor concentration (Supporting Information, Figure S2). Ibuprofen exhibited a $K_i$ of 80 ± 20 μM, whereas mfenamic acid exhibited a $K_i$ of 10 ± 5 μM.

Oxygenation of 2-AG was completely inhibited by the lowest concentrations of inhibitors used in the experiments with AA (ibuprofen-25 μM, mfenamic acid-5 μM), so a much lower concentration range was evaluated. In contrast to the results with AA, increasing concentrations of inhibitor did not increase the $K_m^{app}$ but rather decreased $V_{max}$ (Figure 1B for ibuprofen and Supporting Information, Figure S1 for mfenamic acid). The general appearance of the $V$ vs $[2-AG]$ plots at different inhibitor concentrations was not consistent with competitive inhibition but was suggestive of noncompetitive inhibition. Thus, the $K_i$ for ibuprofen was calculated from a secondary plot of $1/V_{max}^{app}$ vs [ibuprofen] and found to be 1.2 μM (Supporting Information, Figure S3).

The concentrations of mfenamic acid that inhibited 2-AG oxygenation were in the range of the enzyme concentration so a $K_i$ could not be calculated from the inhibitor data. Fortunately, mfenamic acid quenches the intrinsic protein fluorescence of apoCOX-2 (without heme) so experiments were conducted using fluorescence quenching to monitor inhibitor association (6). The equilibrium constant for dissociation ($K_D$) could be measured by titration of mCOX-2 with mfenamic acid, as previously described for darbufelone (7). Fitting the corrected data to a logarithmic plot of fluorescence vs inhibitor concentration yielded an $EC_{50}$ value representing the apparent $K_D$ of quenching (Supporting Information, Figure S4). The calculated $EC_{50}$ was in the range of the protein concentration so it is only an approximation of the $K_D$. Therefore, the experiment was repeated at varying enzyme concentrations, and a plot of $EC_{50}$ versus enzyme...
inhibitor suffices to inhibit 2-AG oxygenation (Figure 2). The 2-AG at the other subunit. Therefore, a single molecule of bound mefenamate at one subunit prevents productive binding of protein are not identical once substrate or inhibitor is bound (2) establishing that the two subunits of the homodimeric COX-2 oxygenation of AA and 2-AG by different mechanisms for the fluorescence quenched by mefenamic acid.

The kinetics of ibuprofen and mefenamate inhibition of COX-2 oxygenation of AA and 2-AG by ibuprofen at 0 μM ( ■), 0.5 μM ( ▽), 1.25 μM ( ●), and 2.5 μM ( ●). (B) Instantaneous COX-2 inhibition of 2-AG oxidation by ibuprofen at 0 μM ( ■), 0.5 μM ( ▽), 1.25 μM ( ●), and 2.5 μM ( ●).

Figure 2: Model for differential inhibition 2-AG and AA oxygenation by COX-2. The uninhibited mCOX-2 homodimer (blue) is able to effectively metabolize both AA and 2-AG to form PGG2 and PGG2-G. Binding of an inhibitor (yellow) to a single monomer (green) precludes the productive binding of 2-AG in the partner monomer (red) but still allows for AA oxygenation. Metabolism of AA is inhibited only when an inhibitor occupies both active sites of the COX dimer as shown on the far right.

concentration was constructed. The y-intercept of this plot (equivalent to the EC50 at infinitely small enzyme concentration) provided an estimate of the true Kd (4 nM).

These data suggest that ibuprofen and mefenamic acid inhibit COX-2 oxygenation of AA and 2-AG by different mechanisms and with different potencies. An interpretation of the experimental findings can be made that is consistent with recent results establishing that the two subunits of the homodimeric COX-2 protein are not identical once substrate or inhibitor is bound (2). In the case of 2-AG oxygenation, binding of ibuprofen or mefenamate at one subunit prevents productive binding of 2-AG at the other subunit. Therefore, a single molecule of bound inhibitor suffices to inhibit 2-AG oxygenation (Figure 2). The Kd for inhibition by ibuprofen and the Kd for binding of mefenamate suggest high affinity binding at the first COX-2 subunit. The kinetics of ibuprofen and mefenamate inhibition of 2-AG oxygenation are typical of noncompetitive inhibition, consistent with the hypothesis that binding of the inhibitor at one subunit inhibits productive binding of 2-AG at the other subunit. We cannot rule out the possibility that a separate, high affinity allosteric binding site exists for these compounds outside of the active site, but it seems unlikely given (a) the high affinity of the inhibitors for the protein, (b) the existence of crystal structures demonstrating the presence of arylcarboxylic acid inhibitors in the active sites of both subunits, and (c) the likelihood that the active site residue, Trp-387, is responsible for the fluorescence quenched by mefenamic acid.

In contrast to the observations with 2-AG, inhibition of COX-2 oxygenation of AA by ibuprofen or mefenamate requires much higher concentrations of inhibitor and displays kinetic behavior typical of competitive inhibition. The most straightforward interpretation of these results is that inhibition of AA oxygenation requires inhibitor molecules to bind in both active sites (Figure 2). Binding in the first active site is necessary but not sufficient to inhibit AA oxygenation; inhibition is only observed when the second molecule of inhibitor binds. Binding in the first active site must decrease the affinity of the unoccupied active site for the second molecule of inhibitor so that higher concentrations are required and their binding is competitive with that of AA.

Several arylcarboxylic acids or diarylheterocycles are slow, tight-binding inhibitors of COX-2 (8). These compounds exhibit low Kd’s for binding and potent inhibition but only after a lengthy preincubation period. The indoleacetic acid derivative, indomethacin, is a classic slow, tight-binding inhibitor of both COX-2 and COX-1 (9). Inhibition of AA oxygenation by COX-2 requires a preincubation period of up to 15 min, and its inhibition potency increases dramatically during this time. Binding of a single molecule of indomethacin to a COX homodimer is sufficient to inhibit AA oxygenation (10). Following a 15 min preincubation, indomethacin displayed an IC50 of 2 μM for inhibition of AA oxygenation and 5.5 μM for inhibition of 2-AG oxygenation (Figure 3). Thus, a single indomethacin molecule bound in one subunit is sufficient to inhibit the oxygenation of either a fatty acid or fatty acid ester substrate in the other subunit.

A major determinant of the slow, tight binding of indomethacin to COX-2 is insertion of the 2'-methyl group on the indole ring into a hydrophobic depression in the side of the COX-2 active site (11). Removal of the 2'-methyl group generates a molecule, 2'-des-methylindomethacin, which exhibits rapid reversible inhibition of AA oxygenation with a much higher IC50. Figure 3 demonstrates that removal of the 2'-methyl group from indomethacin increases the IC50 for inhibition of AA oxygenation from 2 μM for indomethacin to ~500 μM for 2'-des-methylindomethacin. In contrast to the results with AA, removal of the 2'-methyl group from indomethacin has no effect on the
inhibition of 2-AG oxygenation; the IC$_{50}$ for inhibition of 2-AG oxygenation by 2'$\alpha$-methylindomethacin is 6.8 $\mu$M, essentially the same as the IC$_{50}$ of indomethacin (Figure 3).

Rome and Lands first demonstrated that some COX inhibitors display rapid, reversible inhibition, whereas others exhibit slow, tight-binding inhibition after the initial rapid, reversible interaction with the enzyme (9). The rapid, reversible inhibitors are relatively weak inhibitors of AA oxygenation, whereas the slow, tight-binding inhibitors are more potent. The slow, tight-binders exhibit very low dissociation rates so they are poorly reversible even in the presence of saturating concentrations of AA. The validity of the two-step mechanism of inhibition has been demonstrated repeatedly and in some cases extended as exemplified by certain diarylheterocycles, which demonstrate a second time-dependent step responsible for COX-2 selective inhibition (12–14). Subsequent work has revealed that for several slow, tight-binding inhibitors (e.g., indomethacin, flurbiprofen) association of only a single molecule of inhibitor is sufficient to inhibit the activity of both subunits, and this can be understood by the recent discovery that the two subunits communicate through the dimer interface (2).

The results of the present experiments demonstrate that both reversible and time-dependent inhibitors can potently inhibit oxygenation of the fatty acid ester substrate 2-AG and that some of the reversible inhibitors (e.g., ibuprofen and mefenamate) actually display much higher affinity for the enzyme than originally thought as judged by their $K_i$'s for inhibition and $K_d$'s for binding. The $K_d$ for ibuprofen and the $K_i$ for mefenamic acid establish these compounds as high affinity ligands for first subunit binding. The differential potency and mechanism of inhibition of 2-AG oxygenation compared to AA oxygenation displayed by ibuprofen and mefenamic acid adds a new dimension to our understanding of COX inhibition by this class of compounds. They actually associate much more tightly with COX-2 than previously appreciated and prevent the productive binding of 2-AG but not AA. Their status as relatively weak, rapidly reversible COX inhibitors derives from the fact that they have to bind to both subunits of COX-2 (and presumably COX-1) to inhibit productive binding of AA and that binding in the second subunit is competitive with AA.

Our data suggest that binding of a single molecule of ibuprofen, mefenamic acid, indomethacin, or 2'$\alpha$-methylindomethacin is sufficient to cause noncompetitive inhibition of 2-AG oxygenation but only indomethacin inhibits AA oxygenation under these conditions. These observations indicate that although all of the inhibitors induce conformational changes in the second subunit following binding in the first subunit, there are differences in the nature of the conformational changes induced as judged by the differential effects on 2-AG and AA binding in the second subunit. In addition, this differential sensitivity to inhibition reveals differences in the binding of 2-AG and AA that are not anticipated by the similarities in the $k_{cat}/K_m$ for oxygenation of the two substrates (4).

Inhibition of 2-AG oxygenation provides a powerful tool with which to investigate the binding of NSAIDs to COX-2. It will be interesting to examine a broader range of inhibitors than described here for their ability to differentially inhibit the oxygenation of 2-AG and AA and to compare the molecular determinants for this differential inhibition. It is also intriguing to consider the possibility that the greater potency for inhibition of 2-AG oxygenation exhibited by certain NSAIDs against purified protein has implications for understanding the pharmacological properties of these compounds in vivo.

SUPPORTING INFORMATION AVAILABLE

Figures S1–S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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