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A Binding Site for Nonsteroidal Anti-inflammatory Drugs in Fatty Acid Amide Hydrolase

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Supporting Information

ABSTRACT: In addition to inhibiting the cyclooxygenase (COX)-mediated biosynthesis of prostanoids, various widely used nonsteroidal anti-inflammatory drugs (NSAIDs) enhance endocannabinoid signaling by blocking the anandamide-degrading membrane enzyme fatty acid amide hydrolase (FAAH). The X-ray structure of FAAH in complex with the NSAID carprofen, along with site-directed mutagenesis, enzyme activity assays, and NMR analysis, has revealed the molecular details of this interaction, providing information that may guide the design of dual FAAH−COX inhibitors with superior analgesic efficacy.

Nonsteroidal anti-inflammatory drugs (NSAIDs), one of the most widely used classes of therapeutic agents, alleviate pain and inflammation[1] by inhibiting the enzymes cyclooxygenase-1 (COX-1) and COX-2,[2] which catalyze the conversion of membrane-derived arachidonic acid into the prostaglandin endoperoxides PGG2 and PGH2.[3] This reaction is the first committed step in the biosynthesis of the prostanoids,[4] lipid messengers that cause pain and inflammation by engaging G protein-coupled receptors present on the surface of innate-immune and neural cells.[5]

Evidence indicates that the analgesic actions of the NSAIDs are enhanced in a synergistic manner by drugs that inhibit fatty acid amide hydrolase (FAAH),[6] a serine enzyme responsible for the deactivation of the endogenous cannabinoid receptor agonist anandamide.[7] By increasing anandamide levels, FAAH inhibitors[8] heighten the ability of this compound to control emerging nociceptive signals[9] such as the prostanoids, resulting in a superadditive potentiation of NSAID-mediated analgesia. In addition to magnifying the analgesic actions of the NSAIDs, FAAH inhibitors reduce the frequency and severity of gastric side effects exerted by those compounds.[10]

These data suggest that dual inhibitors of FAAH and COX might provide superior efficacy and greater safety than current non-narcotic analgesics.[11] This possibility is supported by recent studies that have implicated FAAH blockade in the analgesic properties of indomethacin and ibuprofen, two clinically important NSAIDs.[12] Despite the therapeutic relevance of this hypothesis, the molecular mechanism through which NSAIDs inhibit FAAH remains unknown. To fill this knowledge gap, in the present study we solved the crystal structure of FAAH in complex with the ibuprofen analogue carprofen and investigated this interaction using a combination of site-directed mutagenesis, enzyme activity assays, and NMR analysis.

In a first set of experiments, we tested a representative set of commercially available NSAIDs for their ability to inhibit FAAH and identified one, (*R*,*S*)-2-(6-chloro-9*H*-carbazol-2-yl)propanoic acid (carprofen), which reduced the FAAH activity in rat-brain homogenates with a median effective concentration (IC_{50}) of 79 ± 20 μM [mean ± standard error of the mean, n = 3; assays were conducted at pH 7.4; see Methods in the Supporting Information (SI)]. Carprofen was approximately as potent as indomethacin (IC_{50} = 68 ± 4 μM) and more potent than ibuprofen (IC_{50} = 711 ± 44 μM), two NSAIDs that have been previously shown to inhibit FAAH.[13] As expected from studies with other NSAIDs, carprofen's inhibition of FAAH activity was weaker under neutral conditions than under acidic conditions (IC_{50} = 15.5 ± 0.1 μM at pH 6.0; Supplementary Figure 1 and Methods in the SI). To investigate the mechanism through which carprofen inhibits FAAH, we crystallized recombinant rat FAAH in complex with this drug and solved the structure at 2.25 Å resolution (Table 1 in the SI). Diffraction-quality crystals of the FAAH/carprofen complex were obtained by preincubating FAAH with the *O*-arylcarbamate inhibitor 3-(3-carbamoylphenyl)phenyl *N*-cyclohexylcarbamate (URB597).[14]

The electron density map revealed that carprofen occupies a space located at the entrance of the membrane access (MA) channel of FAAH (Figure 1a), an elongated cavity that allows substrates to enter the enzyme's active site (Figure 1b). The propanoic acid group of carprofen remains partially exposed to the solvent, where its higher mobility produced a weaker and less defined electron density map (Figure 1a). This group, which is likely to be ionized at the pH used for crystallization (pH 7.5), forms a hydrogen bond with the side-chain nitrogen of Trp531 (Figure 1b). On the other hand, the carbazole ring and chloride atom of carprofen are positioned within the MA channel and enshrouded by hydrophobic amino acid residues,

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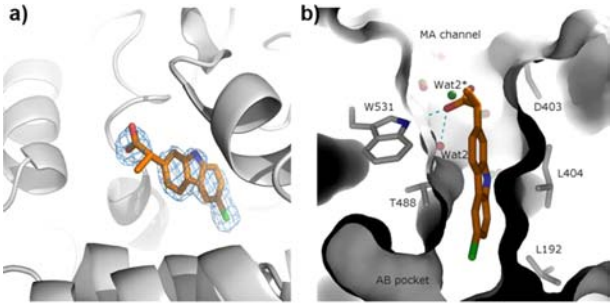

Figure 1. Structure of carprofen bound to FAAH. (a) Position of carprofen (C atoms shown in orange) at the entrance of the active-site gorge of FAAH. The 2-arylpropionic acid group of carprofen protrudes from the enzyme active-site cleft. The electron density map ($2F_o - F_c$) corresponding to the position of the drug (in sky-blue) is contoured at 1.0σ. (b) Binding of carprofen (C atoms shown in orange) in the membrane access (MA) channel of FAAH. Water molecules are depicted as red spheres. H-bonds involving the carprofen carboxylate, Wat2, and W531 are represented as cyan dashed lines. Green spheres represent superimposed water molecules in the structure of the FAAH/URB597 complex (PDB entry 3LJ7) and show the different position of the corresponding Wat2 (highlighted by an asterisk) upon inhibitor binding. Single-letter abbreviations of amino acids have been used for clarity. AB denotes acyl binding.

which form a tight and well-modeled binding site (Figures 1b and 2a) at ideal interaction distances (Figure 2b).

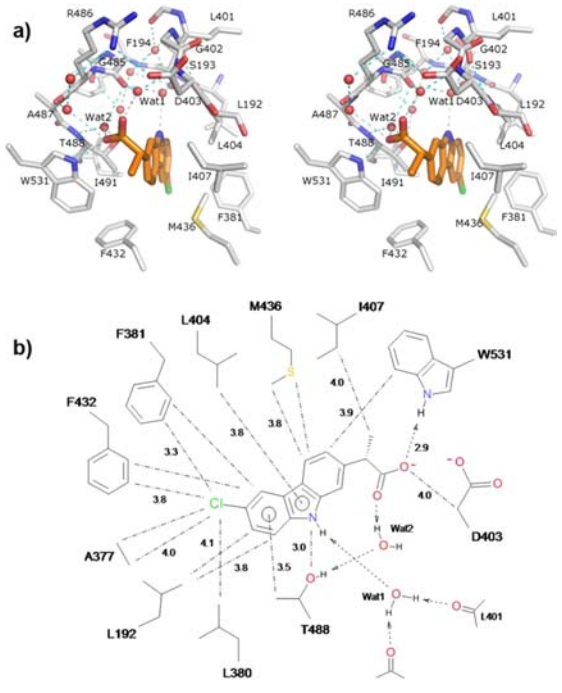

Figure 2. Interactions between carprofen and FAAH. (a) Stereoview showing the interactions between carprofen (C atoms shown in orange) and residues of the FAAH MA channel. Selected residues and water molecules facing carprofen are shown as sticks and red spheres, respectively, and their H-bonds are represented as cyan dashed lines. Water molecules Wat1 and Wat2 form H-bonds involving the carbazole nitrogen and the carboxylate of carprofen, respectively. Single-letter abbreviations of amino acids have been used for clarity. (b) Interaction distances (in Å) between carprofen and FAAH active-site residues.

An intense H-bond network of water molecules further stabilizes the complex, filling the space between carprofen and the amino acid residues flanking the MA cavity (Figure 2a). These water molecules occupy positions similar to those previously reported for the structure of the FAAH/URB597 complex.[15] One notable exception is represented by water molecule 2 (Wat2), which in the FAAH/carprofen complex bridges the propionic acid moiety of carprofen and the residues Thr488 and Gly485 through H-bonds (Figure 2a). Wat2 is located ~3.5 Å away from the site occupied by its counterpart in the FAAH/URB597 complex (Figure 1b). Comparison of the FAAH/carprofen and FAAH/URB597 complexes revealed that carprofen binding is associated with a marked structural rearrangement of the MA channel. For example, Phe432 and Met436 adopt side-chain conformations that are substantially different in the two complexes (Supplementary Figure 2a). This restructuring of the MA cavity may contribute to maximizing the interaction between the NSAID and FAAH.

To determine whether the binding interaction identified by X-ray crystallography might influence the FAAH activity, we measured the inhibitory potencies of carprofen on wild-type FAAH and a FAAH mutant in which Thr488, one of the residues most directly involved in the interaction with carprofen (Figures 1b and 2a), was replaced with alanine. In agreement with the X-ray complex outlined above, we found that carprofen is a more potent inhibitor of purified recombinant wild-type FAAH (IC_{50} = 74 ± 8 μM) than of the Thr488Ala mutant (IC_{50} = 165 ± 7 μM) (Figure 3; assays

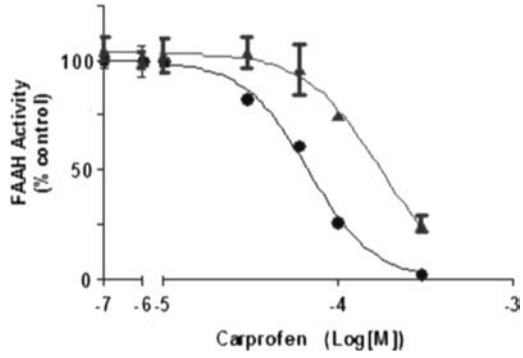

Figure 3. Profiles for carprofen inhibition of (●) wild-type and (▲) Thr488Ala FAAH using the [^{3}H]anandamide hydrolysis assay. Data are expressed as percent of control activity, which was 81364.96 pmol min^{-1} (mg of protein)$^{-1}$ for wild-type FAAH and 12605.33 pmol min^{-1} (mg of protein)$^{-1}$ for the mutant. The results suggest that the interaction of carprofen with Thr488 is important for FAAH inhibition.

conducted at pH 6). A similar difference was observed when the activity assays were carried out under neutral conditions (pH 7.4; IC_{50} = 381 μM for wild-type FAAH and 647 μM for Thr488Ala FAAH). In contrast with carprofen, the *O*-arylcarbamate derivative URB597 inhibits wild-type and Thr488Ala FAAH with similar potencies (3.1 ± 0.96 and 3.4 ± 1.04 nM, respectively, at pH 7.4), suggesting that the two compounds interact with distinct binding sites of FAAH. In agreement with this conclusion, previous[15] and present X-ray crystallography studies have shown that URB597 reacts with the catalytic nucleophile Ser241 to form a cyclohexyl carbamate adduct that is positioned at the inner extremity of the MA channel (Supplementary Figure 2b).

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To assess whether the formation of this adduct might impair the ability of carprofen to interact with FAAH, we measured the binding of this compound in the presence and absence of URB597 using WaterLOGSY[16a] NMR spectroscopy (BOX 1 in the SI). Consistent with the structural data, positive WaterLOGSY signals indicated that carprofen can bind FAAH and that preincubating the enzyme with URB597 does not alter such binding (Supplementary Figure 3).

Finally, we asked whether the structure of the carprofen complex reported here might provide insights into the interaction of FAAH with other NSAIDs. We used a highly sensitive NMR method, fluorine atoms for biochemical screening (FABS)[16b] (BOX 1 in the SI), to monitor the FAAH-mediated hydrolysis of ARN1203, a specifically designed fluorinated analogue of anandamide[16c] (Figure 4a inset and

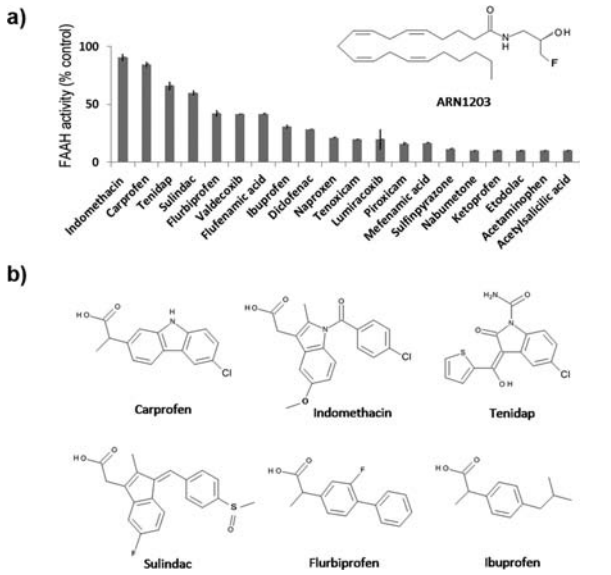

Figure 4. NSAIDs that inhibit FAAH activity share a common chemical signature. (a) FAAH inhibition (% of control) of representative NSAIDs assessed using the NMR-based FABS method. Compounds were tested at 200 μM. Inset: Chemical structure of the fluorinated substrate. (b) Chemical structures of the NSAIDs that were most active at inhibiting FAAH. The presence of a planar bicyclic 5−6 fused system, a chlorine atom linked to one of phenyl ring, and a carboxylate group should be noted. The FAAH/carprofen complex (Figure 2) points to possible interactions of these chemical groups with FAAH.

Methods in the SI), in the presence of a representative set of commercially available NSAIDs (Figure 4a, Supplementary Figure 4, and Table 2 in the SI). Hydrolytic cleavage of ARN1203 by FAAH produces changes in the ^{19}F NMR signal, which were used to measure enzyme inhibition. FABS analyses on 20 NSAIDs yielded the following rank-order potency of FAAH inhibition: indomethacin ≈ carprofen > tenidap > flurbiprofen > ibuprofen. This is in line with the rank-order potency obtained for carprofen, indomethacin, and ibuprofen using a standard enzyme assay (above) and points to the presence of common pharmacophore functions in the NSAID class that might be responsible for FAAH inhibition. Indeed, the three most active compounds (indomethacin, carprofen, and tenidap) share a common planar 5,6-fused bicyclic system and a chlorine atom linked to one of the phenyl rings (Figure 4b). Possible interactions of these structural elements with the MA channel of the enzyme may be gleamed from the FAAH/carprofen complex (Figure 2).

Carprofen is currently utilized in veterinary medicine because of its high analgesic effectiveness and relative lack of gastric side effects.[17] The present study shows that this NSAID binds to a set of amino acid residues located at the entrance to the active site of FAAH, with the carboxylate group of the molecule protruding from the enzyme. This interaction (a) appears to influence FAAH function, as removal of one of its key components (Thr488) markedly weakens the ability of carprofen to inhibit FAAH activity, and (b) distinguishes carprofen from other known FAAH inhibitors, which occupy either the core of the substrate-binding cavity (URB597, OL-135, and PF-3845)[18] (Supplementary Figure 5a) or, less frequently, the entirety of the MA channel (ketobenzimidazoles)[18d] (Supplementary Figure 5b). Furthermore, our FABS experiments revealed several chemical commonalities that might underpin the ability of clinically important NSAIDs such as indomethacin to inhibit FAAH activity. This structural information provides novel insights into the mode of action of the NSAIDs and may help to design superior analgesic agents that act by simultaneously targeting FAAH and COX.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures; experimental procedures for construct generation, mutagenesis, protein expression and purification, NMR studies, in vitro assays, and structure determination; and a table of crystallographic analysis and refinement statistics. This material is available free of charge via the Internet at http://pubs.acs.org. The atomic coordinates and crystallographic structure factors of FAAH in complex with carprofen have been deposited in the Protein Data Bank as entry 4DO3.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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