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Design of On-Target FAAH Inhibitors

In this issue of *Chemistry & Biology*, Alexander and Cravatt [1] propose a model for the binding of carbamate inhibitors to fatty acid amide hydrolase (FAAH), the enzyme that breaks down signaling lipids. Using competitive activity-based protein profiling and click chemistry, they designed potent and selective FAAH inhibitors and characterized their off-target reactions.

Marijuana, Cannabinoid Receptors, Anandamide, and FAAH. Marijuana has been used for thousands of years for the physiological effects that occur when its active component THC [2] overwhelms the brain's cannabinoid receptors [3–5] that normally are occupied by endogenous ligands including anandamide [6]. Fatty acid amide hydrolase (FAAH) is the key integral membrane enzyme involved in the hydrolysis of anandamide [7] and other fatty acid amides [8] including oleamide, a sleep factor, and palitoylethanolamide, an anti-inflammatory substance [9].

The field made a quantum jump in 1996 when the Cravatt laboratory cloned FAAH. They did it the old-fashioned way—by purifying it first [10]. Three independent laboratories found the catalytic amino acids in FAAH to be unique in that, unlike most serine hydrolases, there were two serines instead of the typical serine-histidine-aspartic acid catalytic triad. The whole story was eventually elucidated by Cravatt laboratory, using mutagenesis, kinetics, and labeling experiments through which they described a unique catalytic mechanism involving a serine-serine-lysine triad. Serine 241 is the nucleophile binding to anandamide and inhibitors.

Later work showed that FAAH knockout mice had elevated levels of anandamide and other fatty acid ethanolamides in the central and peripheral nervous system [11]. From these and other transgenic experiments, it was concluded that in $FAAH^{-/-}$ mice there was a cannabinoid receptor-dependent reduction in pain sensation caused by higher anandamide levels and reduced inflammation, which, in part, may be mediated by other fatty acid amides normally metabolized by FAAH [12]. In 2002, the crystal structure of FAAH bound to an inhibitor revealed a dimeric protein with a hydrophobic cap that constitutes a membrane binding region, presumably reinforcing the binding of the N-terminal transmembrance binding domain (deleted for practical purposes during crystallization). Most interestingly, the crystal structure revealed an acyl chain binding (ACB) channel for entry of hydrophobic substrates from the membrane and a cytoplasmic access (CA) channel for both entrance of water for the hydrolysis of substrate and exit of the hydrophilic breakdown products. For example, anandamide would enter via the membrane through the ACB to the active site where it would be hydrolyzed, and the liberated hydrophobic fatty acid would exit through the ACB to the membrane while hydrophilic ethanolamide would exit to the cytoplasm by the CA channel (see McKinney and Cravatt [9] for a comprehensive review of this work).

First Generation FAAH Inhibitors Were Potent but Nonselective. Enzymes are important targets for many drugs such as cyclooxygenase (COX) inhibitors, angiotension-converting enzyme (ACE) inhibitors, and HIV protease inhibitors. Starting with the serendipitous discovery that phenylmethylsulfonylfluoride (PMSF) [7, 13] is a FAAH inhibitor, hundreds of compounds have been synthesized targeting FAAH. Already in 1994, based upon studies with trifluormethylketones, α-keto esters, and α-keto amides, it was predicted that FAAH inhibitors would have significant therapeutic value in the areas of analgesia, mood, nausea, memory, appetite, sedation, locomotion, glaucoma, and immune function [14], and some of this has already been borne out with a variety of compounds in animal studies [15]. However, many of these compounds, although potent, could not be checked for specificity against the large number of other enzymes that they may also inhibit. Interestingly it was discovered that NSAIDs are weak FAAH inhibitors [16, 17].

Cravatt's group pioneered new methods to measure the potency and specificity of FAAH inhibitors against the proteome (proteins expressed in different tissues) [18]. Termed competitive activity-based protein profiling (ABPP), the technique employs a fluorescent-labeled fluorophosphonate that reacts with the broad family of serine hydrolases including FAAH in tissue homogenates. When putative FAAH inhibitors are incubated with the probe, the off-target interactions (e.g., in the brain, liver, and kidney) become evident as well as the strength of the inhibitor for FAAH. Competitive ABPP has been used successfully to show that many of the inhibitors described above had off-target reactions [19]. In addition, in collaboration with the Boger group at Scripps, many new potent and selective inhibitors have been described, such a OLE135, that have potent analgesic effects [20].

Putting It All Together. However, ABPP cannot be applied to live animals or cells in culture because the probe with the fluorescent tag is too bulky. Click chemistry (CC) provided the solution to this problem through a FAAH inhibitor bearing a stable small reactive group (e.g., an alkene). The molecule gets into cells and hits its target(s). The tissue is then isolated and reacted with an azide-tagged fluorescent group, and FAAH and other reactive proteins are separated and identified (Figure 1). The paper by Alexander and Cravatt [1] brings it all together. They now describe a new series of selective and potent FAAH inhibitors whose structures are based upon carbamates such as URB597, recently described to modulate anxiety in an animal model [21–23]. It was proposed that URB-related compounds carbonylate Ser241 and that the compound resides in the enzymatic pocket of the protein such that the aliphatic part of the molecule faces the cytoplamic channel [21, 22]. However, Alexander and Cravatt found that the URB inhibitors are, in fact, oriented in the opposite direction. That is, the biaryl part of URB597 is in the cytoplasmic channel, and the aliphatic part of the molecule is in the hydrophobic acyl

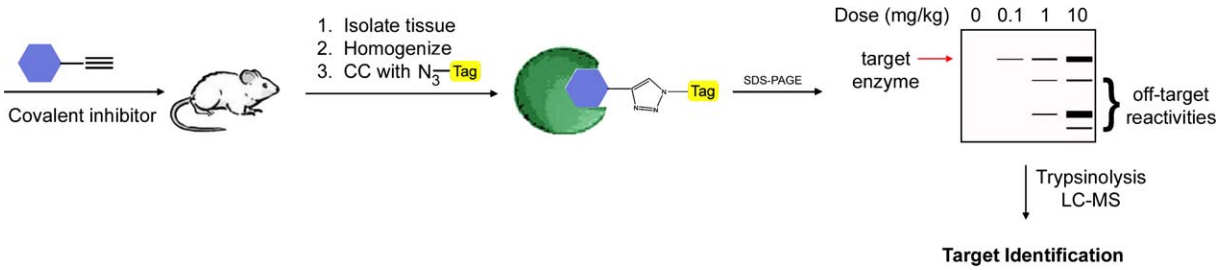

Figure 1. In Vivo Proteomic Profiling of Inhibitor/Drug Selectivity by Click Chemistry

Mice were administered an alkyne-modified variant of the inhibitor at increasing doses. After a period of time, the animals were sacrificed, the tissue was isolated and homogenized and then reacted under CC conditions with an azide-modified reporter tag. Labeled proteins were separated by SDS-PAGE and visualized by in-gel fluorescence scanning. Off-target reactivities were identified by trypsinolysis followed by LC-MS. The figure was kindly provided by Jessica Alexander.

chain binding channel. This model for URB597 binding and orientation was consistent with the carbamylation of Ser 241, observed by MALDI-TOF mass spectromeric mapping of a tryptic peptide. To further confirm their model, they synthesized other carbamates with longer aliphatic side chains (similar to those found on known FAAH inhibitors) that were more potent than URB597.

Using one of these FAAH inhibitors (JP104), they showed, in an in vitro ABPP assay, that it was specific for brain FAAH. Experiments using FAAH knockout mice then showed that even at high concentration of JP104 there were no other targets in the brain, but that there were heretofore unknown targets in other organs such as liver and kidney. The authors isolated and identified some of these off-target reactants including carboxylesterase 6 and esterase 31; neither of which share any sequence homology with FAAH. Using JP104 as a click chemistry probe in mice, they found a window where its concentration could be adjusted to bind brain FAAH selectively (1 mg/kg) while the reactions in liver and kidney with proteins other than FAAH could be minimized.

Using JP104, for example, it will now be possible to search for other, even more specific FAAH inhibitors that have even fewer side reactions in peripheral tissues. These authors are "on-target" with their speculation that covalent enzyme inhibitors will be useful as in vivo functional probes, paving the way for the design of therapeutic agents in other areas such as cancer invasiveness [24].

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