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Identification and optimization of soluble epoxide hydrolase inhibitors with dual potency towards fatty acid amide hydrolase

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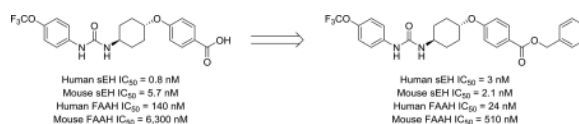
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Abstract

Multi-target inhibitors have become increasingly popular as a means to leverage the advantages of poly-pharmacology while simplifying drug delivery. Here, we describe dual inhibitors for soluble epoxide hydrolase (sEH) and fatty acid amide hydrolase (FAAH), two targets known to synergize when treating inflammatory and neuropathic pain. The structure activity relationship (SAR) study described herein initially started with *t*-TUCB (*trans*-4-[4-(3-trifluoromethoxyphenyl-1-ureido)-cyclohexyloxy]-benzoic acid), a potent sEH inhibitor that was previously shown to weakly inhibit FAAH. Inhibitors with a 6-fold increase of FAAH potency while maintaining high sEH potency were developed by optimization. Interestingly, compared to most FAAH inhibitors that inhibit through time-dependent covalent modification, *t*-TUCB and related compounds appear to inhibit FAAH through a time-independent, competitive mechanism. These inhibitors are selective for FAAH over other serine hydrolases. In addition, FAAH inhibition by *t*-TUCB appears to be higher in human FAAH over other species; however, the new dual sEH/FAAH inhibitors have improved cross-species potency. These dual inhibitors may be useful for future studies in understanding the therapeutic application of dual sEH/FAAH inhibition.

Graphical abstract



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Competing financial interests declaration: SDK and BDH have filed a patent through the University of California for compounds described in this text for use as dual sEH/FAAH inhibitors. BDH is founder and SHH is an employee of Eicosis Animal Health, a company developing *t*-TUCB for use in companion animals.

Keywords

Soluble Epoxide Hydrolase; Fatty Acid Amide Hydrolase; Urea Inhibitors; Neuropathic Pain

Chronic pain is poorly managed by current treatment options. The available therapies, including non-steroidal anti-inflammatory drugs (NSAIDs) and opioids, are not effective on all types of pain, can be debilitating or have a high potential for abuse.¹ Furthermore, few new therapies have come to the market in the past decade. One recent approach towards designing analgesics with high efficacy and reduced side effects has been the combination of inhibitors for two or more targets known to regulate pain, known as poly-pharmacology.² These multi-target inhibitors have the potential for higher efficacy and reduced drawbacks arising from the use of a single-target drug or a combination of multiple drugs.³ In particular, enzymes involved in the regulation of signaling lipids, including soluble epoxide hydrolase (sEH) and fatty acid amide hydrolase (FAAH), have been proposed as suitable targets for the application of poly-pharmacology for pain treatment.^{4,5}

The sEH is responsible for the regulation of lipid epoxides acting as potent chemical mediators such as epoxyeicosatrienoic acids (EETs).⁶ These signaling lipids are responsible for mediating a number of biological processes including nociception,^{7,8} inflammation⁹ and hypertension.^{10,11} By converting the biologically active epoxides to their respective largely inactive diols, sEH negatively regulates the activity of the EETs. The *in vivo* stability of EETs and other chemically stable epoxy-fatty acids is low due to the high catalytic efficiency of sEH.¹² Thus, sEH inhibition has been the major approach for studying the biological role of lipid epoxides in numerous disease states including neuropathic and inflammatory pain.^{13,14} Treating with sEH inhibitors reduces both forms of pain in a manner that may be dependent, in part, on cannabinoid signaling,¹⁵ endoplasmic reticulum stress¹⁶ and/or other mechanisms. Several of these inhibitors have been developed as IND candidates that have reached Phase I (GSK2256294A) and Phase II (AR9281) clinical trials for COPD and hypertension, respectively (Figure 1).¹⁷⁻¹⁹ GSK2256294A has not progressed to further stages of clinical trials and AR9281 was unable to demonstrate efficacy in human patients.

FAAH is a separate enzyme that is studied as a potential therapeutic target for neuropathic and inflammatory pain.²⁰⁻²² This enzyme hydrolyzes arachidonoyl ethanolamide (AEA), an endocannabinoid that regulates nociception and other physiologies through activation of the cannabinoid receptors.^{22,23} Like EETs, AEA is quickly metabolized *in vivo* by FAAH and therefore *in vivo* studies investigating AEA require FAAH inhibitors. Although activation of cannabinoid receptors has numerous undesired effects including hypothermia, catalepsy and hyperphagia, treatment with FAAH inhibitors or AEA alone is not sufficient for producing these effects.^{24,25} Several FAAH inhibitors have been developed. Among them, PF-04457845 reached Phase II clinical trials without success due to lack of efficacy despite its excellent target engagement.²⁶ Recently, BIA 10-2474 was also pulled from a Phase I clinical trial after the death of a study subject,²⁷ which was independent of FAAH inhibition.²⁸

Concurrent inhibition of both sEH and FAAH synergistically reduces both inflammatory and neuropathic pain.²⁹ Interestingly, the sEH inhibitor *trans*-4-[4-(3-trifluoromethoxyphenyl)-

lureido)-cyclohexyloxy]-benzoic acid (*t*-TUCB), which was thought to be a selective potent sEH inhibitor ($IC_{50} = 0.4$ nM), was recently identified as a weak FAAH inhibitor (human FAAH $IC_{50} = 260$ nM). *t*-TUCB demonstrates excellent efficacy with multiple indications including neuropathic pain, but it was not clear whether its high efficacy is derived from its poly-pharmacology. This excellent efficacy has led to its use as a tool to treat various diseases^{30–32} in several animal species.^{33,34} Despite the extensive use of this compound for studying sEH biology, the contribution of FAAH inhibition to results in the experimental or clinical disease models has not been explored. Thus, our primary goal was to produce novel inhibitors with improved potency towards sEH and FAAH. Our secondary goal was to test the plausibility of FAAH inhibition contributing to the observed beneficial effects of *t*-TUCB and related compounds by defining the potency for dual inhibition in other species.

Synthesis of all inhibitors was done according to established procedures (described in detail in the Supplementary Material). Recombinant enzyme preparations were used with fluorescent-based substrates to quantify potency of inhibitors on sEH and FAAH (described in the Supplementary Material). All of the newly synthesized inhibitors are relatively potent towards sEH ($IC_{50} < 50$ nM) as expected. Thus, we primarily focused on determining the chemical structures essential for optimizing potency on FAAH. Compared to the known FAAH inhibitors PF-3845 and URB597, *t*-TUCB is 233-fold and 6-fold less potent, respectively (Table 1).^{20,35} Rings “A” and “B” and substituents on the 4-position of “C” were modified on *t*-TUCB to determine the portion of the structure that primarily confers potency on FAAH (Figure 1B). Urea-based FAAH inhibitors described previously have an aromatic substitution on one side of the urea, similar to ring “A” on *t*-TUCB. Since these compounds had higher potency for the 4-fluoro or unsubstituted rings than the 4-trifluoromethoxy substituent,³⁶ the 4-trifluoromethoxy group on *t*-TUCB was replaced by a hydrogen (**2**), fluoride (**3**) or chloride (**4**). Potency on FAAH decreased as the size and hydrophobicity of the *para* position substituent increased, with 4-trifluoromethoxy (**1**) being the most potent. Substituting the aromatic ring for a cyclohexane (**5**) or adamantane (**6**) resulted in a complete loss in activity against FAAH. Switching the cyclohexane linker of ring “B” to a *cis* conformation (*c*-TUCB) resulted in a 20-fold loss of potency while replacing it with a butane chain (**9**) resulted in a completely inactive compound. Modification of the cyclohexane to an aromatic linker (**10**) had essentially no effect on potency for FAAH relative to *t*-TUCB. Although many potent urea-based FAAH inhibitors use a piperidine as the carbamoylating nitrogen,^{21,37,38} the modification to piperidine-incorporated tri-substituted urea reduced potency 13-fold (**13**). Together, these changes on ring “B” indicate the *trans*-cyclohexyl ring provides the exact fit in the active site of FAAH essential for *t*-TUCB’s inhibitory potency.

To further explore the relationship between structure and function on the FAAH enzyme, we focused on the substitutions on the 4-position of ring “C” (Table 2). The importance of the terminal carboxylic acid group was explored by testing the potency of the corresponding aldehyde (**15**) and alcohol (**16**) in addition to the amide (**21**) and nitrile (**14**). Generally, the higher oxidation state of the terminal portion correlates with higher potency towards FAAH. *t*-TUCB was 10-times more potent than **15** and 50-times more potent than **16**. Similarly, the amide (**21**) was over 100-times more potent than the nitrile (**14**). Converting the benzoic acid

to the phenol (**18**) had a minor effect on potency. Interestingly, modifying the phenol to the anisole (**19**) completely removed activity while creating an acetate ester (**20**) is equipotent to the phenol. Since the substrates for FAAH tend to be relatively hydrophobic lipids, we speculated conversion of the acid and primary amide to the corresponding esters or substituted amides, respectively, would result in improved potency by introducing hydrophobic groups. As expected, the methyl ester (**22**) had 4-fold improved potency relative to the corresponding acid (*t*-TUCB). However, incorporating a bulkier substitution than the methyl group such as isopropyl ester (**23**) showed 11-fold less potency compared to the methyl ester (**22**). Interestingly, the benzyl ester (**24**) gives approximately the same potency as the methyl ester, suggesting that π - π stacking between benzylic group and a residue at the active site may be important. Relative to *t*-TUCB, the methyl (**25**), ethyl (**26**) and glyciny (**27**) amides all had essentially the same potency; however, the benzyl amide (**29**) was substantially less potent (16-fold). Between the methyl- (**22** and **25**) and benzyl-substituted (**24** and **29**) compounds, the esters generally appear to be more potent than the amides. This difference in the potency may be due to the specific angle of the substituted groups or the increased electron density of the ester carbonyl compared to the amide carbonyl. Generating the methyl ester of the glyciny amide (**28**) increased the potency 4-fold compared to the corresponding free acid. Since the amide and esters appeared to be active, the amide bioisostere oxadiazole (**17**) was tested and had 38-fold less potency than the initial compound. Many compounds in this series could act as prodrugs following esterase or amidase catalyzed hydrolysis.³⁹

Many FAAH inhibitors work through carbamoylation of the catalytic serine residue.^{20,36,40} Inhibition through this mechanism is time-dependent because the inhibitory potency depends on the rate of carbamoylation. However, to our surprise, the potency of *t*-TUCB and two new inhibitors (**18** and **24**) does not change with time (Figure 2A), suggesting that these inhibitors are unlikely to inhibit through the formation of a covalent intermediate. URB597, an inhibitor known to carbamoylate FAAH, has a 3-fold increase in potency over the same period of time. To further test the mechanism of action of these inhibitors we determined the effect of varying substrate concentrations on the inhibitory potency of *t*-TUCB (Figure 2B). Results showed an increase in K_M^{app} with only a minor change in v_{max}^{app} indicating that inhibition primarily occurs through a competitive mechanism. Assuming a simple competitive model, the calculated K_i for *t*-TUCB is 156 ± 85 nM. Using the same analysis, the K_i for **18** and **24** are 9.1 ± 2.5 nM and 43 ± 25 nM, respectively.

Since the endogenous substrates, EETs and AEA, for sEH and FAAH, respectively, share the arachidonic acid backbone, it is reasonable to speculate *t*-TUCB may competitively inhibit FAAH by mimicking and displacing the substrate. Although these lipids are linear carbon chains, a folded orientation in the substrate tunnel may resemble the linked-ring structure of *t*-TUCB. The crystal structure of methoxy arachidonoyl phosphonate (MAP) bound to FAAH demonstrates the bound substrate is not linear in the active site but bends around the unsaturated bonds.⁴¹ To investigate this possibility, we docked *t*-TUCB, **18** and **24** into the FAAH active site using AutoDock Vina (Figure 3A and Supplementary Figure 1). Docking in the FAAH active site, the polar aromatic groups on *t*-TUCB, **18** and **24** form hydrogen-bond interactions with the catalytic serine and orient in a manner similar to its lipid

substrates when docked in the FAAH active site. In the case of *l*-TUCB and **18**, the A ring occupies the acyl-binding pocket of the active site; while **24** stays in the membrane access channel. The binding orientation from docking *l*-TUCB and **18** is similar to the co-crystal structure described for covalently bound PF-3845²⁰ and is consistent with the SAR described in this manuscript. If the 4-trifluoromethoxyphenyl group on ring A occupies the acyl-binding pocket, then modification of this group to bulky lipophilic groups such as an adamantyl group will be not suitable for this site and modification to smaller groups such as an unsubstituted phenyl will decrease the ability to stably occupy that pocket. Further, modification of ring B will either decrease the stability of that conformation in the case of the butane chain or force the molecule to unfavorable conformation in the case of the *cis* form of TUCB (*c*-TUCB) and the tri-substituted urea (**13**). Finally, modifications to the 4-substituent on ring C determine potency based on the interactions required to generate an acyl-intermediate; thus, we would predict a highly electrophilic organophosphate or trifluoromethylketone analogues that mimic these acyl-intermediates may improve the potency of future inhibitors.^{42, 43}

By comparison, the disubstituted urea on *l*-TUCB is known to form strong hydrogen bonds with the catalytic aspartate residue and the H-bond donating tyrosine residues in the active site of the sEH enzyme.⁴⁴ In the co-crystallization of *l*-TUCB in the active site of sEH (Figure 3B) the urea fits between the aspartate and the two tyrosine residues. The flanking pockets on either side of the catalytic site are large and can accommodate a variety of shapes. Thus, consistent with the SAR, a large variety of shapes may be accommodated by sEH on either side of the urea and potency will remain relatively high (IC₅₀ < 50 nM). The atomic coordinates and structure factors (code 6AUM) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

To test the selectivity of these inhibitors toward FAAH, we investigated their ability to inhibit a series of related enzymes, especially serine hydrolases (Table 3). *l*-TUCB, **18**, or **24** did not inhibit the tested carboxylesterases (hCE1 and hCE2), hydrolases involved in xenobiotic detoxification, or paraoxonases (PON1, PON2 and PON3), esterases involved in the regulation of atherosclerosis,⁴⁵ but **18** did partially inhibit arylacetamide deacetylase (AADAC), a poorly characterized enzyme that is known to metabolize several xenobiotics.⁴⁶ These results indicate that, unlike most known FAAH inhibitors that have poor selectivity against other serine hydrolases,^{47, 48} broad off-target inhibition of other serine hydrolases by *l*-TUCB, **18** and **24** is unlikely.

l-TUCB has been used extensively for studying the role of sEH in multiple animal models of pain.^{33, 34, 49} To test whether potency towards FAAH may account for the efficacy in these animal models, the effectiveness of *l*-TUCB, **18** and **24** to inhibit FAAH was measured on other species than human using brain microsome preparations. As positive controls, the IC₅₀ values of two well characterized FAAH inhibitors, PF-3845 and URB-597 were measured also (Table 4, Supplementary Data). These two inhibitors are structurally distinct and have excellent selectivity in rat brain microsomes for the FAAH enzyme over other hydrolases.^{20,38} As expected, both inhibitors blocked >80% of the activity in the microsomes of the different species tested (Supplementary Figure 2). Interestingly, both *l*-TUCB and **24** are less

potent against the FAAH of all other species tested than human FAAH. **18** had comparable IC₅₀s for FAAH from all species tested except from rat, where **18** is 10-fold less potent. The hypothesized mechanism of inhibition based on docking relies on a relatively specific fit in the enzyme active site. Thus, we speculate small modifications to this acyl-binding pocket across species accounts for the substantial species selectivity observed for these inhibitors. By comparison, sEH inhibition relies primarily on strong hydrogen-bonding interactions and less on enzyme fit. *t*-TUCB, **18** and **24** also had species differences on sEH but all three inhibitors were still relatively potent in all species tested (IC₅₀ < 100 nM). Due to the low potency towards FAAH relative to sEH (Table 4), it is unlikely that FAAH inhibition contributes to the previously observed effects of *t*-TUCB on pain in a mouse model⁴⁹.

In conclusion, we have designed dual sEH/FAAH inhibitors with nanomolar potency towards both enzymes. These inhibitors have potency independent of incubation time and a mechanism consistent with competitive inhibition. Furthermore, these inhibitors are selective for FAAH over other serine hydrolases and have relatively low species selectivity. Use of these inhibitors will support future biological studies investigating the importance of dual sEH/FAAH inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations and definitions

AADAC	arylacetamide deacetylase
AEA	arachidonoyl ethanolamide
EETs	epoxyeicosatrienoic acids
FAAH	fatty acid amide hydrolase
hCE	human carboxylesterase
MAP	methoxy arachidonoyl phosphonate
NSAIDS	non-steroidal anti-inflammatory drugs
PF-3845	N-(pyridin-3-yl)-4-(3-((5-(trifluoromethyl)pyridin-2-yl)oxy)benzyl)piperidine-1-carboxamide

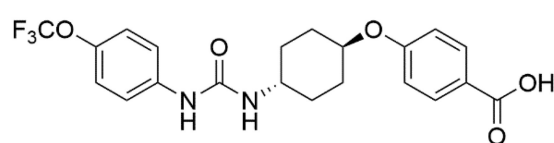
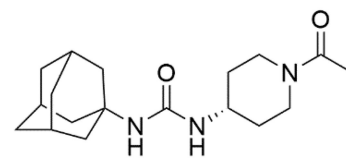
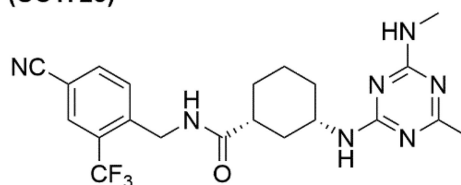
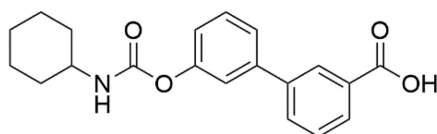
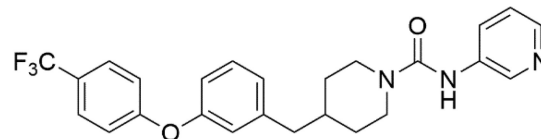
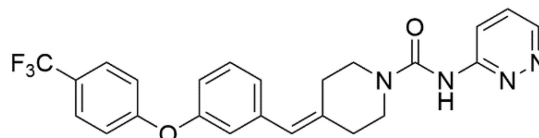
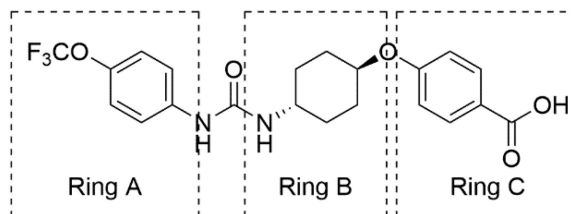
PON	paraoxonase
sEH	soluble epoxide hydrolase
<i>t</i>-TUCB	<i>trans</i> -4-[4-(3-trifluoromethoxyphenyl-1-ureido)-cyclohexyloxy]-benzoic acid
URB597	3'-carbamoyl-[1-1'-biphenyl]-3-yl cyclohexylcarbamate

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A**sEH Inhibitors*****t*-TUCB
(UC1728)****AR9281
(UC1153)****GSK2256294A****FAAH Inhibitors****URB597****PF-3845****PF-04457845****B****Figure 1.**

A. Structures of several sEH inhibitors (*t*-TUCB, TPPU, GSK2256294A, AR9281) and FAAH inhibitors (URB597, PF-3845, PF-04457845) **B.** Modifications of *t*-TUCB skeleton were tested at the trifluoromethoxyphenyl group (“Ring A”), the *trans* cyclohexyl group (“Ring B”) or the benzoic acid (“Ring C”).

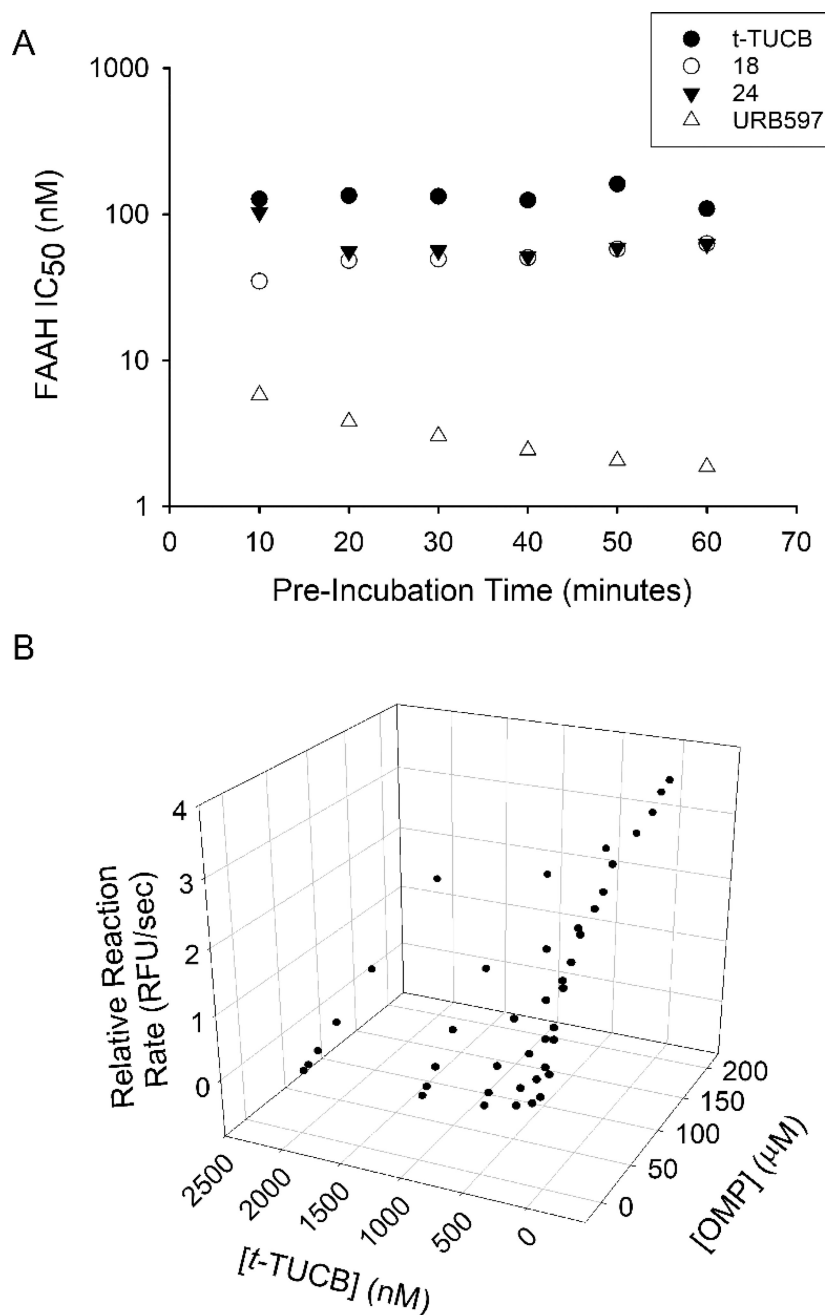


Figure 2.

A. *t*-TUCB, **18**, **24** and URB597 were pre-incubated with FAAH enzyme at various time intervals followed by addition of substrate ($[S]_{\text{final}} = 5 \mu\text{M}$). Potency of *t*-TUCB, **18** or **24** inhibition is independent on pre-incubation time while inhibition by URB597 is time dependent. **B.** Reaction kinetics of OMP hydrolysis by FAAH was measured at various [OMP] and [*t*-TUCB] to determine inhibition mechanism. As [*t*-TUCB] increases the K_M^{app} substantially increases (from $K_M^{\text{app}} = 30 \mu\text{M}$ to $K_M^{\text{app}} > 200 \mu\text{M}$) and the v_{max} marginally increases (4.0 to 5.4 RFU/sec), consistent with a primarily competitive mechanism of inhibition.

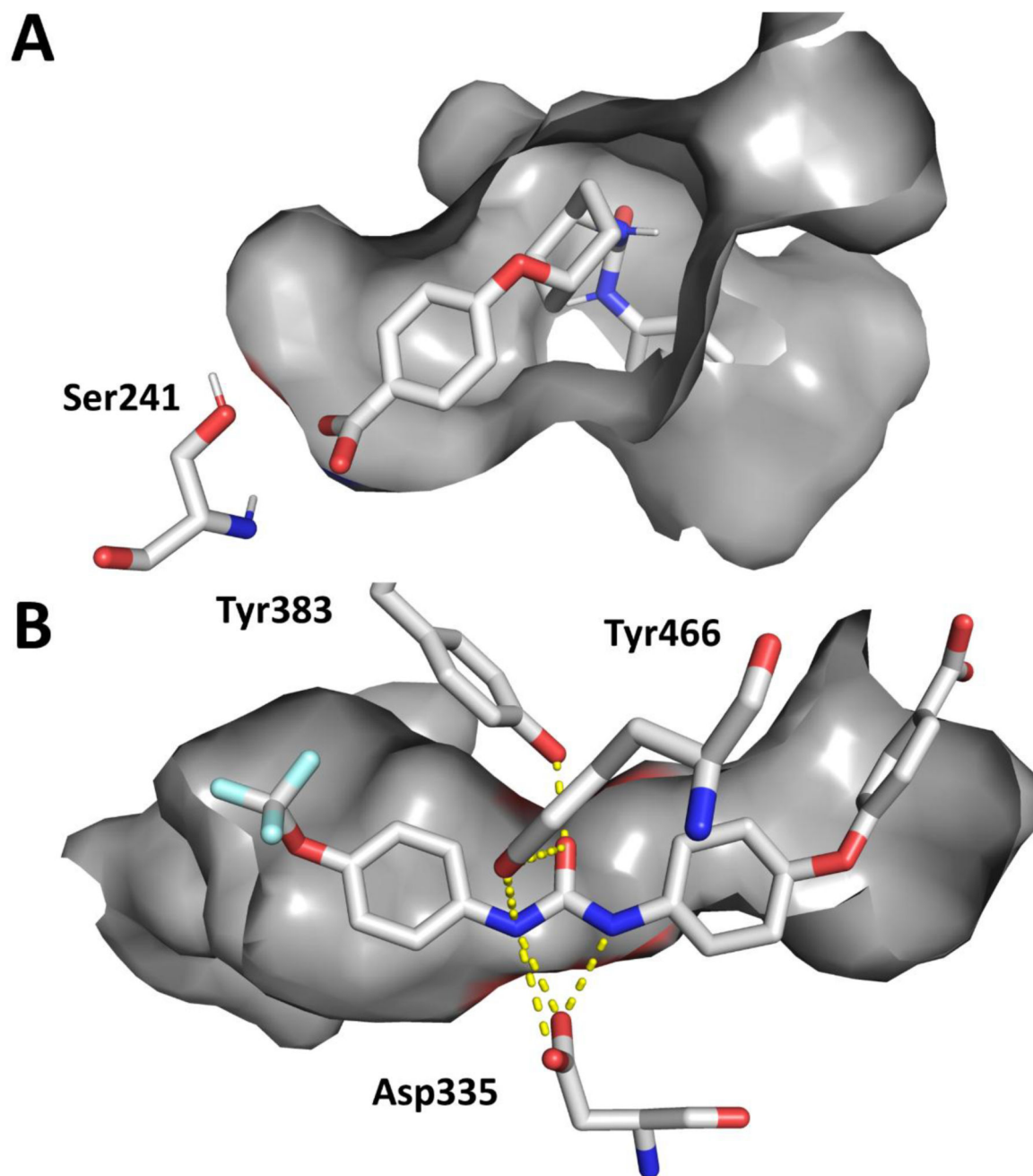
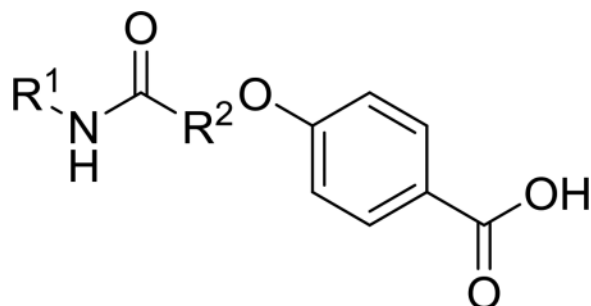


Figure 3.

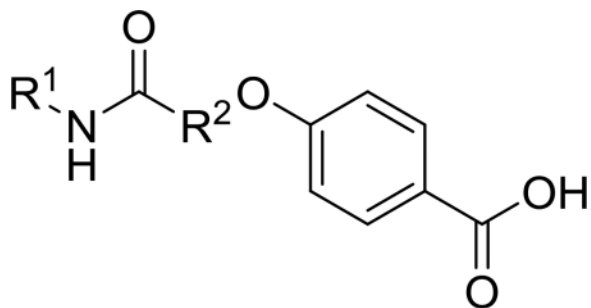
A. Docking of *l*-TUCB (yellow) in the active site of FAAH (PDB: 1MT5) using AutoDock Vina and **B.** Co-crystal structure of *l*-TUCB (yellow) in the active site of sEH. The key catalytic residues for FAAH (Ser241) and sEH (Asp335) are represented in addition to the proton-donating residue on sEH (Tyr 383 and Tyr466). Hydrogen bonds are represented on the co-crystal structure as yellow dashed lines.

Table 1

Potency of inhibitors with modifications on Ring A (R^1) and Ring B (R^2) against both human sEH and human FAAH.



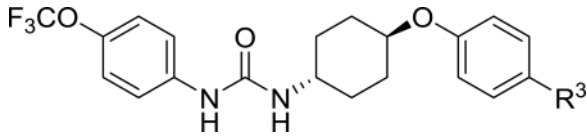
	R^1	R^2	IC_{50} (nM)	
			hsEH	hFAAH
URB597			1100	23
PF-3845			>10,000	0.6
<i>l</i> -TUCB			0.8	140
2			30	9,200
3			18	4,600
4			7	680
5			6	>10,000
6			3	>10,000
<i>c</i> -TUCB			2	2,800
9			15	>10,000

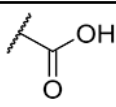

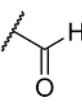
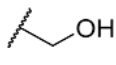
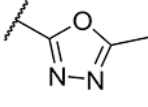


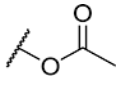
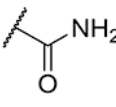
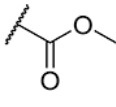
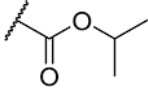


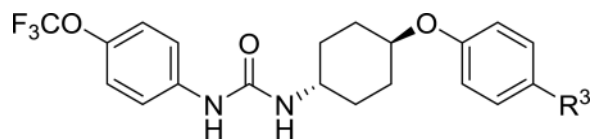
	R ¹	R ²	IC ₅₀ (nM)	
			hsEH	hFAAH
10			7	170
13			8	1,800

Table 2

Potency of inhibitors with modifications on Ring C (R^3) against both human sEH and human FAAH.



	R^3	IC_{50} (nM)	
		hsEH	hFAAH
<i>ε</i> -TUCB		0.8	140
14		5	>10,000
15		4	1,100
16		3	5,800
17		4	5,300
18		2	120
19		3	>10,000
20		4	120
21		2	70
22		7	35
23		5	400



	R ³	IC ₅₀ (nM)	
		hsEH	hFAAH
24		3	24
25		2	170
26		2	100
27		2	130
28		3	30
29		5	1,100

Table 3IC₅₀ values of compounds *t*-TUCB, **18** and **24** on other human serine hydrolases.

Enzyme	IC ₅₀ (nM)		
	<i>t</i> -TUCB	18	24
FAAH	140	120	24
sEH	0.8	2	3
mEH	>10,000	>10,000	>10,000
hCE1	>10,000	>10,000	>10,000
hCE2	>10,000	>10,000	>10,000
PON1	>10,000	>10,000	>10,000
PON2	>10,000	>10,000	>10,000
PON3	>10,000	>10,000	>10,000
AADAC	>10,000	5,400	>10,000

Table 4
 IC₅₀ values of compounds ι -TUCB, **18** and **24** on sEH and FAAH in multiple species.

Enzyme	Species	IC ₅₀ (nM)				
		ι -TUCB	18	24	PF-3845	URB597
FAAH	Human	140	120	24	0.6	23
	Mouse	6,300	350	510	12	11
	Rat	>10,000	1,700	>10,000	4.4	12
	Cat	1,200	92	240	<0.1	12
	Dog	3,900	280	280	0.4	22
	Horse	3,300	270	87	0.3	7
sEH	Human	0.8	2	3	-	-
	Mouse	5.7	0.4	2.1	-	-
	Rat	54	1.0	1.6	-	-
	Cat	2.0	4.3	42	-	-
	Dog	5.6	11	28	-	-
	Horse	19	21	77	-	-