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

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ABSTRACT

Multi-target inhibitors have become increasing 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-l-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.

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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] inflammation[9] 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 stress[16] 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 (Fig. 1).[17–19] GSK2256294A has not progressed to further stages

Abbreviations: 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; *t*-TUCB, *trans*-4-[4-(3-trifluoromethoxyphenyl-l-ureido)-cyclohexyloxy]-benzoic acid; URB597, 3′-carbamoyl-[1,1′-biphenyl]-3-yl cyclohexylcarbamate.

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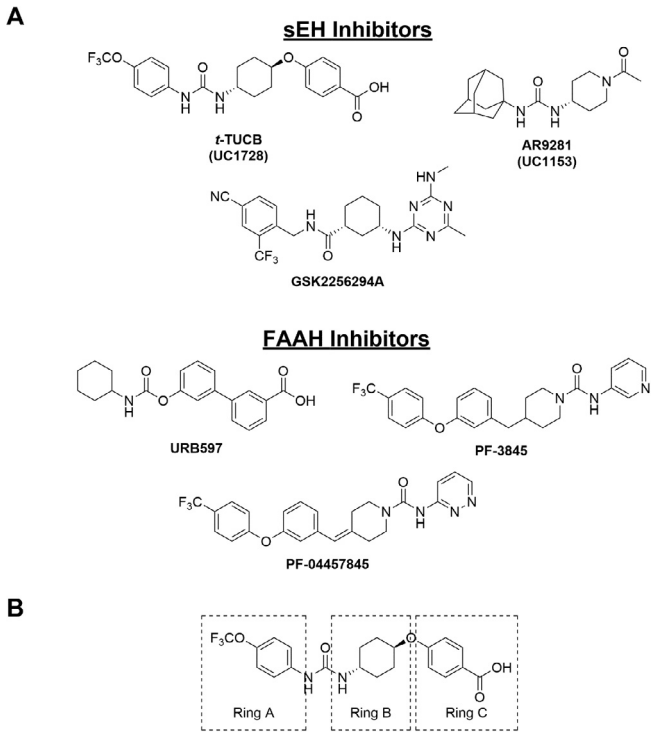

Fig. 1. A. Structures of several sEH inhibitors (*t*-TUCB, TPPU, GSK2256294A, AR9281) and FAAH inhibitors (URB597, PF-3845, PF-04457845) B. Modifications of *t*-TUCB skeleton were tested at the trifluoromethoxyphenyl group ("Ring A"), the *trans* cyclohexyl group ("Ring B") or the benzoic acid ("Ring C").

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-l-ureido)-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 (Fig. 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 glycinyl (**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

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