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Fatty acid amide hydrolase as a potential therapeutic target for the treatment of pain and CNS disorders

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Abstract

Background—Fatty acid amide hydrolase (FAAH) is an integral membrane enzyme that hydrolyzes the endocannabinoid anandamide and related amidated signaling lipids. Genetic or pharmacological inactivation of FAAH produces analgesic, anti-inflammatory, anxiolytic, and antidepressant phenotypes without showing the undesirable side effects of direct cannabinoid receptor agonists, indicating that FAAH may be a promising therapeutic target.

Objectives—This review highlights advances in the development of FAAH inhibitors of different mechanistic classes and their *in vivo* efficacy. Also highlighted are advances in technology for the *in vitro* and *in vivo* selectivity assessment of FAAH inhibitors employing activity-based protein profiling (ABPP) and click chemistry-ABPP, respectively. Recent reports on structure-based drug design for human FAAH generated by protein engineering using interspecies active site conversion are also discussed. Methods: The literature searches of Medline and SciFinder databases were used.

Conclusions—There has been tremendous progress in our understanding in FAAH and development of FAAH inhibitors with *in vivo* efficacy, selectivity, and drug like pharmacokinetic properties.

1. Introduction

Endocannabinoids are endogenous lipid ligands that activate the cannabinoid G-protein coupled receptors CB1 and CB2. Two endocannabinoids have been identified in mammals, *N*-arachidonoyl ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) (Figure 1), and the biological actions of these signaling lipids are terminated by enzymatic hydrolysis (1-4). Two principal enzymes that are responsible for the metabolism of AEA and 2-AG are fatty acid amide hydrolase (FAAH) (5,6) and monoacylglycerol lipase (MAGL) (7) (Figure 1). CB1 and CB2 are also activated by Δ^9-tetrahydrocannabinol (THC), the psychoactive component of marijuana (8,9). THC and other direct CB1 agonists have long been recognized to possess medicinally beneficial properties including pain relief. However, these agents also produce undesirable side effects including impairments in cognition and motor control, which limit their utility as therapeutic agents. One attractive approach to retain the beneficial effects of cannabinoid activation, while avoiding the undesirable effects of global cannabinoid activation, is to elevate "endogenous cannabinoid (endocannabinoid) tone" by inhibition of hydrolytic degradation. FAAH, in addition to hydrolyzing AEA, regulates other lipid signaling molecules with anti-inflammatory and analgesic properties such as *N*-palmitoyl ethanolamine

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Declaration of interest
KA and DSJ are employees of Pfizer.

(PEA), which exerts its biological activity via non-cannabinoid pathways (10). In contrast to CB1 agonists, FAAH inhibitors and FAAH knockout (−/−) mice have been found to display analgesia (11-15), anti-inflammation (16-18), anxiolysis (12,19,20) and anti-depression (19, 21) without disruptions in motility, cognition, or body temperature (11,12,22,23). These findings suggest that FAAH may represent an attractive therapeutic target for treatment of pain, inflammation, and other central nervous system (CNS) disorders.

Over the past several years, multiple excellent reviews have appeared that discuss endocannabinoid metabolism and signaling, as well as pharmacological inhibition of FAAH and its therapeutic application (1,2,24-30). In this review, we will focus on recent advances in the development of potent and selective FAAH inhibitors with *in vivo* efficacy. In addition we will also discuss new technologies that allow the assessment of *in vitro* and *in vivo* selectivity of FAAH inhibitors as well as the recent disclosure of the crystal structure of "humanized" rat FAAH in complex with small molecule inhibitors that facilitates structure-based inhibitor/drug design (31,32).

2. FAAH as the principal AEA-hydrolyzing enzyme and its molecular characterization

AEA was the first identified endogenous ligand for the CB1 receptor (33). Unlike hydrophilic neurotransmitters, whose action involves the uptake and storage of these small molecules into synaptic vesicles, AEA and other *N*-acyl ethanolamines (NAEs) are produced upon demand through activity-dependent cleavage of membrane lipid precursors (29,34). The biological activity of AEA in the central nervous system and in peripheral tissues is terminated by its removal from the extracellular space via cellular uptake potentially involving the action of a protein transporter (3,35,36), followed by expeditious enzymatic degradation. The principle enzyme responsible for AEA degradation has been identified as the integral membrane protein fatty acid amide hydrolase (FAAH) (6). FAAH terminates AEA signaling by hydrolyzing this lipid to arachidonic acid and ethanolamine, two metabolites that do not activate CB1 or CB2 receptors (Figure 1).

Prior to the molecular characterization of FAAH, there were several reports on a membrane-associated enzyme activity that hydrolyzes NAEs. In 1985, Schmid and colleagues first described an enzyme activity from rat liver that hydrolyzes saturated and monounsaturated NAEs (37). An enzyme activity with similar properties that converted AEA to arachidonic acid was also reported from N18TG2 neuroblastoma cells (38) and from rat and porcine brain tissue (39-41). Despite these early reports, it was not until 1996 when Cravatt and colleagues identified FAAH as the AEA hydrolyzing enzyme. Following the identification of the fatty acid primary amide oleamide as a sleep-inducing substance, an oleamide hydrolase activity was described (42) and suggested to represent the same enzyme as the AEA/NAE hydrolase (43). The researchers isolated this hydrolase from rat liver membranes using affinity purification with a column covalently modified with an oleoyl trifluoromethyl ketone inhibitor derivative (see compound **5**,Figure 4) (5). Cloning and transfection of the corresponding cDNA confirmed that the enzyme displayed robust hydrolysis activity for numerous fatty acid amides, including AEA and oleamide. The enzyme was therefore named fatty acid amide hydrolase (FAAH). Human (44), mouse (44), and pig (45) *FAAH* genes have since been characterized and shown to be highly conserved in primary structure. FAAH possesses a single predicted NH_2-terminal transmembrane domain, an amidase signature domain, and a polyproline sequence predicted to interact with Homer and SH3 domain proteins (Figure 2A). Both the human and rat FAAH enzymes are expressed at high levels in the nervous system, but show differences in their relative distribution among peripheral tissues (5,44). Immunohistochemical and immuno-electron microscopy studies have revealed that FAAH is broadly expressed in the nervous system, where the enzyme localizes predominantly to intracellular membranes (e.g.,

smooth endoplasmic reticulum, outer membrane of the mitochondria) in the somatodendritic compartment of neurons (46,47).

FAAH belongs to a large group of enzymes termed the amidase signature (AS) family (48). There are more than 100 members of this enzyme family and FAAH was the first characterized mammalian member. AS enzymes are characterized by a highly conserved region that is rich in serine, glycine, and alanine residues comprising approximately 130 amino acid residues. Despite sharing significant sequence homology, members of this enzyme class exhibit markedly different substrate specificities. Unlike most other AS enzymes, FAAH is an integral membrane protein with a single predicted NH_2-terminal transmembrane domain (amino acids 9−29). However, deletion of the first 29 amino acids of rat FAAH generated a catalytically active truncated enzyme, termed transmembrane domain-deleted-FAAH (ΔTM-FAAH), that still tightly bound to cellular membranes (49). These results indicated that FAAH possesses other regions for membrane-association beyond the predicted NH_2-terminal transmembrane domain.

3. Recombinant expression and purification of FAAH

To obtain sufficient quantities of purified FAAH for its detailed mechanistic and structural characterization, an *E. coli* expression system was developed for both the full-length (FL) and ΔTM-rat FAAH (ΔTM-rFAAH), which were expressed with COOH- and NH_2-terminal His_6 tags, respectively, in *E. coli* and purified to near homogeneity with typical yields of 1−1.5 mg /liter culture (49). Despite having the transmembrane domain deleted, ΔTM-rFAAH still required detergent for solubilization, but possessed similar catalytic properties to the FL enzyme. Analytical ultracentrifugation demonstrated that ΔTM-rFAAH existed as a single 11S detergent-protein complex, but the FL enzyme was distributed as a heterogeneous mixture of larger species in the range of 15S-28S in size. These data supported ΔTM-FAAH as the superior form of the FAAH protein for X-ray crystallographic studies.

Human FAAH (hFAAH), despite sharing ~82% sequence identity with rFAAH, has proven more difficult to express and purify. Over the many years since the report of the *E. coli* expression of rFAAH described above, there have been only a few reports on the recombinant expression of hFAAH using baculovirus-insect cell (50,51) and bacterial systems (50); however, in these cases, hFAAH expression levels were not reported. Only recently has an hFAAH expression protocol that generates a modest quantity of hFAAH (~1 mg purified protein/liter *E. coli* culture) been reported (31). This optimized protocol also produced much higher yields of rFAAH (~20 mg purified enzyme/liter culture) (31).

Although modest quantity of hFAAH could be obtained, this protein was much less stable and more prone to aggregation than rFAAH, limiting its utility for crystallization. As an alternative strategy, a “humanized” version of rFAAH, where the protein's active site was converted to match the human enzyme was created. Active site residues were identified based on the crystal structure of rFAAH, and sequence comparisons identified six of these amino acids that differed between rFAAH (L192, F194, A377, S435, I491, and V495) and hFAAH (F192, Y194, T377, N435, V491, and M495) (31). In the rFAAH structure, all of these residues except Ser435 interact with the arachidonyl chain of the bound methyl arachidonyl phosphonate (MAP) inhibitor. S435 was found to be in close proximity to, but not directly contacting the arachidonyl chain. Mutation of each of these six amino acids in rFAAH to the corresponding residues in hFAAH generated a humanized rat (h/r) FAAH protein that is expressed at levels similar to rFAAH in *E. coli* (~10 mg purified protein/liter culture), but showed inhibitory sensitivity profiles that matched the human enzyme. This h/rFAAH was used as a surrogate for hFAAH for structural studies (31).

4. X-ray crystallographic structure determination of FAAH

In 2002, the first X-ray crystal structure of FAAH was determined for the rat enzyme at 2.8 À resolution using ΔTM-rFAAH in complex with an irreversible inhibitor, methyl arachidonyl fluorophosphonate (MAFP) (52). FAAH crystallized as a dimeric enzyme, consistent with chemical cross-linking and analytical ultracentrifuge studies (49). The FAAH structure revealed multiple channels that appear to grant the enzyme simultaneous access to both the membrane and cytosolic compartments of the cell. One channel leads from the putative membrane-binding surface of the protein to the enzyme active site and was occupied by the bound MAP inhibitor. This channel, dubbed the "acyl chain-binding channel", is comprised almost entirely of hydrophobic residues and is thought to participate in substrate recognition. A second channel emerges from the active site at an angle of approximately 80° from the substrate-binding cavity to create a solvent-exposed "cytoplasmic port." These structural features suggest that the hydrophobic fatty acid and hydrophilic amine products of FAAH-catalyzed substrate hydrolysis may exit via the membrane-access and cytosolic-access channels, respectively. The FAAH structure in combination with extensive mutagenesis studies (53-55) also revealed that this enzyme possesses an unusual serine-serine-lysine (Ser241-Ser217-Lys142) catalytic triad that is conserved among enzymes in the AS class and is distinct from the typical Ser-His-Asp catalytic triad utilized by the majority of serine hydrolases. Ser241 was determined to be the FAAH nucleophile and Lys142 of FAAH appears to serve as a key acid and base in distinct steps of the catalytic cycle (55,56). As a base, Lys142 first activates the Ser241 nucleophile for attack on the substrate amide carbonyl. As an acid, Lys142 participates in the protonation of the substrate leaving group, which appears to occur early in the acylation reaction (i.e., concomitant with nucleophilic attack). This mechanism allows FAAH to exhibit an unusual ability to hydrolyze amides and esters at equivalent rates. Ser217 was also found to play a key role in catalysis (54,55), likely acting as a "proton shuttle" between Ser241 and Lys142 as shown in Figure 2B.

The MAP-rFAAH structure remained as the only crystal structure of FAAH for many years. However, the high levels of expression of the h/rFAAH protein offered a new opportunity to solve structures of the enzyme that would be of great relevance to structure-based drug design. Indeed, as noted above, h/rFAAH exhibited an inhibitor sensitivity profile that matched closely with that of hFAAH regardless of inhibition mechanism (reversible or irreversible) or species specificity (higher potency for hFAAH or rFAAH). This strategy finally led to the 2nd crystal structure of FAAH reported by Mileni *et al.* in 2008 (31). The crystal structure of h/rFAAH protein bound to a small molecule inhibitor PF-750 was determined at 2.75-Å resolution. PF-750 is an irreversible and highly selective inhibitor of FAAH (57). Not surprisingly, the overall fold of the h/rFAAH structure was essentially identical to rFAAH (52), with both enzymes crystallizing as a dimer. As expected based on previous mechanistic studies, PF-750 was covalently attached to the Ser241 nucleophile of h/rFAAH through a carbamate linkage (57), with the remaining portion of the parent inhibitor occupying the acyl chain-binding pocket (Figure 3A). The aniline leaving group is not observed, indicating that this portion of the inhibitor detaches from the enzyme following covalent inactivation. Despite obvious structural differences between PF-750 and MAFP, these inhibitors showed remarkable overlap in their respective binding modes in the h/rFAAH and rFAAH active sites (Figure 3B). Both inhibitors traversed a substantial portion of the FAAH acyl chain-binding pocket, making contacts with side chains on several of the residues that form the surface of this hydrophobic channel. One striking difference, however, was observed at the distal end of the acyl chain-binding pocket, where the presence of the shorter PF-750 group allowed Phe432 to undergo a conformational shift that moved this residue out of the membrane-access channel and into the acyl chain-binding channel. The net effect of this structural rearrangement is a substantial expansion of the size of the membrane-access channel (See reference (31) for details). This channel has been postulated to serve as a portal for lipid substrates to enter FAAH from the cell membrane,

suggesting that Phe432 may act as a dynamic paddle that directs and orients substrate molecules towards the active site during catalysis.

Multiple key interactions were observed between PF-750 and h/rFAAH that could explain the distinct inhibitor sensitivity profiles of the human and rat enzymes. For instance, an aromatic-CH...π-interaction was observed between Phe192 and the quinoline ring of PF-750 (Figure 3C). This interaction would only occur in the human active site, since, in rFAAH, the corresponding amino acid residue is a leucine, resulting in an increased distance between the residue and bound inhibitor and loss of the hydrophobic interaction. An additional interaction with Val491 also could contribute to the enhanced potency of PF-750 for hFAAH. This improvement may reflect better steric accommodation of PF-750 by Val491 as opposed to the corresponding Ile491 residue in rFAAH (Figure 3B). Val491 is in good Van der Waal's distance from PF-750, but replacement of this residue with an isoleucine would be predicted to bring the side chain too close to the quinoline ring of the inhibitor (below 3Å), resulting in steric repulsion.

Based on the PF-750-h/r-FAAH structure, it was predicted that extension of the inhibitor's length would allow additional contacts with the acyl chain-binding channel and lead to higher potency. The 3rd crystal structure of FAAH recently reported by Ahn *et al.* in 2009 was accomplished with h/rFAAH in complex with PF-3845, which has 20-times higher potency for hFAAH compared to PF-750 (58). As expected from its irreversible mechanism of FAAH inhibition, PF-3845 was also found to be covalently attached to the catalytic Ser241 nucleophile of h/rFAAH through a carbamate linkage. The 3-aminopyridine leaving group was not observed in the h/rFAAH structure, but the remaining piperidine portion of the parent molecule occupied the acyl chain-binding pocket (Figure 3A). As was observed in the PF-750-h/rFAAH structure, a strong aromatic C-H...π-interaction can be seen between Phe192 and the phenyl aromatic ring of PF-3845. The ~20-fold improvement in potency for PF-3845 appears to derive from a more extended set of van der Waals interactions between this inhibitor's 4-trifluoromethyl-2-pyridyl group and the hydrophobic acyl chain-binding pocket of FAAH (Figure 3A). The overlap between the biaryl ether piperidine moiety of PF-3845 and the arachidonyl chain of MAP is striking considering their large structural differences between PF-3845 and MAFP. Notably, this overlap in the acyl-chain-binding pocket allows Phe432 and the adjacent Met436 residues to occupy similar overlapping spaces resulting in similar sizes in the acyl chain-binding pocket and membrane access channel in the PF-3845-h/rFAAH and MAP-rFAAH crystal structures (Figure 3D).

As the PF-750- and PF-3845-h/rFAAH structure facilitated a deeper understanding of features required for inhibitor potency, it is expected that these and other crystallographic structures of FAAH in complex with small molecule inhibitors will greatly assist structure-based inhibitor/drug design.

5. FAAH inhibitors

A thorough review of the medicinal chemistry and SAR of FAAH inhibitors has recently been published (28). This section will provide a brief review on some of early inhibitors that served as important chemical tools and then will focus on the development of FAAH inhibitors that have been shown to exhibit *in vivo* activity. Several classes of FAAH inhibitors have been reported, including reversibly (e.g., trifluoromethyl ketonesα-ketoheterocycles) and irreversibly (e.g., fluorophosphonates; carbamates; ureas) acting agents. These inhibitors each possess distinct sets of attributes and deficiencies that reflect some of the most challenging aspects of FAAH inhibitor development.

5.1 Substrate-derived inhibitors

Deutsch and colleagues prepared a trifluoromethyl ketone analog (**3**) of AEA and showed that it inhibited AEA hydrolysis (Figure 4) (59). Meanwhile, researchers at The Scripps Research Institute isolated and determined the structure of the fatty acid amide oleamide from the cerebrospinal fluid of sleep-deprived cats and found that it was rapidly degraded by an integral membrane protein that generates oleic acid (42). The trifluoromethyl ketone analog of oleamide (**4**) was shown to be a potent inhibitor of this enzymatic activity (K_i = 82 nM) (60,61). The enzyme responsible for the hydrolysis of AEA and oleamide was identified and characterized from studies using trifluoromethyl ketone analog **5** where a thiol was incorporated at the terminus of the acyl chain and linked through a disulfide bond to beads. Affinity chromatography was used to isolate the native enzyme responsible for the hydrolysis of these fatty acid amides and this enzyme was given the name fatty acid amide hydrolase (FAAH) (5). Another prominent substrate-derived inhibitor is MAFP (**6**), which was orginally prepared as an irreversible $cPLA_2$ inhibitor (62), but was later shown to be a potent irreversible inhibitor of FAAH and several other serine hydrolases (52,63,64). As described in section 7, activity-based probes to profile the proteomic selectivity of FAAH inhibitors against the serine hydrolase class of enzymes have been developed by linking a reactive fluorophosphonate group to reporter tags, such as fluorophores or biotin (65-68). As described above, these substrate-based inhibitors have served as useful chemical tools, but they are not viable templates for drug design because they are highly hydrophobic and display low selectivity.

5.2 Electrophilic ketone inhibitors

Figure 5 shows a series of α-ketoheterocycle inhibitors (**7**−**18**) that Boger and colleagues have introduced that, like the trifluoromethyl ketones, reversibly form an enzyme-stabilized hemiketal between the active-site Ser241 and the electrophilic carbonyl (23). Initially, various monocyclic and bicyclic heterocycles including oxazole, pyridazine, pyrimidine, and benzoxazole were appended to the oleoyl acyl side-chain to give α-ketoheterocycles with similar potency to trifluoromethyl ketone **4** (23). The introduction of a nitrogen into the benzoxazole resulted in oxazolopyridine **7**, which enhanced the potency more than 50-fold (K_i = 2.3 nM) presumably as a result of favorable H-bonding interactions in the cytoplasmic access channel of FAAH. The corresponding oxazolopyridine with an arachidonyl acyl side-chain was slightly more potent (K_i = 1 nM), but proved to be unstable and decomposed rapidly under storage. It was also found that the oleoyl or arachidonyl acyl side chain could be replaced by a structurally simpler phenbutyl, phenpentyl, or phenhexyl side-chain to give **8** − **10**. These compounds showed improved potency (K_i = 0.2−0.3 nM) and were much more amenable to further structural modification. Efforts to optimize the heterocycle resulted in the unfused pyridyl oxazole **11** (K_i = 18 nM) and **12** (OL-135, Ki = 4.7 nM) (69,70). While the potency decreased somewhat, activity-based protein profiling (ABPP) against the serine hydrolase superfamily (For details on selectivity assessment using ABPP, see section 7 below) distinguished **11/12** as having superior selectivity over the predecessor oxazolopyridines (**7/10**) and trifluoromethyl ketones (**4**) (65,71). OL-135 (**12**) exhibited >10,000-fold selectivity for FAAH over KIAA1363 and 60-fold selectivity over triacylglycerol hydrolase (TGH), reversing the intrinsic selectivity for KIAA1363 and TGH seen with many of the trifluoromethyl ketones. Notably, the potency was recovered and the selectivity was improved further with pyridyl oxadiazole **13** (K_i = 0.29 nM) (71,72). The electrophilic character of the ketone appears to be critical for FAAH activity. Boger and colleagues have further prepared a series of oxazoles with small substituents (i.e. **14**−**16**) and showed that FAAH inhibition potency tracked with the electron-withdrawing properties of the R group (73). For example, trifluoromethyl oxazole **16** (K_i = 0.8 nM) is 100-fold more potent than the corresponding methyl oxazole **14** (K_i = 80 nM). This observation aided the design of more potent FAAH inhibitors within this series, but also highlights one of the key challenges in that activated ketones are known to have metabolic liabilities. Introducing conformational constraints into

the flexible C2 acyl chain of OL-135 (**12**) by removing many of the rotatable bonds and introducing π-unsaturation resulted in several potent FAAH inhibitors exemplified by the biphenyl ethyl compound **17** (K_i = 0.75 nM) and 4-(phenoxymethyl)phenethyl compound **18** (K_i = 1 nM) (74,75). Johnson & Johnson has published on a series of ketooxazole FAAH inhibitors where they have incorporated a piperidine into the acyl side-chain resulting in improved solubility. Compound **19** (IC_{50} = 3.6 nM) was efficacious in the spinal nerve ligation (SNL) model of neuropathic pain (76).

Reversible inhibitors, such as the α-ketoheterocycle OL-135, have been found to display good *in vitro* potency and selectivity for FAAH relative to other serine hydrolases in mammalian proteomes (22,71), but produce only transient elevations in AEA *in vivo* (14,22,76). The submaximal efficacy of reversible FAAH inhibitors may be due to their rapid metabolism, as well as the fact that near-complete (>85%) blockade of FAAH activity is required to maintain elevated AEA levels *in vivo* (77). Another challenge with developing reversible inhibitors is that inhibition of FAAH leads to elevated levels of several NAE substrates, which can diminish the efficiency and potency of the inhibitor by mass-action competition with the substrates (78). A key challenge that still remains is to improve the pharmacokinetic drug-like properties of this class of α-ketoheterocycle inhibitors to be able to achieve oral activity with a sufficient *in vivo* half-life such that FAAH inhibition can be maintained over a longer period of time.

5.3 Carbamate inhibitors

The first carbamate inhibitors of FAAH were designed by modifying the structure of a known inhibitor of the serine hydrolase acetylcholinesterase. Carbamate **20** had moderate activity against FAAH (IC_{50} = 324 nM), while the corresponding urea **21** was inactive (Figure 6) (79). The meta-biphenyl derivative **22** showed a 5-fold improvement in potency (IC_{50} = 63 nM) and addition of an amide on the biphenyl resulted in an additional 10-fold boost in potency to give URB597 (**23**) (IC_{50} = 4.6 nM) (80). It has been reported that URB597 at 10 μM was selective against a broad panel of receptors, ion channels, neurotransmitter transporters and enzymes (81).

Recently it was shown that the potency could be enhanced further by replacing the cyclohexyl ring of **23** with β-naphthylmethyl to give URB880 (**24**) (IC_{50} = 0.63 nM) (82). Dialysis experiment with URB597 supported an irreversible mechanism. It was presumed that carbamates inhibit FAAH through a covalent mechanism involving carbamylation of the active site Ser241 nucleophile. Later Cravatt and colleagues provided direct evidence for this and showed that the phenol was acting as the leaving group (83). They also prepared a hybrid of OL-135 and URB597 where the cyclohexyl was replaced with phenhexyl to give JP83 (**25**) (IC_{50} = 7 nM vs 48 nM for URB597 under these assay conditions) (83). This strongly suggested a binding mode where the cyclohexyl moiety of URB597 is located in the acyl chain-binding channel of FAAH. These data facilitated the design of a "clickable" carbamate activity-based probe (JP104, **26**) to profile the proteome reactivity of FAAH-directed carbamates *in vivo* (see section 7.2) (83). A recent computational modeling study also provided a theoretical explanation showing that carbamylation of Ser241 is more favorable in the binding orientation with the cyclohexyl moiety located in the acyl chain-binding channel (84).

Sanofi-Aventis has several patent applications detailing a series of 2-(methylamino)-2-oxoethyl carbamates as FAAH inhibitors. These compounds could be divided into two general classes – aryl/heteroaryl alkyl carbamates as exemplified by SA-47 (**27**) (85) and piperazine/piperidine carboxylates as exemplified by **28** (86). SA-47 (**27**) at 10 μM has been reported to be selective for FAAH by ABPP in multiple rat and human tissues (87). Compounds in the patents are reported to be active in a visceral pain model (PBQ-induced writhing).

Bristol-Myers Squibb has also reported a class of bisarylimidazole phenyl carbamate FAAH inhibitors (88). Compound **29** was the most potent compound in this series (IC_{50} = 2 nM) and showed activity in models of persistent and neuropathic pain (see section 6.2). The corresponding *N*-ethyl carbamate **30** and urea analog were inactive giving evidence that the phenyl carbamate is functioning as an electrophile resulting in covalent modification of FAAH. Carbamate **29** has been reported to be quite promiscuous, displaying multiple off-targets by ABPP in various rat and human tissues (87). Astellas has patented a series of 3-pyridyl carbamates exemplified by **31** which are reported to be very potent FAAH inhibitors (IC_{50} < 1 nM in their assay) (89). It should be noted that comparing IC_{50} values of irreversible inhibitors between different assays are not useful as they show time dependence and are highly dependent on the assay conditions (preincubation time, enzyme concentration, and substrate concentrations, etc.).

Irreversible phenyl carbamate inhibitors, such as the URB597 (**23**) and **29**, inhibit other hydrolases in peripheral tissues, including several carboxylesterases (22,57,83,87). 2-(methylamino)-2-oxoethyl carbamates SA-47 (**27**) appears to be more selective for FAAH than the phenyl carbamates (87), so the design of selective carbamate FAAH inhibitors may be a tractable problem.

5.4 Urea inhibitors

Since 2006, Takeda (90), Johnson & Johnson (91-93), Pfizer (57,58,94) and Astellas (95) have reported piperazine/piperidine aryl ureas as an emerging class of FAAH inhibitors (Figure 7; compounds **32**–**40**). This was initially quite surprising since the direct urea analogs of the carbamates **20** and **29** had previously been found to be completely inactive (79). Takeda patented a series of thiadiazolylpiperazine carboxamides (i.e. **32**) (90) which was followed by a very similar patent from Johnson & Johnson (92). Johnson & Johnson also patented a series of benzyl piperazine ureas represented by compound **33** (91). In 2007, Pfizer and colleagues at The Scripps Research Institute reported that the quinoline piperidine urea PF-750 (**36**) inhibited FAAH in a time-dependent manner (IC_{50} = 52 nM with 30 min preincubation) by covalently modifying the enzyme's active site serine nucleophile (57). PF-750 was confirmed to be covalently attached to the Ser241 of FAAH through a carbamate linkage by the PF-750-h/rFAAH crystal structure as discussed (31). The irreversible covalent inhibition by PF-750 was rather surprising considering the stability of the urea functional group. Despite the covalent mechanism, PF-750 selectively inhibited FAAH relative to other mammalian serine hydrolases as determined by ABPP. A proposed mechanism that could explain this exquisite selectivity is a specific binding-induced activation of the urea in the FAAH active site, which renders the reactivity of urea similar to an amide (57). Given the covalent, irreversible inhibition of FAAH, the potency of irreversible inhibitors were measured by the second order rate constants (k_{inact}/K_i) as k_{inact}/K_i values are the best measure of potencies of irreversible inhibitors (96). Using this measure, **36** was determined to have a moderate potency (k_{inact}/K_i = 791 M^{-1} s^{-1}) for FAAH. More recently a series of biaryl ether urea analogs with improved potency have been reported (58). PF-3845 (**37**) was the most potent inhibitor (k_{inact}/K_i = 14,310 M^{-1} s^{-1}). Structural studies support that PF-3845 gains its potency from a more extended set of van der Waals interactions between the biaryl ether piperidine moiety and the hydrophobic acyl chain-binding pocket of FAAH based on a crystal structure of a PF-3845-h/rFAAH complex (58). In addition, the trifluoromethyl group of **37** was replaced with an alkyne to generate “clickable” ABPP probe **38** that was used to profile the *in vivo* selectivity of this inhibitor (section 7.2). FAAH-2 is a recently identified FAAH homolog that is selectively expressed in higher mammals, but not in rodents (97). In contrast to URB597, which has been found to inhibit human FAAH-2 with potency equivalent to that for FAAH (97), PF-3845 was shown to have negligible activity against this FAAH variant (IC_{50} > 10 μM) (32).

Johnson & Johnson has reported a detailed characterization of **32** (IC_{50} = 33 nM with 20 min preincubation) and has shown that it is also a time-dependent inhibitor of FAAH (93,98). Incubation of **32** with FAAH followed by mass spectrometry analysis indicated carbamylation of the enzyme with loss of aniline. Furthermore, dialysis experiments showed that the covalent adduct can be slowly hydrolyzed with recovery of enzyme activity. Pfizer (e.g., **39**) (94), Astellas (e.g., **40**) (95) and Johnson & Johnson (e.g., **34** and **35**) (99) have reported additional piperazine/piperidine aryl urea FAAH inhibitors in the patent literature in 2008.

Lilly has reported that the "activated" urea **41** is an inhibitor of the putative AEA transporter (100). However it was later shown to be a potent inhibitor of FAAH, as well as several other serine hydrolases (101). It appears that the increased reactivity of the tetrazole carboxamide group in compound **41** results in high target promiscuity in contrast to much higher selectivity of piperazine/piperidine ureas described above. Interestingly, Sanofi-Aventis has recently patented a series of triazolopyridine carboxamides (i.e. **42**) (102). It will be interesting to find out if these compounds possess improved selectivity compared to **41**.

5.5 Boronic acid inhibitors

Recently Infinity has described a series of aryl boronic acids (i.e. **43**) as inhibitors of FAAH in a patent application (Figure 8) (103). It is hypothesized that the boronic acids form a reversible covalent complex with the active site serine nucleophile of FAAH and the aryl ring is directed toward the hydrophobic acyl chain-binding channel. A publication from the University of Kuopio in Finland has also reported a series of boronic acid FAAH inhibitors with alkenyl boronic acid **44** being the most potent (IC_{50} = 14 nM) (Figure 8) (104). Boronic acids have previously been reported as covalent inhibitors of serine proteases (105), so it will be important to assess the selectivity of these compounds against other serine hydrolases.

5.6 Other FAAH inhibitors

There are a few additional types of FAAH inhibitors that are difficult to categorize with the information available to date (Figure 8; compounds **45**–**49**). Microbia has patented several series of substituted indoles related to the NSAID indomethacin that are reported to act as dual COX/FAAH inhibitors. A representative example is indole **45** which is reported to have IC_{50} values of 0.235 and 0.66 μM for FAAH from the rat and human brain, respectively (106). In this patent, all the compounds with IC_{50}'s < 1 μM contain an ester functionality so it will be important to test whether these compounds are acting as substrates. Abbott has recently disclosed a series of benzothiazole sulfonamides with no obvious serine-interacting groups (107). In contrast to the carbamate and urea FAAH inhibitors, **46** (IC_{50} = 18 nM) did not show a time-dependent improvement in potency suggesting that it might be a reversible inhibitor. Compound **46** was selective against other serine hydrolases as revealed by ABPP in rat tissues. Compound **47** was the most potent analog in this series. A recent patent application by Renovis detailed a series of tetrahydro-naphthyridine FAAH inhibitors including compound **48** and **49** (IC_{50} < 100 nM) (108). These structures also do not contain any obvious serine-interacting groups. It still remains to be determined how these compounds inhibit FAAH.

6. *In vivo* efficacy of FAAH inhibitors

There is a rich literature on the endocannabinoid system as a therapeutic target in pathophysiological conditions such as pain and inflammation, central nervous system disorders such as multiple sclerosis, Parkinson's disease, Alzheimers's disease, insomnia, anxiety and depression, as well as hypertension, cancer and inflammatory bowel disease to name just a few (2,4,29). FAAH inhibitors enhance the action of the endocannabinoid AEA and other fatty acid amides indirectly through blocking their metabolism and could serve as potential therapeutic agents for the treatment of diseases where the endocannabinoid activation is beneficial. FAAH

inhibitors may provide a functionally selective way of enhancing endocannabinoid tone only in those tissue and cells with active synthesis and release of endocannabinoids (29). It should be noted that FAAH inhibitors also increase levels of other fatty acid amides including oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) which act on non-cannabinoid receptors and could work to synergize or antagonize the effects of AEA (109). Nonetheless, promising results have been obtained for FAAH inhibitors in preclinical models of acute, inflammatory, and neuropathic pain (14,15,18,22), as well as anxiety (12,19,20), depression (21,110), nausea (111), hypertension (112), pruritus (113), smoking cessation (114) and Parkinson's disease (115) as detailed below. In this section, we will focus on a summary of several FAAH inhibitors which have shown *in vivo* efficacy.

6.1 OL-135

OL-135 (**12**) has demonstrated *in vivo* activity, producing analgesic effects in acute thermal and noxious chemical pain assays in mice (22). OL-135 produced analgesic effects in tail immersion (10 mg/kg, ip), hot plate (10 mg/kg, ip) and phase 1 (ED_{50} = 7.9 mg/kg, ip) and phase 2 (ED_{50} = 7.5 mg/kg, ip) of the formalin test, which were completely reversed by the CB1 antagonist SR141716. These pharmacological effects of OL-135 have been correlated with ~three-fold elevations in brain AEA levels. OL-135 also resulted in a dose-responsive reversal of mechanical allodynia in both mild thermal injury (MTI) and spinal nerve ligation (SNL) models in the rat (ED_{50} = 6−9 mg/kg, ip) (14). It was reported that the analgesic effect of OL-135 (20 mg/kg, ip) in SNL was blocked by the CB2 selective antagonist SR144528, however neither SR141716 or SR144528 reversed the analgesia produced by OL-135 in MTI. These results suggest that FAAH inhibitors may produce analgesia by multiple mechanisms depending on the model used, which could involve the actions of other fatty acid amides such as PEA on noncannabinoid receptors. Researchers at Johnson & Johnson have adapted the MTI model of acute pain for the mouse and showed that OL-135 (10, 30, 100 mg/kg, ip) significantly reversed the tactile allodynia in a dose-dependent manner (116). Johnson & Johnson has also reported that α-ketoheterocycle **19** is efficacious in the SNL model of neuropathic pain (76). The efficacy was maintained over 6 hr when rats were given a bolus dose (4 mg/kg, iv) followed by infusion from an implanted mini pump (6 mg/kg/hr).

More recently, OL-135 has shown activity in the compound 40/80 acute allergenic murine model, which is described in more detail along with URB597 described below (117).

6.2 Carbamates

URB597 (**23**) has been shown to display activity in rodent models of acute (12), inflammatory (18,118), and neuropathic pain (15,119). The first report of the antinoceptive actions of URB597 were in the mouse hot-plate test (12). URB597 (0.5 mg/kg, ip) increased the response latencies and these effects were blocked by the CB1 antagonist rimonabant. URB597 has also been shown to dose-dependently reduce oedema formation (ED_{50} = 0.3 mg/kg, ip) in the carrageenan model of acute inflammation, and these effects were blocked by the CB2 antagonist SR144528 (18). Furthermore, PEA has been shown to reduce the magnitude of paw edema when dosed before carrageenan (120) suggesting that the effects of FAAH inhibitors in the carrageenan-induced paw edema model are at least partially mediated by elevation of PEA.

URB597 (0.3 mg/kg, ip) reduced allodynia and hyperalgesia in the CFA model of inflammatory pain. The effects of URB597 were partially reduced by the CB1 antagonist AM251 or the CB2 antagonist SR144528 and completely reversed by a combination of AM251 and SR144528 (118). URB597 also demonstrated analgesic effects in the phenyl-*p*-quinone (PPQ)-induced writhing pain model in mice (ED_{50} = 22.9 mg/kg, ip), which was blocked by a CB1 antagonist (121). URB597 (ED_{50} = 2.1 mg/kg) produced antinociception in the acetic acid-induced

abdominal stretching model of visceral pain in mice (122). Combinations of URB597 and the nonselective cyclooxygenase (COX) inhibitor diclofenac yielded synergistic analgesic interactions, and URB597-treated mice displayed significant reductions in the severity of gastric irritation caused by diclofenac (122). These data suggest a potential benefit of dual FAAH-COX inhibitors by inhibiting both the hydrolytic degradation and oxidative metabolism of AEA through FAAH and COX, respectively. URB597 (0.3 mg/kg, ip) has also been found to enhance non-opioid, stress-induced analgesia, which was prevented by the CB1 antagonist rimonabant (123).

URB597 (5 mg/kg, ip) dosed once or twice daily over 3 days attenuated trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice as evaluated by macroscopic damage score, myeloperoxidase levels and colon length (124). Thus, FAAH inhibitors might offer therapeutic potential for the treatment of inflammatory bowel diseases (125).

The effects of URB597 on neuropathic pain are complex, as spinal administration of the agent attenuated evoked responses on neurons in neuropathic rats (119), but systemic administration was not able to reduce mechanical allodynia in the rat partial sciatic nerve-ligation model of neurophatic pain (118). On the other hand, oral administration of URB597 (10 mg/kg, po) for 4 days did reduce hyperalgesia in the mouse chronic constriction injury model of neuropathic pain and these effects were prevented by the CB1 antagonist rimonabant (15).

In vivo efficacy in pain models has also been reported for carbamates from Sanofi-Aventis and BMS. Sanofi-Aventis has reported analgesic effects (1−30 mg/kg) in a PBQ-induced writhing mouse model for their patented 2-(amino)-2-oxoethyl carbamates (i.e. **27** and **28** in Figure 6) (85,86). The BMS carbamate **29** (20 mg/kg, iv) demonstrated activity in the formalin test which was inhibited by the CB1 antagonist SR141716A. Also **29** produced significant reversal of mechanical allodynia in the Chung model of neuropathic pain (20 mg/kg, iv) (88).

URB597 (**23**) has been shown to display anxiolytic (12,20,126,127) and antidepressant-like (21,110,128) activities in rodents. Initially, the anxiolytic effects of URB597 (0.1 mg/kg, ip) were shown in the elevated zero-maze test in adult rats and the isolation-induced ultrasonic vocalization emission model in rat pups (12). More recently, FAAH(−/−) mice and URB597-treated mice have also been shown to display anti-depressant phenotypes in the tail suspension test, although substantial methodological changes needed to be made to observe these effects (19). The authors speculated that the effectiveness of FAAH inactivation may depend on the levels of stress associated with the environmental conditions. Consistent with this hypothesis, Goldberg and colleagues have recently reported that URB597 (0.1−0.3 mg/kg, ip) does not affect anxiety under mildly stressful conditions, but produces robust anxiolytic effects when the testing procedure involves aversive conditions (eliminating habituation or employing bright lighting conditions) (129). This suggests that FAAH inhibitors may be effective at counteracting the anxiogenic effects of stressful stimuli. URB597 (1 mg/kg) has showed anxiolytic-like effects in C57BL/6N mice in the elevated plus maze, which was prevented by rimonabant (20). In addition to the experimental environment, the genetic background of the mice may be another important consideration when examining the anxiolytic effects of FAAH inhibitors. Furthermore, URB597 suppressed anxiety-like behavior in the elevated plus maze but did not alter submissive/defensive behavior in the social defeat model in hamsters (130) again highlighting the subtleties with observing anxiolytic effects with FAAH inhibitors. URB597 (0.1 mg/kg, ip) has also been reported to enhance social play behavior in adolescent rats (131,132).

More elaborate experimental protocols have also been conducted with URB597 to assess effects on emotionality. For example, URB597 (0.3 mg/kg, iv) and AEA (0.03−3 mg/kg, iv) were reported to have no motivational effects in place-conditioning experiments, however,

when URB597 (0.3 mg/kg, ip) was administered followed by AEA (0.3 and 3 mg/kg, iv), dose-related anxiogenic effects and conditioned place aversions were observed (127). Daily administration of URB597 (0.3 mg/kg, ip) for 5 weeks corrected the reduction in body weight gain and sucrose intake in rats induced by chronic mild stress, a behavioral model of depression (128). This treatment also resulted in a significant, but incomplete inhibition of brain FAAH activity with a concomitant increase in AEA levels in midbrain, striatum, and thalamus. Furthermore, injection of URB597 (0.01 nmol) aimed at the dorsolateral periaqueductal gray (dlPAG) in rats produced anxiolytic-like effects in the Vogel conflict test, which was prevented by the CB1 antagonist AM251 (133).

Studies with FAAH inhibitors and FAAH(−/−) mice have revealed additional physiological processes that are regulated by the endocannabinoid system. In spontaneously hypertensive rats, URB597 reduced blood pressure, cardiac contractibility, and vascular resistance to levels in normotensive rats, and these effects were blocked by a CB1 antagonist (112). Age-associated declines in cardiac function and changes in inflammatory gene expression, nitrative stress, and apoptosis were also attenuated in FAAH(−/−) mice (134). These results suggest that the pharmacological blockade of FAAH may represent a protective strategy to counter cardiovascular aging and atherosclerosis.

URB597 and OL-135 have been evaluated in the compound 40/80 acute allergenic murine model (113). Mice treated with URB597 and OL-135 displayed comparable reductions in scratching to mice treated with common non-sedative allergenic treatments (loratadine & dexamethasone), but without affecting locomotor behavior. The antiscratching phenotype of FAAH-compromised mice was completely blocked by either genetic deletion or pharmacological antagonism of the CB1 receptor. Furthermore, URB597 reduced compound 48/80-induced scratching in FAAH-NS mice, which have FAAH exclusively restricted to neural tissues, but it did not produce any further reduction in FAAH (−/−) mice indicating that neuronal FAAH suppression reduces the scratching response through activation of CB1 receptors.

Preliminary results in additional preclinical models suggest that FAAH inhibitors may hold promise for the treatment of other therapeutic indications. For instance, URB597 (0.3 mg/kg, ip) has also been shown to suppress conditioned gaping (a model of nausea) elicited by a lithium-paired context in the rat, which was reversed by CB1 antagonists (111). In addition, URB597 rescued indirect-pathway eCB-LTD in dopamine-depleted animals and, when administered (1 mg/kg, ip) together with quinpirole, improved the motor deficits in mice treated with reserpine or 6-OHDA (animal model of Parkinson's disease) (115). Furthermore, URB597 (0.3 mg/kg) reduced nicotine self-administration in rats (114). *In vivo* microdialysis experiment showed that URB597 reduced nicotine-evoked dopamine release, while not altering dopamine levels when administered alone. Another study showed that pretreatment with URB597 (0.1 mg/kg, iv) prevented the nicotine-induced increases in firing rate and burst firing of VTA dopamine neurons (135). Interestingly, administration of OEA and PEA mimicked this effect. URB597 (0.3 mg/kg, ip) was also shown to minimize the retinal damage observed in ischemic–reperfused samples after high intraocular pressure–induced ischemia in rats (136).

Despite producing a provocative number of behavioral effects, OL-135 and URB597 display rather short durations of actions *in vivo* (22,32,77). . Furthermore, variable degrees of selectivity have been reported for α-ketoheterocycle and carbamate FAAH inhibitors when tested against other members of the serine hydrolase family (see Section 7). Therefore, more selective inhibitors are needed with longer duration of action to fully explore the role of the endocannabinoid system in chronic pathological syndromes that may require sustained elevations in endocannabinoid tone.

6.3 Ureas

JNJ-1661010 (**32**, 20 mg/kg, ip) reversed the tactile allodynia in the rat mild thermal injury (MTI) model and in the Chung spinal nerve ligation (SNL) model of neuropathic pain (98). FAAH activity in the brain was inhibited by at least 85% for up to 4 h. Also compound **32** (50 mg/kg, ip) exhibited a significant attenuation of thermal hyperalgeisa in the inflammatory rat carragenan paw model. Full inhibition of FAAH in rat brain was achieved at the 30 mg/kg dose (ip) which resulted in a modest 1.5-fold increase in brain levels of AEA and a 3-fold increase in PEA and OEA compared to vehicle levels. The analgesic activity of **32** was blocked by naloxone in both the MTI and the SNL models, suggesting a downstream opioid component in these models (119). Furthermore, the CB2 antagonist SR144528 almost completely reversed the analgesia in the SNL model.

PF-3845 (**37**) was efficacious in a complete Freund's adjuvant (CFA) rat model of inflammatory pain when orally dosed (32). PF-3845 (3 − 30 mg/kg, po) caused a dose-dependent inhibition of mechanical allodynia with a minimum effective dose of 3 mg/kg. At higher doses (10 and 30 mg/kg), PF-3845 inhibited pain responses to an equivalent, if not greater degree than the nonsteroidal anti-inflammatory drug naproxen (10 mg/kg, po). Robust, near-complete inhibition of FAAH activity with concomitant elevations in AEA and other NAEs were observed in brain, peripheral blood leukocytes/plasma, and liver from PF-3845-treated animals (1−30 mg/kg). Interestingly, a slightly left-shifted dose-response profile for measurements of FAAH activity and NAEs was observed compared to efficacy in the CFA model. These data may indicate the need to fully block FAAH (and fully elevate AEA levels) in order to observe anti-allodynic effects in the CFA model. The maximal elevation of AEA in PF-3845-treated rats was approximately 10-fold in brain and plasma. Specific antagonists for central CB1 (SR141716) and peripheral CB2 (SR144528) receptors each partially, but significantly reduced the anti-allodynia activity of PF-3845. Notably, dual treatment with SR141716 and SR144528 produced a near-complete ablation of the efficacy of PF-3845 in the CFA model. These data indicate that PF-3845 blocks inflammatory pain responses in the CFA model by a cannabinoid receptor-dependent mechanism that likely involves both CB1 and CB2 receptors, as has been shown for other FAAH inhibitors (14,118). Mice treated with PF-3845 (10 mg/kg, ip) also showed dramatic (>10-fold) elevations in brain levels of AEA, PEA, and OEA, while no changes were observed in 2-AG levels in brain or liver (32), consistent with findings indicating that distinct enzymes regulate 2-AG *in vivo* (12,137). PF-3845-induced elevations of AEA, PEA, and PEA in brain were long lasting as they peaked at ~3 hr and were maintained at maximal levels for up to 7−12 hr. Even after 24 hr posttreatment with PF-3845, significant elevations of NAEs in brain were observed (32).

7. Assessment of global selectivity of FAAH inhibitors by ABPP

Selectivity assessment is critical in order to determine whether or not FAAH inhibitors could serve as valuable pharmacological tools and potential drugs. FAAH belongs to the serine hydrolase superfamily of enzymes, of which there are more than 200 members in human. Determining the selectivity of FAAH inhibitors by conventional substrate-based assays for these individual enzymes would be a daunting task. In addition, a large portion of these enzymes have not yet been characterized and therefore lack cognate substrate assays. To overcome these limitations, a functional proteomic screen based on ABPP (138,139) has been employed to evaluate the selectivity of FAAH inhibitors *in vitro* as well as *in vivo*.

Competitive ABPP for serine hydrolases involves the coordinated application of a candidate inhibitor and a reporter-tagged fluorophosphonate (65), which serves as a general ABPP probe for the serine hydrolase superfamily (67,68) (Figure 9). Serine hydrolases that show significant reductions in probe labeling intensity in the presence of inhibitor are scored as targets of the compound. In this way, competitive ABPP provides a global view of the proteome-wide

selectivity of serine hydrolase inhibitors. Importantly, competitive ABPP has been successfully employed for the assessment of selectivity of both reversible and irreversible FAAH inhibitors (22,57,65,69,71,83,87,107).

7.1 *In vitro* selectivity

The *in vitro* selectivity of FAAH inhibitors has been tested by competitive ABPP in multiple rat, mouse, and human tissues. Most inhibitors have displayed selectivity for FAAH in brain tissue, but possess additional targets in peripheral tissues such as liver and kidney. The widely used FAAH inhibitor URB597 falls into this category, showing specificity for FAAH in brain, but inhibiting multiple serine hydrolases with near-equivalent potency in peripheral tissues (57). Similarly, BMS1 (1−10 μM), OL-135 (100 μM), and CAY-10402 (10−100 μM) were all shown to inhibit multiple serine hydrolases in peripheral tissues, including FAAH and several members of the carboxyesterase clan (22,57,65,69,71,87,107). These carboxylesterase targets were especially sensitive to the aryloxy carbamates URB597 and BMS1. In contrast, the alkoxy carbamates SA-47 and SA-72 (87), OL-135 (22) and benzothiazole-based compound **46** (107), showed much higher selectivity for FAAH, showing no off targets in brain and peripheral tissues at 10 μM The selectivity of the piperidine/piperazine ureas represented by PF-750 and PF-3845 were also assessed by competitive ABPP in multiple human and mouse tissue proteomes (57,58). The results indicated that PF-750 and PF-3845 are remarkably selective FAAH inhibitors, showing no discernible activity against other serine hydrolases at concentrations up to 500 μM and 100 μM, respectively.

7.2. *In vivo* selectivity

In order for FAAH inhibitors to serve as highly selective pharmacological tools or potential drug candidates, their selectivity in the native system (*in vivo*) becomes critical. Although competitive ABPP can be applied to assess the targets of irreversible inhibitors *in vivo*, by treating animals with inhibitors and performing ex vivo competitive ABPP studies in excised tissue extracts (57,58,83,137), this method will only reveal inhibited proteins within the serine hydrolase family. To determine whether FAAH inhibitors, might covalently modify proteins outside of the serine hydrolase class *in vivo*, a click chemistry (CC) version of ABPP was employed (58,83). Alkyne analogues of URB597 (**23**) and PF-3845 (**37**), JP104 (**26**) and PF3845yne (**38**), respectively, maintained their potency for FAAH and served as useful "clickable" ABPP probes (Figure 10). After the treatment of mice with alkyne probes at 10 mg/kg (ip), brain and liver proteomes were isolated and reacted with an azide-rhodamine tag under CC conditions to generate the corresponding triazole products, and the individual targets were visualized by in-gel fluorescence. Consistent with the results of previous competitive ABPP experiments, both PF3845yne and JP104 selectively reacted with a single brain protein, which was confirmed to be FAAH based on its absent from FAAH(−/−) mice. In liver, however, PF3845yne and JP104 showed strikingly different profiles, with the former agent once again showing selective reactivity with FAAH and the latter inhibitor labeling a number of proteins. These data indicate that PF-3845 inhibits FAAH *in vivo* with exceptional efficacy and selectivity (32).

8. Expert Opinion

There has been tremendous progress in our understanding of FAAH and development of FAAH inhibitors. Since the cloning and molecular identification of FAAH in 1996 (5), the availability of FAAH(−/−) mice and FAAH inhibitors with reasonable potency and selectivity contributed to this rapid progress by allowing the confirmation of its prominent roles in the termination of AEA signaling *in vivo*.

The application of ABPP for assessing the selectivity of FAAH inhibitors both *in vitro* and *in vivo* has played a significant role in the advancement of FAAH inhibitor development as potential drug candidates. This technology permits evaluation of FAAH inhibitor selectivity directly against serine hydrolases in human tissues from early to late stages of a drug discovery program. This global view of selectivity assessment against an entire class of enzymes will likely continue to play a key role in the development of inhibitors and drug candidates well beyond FAAH in the future. Of course, ABPP is not without limitations. First, off-targets identified *in vitro* may not be inhibited by a compound *in vivo*. Second, certain hydrolases may prove too low in abundance to detect in native proteomes (64). Third, a certain level of off-target activity may prove tolerable for *in vivo* pharmacology and even drug discovery applications. This is especially important to consider for poorly characterized enzymes, such as carboxylesterases, whose endogenous substrates are not well-understood (81).

Structural biology has become an integral part of modern drug design programs. A major hurdle to the routine implementation of structure-based drug design is provided by challenges that are commonly encountered with the expression of human proteins. Integral membrane proteins such as FAAH can be particularly troublesome. An alternative approach has been employed for FAAH, where the active site of the highly expressed rat enzyme was mutagenically converted to the human enzyme. This h/rFAAH protein is providing penetrating insights into the structures of FAAH-inhibitor complexes (31,58), which should help drive future drug design decisions.

The genetic and pharmacological inactivation of FAAH leads to the elevation of endogenous levels of fatty acid amides and produces analgesic, anxiolytic, antidepressant, sleep-inducing, and anti-inflammatory phenotypes. Importantly, these behavioral phenotypes occur in the absence of alterations in motility, weight gain, cognition, or body temperature that are typically associated with direct CB1 agonists. Therefore, FAAH inhibition offers an attractive strategy to induce the beneficial properties of CB1 receptor activation without undesirable side effects that are observed with direct receptor agonists. In addition, chronic inhibition of FAAH does not appear to cause deleterious effects, as FAAH(−/−) mice have been shown to be viable and fertile and largely indistinguishable from their wild type littermates. The identification of a human FAAH polymorphism linked to problem drug use and obesity (140-142) raises a potential concern for FAAH inhibitors. However, URB597 has been shown to have no effect in two rat models of abuse liability, conditioned place preference test and the drug discrimination test (21), and has displayed no reinforcing properties in monkeys (143). Recent studies have also questioned the significance of the association of human FAAH polymorphisms to obesity (144). Other potentially undesirable effects of FAAH inactivation include higher alcohol consumption shown in FAAH(−/−) mice (145,146) and a potential role for FAAH and AEA in reproduction (147-149). The latter concern is something that should be monitored in future studies. Although FAAH(−/−) mice are fertile, they have been found to exhibit defects in litter size, especially in breeding where both males and females lack the FAAH enzyme (150).

In summary, it is evident that FAAH represents a novel therapeutic target with potential to impact various diseases with significant unmet medical needs. Despite some potential risks associated with FAAH inactivation discussed above, clinical results of FAAH inhibitors for the potential treatment of human diseases are awaited with great anticipation. It is exciting to see that two FAAH inhibitors, SSR-411298 and PF-04457845, are currently under clinical development for the treatment of major depressive disorder and osteoarthritis pain, respectively (see www.clinicaltrials.gov).

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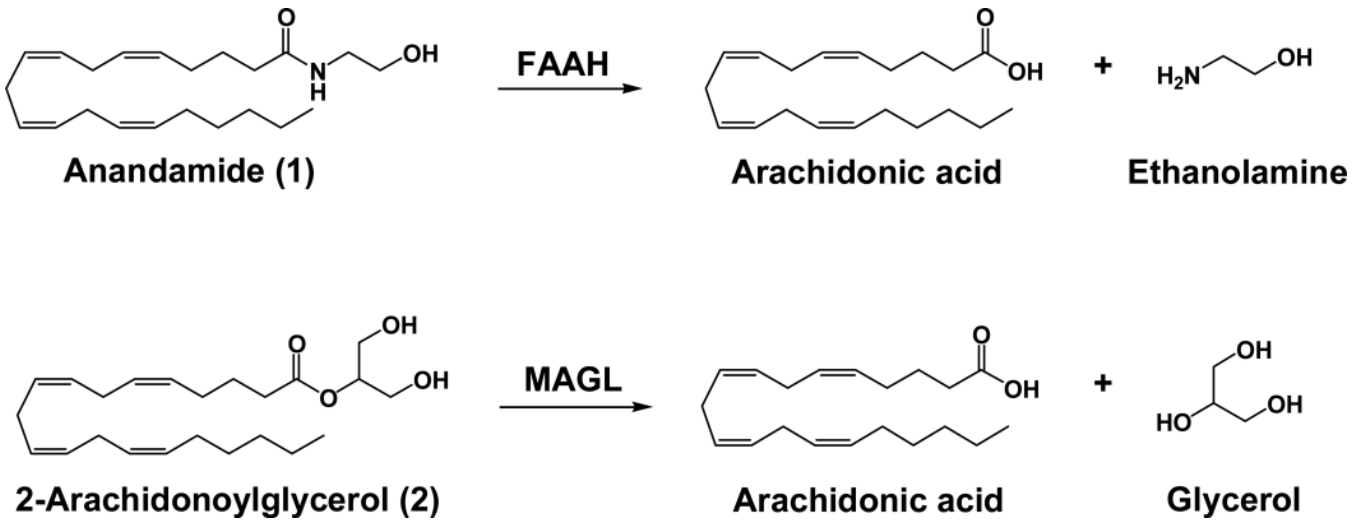


Figure 1.

Structures of two principal endocannabinoids, *N*-arachidonoyl ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). AEA is hydrolyzed to arachidonic acid and ethanolamine by FAAH. MAGL is a principal enzyme that hydrolyzes 2-AG to arachidonic acid and glycerol.

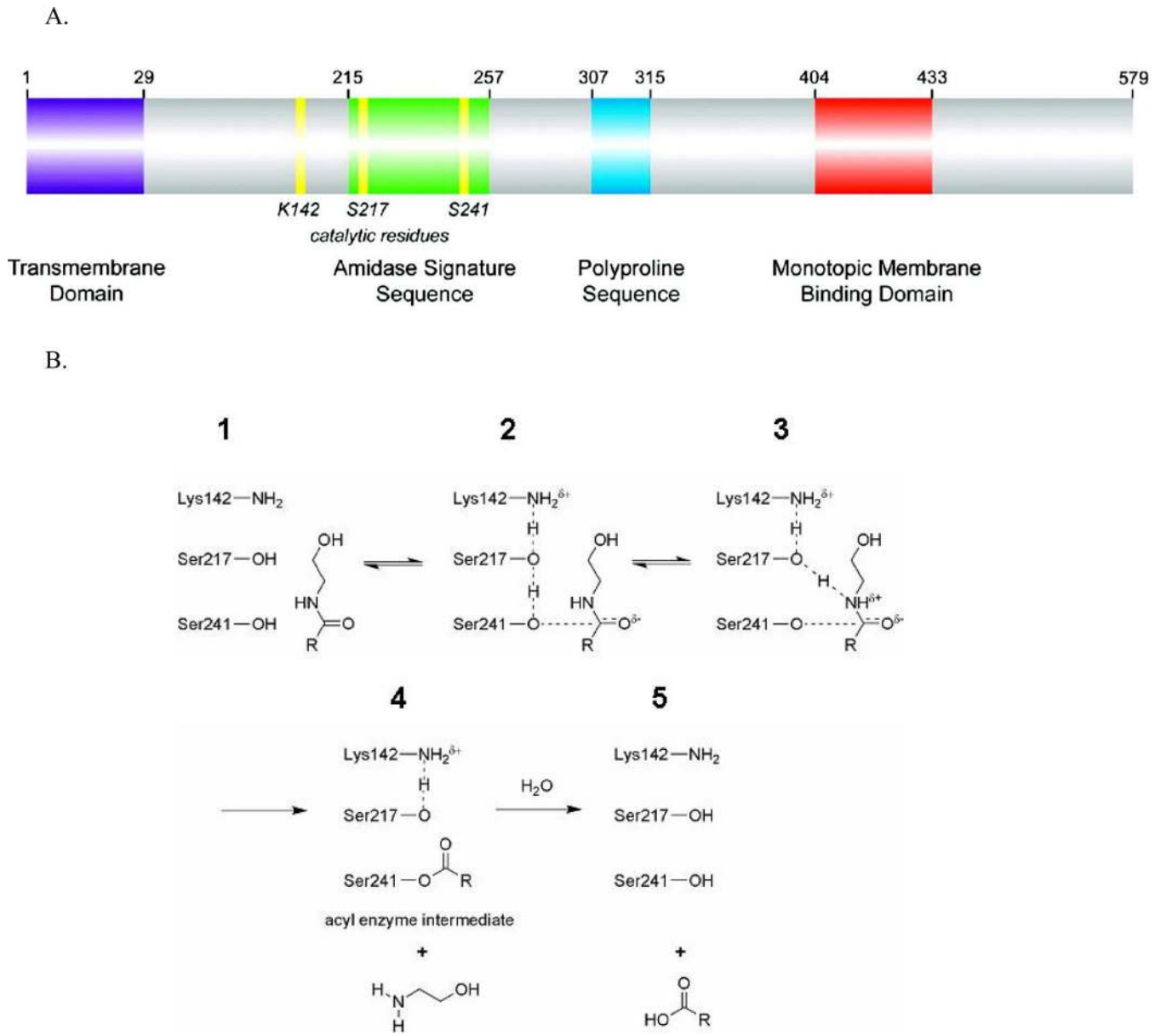
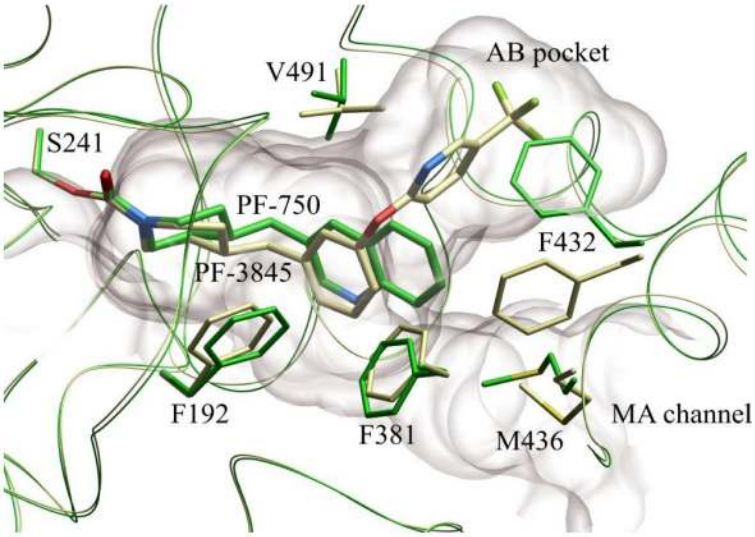


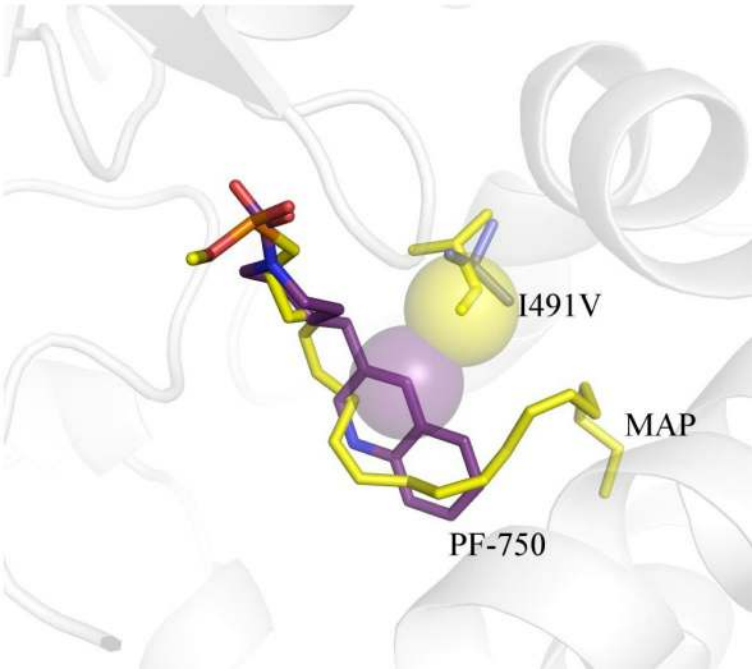
Figure 2.

(A) Structural domains of FAAH. Primary sequence analysis reveals a predicted NH_2-terminal transmembrane domain (purple), an amidase signature sequence rich in glycine and serine residues (green), a polyproline sequence predicted to interact with Homer and SH3 domain-containing proteins (blue), and a monotopic membrane binding domain that enables FAAH to bind the membrane (red). (B) Hydrolytic mechanism of amide and ester substrates involving catalytic triad of FAAH (shown for amides). (1) Lys142, initially in a deprotonated state, (2). abstracts a proton from Ser217, which in turn abstracts a proton from the Ser241 nucleophile. (3). Attack of the nucleophile on the substrate carbonyl is proposed to occur in a coupled manner with proton donation from Ser217 to the nitrogen atom of the amide substrate. This latter step requires the concurrent donation of a proton from Lys142 to Ser217, resulting in (4) the formation of an acyl−enzyme intermediate where both Lys142 and Ser217 have returned to their initial protonation states. (5) Deacylation results in release of the free fatty acid product.

A.



B.



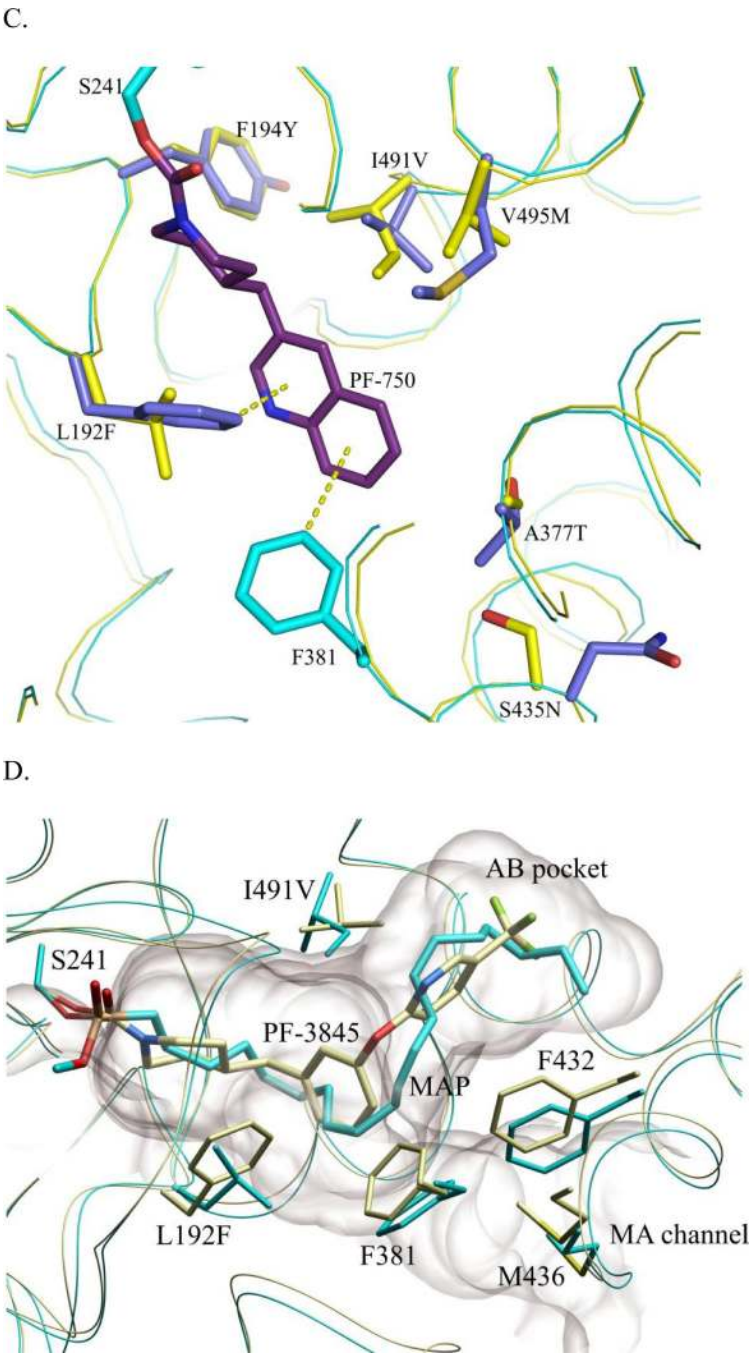


Figure 3.

Crystal structure of PF-750-h/rFAAH and PF-3845-h/rFAAH complexes. (A) Overlap of the crystal structures of the PF-3845 (gray) and PF-750 (green) complexes with h/rFAAH, showing the S241-carbamylated adduct and the different modes of binding that lead to distinct conformations for the F432 residue that toggles between the membrane access (MA) channel (F432 in gray) and AB pocket (F432 in green). (B) Overlap of the crystal structures of PF-750-h/rFAAH and MAP-rFAAH complexes. The MAP adduct (yellow) is shown to indicate its different arrangement compared to the PF-750 adduct (purple and blue). Potential steric hindrance between I491 (yellow sticks) from the rFAAH structure (PDB code 1MT5) and the inhibitor PF-750 (purple and blue) from the h/rFAAH structure. The residue V491 from the h/

rFAAH structure is shown in violet sticks. The spheres indicate van der Waal's radii of carbon in position 4 (purple) and distal carbon of the I491 side chain (yellow). (C) Structual analysis of PF-750 bound to FAAH. The weak H-bonds beween F192 and F381 and the π-ring of the quinoline moiety are shown as yellow dashed lines. The following moieties are shown in stick representation: :rFAAH residues (yellow), PF-750 (purple), h/rFAAH residues (violet), and conserved (cyan) residues. (D) Overlap of crystal structures of PF-3845-h/rFAAH and MAP-rFAAH complexes, showing similar binding modes for PF-3845 (gray) and MAP (blue).

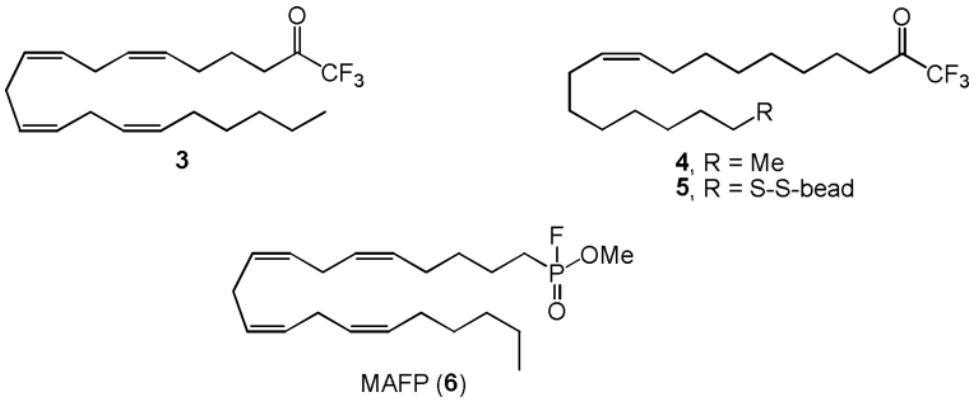


Figure 4.
Substrate-derived FAAH inhibitors.

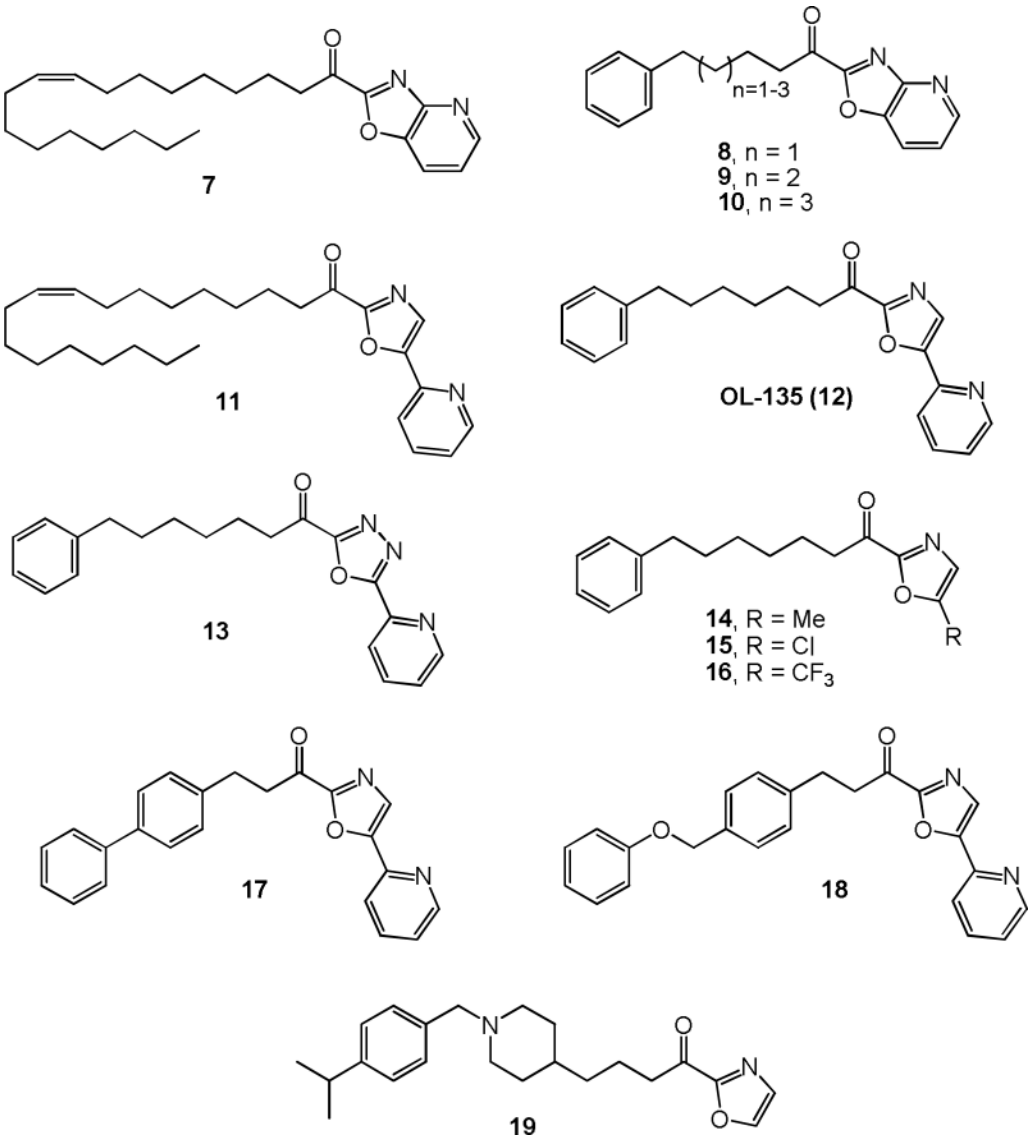


Figure 5.
α-Ketoheterocycle FAAH inhibitors.

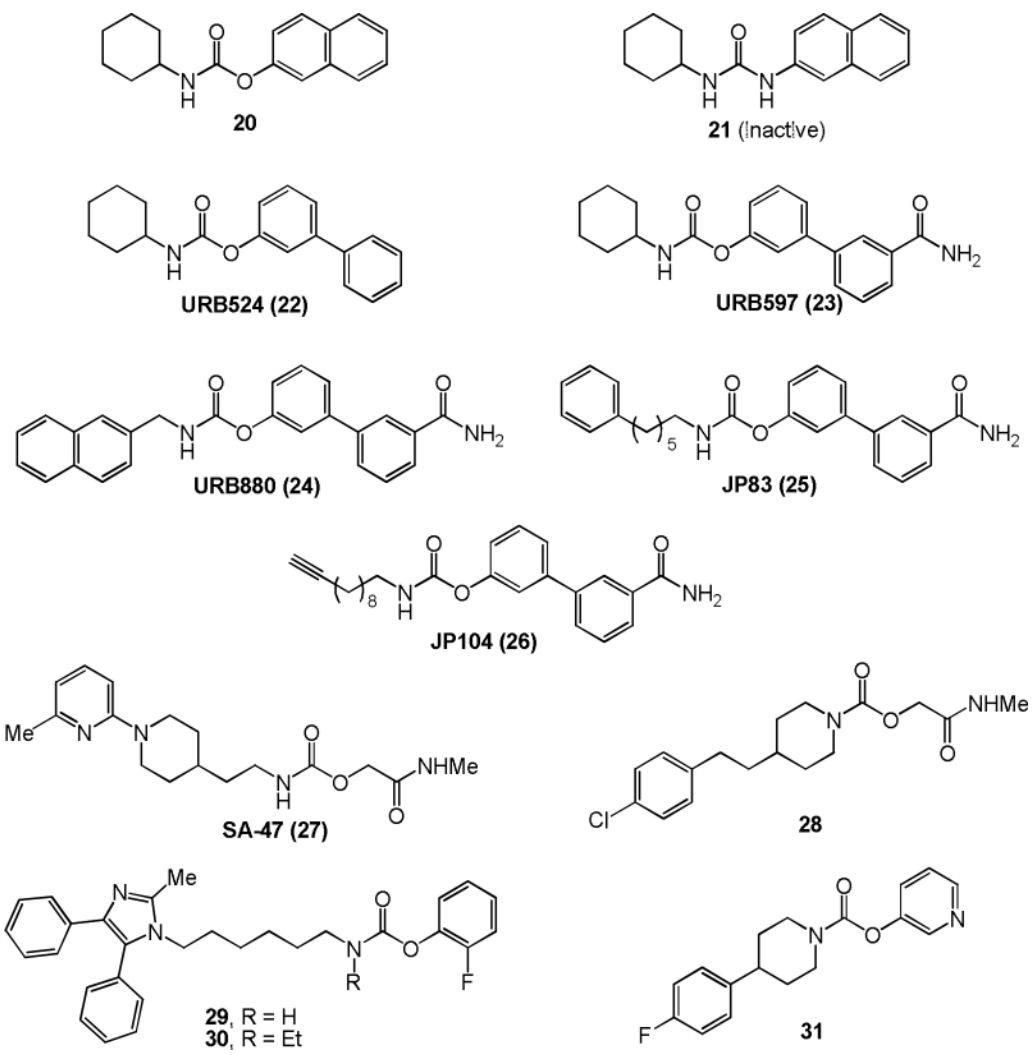


Figure 6.
Carbamate FAAH inhibitors.

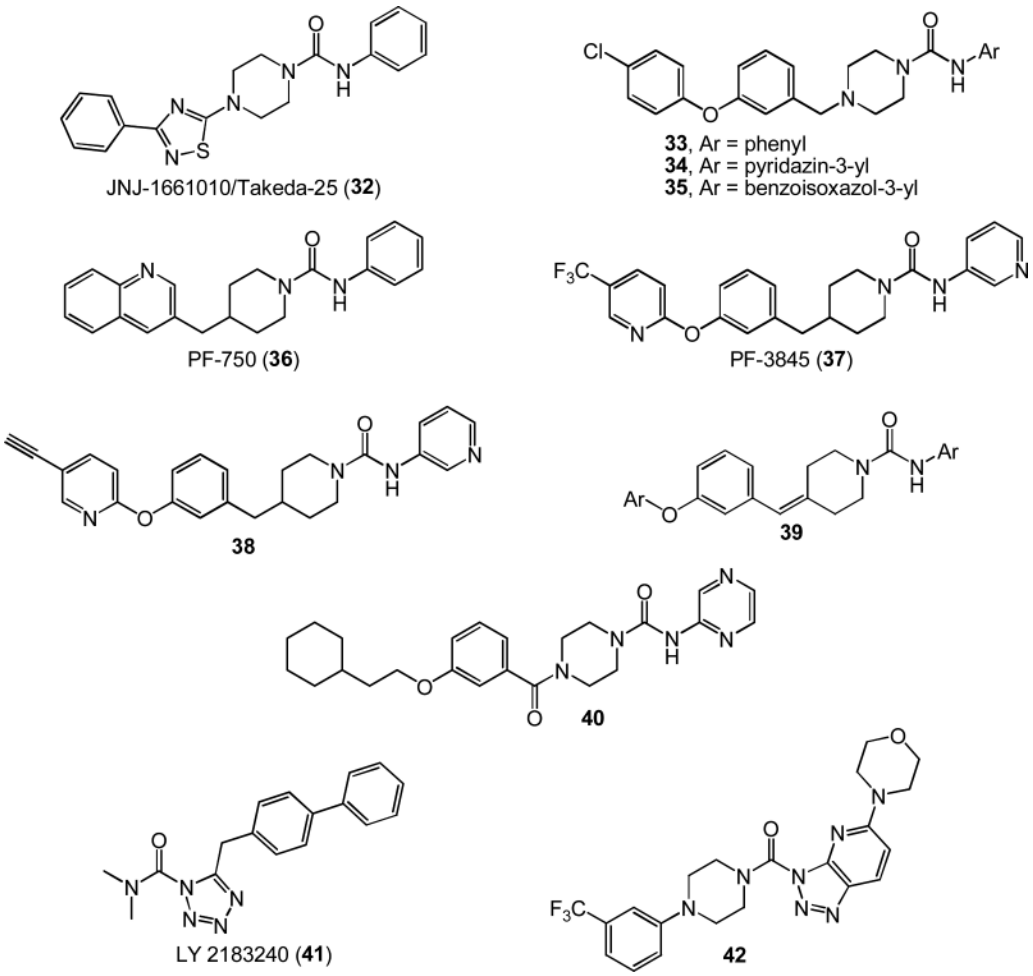


Figure 7.
Urea FAAH inhibitors

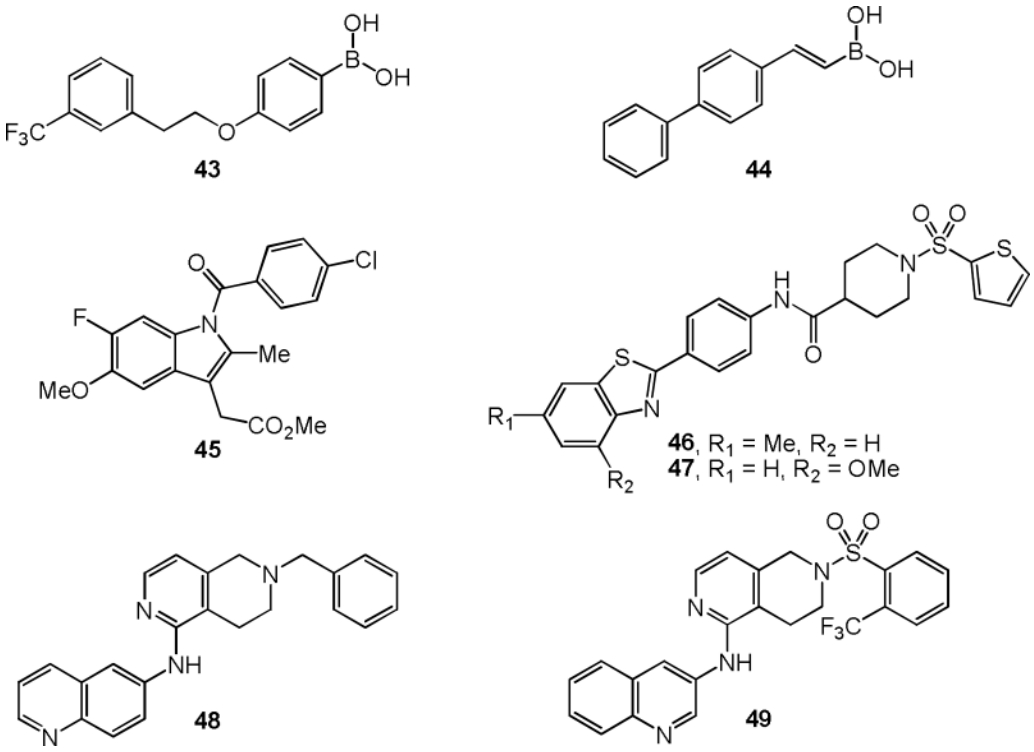


Figure 8.
Additional FAAH inhibitors.

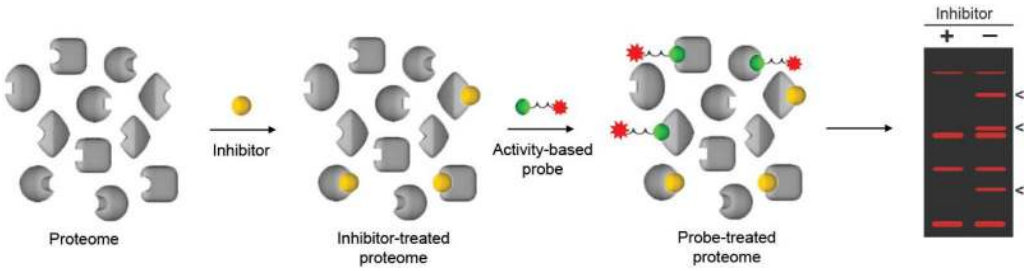


Figure 9.

Competitive activity-based protein profiling (ABPP). To determine the selectivity of an inhibitor against serine hydrolases, a proteome is reacted with inhibitor and subsequently labeled with a rhodamine-tagged fluorophosphonate. Reacted proteomes are then analyzed by 1-D SDS-PAGE. A decrease in fluorescent intensity of the probe in the presence of inhibitor indicates a target.

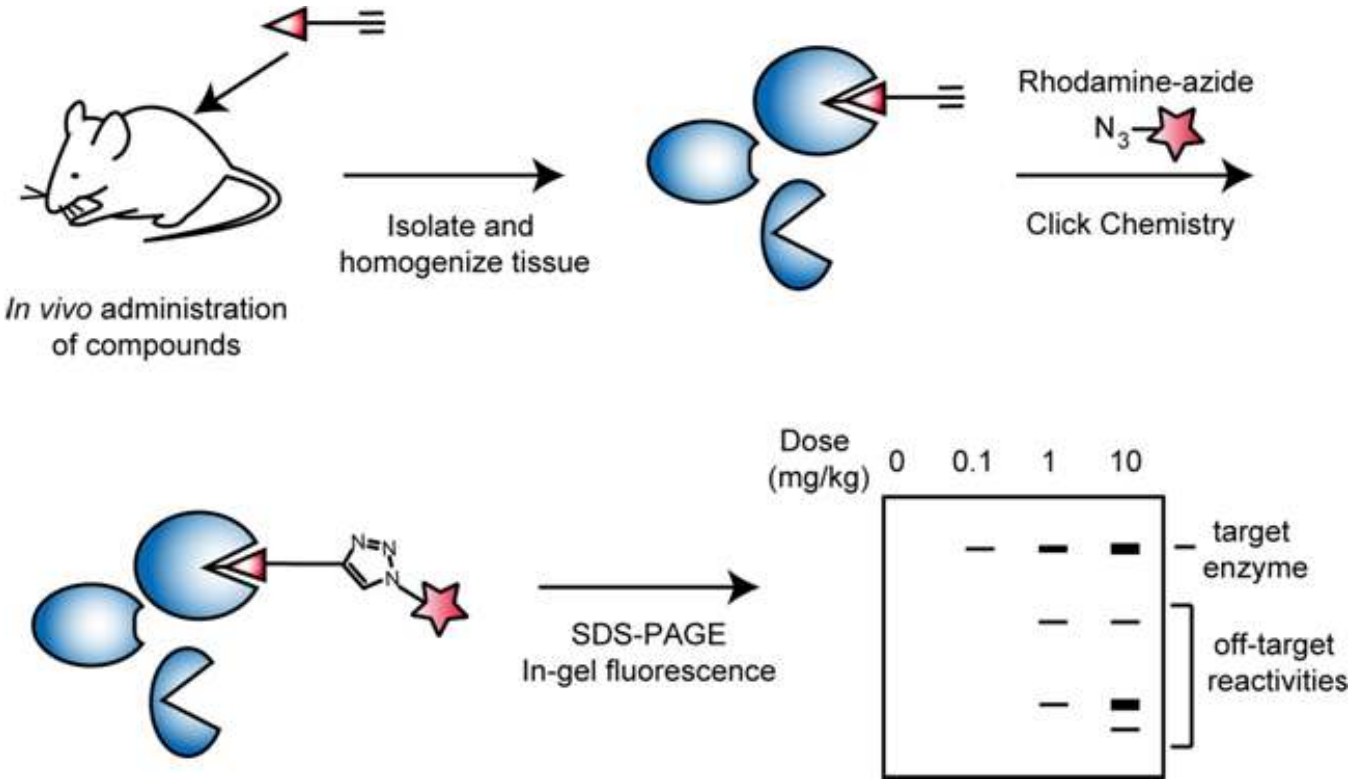


Figure 10.

Direct analysis of *in vivo* protein targets by CC-ABPP. Covalent inhibitors that were converted into activity-based probes via addition of a bio-othorgonal 'handle' (alkyne group) are dosed to animals and given time to react with protein targets. Probe-labeled proteins are then captured and identified from proteomes (isolated tissues) using bio-orthogonal chemistry and LC-MS–based proteomic methods, respectively. Using this approach, off-target reactivity at 1 and 10 mg/kg is shown.