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The Discovery and Development of Inhibitors of Fatty Acid Amide Hydrolase (FAAH)

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Abstract

A summary of the discovery and advancement of inhibitors of fatty acid amide hydrolase (FAAH) is presented.

Introduction

The discovery and characterization of fatty acid amides,[1] including anandamide[2] and oleamide,[3] as a fundamental class of endogenous signaling molecules led to the identification of the enzyme fatty acid amide hydrolase (FAAH).[4] The enzyme serves as the major metabolic regulator of many of the endogenous fatty acid amides, exhibiting a distribution[5] consistent with its role in regulating (terminating) their effects at their released sites of action. Although FAAH acts on a wide range of fatty acid amides or esters,[4,6] it preferentially hydrolyzes arachidonoyl and oleoyl substrates (arachidonoyl > oleoyl, 3-fold) and primary amides are hydrolyzed 2-fold faster than ethanolamides.[7] Although FAAH is a member of the amidase signature family of serine hydrolases, it is the only well-characterized mammalian enzyme bearing their unusual Ser–Ser–Lys catalytic triad. Its catalytic mechanism involves the formation of a tetrahedral intermediate, derived from nucleophilic attack of the catalytic Ser241 on the carbonyl group of the substrate. The tetrahedral intermediate collapses to release the amine and the enzyme-bound acyl intermediate. Lys142 acts as a general base-general acid, mediating the deprotonation of the Ser241 and subsequent protonation of the leaving group that are shuttled through Ser217. The reaction terminates with a water-mediated deacylation of the enzyme-bound acyl intermediate and release of the free fatty acid with restoration of the active enzyme.[8] In addition to possessing an atypical catalytic core, integrally membrane-bound FAAH bears a series of channels and cavities that are involved in substrate or inhibitor binding. These include a membrane access channel (MAC) that connects the active site to an opening located at the membrane anchoring face of the enzyme, a cytosolic port that may allow for the exit of hydrophilic products from the active site to the cytosol, and an acyl chain-binding pocket (ABP), which interacts with the acyl chain during the catalytic reaction.[9]

A series of seminal studies summarized in recent reviews[10] have detailed the discovery of FAAH and defined its potential to serve as a new therapeutic target for the treatment of a range of clinical disorders including pain, inflammation, and sleep disorders. Herein, we summarize the present state of the discovery and development of FAAH inhibitors, many of

which have been used to validate the therapeutic opportunities for the enzyme target, enroute to their potential clinical introduction.[11] As an attractive alternative to developing agonists of GPCR's at which some of the endogenous fatty acid amides are now known to signal (e.g., cannabinoid receptors for anandamide), the development of FAAH inhibitors that raise their endogenous levels and sustain their duration of action by blocking their hydrolysis, has emerged as an approach that may avoid the undesired side effects of a conventional cell surface receptor agonist. Since FAAH inhibition only potentiates an activated signaling pathway, increasing the endogenous levels of the released lipid signaling molecules at their sites of action, it provides a temporal and spatial pharmacological control not available to a classical blunt force receptor agonist.

Reversible FAAH Inhibitors

A major class of reversible fatty acid amide hydrolase (FAAH) inhibitors is the α-ketoheterocycle-based inhibitors that bind to FAAH by reversible hemiketal formation with an active site serine. Many of these reversible competitive inhibitors have been shown to be potent and selective for FAAH versus other mammalian serine hydrolases, and members of this class have been shown to be efficacious in preclinical animal models of pain. Additional classes of reversible FAAH inhibitors have been reported including substituted (thio)hydantoins and imidazolidinediones, oxime and enol carbamates, benzothiazoles and benzoxazoles, arylboronic acids, selected sulfonamides, and cyclic ureas and lactams.

Early Inhibitors: Activated Carbonyl Inhibitors

Early studies following the initial characterization of FAAH led to the discovery that the endogenous sleep-inducing molecule 2-octyl α-bromoacetoacetate[12] is a potent, reversible inhibitor of FAAH (K_i = 0.8 μM).[13] In addition to suggesting that 2-octyl α-bromoacetoacetate may serve as a potential endogenous regulator of FAAH, this unusual inhibitor may embody Nature's design of an electrophilic carbonyl capable of reversible serine hydrolase inhibition (Figure 1). A series of related analogues of this inhibitor were prepared and examined, including oleoyl derivatives, and improvements in potency were achieved.[13]

Similarly, the first series of reversible competitive FAAH inhibitors reported possessed an electrophilic carbonyl within substrate inspired, oleoyl-based inhibitors, including aldehydes, α-ketoamides, α-ketoesters, and trifluoromethyl ketones.[14] The relative potency of the inhibitors followed the expected trends of the electrophilic carbonyls, culminating with the α-ketoesters and trifluoromethyl ketones, and the profile of active/inactive designs established FAAH as a serine (vs cysteine or metallo) hydrolase (Figure 2). The oleyl trifluoromethyl ketone disclosed in these studies was also immobilized through a disulfide bond to Sepharose beads and used to purify the rat enzyme by affinity chromatography, permitting its characterization, sequencing, cloning and expression.[4a] An analogous series of trifluoromethyl ketone, α-ketoester, and α-keto ethanolamide derivatives of arachidonic acid and simpler fatty acids was also independently examined for inhibition of anandamide hydrolase before the two enzymes (oleamide hydrolase and anandamide hydrolase) were recognized as being same (FAAH).[15]

Prior to the availability of the enzyme X-ray structure, a more extensive series of trifluoromethyl ketones was examined to define structural and conformational properties that contribute to enzyme binding and inhibition.[16] Variation of the nature of the lipophilic group had significant effects on the enzyme inhibition and the inhibitors exhibited a parabolic relationship with regard to the alkyl chain length. The incorporation of the oleamide cis double bond increased the potency of the inhibitors, and its replacement with a trans double bond or its removal resulted in incremental reductions in potency. The

synthesis of the corresponding inactive methyl ketone (K_i > 100 μM) illustrated the importance of the electrophilicity of the carbonyl for enzyme inhibition. The identification of the phenhexyl group as a simplified alternative to long chain fatty acid acyl groups was an important finding disclosed in these early studies and was used extensively in later inhibitor designs (Figure 3). Activity based protein profiling (ABPP), a powerful technique developed to profile such reversible enzyme inhibitors directly in complex proteomes,[17] demonstrated that trifluoromethyl ketones exhibit a low selectivity for FAAH and displayed more potent activity against two other serine hydrolases (TGH and KIAA1363).[18]

α-Ketoheterocycle Inhibitors

The replacement of the oleyl trifluoromethyl ketone by α-ketoheterocycles led to the discovery of the first highly potent and selective reversible FAAH inhibitors.[19] A range of 5- and 6-membered monocyclic and bicyclic heterocycles were incorporated into the oleyl α-ketoheterocycles. In this extensive and systematic study, oxazoles were found to be more potent than thiazoles, imidazoles and the corresponding oxazolines. Benzoxazoles were more modest FAAH inhibitors; however, conversion to the oxazolopyridines bearing a fused pyridine afforded a significant increase in potency (>100-fold increase). This increase in potency was relatively insensitive to the position of the pyridyl nitrogen although the N4 incorporation provided the most potent inhibitor (Figure 4). These results reflect principally the enhanced electron-withdrawing properties of oxazolopyridines versus benzoxazoles that increase the electrophilic character of the reactive carbonyl. However, it is also now known that the second basic nitrogen of the fused pyridines also likely participates in a key stabilizing hydrogen bond to a cytosolic port ordered water molecule engaged in an intricate hydrogen-bond array with the active site catalytic residues that further enhance their affinity. Moreover, it is also now recognized that the activating heterocycles themselves do not hydrogen bond to active site catalytic residues through their basic nitrogen allowing them to flip 90º in order to present any of isomeric pyridyl nitrogens with the opportunity to engage this cytosolic port water hydrogen bond.

Inhibitors containing the cis double bond of the oleoyl side chain were more potent than the trans isomers that were in turn more potent than inhibitors where the double bond was removed. Systematic variation in the fatty acid saturated side chain of the oxazolopyridine α-ketoheterocycles showed the greatest potency with straight chain lengths of C12–C8 and exhibited a now characteristic parabolic relationship with chain length (Figure 5). Incorporating unsaturation into the fatty acid chain increased inhibitor potency and the incorporation of a benzene ring provided inhibitors with subnanomolar K_i's (e.g., 200 pM). The chain length linking a phenyl group and the oxazolopyridine exhibited an optimal length indicating that introduction of π-unsaturation at the position corresponding to the oleyl double bond was beneficial. Despite these advances and although not extensively examined, the oleoyl-based oxazolopyridines failed to demonstrate activity (analgesia) in vivo. A set of the α-hydroxy precursors to the initial inhibitors were examined and proved inactive as FAAH inhibitors reflecting the requirement of the electrophilic carbonyl for potent enzyme inhibition.

A further optimization of the parent oxazole-based inhibitors, simultaneously optimizing potency and proteome-wide FAAH selectivity using the newly developed ABPP technology, profiled the impact of the C2 acyl chain length separating the phenyl ring and the oxazole and disclosed the incorporation of a 1,3,4-oxadiazole versus oxazole activating heterocycle.[20] This represented the first implementation of a proteome-wide selectivity screen conducted alongside traditional efforts to optimize enzyme (FAAH) inhibition potency and led to the expedited discovery of a class of exceptionally potent (K_i < 300 pM) and unusually selective (>100-fold selective) FAAH inhibitors that lacked the off target activity against TGH and KIAA1363. The iterative inhibitor design and evaluation with

assistance of the selectivity screen served to differentiate otherwise indistinguishable inhibitors permitting the simultaneous optimization of potency and selectivity. Significantly, the simultaneous assessment of all potential competitive enzymes with the selectivity screen does not require the use of expressed or purified enzymes or a competitive substrate, no modification of the inhibitors is required, and the relative potency for competitive enzymes can be quantified (IC_{50}'s) including those that lack known substrates or function.[18,20–22] An examination[21,27] of oxazole C4 and C5 substituents revealed unique enhancements in potency and selectivity with incorporation of a 2-pyridyl group. Again, the position of the nitrogen did not play a significant role in binding, but its removal did. Significantly, the activity of such C5 substituted α-ketooxazoles paralleled their hydrogen bond acceptor properties (Figure 6).

The combination of one of the most active oxazole C5 substituents with the phenhexyl C2 acyl group gave the most widely recognized lead α-ketoheterocycle compound disclosed to date (OL-135, Figure 6).[21] The extensive SAR that accompanied the initial disclosure of OL-135[21,22] benefited from the routine implementation of the proteomics-wide selectivity screen against the serine hydrolase superfamily ensuring selectivity for FAAH coupled with systematic in vivo examinations of the candidate inhibitors. OL-135 is a potent (K_i = 4.7 nM), reversible, competitive, and selective (>60–300 fold)[18] FAAH inhibitor that produces analgesia and increases endogenous anandamide levels in vivo.[22] It exhibits antinociceptive and anti-inflammatory activity in a range of preclinical animal models that include the tail flick, hot plate assay, formalin test of noxious chemical pain (1st and 2nd phase), the mild thermal injury (MIT) model of peripheral pain, the spinal nerve ligation (SNL) and chronic constriction injury (CCI) models of neuropathic pain,[22] and inflammatory models of pruritus[23] and LPS-induced allodynia[24] with efficacies that match or exceed those of morphine (1–3 mg/kg in MTI/SNL), ibuprofen (100 mg in MTI), or gabapentin (500 mg/kg in SNL) and at doses (10–20 mg/kg, i.p.) that approach or are below those of such common pain or anti-inflammatory medications. The compound lacks significant offsite target activity (Cerep assay profiling), does not bind cannabinoid (CB1 or CB2) or vanilloid (TRP) receptors, and does not significantly inhibit common P450 metabolism enzymes or hERG. Importantly, OL-135 did not produce antinociception in FAAH knockout mice establishing that FAAH is the only relevant target responsible for the observed in vivo effects. Moreover, the in vivo effects in the CCI model of neuropathic pain could be blocked by administration of a CB1 or CB2 antagonist, and was unaffected by VR1 or opioid antagonists, consistent with production of increased levels of endogenous anandamide and its selective action at cannabinoid receptors at the sites of injury. Just as importantly, OL-135 had no effect on feeding, mobility or motor control observed with classical CB receptor agonists, and it did not produce respiratory depression or desensitization with chronic dosing observed with opioid agonists.[22] In addition to being one of the best characterized FAAH inhibitors reported to date, its disclosure and characterization validated FAAH as an exciting new therapeutic target for the treatment of pain and inflammation catalyzing subsequent industry-wide investigation.

A series of systematic structure–activity relationship (SAR) studies on OL-135 exploring the 4- and 5-position of the central activating oxazole,[21,25,26,27] the C2 acyl side chain,[21,28,29,30] and the central heterocycle,[20,31] were conducted and each was found to independently impact inhibitor potency or selectivity towards FAAH. These studies demonstrated that incorporation of 2-pyridine at the C5 position of the 2-ketooxazole or related α-ketoheterocycle significantly enhances binding affinity and FAAH selectivity by formation of a hydrogen bonded array between the pyridyl nitrogen and Lys142/Thr236 in the active site. They also defined a role for the central activating heterocycle that is distinct from that observed with serine proteases[32] that explains the unique substituent effects observed. The work illustrated the importance of the electrophilic character of the ketone in driving the

SAR of FAAH inhibition. A well-defined linear correlation between the Hammett σ_p constant of small C5 or C4 substituents on the α-ketooxazole and FAAH inhibition was established that is of a magnitude (ρ= 3.0–3.4, indicating in a 1000-fold increase in K_i per unit change in σ_p) to dominate the behavior of the inhibitors and providing an important new predictive tool for the rational design of α-ketoheterocycle-based serine hydrolase inhibitors (Figure 7).[25,29]

Systematic studies examined the effect of substituents found on the C2 acyl side chain phenyl group,[28] defined the required hydrophobic character of the C2 acyl side chain linker,[28] and defined beneficial conformational constraints in the C2 side chain[21] (Figure 8). The combination of optimized C5 oxazole substituents with optimized C2 acyl side chains provided exceptionally selective and potent FAAH inhibitors.

Janssen (Johnson & Johnson) reported a series of additional α-ketooxazole inhibitors where the phenhexyl group in OL-135 was replaced by alkylated or acylated N-substituted 4-piperidines or 4-propylpiperidines and improvements in solubility relative to OL-135 were achieved.[33] The in vivo efficacy of one such inhibitor was reported in the rat spinal nerve ligation model of neuropathic pain with continuous i.v. administration (6 mg/kg/h to maintain a target plasma concentration of 2 μM) where efficacious activity was maintained over a period of 6 h without loss of potency.[30]

One interesting observation to emerge from the systematic examination of OL-135 was that inhibitors incorporating a 2-pyridyl-6-carboxylic acid as the oxazole C5 substituent productively impact their physical properties (solubility), likely interact additionally with an anion binding site located in the FAAH cytosolic port, exhibit slightly reduced inhibition potency when measured under the assay conditions of pH 9, and display a substantially increased FAAH selectivity versus other serine hydrolases.[26,29] A selected set of reversible α-ketoheterocycles were examined in an assessment of thermal stability (ΔT_m) of FAAH/inhibitor complexes using a thiol-reactive fluorophore (pH = 7.4). In this study and in addition to demonstrating that the observed increased thermal stability qualitatively parallels an inhibitor's potency, the 2-pyridyl-6-carboxylic acid derivatives exhibited a uniquely strong stabilizing effect among the various series examined, suggesting they constitute an especially interesting series to examine in greater detail.[34]

Systematic changes in the central heterocycle of OL-135 were found to significantly influence the inhibitor activity: 1,3,4-oxadiazoles and 1,2,4-oxadiazoles > tetrazoles, the isomeric 1,2,4-oxadiazoles, 1,3,4-thiadiazoles > oxazoles > 1,2-diazines and thiazoles > 1,3,4-triazoles (Figure 9).[20,31] Thus, several activating heterocycles were found to substantially improve the inhibitor potency relative to oxazole and OL-135. Notably, the introduction of an additional heteroatom at position 4 (oxazole numbering, N > O > CH) substantially increased inhibitory activity that may be attributed in part to both the activating hetertocycles increased electron-withdrawing properties and a reduced destabilizing steric interaction at the FAAH active site.

The most recent series of related FAAH inhibitors disclosed contain further conformational constraints in the C2 acyl side chain of OL-135, improving on the drug-like characteristics of the candidate inhibitors and introducing a chiral center adjacent to the electrophilic carbonyl. Only one of the two enantiomers displayed potent FAAH inhibition and their potency is comparable to OL-135 (Figure 10).[35] In vivo characterizations showed that inhibitors in this series raised brain anandamide levels following intraperitoneal (i.p.) or oral (p.o.) administration and exhibited efficacy in models of thermal hyperalgesia and neuropathic pain.[35] Importantly, the inhibitors were found to be orally active, long-acting analgesics significantly attenuating mechanical (>6 h) and cold (>9 h) allodynia for

sustained periods consistent with their long-acting effects in raising the endogenous levels of anandamide (>10-fold) in the CNS when administered orally (>9 h). Notably, studies have demonstrated a requirement for >90% inhibition of FAAH for sustained analgesic effects and the duration of action of members of this recent class of reversible, competitive α-ketoheterocycles exceed that reported for the irreversible carbamate inhibitor URB597 and appear similar to that reported for the irreversible urea inhibitor PF-3845.

The first X-ray structures of reversible α-ketoheterocycle-based inhibitors bound to FAAH were disclosed in 2009.[36] The co-crystal structures of OL-135 and its regioisomer bound to the enzyme confirmed that the active site catalytic Ser241 is covalently bound to the inhibitor electrophilic carbonyl, providing the first structures of FAAH bound to an inhibitor as a deprotonated hemiketal mimicking the enzymatic tetrahedral intermediate. Additional co-crystal structures of representative α-ketoheterocycles systematically probed each of the three active site regions key to substrate or inhibitor binding: (1) the conformationally mobile acyl chain-binding pocket and membrane access channel responsible for fatty acid amide substrate and inhibitor acyl chain binding, (2) the atypical active site catalytic residues and surrounding oxyanion hole that covalently binds the core of the α-ketoheterocycle inhibitors captured as deprotonated hemiketals mimicking the tetrahedral intermediate of the enzyme-catalyzed reaction, and (3) the cytosolic port and its uniquely important imbedded ordered water molecules and a newly identified anion binding site.[37] These structures not only confirmed covalent attachment through nucleophilic attack of Ser241 on the inhibitor electrophlic carbonyl, but they captured the catalytic residues in a unique “in action” state, revealed an unusual Ser217 OH-π hydrogen bond to the activating heterocycle, identified a potential anion binding site in the cytosolic port, defined a distinguishing acyl-chain binding pocket and membrane access channel flexibility, and revealed a prominent role of cytosolic port bound water in stabilizing inhibitor binding. They also revealed that the dominant role of the activating heterocycle is its intrinsic electron-withdrawing properties and defined a key role of an ordered cytosolic port water in mediating the stabilizing hydrogen bonding of optimized oxazole substituents. Additionally, two crystal structures of an exceptionally potent α-ketoheterocycle inhibitor bound to a humanized variant of rat FAAH representing covalently and noncovalently bound states of the same inhibitor with the enzyme were reported.[38] Key to securing the structure of the noncovalently bound state of the inhibitor was the inclusion of fluoride ion in the crystallization conditions that binds the oxyanion hole precluding inhibitor covalent adduct formation with stabilization of the tetrahedral hemiketal. The opportunity to examine the noncovalently bound state of an α-ketoheterocycle inhibitor revealed that they bind in their keto (vs gem diol) state, and that the hydrophobic C2 acyl chain binding in the acyl chain-binding pocket overrides the inhibitor intricate polar interactions in the cytosolic port.

Additional Reversible Inhibitors

A series of reversible FAAH inhibitors have been reported that, on the surface, bear elements that could also result in irreversible enzyme inhibition. A class of substituted (thio) hydantoins and imidazolidinediones were identified as reversible and competitive FAAH inhibitors.[39] The proposed non-convalent binding mode was established by docking studies and compared to reference inhibitors. The alkyl chain of the inhibitor binds to the acyl chain-binding channel (ABP) and one of the phenyl ring points towards the enzyme catalytic triad.[39] Oxime carbamates were also reported as novel competitive reversible FAAH inhibitors.[40] Molecular modeling suggested that a hydrogen bond of the carbamate NH with Met191 contributes to the binding affinity and selectivity. Kinetic Lineweaver Burk analysis confirmed competitive, reversible FAAH inhibition by these derivatives. A member of this series exhibited in vivo activity in an animal pain model (Hargreaves thermal escape test).[40] However, a second series of oxime carbamates has been identified as potent FAAH

inhibitors, and were reported to behave as non-competitive (Lineweaver Burk analysis), but reversible FAAH inhibitors, exhibiting selectivity for FAAH over the other enzymes and receptors in the endocannabinoid system.[41] Enol carbamates have also been reported as analogous non-competitive (Lineweaver Burk analysis), but reversible FAAH inhibitors (Figure 11).[42] Although not yet demonstrated, it is hard to imagine that such classes do not acylate the FAAH catalytic Ser241 as their primary mechanism of FAAH inhibition.

An series of heterocyclic sulfoxides and sulfones related to OL-135 were examined and proved to be 10 to100-fold more potent than the corresponding sulfides with little distinction observed between the sulfoxides and corresponding sulfones, but they were approximately 1000-fold less active than the corresponding α-ketoheterocycles.[43] The potency of the corresponding sulfoxides and sulfones was relatively insensitive to the nature of the attached heterocycle as well as the C2 acyl side chain.[43] Abbott reported a series of benzothiazole-based compounds as potent and selective FAAH inhibitors.[44] Structure–activity relationship studies indicated that the sulfonamide, the piperidine ring and benzothiazole were key components to their activity, but that the amide was not required. A time-dependent pre-incubation study of a representative member of the inhibitors in the series found no potency increase upon extended incubation, suggesting reversible inhibition behavior. Selectivity screening using the ABPP assay revealed exceptional selectivity for FAAH with no off-target activity towards other mammalian serine hydrolases.[44] An additional recent and exciting series of cyclic ureas and lactams disclosed by Amgen were identified as non-covalent, reversible FAAH inhibitors with in vitro potencies comparable to the reversible α-ketoheterocycles and irreversible covalent inhibitors.[45] Starting with designed, irreversible urea-based inhibitors, the optimization of the compounds was conducted using a combination of traditional SAR studies and X-ray co-crystallography with FAAH to identify the potent, reversible, and non-covalently bound inhibitors.[45] To date, no in vivo pharmacological activity of inhibitors in this series has disclosed. Renovis has described two distinct chemical templates as FAAH inhibitors containing either a key sulfonamide or benzoxazole group,[46] Janssen (Johnson & Johnson) recently disclosed a unique amino pyrimidine/triazine scaffold that is reported to inhibit FAAH,[47] and even more recently Merck has disclosed a number of FAAH inhibitor scaffolds that would appear to act as reversible inhibitors[48] (Figure 12).

Additional reversible FAAH inhibitors have been reported including oxadiazolylphenylboronic acids by Infinity,[49] which are in Phase 1/2 clinical trials, extending the observations made in an earlier survey of boronic acids.[50] It was suggested that boronic acids may be used as transition state analogues of carbonyl containing substrates for reversible enzyme inhibitor design, although it is possible such inhibitors simply form reversible covalent adducts with the FAAH catalytic Ser241.[49] Ironwood disclosed a series of FAAH inhibitors incorporating an activated carbonyl (α-ketoamides) as either mixed inhibitors of COX2/FAAH/CRTH2/DAO or selective inhibitors for FAAH for treatment of pain, inflammation, and anxiety.[51] Compound IW-6118 was evaluated in a Phase 1 clinical study and demonstrated favorable pharmacokinetics and dose-related elevation of biomarkers confirming that IW-6118 inhibits FAAH in humans. This compound has entered Phase 2 clinical trials for evaluation of efficacy and safety in patients undergoing third molar dental extraction (Figure 13).[52]

Selected β-lactams have also been identified as a class of FAAH inhibitors that exhibited good potency and selectivity for hFAAH. Moreover, they were found to exhibit a competitive reversible mechanism of enzyme inhibition, an unusual result since β-lactams typically irreversibly (versus reversibility) acylate nucleophilic serine residues to form stable acyl-enzyme intermediates with serine hydrolases (Figure 14).[53]

Irreversible FAAH Inhibitors

Almost 30% of the marketed drugs whose molecular targets are enzymes act by irreversible inhibition.[54] In spite of this, there remains a strong industry bias against pursuing irreversible inhibitors as clinical drug candidates. One of the main rationales behind this is the high inherent reactivity of functional groups generally associated with covalent modification of proteins. Excessively reactive covalent modifiers can form covalent bonds with a large number of enzymes, often within the same mechanistic class. However, much research by numerous groups has focused on developing irreversible covalent inhibitors of FAAH largely because of the ease of achieving long acting pharmacological activity in vivo even without extensive inhibitor optimization. Because the proteome-wide selectivity of such inhibitors can be routinely monitored and because the off-site targets of the identified reacting functionality often would be expected to be low, several classes of such FAAH inhibitors have been extensively examined.

Carbamate Inhibitors

Carbamate-based inhibitors are a well-studied class of FAAH inhibitors. Mor and coworkers were the first to disclose this type of FAAH inhibitor discovered through modification of the known AChE inhibitor **1**, Figure 15.[55] Although this carbamate is not an inhibitor of FAAH, simple modifications led to the *O*-aryl carbamate URB524 and later to URB597,[56] IC_{50} = 63 and 4.6 nM respectively. These early studies demonstrated the need for an activated carbonyl in order to achieve FAAH inhibition. Other less electrophilic isosteres were not effective FAAH inhibitors. Additionally, kinetic studies showed the compounds were noncompetitive and nondializable, suggesting that these compounds irreversibly and covalently modified FAAH. A follow up study of the mass spectral properties of carbamate fragmentation upon ESI, suggested the SAR might be driven by the leaving-group ability of the phenol.[57]

Subsequent studies on a larger set of compounds afforded a QSAR model in which the activity of the compounds was inversely correlated with the lipophilicity of the phenol. Mass spectral studies of URB597 bound to FAAH conducted by Cravatt and coworkers later confirmed that the phenol was the leaving group, and established that the carbamate is binding to the active site Ser241, Scheme 1.[58] In these studies, support for an alternative binding model having the cyclohexyl ring occupy the lipophilic channel in FAAH was established with the synthesis of analogues in which the cyclohexyl ring was replaced with a lipophilic side chain such as phenhexyl and oleyl to create potent FAAH inhibitors, Figure 16.

URB597 is one the best studied carbamate-based inhibitors. Early studies showed that the compound did not inhibit or bind related biological targets (AChE, BCh, MGL, CB1 or CB2).[59] Administration of URB597 to rats and subsequent in vivo evaluation of brain FAAH activity showed the compound elevated endogenous anandamide levels.[59] Importantly, the compound did not produce catalepsy, hypothermia, or hyperphagia, three of the typical effects of exogenous cannabinoids. The compound did produce antinoceptive effects in the mouse hot-plate test, which were reversed by the CB1 antagonist rimonabant. These findings again support the expectation that inhibition of FAAH produces pharmacology distinct from an exogenous CB1 agonist. A more detailed study was published later showing time-course data in mice demonstrating elevation of anandamide, oleamide, and *N*-palitoyl ethanolamine for 2–6 h after administration of URB597.[60] In vivo administration of URB597 showed almost complete inhibition of FAAH by the compound, and the FAAH inhibition was still approximately 70% after 16 h. Complete recovery of CNS FAAH activity was observed 24 h after administration of URB597, reflecting a time frame consistent with FAAH resynthesis.

A number of studies have reported analgesic efficacy with URB597. In vivo administration of URB597 or the cannabinoid receptor agonist HU210 reduced both mechanical allodynia and thermal hyperalgesia in the CFA model of inflammatory pain. However, URB597 had no effect in the partial sciatic nerve-ligation model of neuropathic pain in rats.[61] Effects in the inflammatory model were partially reversed by CB1 and CB2 antagonists. In a related study, the compound produced analgesic effects in the mouse CCI model (neuropathic) when administered orally. These effects were also reversed by both CB1 and CB2 antagonists. Motor impairment was not observed at efficacious doses of URB597.[62] With regards to anxiety and depression, the data are more controversial. Early reports with the compound demonstrated dose-dependent effects in both the zero-maze and isolation-induced vocalization in rats. However, subsequent studies in plus-maze, forced swim, and tail suspension tests showed no effect at doses that potentiate anandamide-mediated analgesia.

Other groups have since refined the structure and produced a second generation series of *O*-aryl carbamate inhibitors demonstrating greater plasma stability, prolonged half lives in vivo, and decreased activity toward liver carboxylesterases in comparison to URB597.[63] It was found that addition of small electron-donating stubstituents at conjugated positions of the *O*-aryl moiety increased the overall hydrolytic stability of the carbamate group without affecting FAAH inhibitory potency.

An additional early series of carbamate inhibitors was reported by Sanofi-Aventis in a number of patent applications, Figure 17. A report on the selectivity of two examples from these patents, SA-47 and SA-73, demonstrated that they are highly selective for FAAH in proteomic analysis and carboxyesterase screening.[64] Very little has been published on the pharmacology of these inhibitors, although it was been stated that these compounds reduced visceral pain in mice. Two such inhibitors entered clinical trials for what appears to be anxiety and depression; however, no data have been released.

Bristol-Myers Squibb reported a unique class of carbamate inhibitors, Figure 18. The initial lead, compound **2**, from a high throughput screening library, was effectively optimized to more potent inhibitors by incorporation of a phenyl carbamate as the putative electrophile.[65,66] Substitution of the ester or carbamate with amides or ureas rendered the compounds inactive against FAAH, supporting the hypothesis of a covalent mechanism of action. BMS-1 (20 mg/kg, i.v.) showed in vivo pharmacological activity in the formalin persistent pain model comparable to that observed with morphine (3 mg/kg, i.v.). In the Chung model for neuropathic pain**,** BMS-1 (20 mg/kg, i.v.) demonstrated a significant effect in reversing mechanical allodynia comparable to the clinically active reference agent gabapentin (100 mg/kg, i.v.).[66] However, BMS-1 was found to be relatively nonselective with respect to other serine hydrolases and esterases by proteomic esterase profiling.[67]

As highlighted earlier, scientists at BMS also reported a series of oxime carbamates (Figure 11).[40,68] The SAR of these compounds suggests that the length of the alkoxy side chain plays a role in their activity, with longer alkyl groups being more active. BMS-469908 was reported to reduce thermal hyperalgesia and paw edema in the rat carrageenan model with i.v. dosing. In dialysis studies, 80% of the enzyme's activity was recovered after 18 h, pre-incubation experiments demonstrated that inhibition was not time-dependent, and kinetic Lineweaver-Burk analysis indicate that BMS-469908 is a competitive, reversible inhibitor of FAAH. Under the same experimental conditions, URB597 inhibited FAAH in a time-dependent manner. These studies make it unclear whether the carbamate plays the same role of covalent inhibition that might be expected of its structure. However, researchers in Italy also synthesized a library of oxime-based FAAH inhibitors structurally similar to BMS-469908. Kinetic studies with Lineweaver-Burk analysis indicated non-competitive

FAAH inhibition consistent with acylation covalent modification of the enzyme, and the compounds also did not exhibit time-dependent inhibition of FAAH.[41,42]

Both Astellas[69] and Kadmus[70] have disclosed additional carbamate inhibitors of FAAH in patent applications, but details of the series have yet to be published. A report from the Helsinki University of Technology attempted to marry the oxazole ketone and the carbamate chemotypes into a new class of FAAH inhibitors, Figure 19.[71] The most potent FAAH inhibitors in this series contained a cyclopentyl carbamate. Inhibition was dependent upon the carbamate, as compounds lacking this moiety were unable to effectively inhibit FAAH. The SAR was insensitive to changes in the ketoheterocycle, indicating the ketone is not acting as a serine trap in these molecules. No pharmacological or mechanistic studies have been reported.

In 2009, Cravatt and coworkers disclosed a series of piperazine carbamate-base inhibitors (Figure 20), several of which were found to inhibit both FAAH and MAGL.[72] JZL-184 was found to be a highly selective and efficacious MAGL inhibitor. However JZL-195, an inhibitor based on this piperazine scaffold, was found to be a dual FAAH/MAGL inhibitor, IC_{50} = 12 and 19 nM respectively.[73] Using ABPP, JZL-195 was found to be highly selective for FAAH and MAGL, inhibiting only one additional serine hydrolase, ABHD6. Mice treated with JZL-195 showed dramatic and sustainable inhibition of brain FAAH and MAGL with 10-fold elevations in endogenous anandamide and 2-AG levels. These effects on the anandamide/FAAH and 2-AG/MAGL systems were similar in magnitude and duration as those observed with selective inhibitors of each individual endocannabinoid pathway. In vivo pharmacology studies have provided insights into the roles that each endocannabinoid pathway plays. Certain behaviors, such as hypomotility and hyperflexia were similarly affected by JZL-184 and JZL-195. However, antinociception was observed in mice treated with JZL-184, but this effect was dramatically enhanced in mice treated with JZL-195. An even more striking effect of dual FAAH/MAGL inhibition was observed in the catalepsy test, in which JZL-184 had no effect, but JZL-195 showed robust activity. These studies confirmed that endocannabinoids anandamide and 2-AG have distinct biological functions even though they bind to and activate CB1 and CB2 receptors. Both JZL-184 and JZL-195 treated mice (i.p.) exhibited analgesic activity in several pain models including the tail immersion test for acute thermal sensation, the acetic acid writhering test of visceral pain, and the formalin test for noxious chemical pain over a period of 8 h.[73]

Aryl Urea Inhibitors

Like the carbamates, aryl ureas have also been explored as inhibitors of FAAH. One of the first examples of an electrophilic urea-based FAAH inhibitor was reported by Lilly. The compound, LY-2183240 (Figure 21), was originally described as an anandamide transport inhibitor.[74] However, it was later found to be a potent inhibitor of FAAH, IC_{50} = 12 nM.[75] Mass spectral studies of the enzyme-inhibitor adduct verified that the inhibition was due to covalent modification of the catalytic Ser241 in a binding mode similar to the previously discussed carbamates. Proteome-wide selectivity analysis indicate that LY-2183240 is not selective for FAAH, since it was found to inhibit several other brain serine hyrolases with IC_{50} values in the low nanomolar range.[75] Despite this, the in vivo administration of the compound did show dose-dependent elevations of anandamide in rat brain and reduced pain in the formalin test without reduction in motor performance.

Sanofi-Aventis has also disclosed a related series of triazolopyridine carboxamides (ureas) as either selective MAGL inhibitors or dual MAGL/FAAH inhibitors,[76,77] and others have disclosed various azol ureas as dual FAAH/MAGL inhibitors, Figure 22. Representative compound **9** was tested in vivo and showed reduced kainic acid-induced seizure severity throughout a 4 h period (5 mg/kg, i.v.). Animals also showed improved rotorod performance

without behavioral impairment.[78] Additional tetrazole-based FAAH inhibitors have been reported by Moore,[75] and additional reactive imidazole-based urea inhibitors were described in patents disclosed from Astellas.[79]

In 2006, both Takeda and Johnson & Johnson disclosed patents for strikingly similar piperazine aryl ureas, Figure 23. In dialysis experiments with JNJ-1661010,[80] enzyme activity remained substantially inhibited after dialysis at 4ºC. However, significant activity was recovered at 22ºC, suggesting a time and temperature-dependent release of the compound from the active site. The recovery of most of the enzyme activity after 18 h at 22ºC rules out true irreversible inhibition, but does reflect a slow reversible covalent modification of the enzyme. Mass spectral analysis of JNJ-1661010 bound to rat FAAH indicated that the urea carbonyl was acting as an electrophile covalently modifying the active site serine with the aniline fragment functioning as the leaving group.[81]

In pharmacological studies, the Takeda compound was reported to produce 100% block of rat FAAH activity at 1 μM and significantly reduced infarct volume in a rat stroke model.[82] JNJ-1661010 exhibited activity in both the mild thermal injury model of acute tissue injury pain and the Chung model of neuropathic pain with no associated motor impairment. In vivo properties for JNJ-1661010 were measured by both ex vivo inhibition of brain FAAH and the elevation of anandamide after dosing the compound at 20 mg/kg (i.p.). Brain FAAH was potently inhibited for an extended period by a single dose of 20 mg/kg. Even after 24 h, FAAH activity had recovered to only 25% of untreated values. In parallel, rats that had been dosed with JNJ-1661010 showed elevated levels of anandamide in brain tissue. Even 4 h post dosing, rat brain anandamide levels increased by up to a factor of 1.4 indicating in vivo inhibition of FAAH.

In 2007, Pfizer reported two related urea-based FAAH inhibitors, Figure 23.[83] This novel mechanistic class of FAAH inhibitors utilizes the urea group as a tempered electrophile that effectively carbamylates FAAH's catalytic Ser241. The time-dependent inhibition by PF-750 and PF-622 combined with results showing that no activity was recovered in rapid dilution studies indicated that their mechanism of inhibition is either slow reversible time-dependent inhibition or covalent irreversible inhibition. Covalent modification of the enzyme was verified by two different methods. Both radioactive labeling studies and mass spectrometry concluded that the aniline is the leaving group, Scheme 2.

Activity based protein profiling (ABPP) was use to evaluate the targets of this inhibitor in multiple human and mouse tissue proteomes. These studies were conducted with other classes of FAAH inhibitors that have been shown to exhibit variable degrees of selectivity. The study revealed that these piperidine/piperazine ureas are highly selective FAAH inhibitors, showing no discernable activity against other serine hydrolases in vitro or in vivo at concentrations up to 500 μM and 30 mg/kg (i.p.), respectively. These results contrast the properties of other classes of irreversible FAAH inhibitors, including URB597 and CAY-10402, which typically target multiple serine hydrolases.

The authors proposed that a binding-induced conformational change in the urea can decrease its hydrolytic stability and account for this acylation mode of action. The catalysis of the acylation reaction could be derived from a binding-induced conformational change[84] in the piperidine/piperizine urea that diminishes the conjugation of the nitrogen lone pair with the carbonyl, activating the urea toward nucleophile attack, Scheme 3. If this binding-induced activation of the urea is FAAH specific, it could help to explain the selectivity of these ureas as inhibitors for FAAH.

An interspecies conversion of FAAH active site residues provided a protein, termed h/rFAAH, that exhibits the inhibitor sensitivity profile of human FAAH and the high-

recombinant expression and stable biochemical properties of rat FAAH. The PF-750-h/rFAAH X-ray structure confirmed that the piperidine/piperazine ureas inhibit FAAH by covalent carbamylation of the catalytic Ser241 nucleophile of the enzyme. This structure also identified key interactions between PF-750 and residues in the h/rFAAH active site that likely account for enhanced potency exhibited by this inhibitor for hFAAH over rFAAH.[85]

Following PF-750, Pfizer has since disclosed a series of additional compounds, including PF-3845 (Figure 25). PF-3845 displays a combination of potent, efficacious in vivo activity and target selectivity. This compound has been utilized as a valuable pharmacological tool for studying FAAH-regulated endocannabinoid pathways. The PF-3845-h/rFAAH crystal structure was disclosed wherein 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. The 20-fold improvement in potency over PF-750 appears to be derived from a more extensive set of van der Waals interactions between the inhibitor 4-trifluoromethyl-2-pyridyl group and the hydrophobic acyl chain-binding pocket of FAAH.[86] In this respect, the biaryl ether piperidine moiety binds in a fashion that more closely resembles the arachidonyl chain of methyl arachidonyl phosphonate MAP-rFAAH.

In pharmacological studies, oral administration of PF-3845-treated mice showed high (>10-fold) and sustained (> 7 h) elevations in brain levels of anandamide and other NAEs. Treated animals also showed significant NEA elevations in the periphery including liver tissue. PF-3845-induced elevations in NAEs peaked at 3 h and were maintained at maximal levels for up to 7–12 h after treatment. Importantly, the compound inhibited the pain response in the Freund's adjuvant (CFA) model in rats to a comparable degree as the nonsteroidal anti-inflammatory drug naproxen at 10 mg/kg. The degree of inhibition in pain response was similar at all efficacious doses likely because near complete FAAH inhibition is necessary to obtain in vivo efficacy, which was achieved at all doses starting at 3 mg/kg. Improvements on PF-3845 resulted in PF-04457845, which has shown excellent pharmacokinetic properties in mice, rats and dogs and which performed better in a rat model of inflammatory (CFA) pain.[87] Oral administration caused a significant decrease in mechanical allodynia in rats measured after 4 h with a minimum effective dose of 0.1 mg/kg, which was 30-fold lower than PF-3845. PF-04457845 progressed into clinical trials for the treatment of osteoarthritis, where administration of the FAAH inhibitor proved safe in Phase I trials, but where human efficacy was not observed in Phase II trials, although detailed results have not yet been published.[87b]

Pfizer has also described the SAR of the series of benzothiophene piperazine ureas initially discovered through high-throughput screening. Activity-based protein profiling revealed that these urea inhibitors are highly selective for FAAH relative to other mammalian serine hyrolases.[88] No off-target activity was observed even when tested at 100 μM. Lead compound, PF-465 (Figure 25), completely inhibited FAAH from both mouse and human tissues. In vivo efficacy was assessed in a rat model of inflammatory pain. Subcutaneous injection of complete Freund's adjuvant (CFA) into the plantar surface of the hind paw produced a significant decrease in mechanical paw weight threshold (PWT) at 5 days post injection. Dose-dependent inhibition of mechanical allodynia with a minimum effective dose of 10 mg/kg was observed.

Vernalis discovered that the racemic azetidine, VER-24052 (Figure 26), was a reasonably potent inhibitor of FAAH. This appears to be the first example of a FAAH inhibitor that contains a tetrasubstituted urea. After chiral resolution, only one enantiomer was found to be a potent inhibitor of FAAH. Interestingly, the resolved compound appeared to be 7-fold less potent against human FAAH that rat FAAH. Species selectivity has been reported

previously for FAAH inhibition with PF-750 being 8-fold more potent in human than in rat FAAH, while several other inhibitors including JNJ1661010 are equipotent.[89]

Conclusions

A remarkable series of potent, selective, and efficacious inhibitors of the enzyme fatty acid amide hydrolase (FAAH) have now been disclosed, many of which have been utilized to mechanistically and structurally characterize the enzyme, define its mechanism of fatty acid amide hydrolysis, and validate the enzyme as a therapeutic target for the treatment of pain/inflammation or sleep disorders. Many of the inhibitors are moving toward or have been advanced into clinical trials where efficacy in humans can now be established by not only monitoring key biomarkers (e.g., anandamide levels), but also critical clinical responses to the candidate drug administration. In this regard, it is interesting to note that 50–70 million Americans annually suffer from chronic disorders in sleep, making sleep second only to pain in the number of patients seeking medical attention. As a result, the potential impact of the clinical introduction of FAAH inhibitors is quite large. A key element of the clinical studies will be the determination of where to expect FAAH inhibitors to exhibit useful therapeutic effects. Here, those that are making decisions on which clinical applications might prove most effective may need to focus not on the most lucrative potential market, but rather those applications with the most precedented expectations of success. Recognizing that cannabinoids already have established clinical uses in a number of important areas, there may be several straightforward paths into and through the clinic for FAAH inhibitors. The distinction is that the efficacious activity expected of FAAH inhibitors, which only potentiate an activated signaling pathway at the immediate site of action, may be observed without the side effects of a classical exogenous cannabinoid. Additional innovative clinical opportunities likely will emerge from a better understanding the fundamental physiological role of the signaling fatty acid amides that FAAH regulates, an area that is still incompletely defined. Similarly, the range of potential clinical applications also defines a range of properties that may be preferred for the clinical drug. Short acting injectable drugs with a rapid onset (e.g., dental pain), i.v. administration for adjustable control of severe pain in hospitalized patients, topical drug administration for contact dermatitis, as well as orally active, long-acting candidate inhibitors for chronic inflammatory or sleep disorders may well find their way into clinical use, but will require a different set of optimized properties for the candidate drug. Consequently, it would be a mistake for those evaluating the field or the inhibitors summarized herein to do so without a clear understanding of the range of clinical applications presently under consideration.

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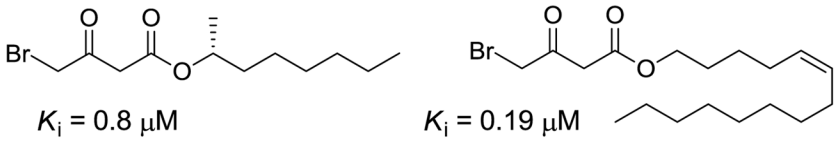


Figure 1.
2-Octyl α-bromoacetoacetate and a more potent synthetic analogue.

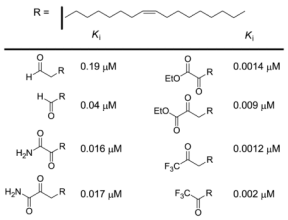
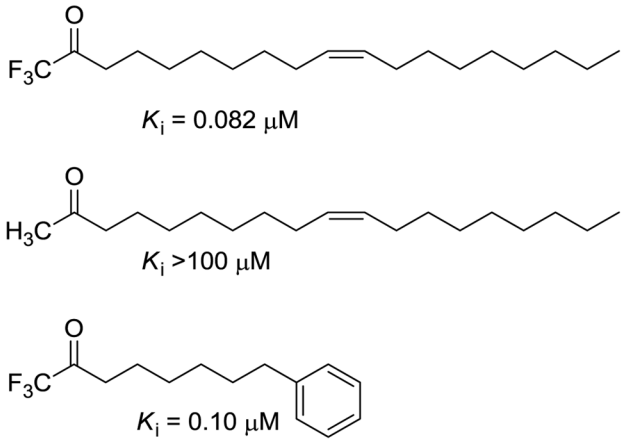


Figure 2.
Representative early inhibitors of FAAH.



O
F3C R
R = (CH2)nCH3

n	K_i
16	0.24 μM
12	0.49 μM
10	0.14 μM
8	0.13 μM
6	1.2 μM
5	10.7 μM
4	26.9 μM

Figure 3.
Representative trifluoromethyl ketone inhibitors.

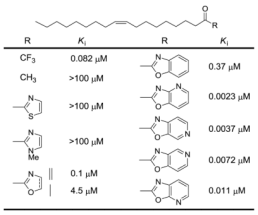


Figure 4.
Representative initial α-ketoheterocycle inhibitors.

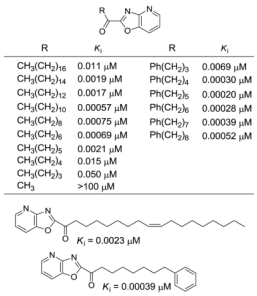


Figure 5.
Characteristic modifications in the fatty acid side chain.

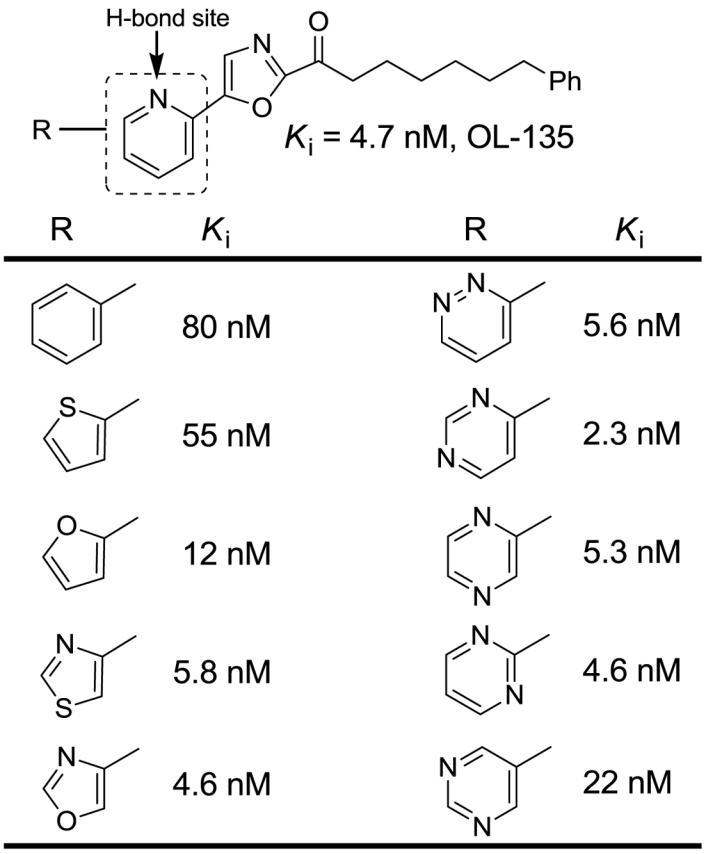


Figure 6.
Representative C5 substituted α-ketooxazoles and structure of OL-135.

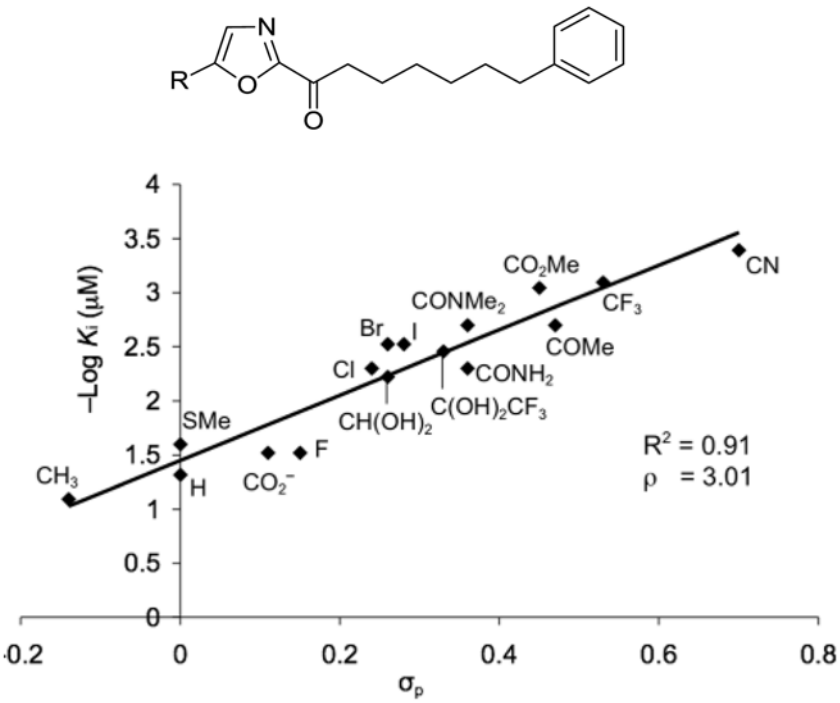
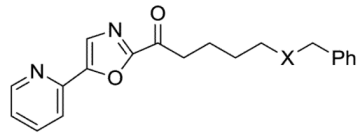
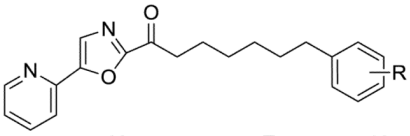


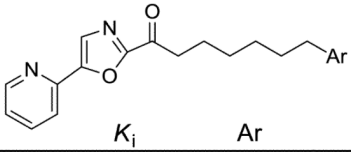
Figure 7.
Plot of–Log K_i versus σ_p.



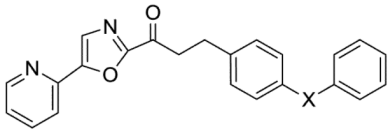
X	K_i	X	K_i
CH_2	4.7 nM	NMe	200 nM
S	25 nM	SO	2.5 μM
O	55 nM	SO_2	>10 μM
CH(OH)	200 nM	CONH	20 μM



R	K_i	R	K_i
2-CH_3	3 nM	2-CF_3	4 nM
3-CH_3	3.3 nM	3-CF_3	1 nM
4-CH_3	2.6 nM	4-CF_3	4 nM



Ar	K_i	Ar	K_i
2-thienyl	4.3 nM	2-naphthyl	11 nM
3-thienyl	5.1 nM	2-pyridyl	120 nM
1-naphthyl	2.6 nM	3-pyridyl	32 nM



X	K_i	X	K_i
OCH_2	1.3 nM	O	3.4 nM
CH_2O	1.0 nM	NH	2.0 nM
CH_2	3.2 nM	–	0.7 nM
S	2.2 nM		

Figure 8.
Representative exploration of the C2 acyl side chain.

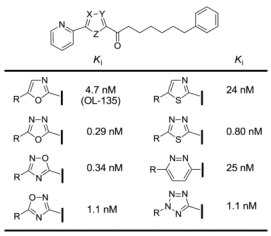
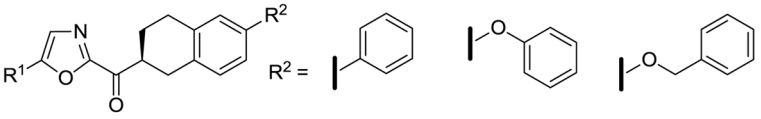


Figure 9.
Key variations of the central activating heterocycle.



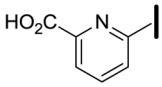
R^1	K_i (nM)	K_i (nM)	K_i (nM)
–H	57	2.2	6.1
	7.2	4.4	3.2
	39	25	34

Figure 10.
Representative further conformational constraints in the C2 acyl side chain.

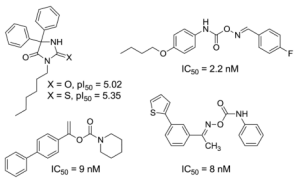


Figure 11.
Additional classes of reversible FAAH inhibitors.

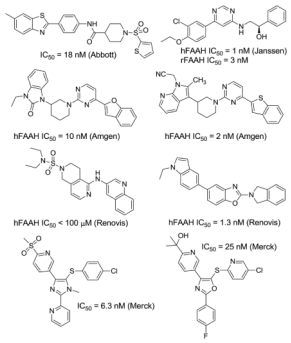


Figure 12.
Recent classes of reversible FAAH inhibitors.

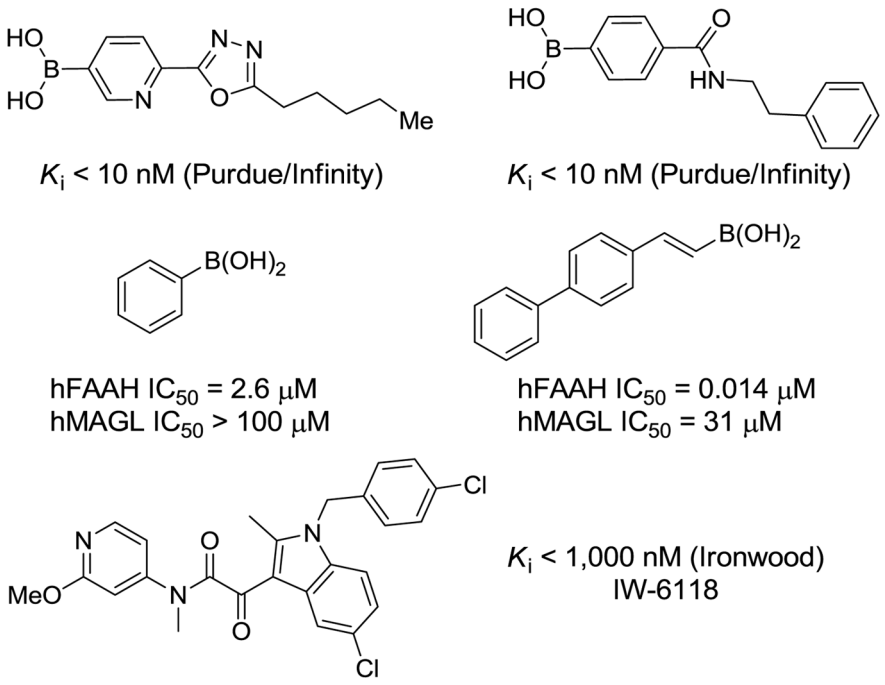


Figure 13.
Additional reversible FAAH inhibitors.

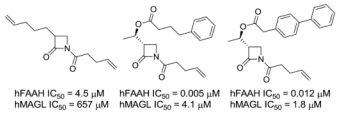


Figure 14.
β-Lactam FAAH inhibitors.

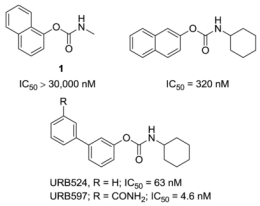


Figure 15.
O-Aryl carbamates.

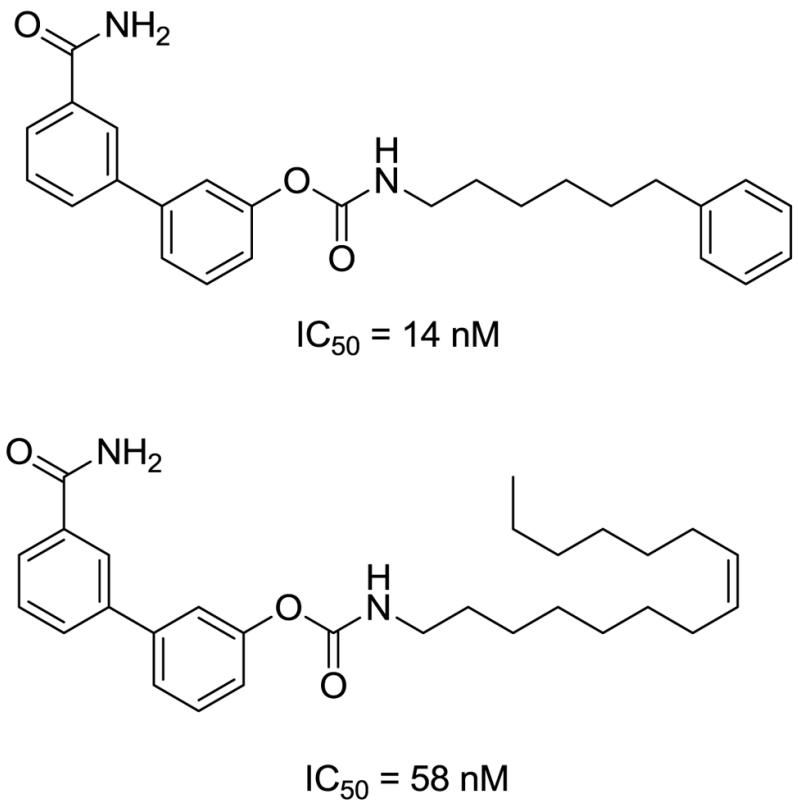


Figure 16.
Representative carbamate hybrids.

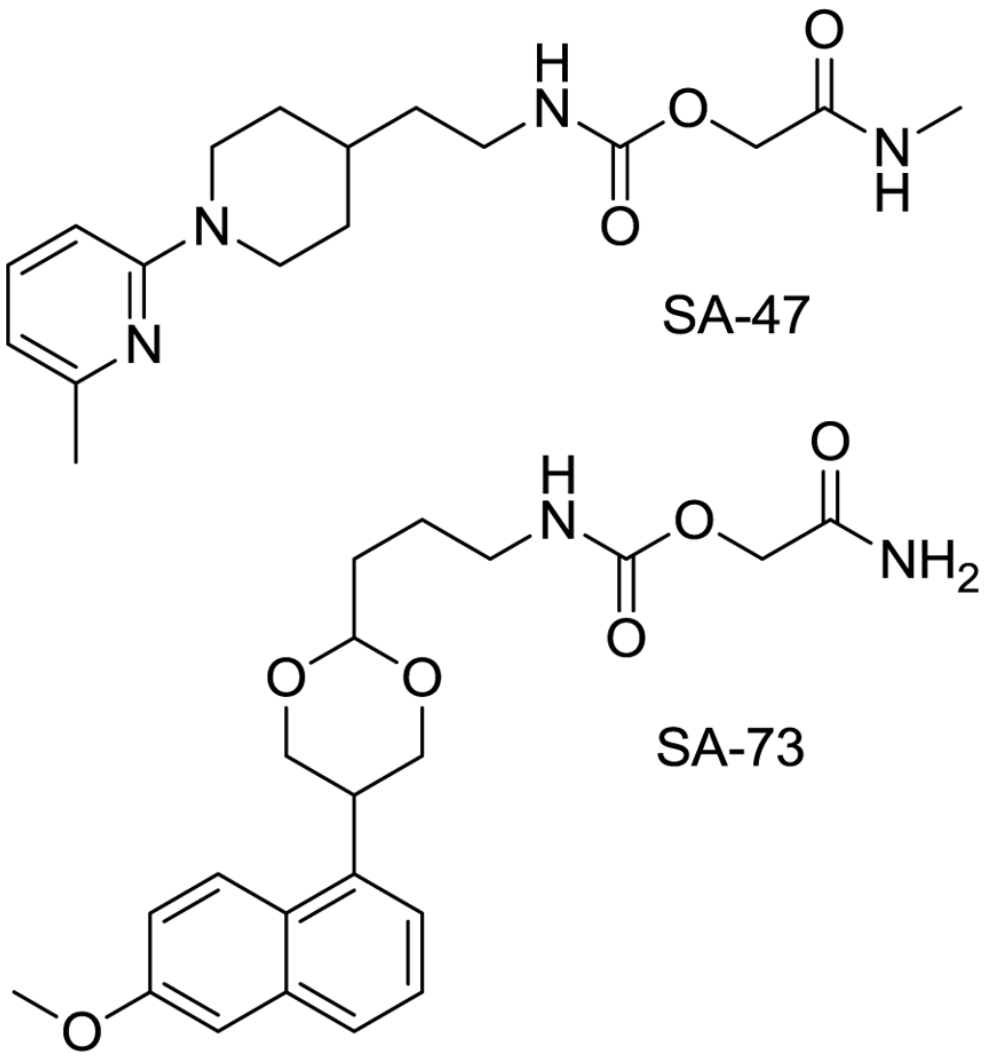


Figure 17.
Additional early carbamates.

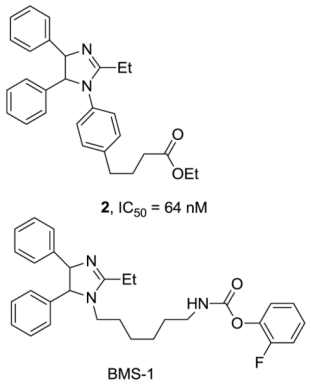
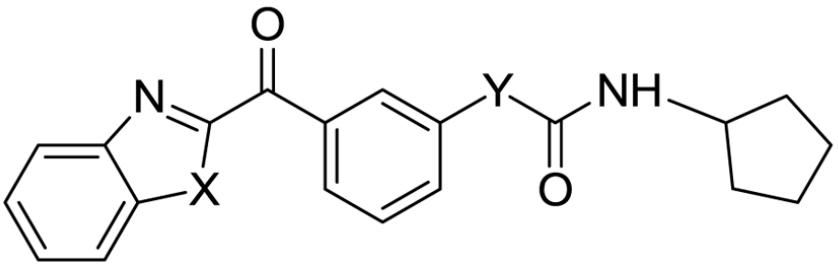


Figure 18.
Subsequent carbamate inhibitors.



X	Y	IC_{50} (μM)
O	O	28
O	NH	> 100
S	O	47

Figure 19.
Oxazole ketone/carbamate inhibitors.

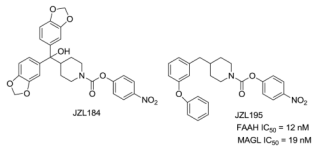


Figure 20.
Representative piperazine carbamates.

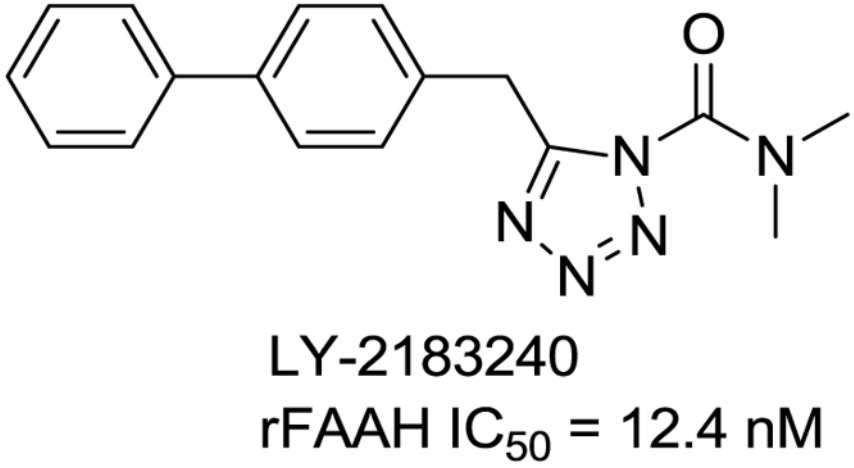


Figure 21.
Tetrazole-based inhibitors.

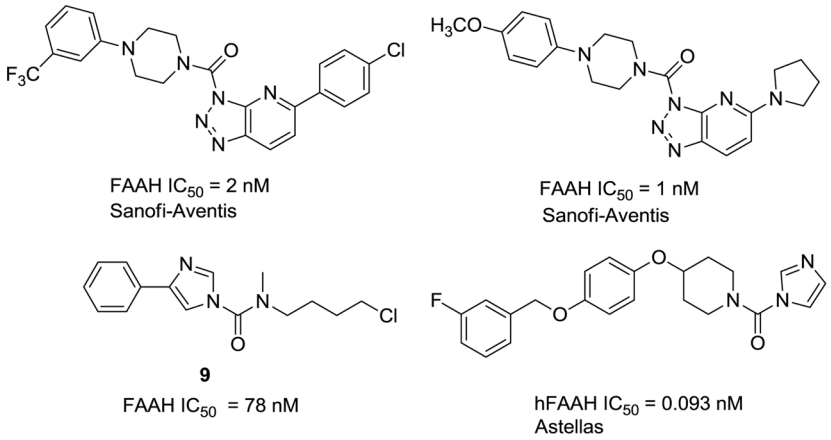


Figure 22.
Additional triazolopyridine and azole FAAH inhibitors.

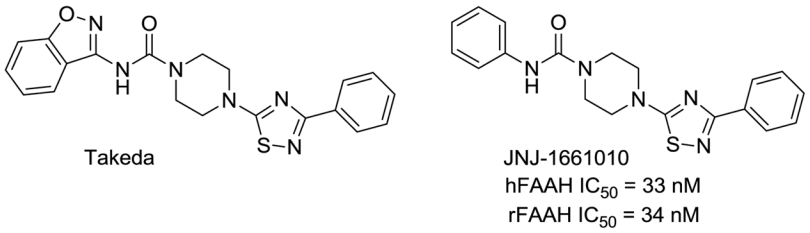


Figure 23.
Piperazine aryl urea inhibitors.

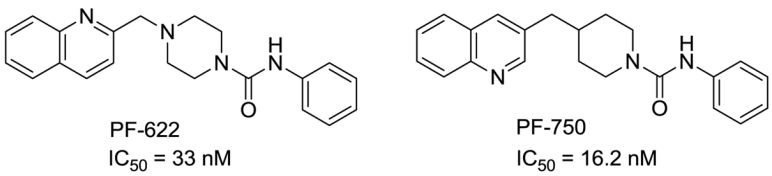


Figure 24.
Piperidine/piperazine urea-based inhibitors of FAAH.

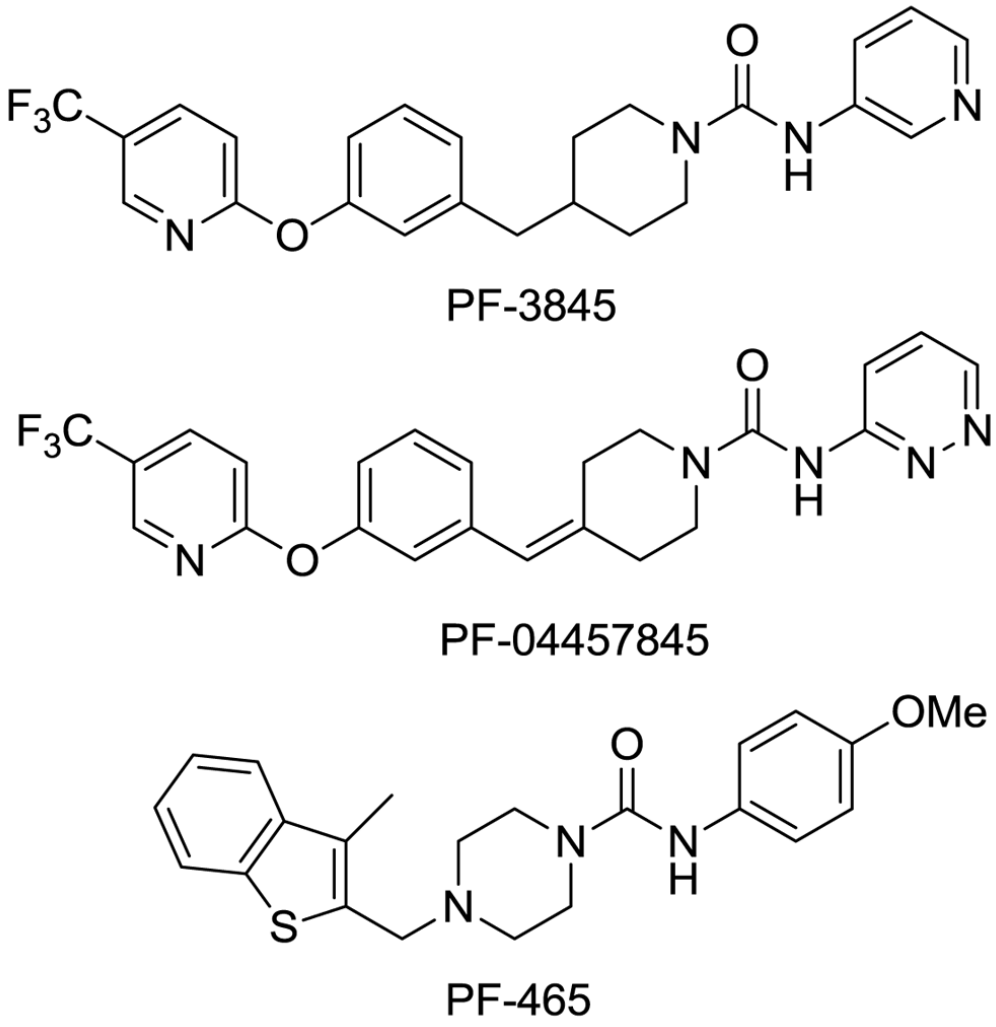


Figure 25.
Additional urea inhibitors.

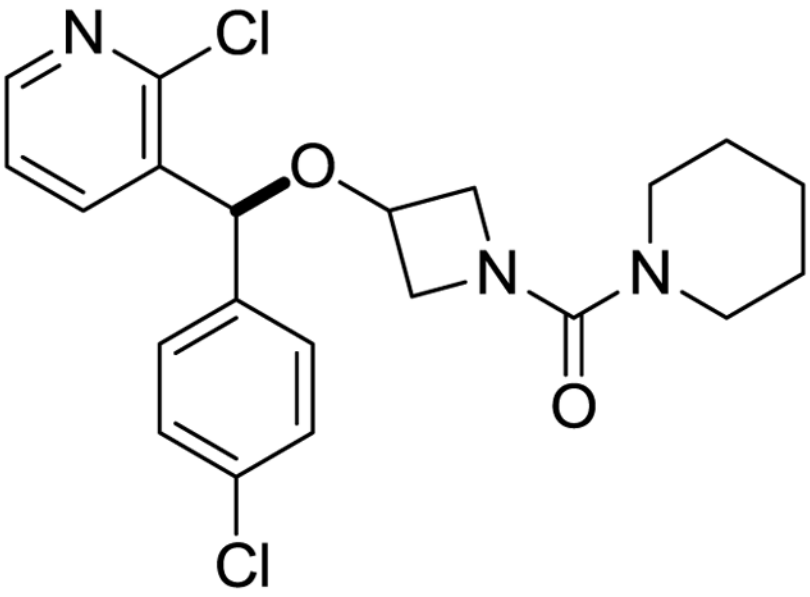


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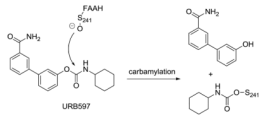
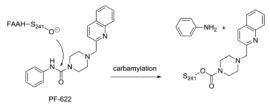
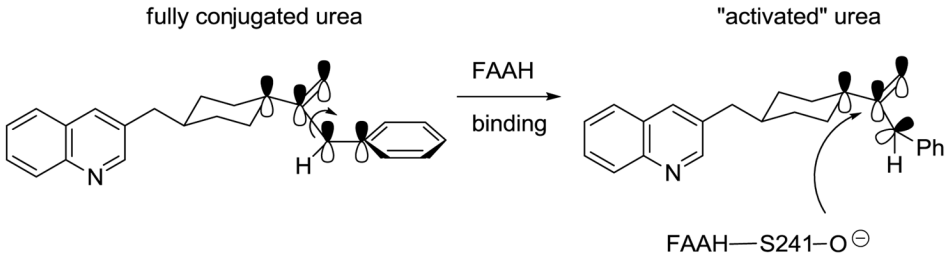
Figure 26.
Tetrasubstituted urea inhibitor.



Scheme 1.
Carbamylation of active site Serine 241.



Scheme 2.
Carbamylation of active site Serine 241.



Scheme 3.
Activating conformational change of urea group.