Title

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4-Phenyl-thiazole-based dual inhibitors of fatty acid amide hydrolase and soluble epoxide hydrolase do not alleviate orofacial inflammatory pain in female rats

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ABSTRACT

Pain arising from trigeminal systems such as headache is common, debilitating, and current treatments (e.g., sumatriptan) are limited. New treatments that target novel mechanisms of action may be required to innovate both short- and long-term pain therapy. Fatty acid amide hydrolase and soluble epoxide hydrolase are two pain-related enzymes that regulate pain and inflammation via independent pathways. We have previously demonstrated that simultaneous inhibition of these enzymes using a novel dual inhibitor alleviates acute inflammatory pain in the hindpaw and does not depress wheel running in rats. Here, we expanded on these findings and performed structure-activity relationships of our lead compound, the 4-phenyl-thiazole-based dual inhibitor SW-17, to generate 18 analogs and tested them for their inhibition at both enzymes. Conversion of the sulfonamide group to a tertiary amine led to a general decrease in the potency for the sEH enzyme, while this change was well-tolerated at the FAAH enzyme yielding several strong inhibitors. Six selected inhibitors were evaluated in mouse and rat sEH inhibition assays and results showed a species difference, i.e. 4-phenyl-thiazole-based analogs are significantly less or not active in mouse sEH compared to human and rat enzymes. The most potent inhibitor, SW-17, was evaluated in a plasma stability assay in human and rat plasma and showed moderate stability. However, SW-17 did not alleviate orofacial inflammatory pain in female rats compared to the traditional anti-migraine agent sumatriptan. Although modification of 4-phenyl-thiazole-based dual inhibitor SW-17 changes potencies at both FAAH and sEH, these approaches may not produce antinociception against trigeminal pain. Key Words: polypharmacology, formalin, inflammation, enzyme inhibition, structure-activity relationship studies

Introduction

Activation of the trigeminal system generates pain in the head and face area and increases sensitivity to various stimuli (e.g., touch, temperature, light) [1]. Further, this activation releases inflammatory mediators that further sensitize the trigeminal system to generate and maintain pain [1]. Treatment options such as nonsteroidal anti-inflammatory drugs (NSAIDs) or migraine-specific treatments (e.g., sumatriptan) provide some relief, but these drugs may lose efficacy with long-term use or produce adverse effects (e.g., ulcers or medication-overuse headache) [2]. As such, it is important to identify new treatments with novel mechanisms of action to develop better short- and long-term therapies for trigeminal pain.

Fatty acid amide hydrolase (FAAH) and soluble epoxide hydrolase (sEH) are two enzymes that regulate both pain and inflammation [3,4]. FAAH degrades endogenous cannabinoids such as anandamide (AEA) produced within the body, thereby preventing the ability of endogenous cannabinoids to bind to their respective cannabinoid receptors to produce antinociception. Inhibiting FAAH alone to increase the concentration of endogenous cannabinoids produces antinociception in many

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different animal models of pain [5–7]. sEH mediates the hydrolysis of epoxyeicosatrienoic acids (EETs) into dihydroxyeicosatrienoic acids (DHETs), which promote inflammation and pain. Like FAAH, inhibition of sEH alone also produces antinociception in many different animal models of pain highlighting its therapeutic utility [4,8,9]. Further, one study demonstrated that inhibition of FAAH and sEH at the same time using their respective inhibitors can produce synergistic pain relief in animal models of inflammatory and neuropathic pain, indicating that simultaneous inhibition of both FAAH and sEH may provide greater antinociception than inhibiting either enzyme alone [10]. Thus, both FAAH and sEH are promising targets for treating pain.

We have previously demonstrated that simultaneous inhibition of FAAH and sEH using *one* compound alleviates formalin-induced inflammatory pain in the hind paw and does not depress wheel running in naïve rats [11]. In this study, we performed structure-activity relationship studies on our lead dual FAAH/sEH inhibitor to generate 18 new analogs (Scheme 1). These analogs were then evaluated in vitro to determine their ability to inhibit human FAAH and human sEH, and five selected analogs were tested as substrates for mouse and rat sEH. The most potent inhibitor identified previously by us, SW-17 [12], was subsequently evaluated in mouse and rat sEH inhibition assays, in stability assays in rat and human plasma and in vivo against formalin-induced orofacial pain mediated by trigeminal ganglia.

Materials and methods

Chemistry

All solvents and reagents were obtained from Sigma–Aldrich, TCI, and Acrōs Organic and used without further purification. Analytical thin-layer chromatography (TLC) was performed on aluminum plates precoated with silica gel, also obtained from Sigma–Aldrich. Flash chromatography was carried out on Teledyne CombiFlash Rf+ system. NMR spectra were recorded with a Bruker 400 MHz NMR spectrometer. Proton chemical shifts are reported relative to the residual solvent peak (chloroform = 7.26 ppm, dimethyl sulfoxide = 2.50 ppm or methanol = 3.31 ppm) as follows: chemical shift (δ), proton ID, multiplicity (s = singlet, bs = broad singlet, d = doublet, bd = broad doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet, integration, coupling constant(s) in Hz). Carbon chemical shifts are reported relative to the residual deuterated solvent signals (chloroform = 77.2 ppm, dimethyl sulfoxide = 39.5 ppm or methanol = 49.00 ppm). All compounds described were of >95% purity. Purity was confirmed on a high-resolution liquid chromatography mass spectrometer (ThermoFisher Scientific system). Elution was isocratic with water (30%, +0.1% formic acid) and acetonitrile (70%, +0.1% formic acid) at a flow rate of 0.4 mL/min. For compounds containing chlorine the ^{35}Cl isotope was measured. Microwave reactions were carried out in a CEM 2.0 Discover microwave synthesizer. Human recombinant FAAH enzyme (Item No. 100101183, Batch No. 0523867) was obtained from Cayman Chemical. Human, rat, and mouse sEH enzyme were obtained from University of California, Davis. The general procedures for the synthesis of the compounds and their characterization are described in the Supporting Information.

In vitro studies

IC_{50} *FAAH Assay Conditions* [13]: N-(6-methoxypyridin-3-yl) octanamide (OMP - synthesized in our lab [14]) was used as the fluorescent substrate. Human FAAH (1 nM) was incubated with the inhibitor for 5 min in pH 8 Bis–Tris/HCl buffer (25 mM) containing 0.1 mg/mL of BSA at 37 °C prior to substrate introduction ([S] = 5 μM). Activity was determined by monitoring the appearance of 6-methoxypyridin-3-amine over 10 min by fluorescence detection with an excitation wavelength of 303 nm and an emission wavelength of 394 nm. Reported IC_{50} values are the average of the three replicates with at least two datum points above and at least two below the IC_{50}.

IC_{50} *sEH Assay Conditions* [15]: Cyano(2-methoxynaphthalen-6-yl) methyl *trans*-(3-phenyloxyran-2-yl) methyl carbonate (obtained from University of California, Davis) was used as the fluorescent substrate. Human sEH (1 nM) or rat (1 nM) or mouse sEH (2 nM) was incubated with the inhibitor for 5 min in pH 7.4 Bis–Tris/HCl buffer (25 mM) containing 0.1 mg/mL of BSA at 30 °C prior to substrate introduction ([S] = 5 μM). Activity was determined by monitoring the appearance of 6-methoxy-2-naphthaldehyde over 10 min by fluorescence detection with an excitation wavelength of 330 nm and an emission wavelength of 465 nm. Reported IC_{50} values are the average of the three replicates with at least two datum points above and at least two below the IC_{50}.

The fluorescent assay as performed here has a standard error between 10 and 20%, suggesting that differences of two-fold or greater are significant [15]. None of the compounds showed a greater error of 20%. To determine the IC_{50}, we measured a total of 8 concentrations, and always used 2 points above and 2 points below 50%, totaling 5 points.

Plasma stability assays

Plasma stability tests was conducted by Cyprotex US, LLC (Framingham, MA). SW-17 (5 μM) was incubated with human or rat plasma (pH 7.4, ± 0.1, adjusted if necessary) at 37 °C. At 0, 15, 30, 60, and 120 min, an aliquot was removed from each experimental reaction and mixed with ice-cold stop solution. Stopped reactions were kept on ice for at least 10 min. Samples were filtered by centrifugation for 1 min at 500 rcf and 4 °C, and the supernatants were analyzed by LC-MS/MS.

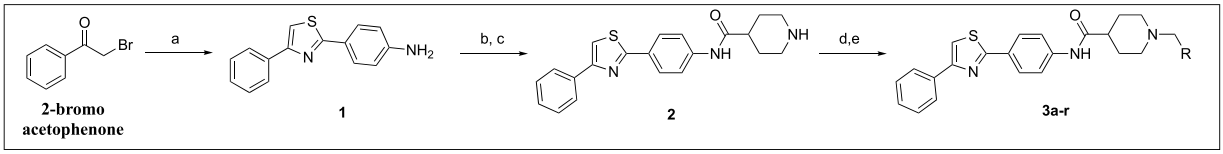
Analysis: Data were calculated as% parent remaining by assuming 0 min time point peak area ratio (analyte/internal standard) as 100% and dividing remaining time point peak area ratios by 0 min time point peak area ratio. Data were subjected to fit first-order decay model to calculate slope and thereby half-life:

$$\mathbf{Eliminationrateconstant(k)} = (-\mathbf{gradient})$$

$$\mathbf{Half-life(t_{1/2})(min)} = \mathbf{0.693 / k}$$

Subjects

Data were collected from 36 female Sprague-Dawley rats purchased from Charles River (Hollister, CA, USA). All rats were at least 50 days old at the start of the study and randomly assigned to treatment groups. Rats were housed in pairs on a 12/12-hour light/dark cycle (lights off at 1500 h). Food and water were available ad libitum except during testing procedures. All procedures were approved by the California State



Scheme 1. *Reagents and conditions:* (a) 4-aminothiobenzamide, iPrOH, 2.0 h, 60–70 °C, 95%; (b) 1-Boc-piperidine-4-carboxylic acid, EDC, DMAP, DCM, 25 min, 80 °C, microwave irradiation, 35%; (c) TFA, DCM, 0 °C - rt, 24 h, 92%; (d) $NaHCO_3$, EtOAc; (e) STAB, R-aldehyde (See Table 1 for R), DCM, 30 min, 80 °C, microwave irradiation, 39–62%.

University, East Bay Institutional Animal Care and Use Committee.

Orofacial formalin test

Rats were briefly anesthetized with isoflurane to reduce stress and improve animal welfare as previous studies have shown that brief anesthesia before formalin injection into the hind paw does not alter behavioral responses in the late phase of the test [16]. Dilute formalin (2%, 100 μL) was injected subcutaneously into the perinasal area lateral to the nose. Rats were immediately placed in Plexiglas chambers on an elevated mesh rack for observation. The time spent exhibiting face-washing behavior, characterized by rubbing the injected area with the forepaws or ipsilateral hind paw was recorded in 3 min intervals for 45 min. Experimenters were blinded to treatment conditions. Rats were euthanized immediately after data collection.

Dual inhibitor effects on formalin-induced orofacial pain

SW-17 was dissolved in vehicle (20% ethanol, 20% cremophor, and 60% saline) and delivered via intraperitoneal injection at a volume of 1 mL/kg using a 26-gage needle. To determine the effects of SW-17 on formalin-induced pain in the orofacial region, rats received one injection of either SW-17 (1 and 3 mg/kg), sumatriptan dissolved in saline (1 mg/kg), or vehicle 30 min prior to injection of formalin. 30 min after injection, rats were injected with formalin and observed as described above. Doses were obtained from our previous work with dual FAAH/sEH inhibitors [17] and migraine pain [18].

Data analysis

All data are expressed as mean ± SEM except where stated. Face-washing behavior produced by formalin was converted to area under the curve and analyzed using one-way analysis of variance (ANOVA) on GraphPad Prism (degrees of freedom = number of data points – number of time intervals). Statistical significance was defined as a probability of < 0.05.

Results

Structure activity relationship studies of 4-phenyl-thiazol-2yl-phenyl analogs

Previously we identified 1-((2-chlorophenyl)sulfonyl)-N-(4-(4-(p-tolyl)thiazol-2-yl)phenyl)piperidine-4-carboxamide (SW-17), a potent FAAH/sEH dual inhibitor with IC_{50} values of 9.8 nM and 2.5 nM in human FAAH and human sEH respectively [12]. Our SAR showed that placement of various groups on the phenyl moiety (Fig. 1– red box) did not affect inhibition potencies and this set of analogs bind to human FAAH as reversible inhibitors. Since our SAR study concluded that piperidine ring linked to the phenyl group via amide bond is important for dual inhibition (Fig. 1– blue box), we decided to further explore SAR space of the left side of these inhibitors, i.e. the importance of the sulfonamide moiety for the inhibition of both enzymes, Fig. 1– red dotted circle. Replacing a sulfonyl with methylene group will decrease the size of that part of the molecule and provides more flexibility of the overall molecule, since sulfonamide group is more rigid. This change will also remove existing hydrogen bonding, i.e. oxygens on sulfonyl groups are potential hydrogen bond acceptors. However, basic nitrogen can be protonated at the physiological pH which can provide new non-covalent interactions with the neighboring amino acid residues in the binding pockets of both enzymes. All analogs **3a-r** were synthesized using 5-steps synthetic route shown in Scheme 1.

Starting from two commercially available compounds, 2-bromoacetophenone and 4-aminothiobenzamide and using Hantzsch thiazole synthesis reaction conditions we were able to obtain aniline **1** in 95% yield. Microwave-assisted EDC coupling of **1** with commercially available 1-boc-piperidine-4-carboxylic acid furnished amide intermediate in moderate yields. The boc-protecting group was removed using trifluoroacetic acid (TFA), which provided the key intermediate **2** in high yield. This salt was subsequently free-based with sodium bicarbonate and immediately used in a last step – a reductive amination with sodium triacetoxyborohydride as a reducing agent, to obtain the final compounds **3a-r** in moderate yields.

All synthesized compounds were biologically evaluated in human FAAH and human sEH inhibition assays, Table 1, and selected inhibitors were evaluated in mouse and rat sEH inhibition assays.

Our SAR study started with the introduction of the cyclohexyl group, **3a**. This analog, although still potent for the FAAH (IC_{50} = 37.7 nM), showed significantly lower potency for sEH. Next, we added the phenyl ring (analog **3b**), and we observed similar activity for FAAH compared to SW-17, but again decreased potency for sEH, with the IC_{50} value of 222.7 nM. However, we were encouraged with these results, since the phenyl ring offers access to many diverse chemical substituents that could improve sEH inhibition. We first synthesized a 3-pyridyl analog, **3c**, and observed again a very good potency for FAAH (IC_{50} = 16.9 nM) and much better potency for sEH (IC_{50} = 38.8 nM) compared to phenyl analog **3b**. Since this analog had very good potencies for both enzymes, we decided to screen it in mouse and rat sEH inhibition assays as well. We previously noticed that certain functional groups show species differences, i.e. compounds were active in human and rat sEH, but significantly less active in mouse sEH [11]. Indeed, this compound showed a similar trend – it was not active for mouse sEH and was very potent inhibitor in rat sEH assay. It will be interesting to follow up on this analog and introduce 2- or 4- pyridyl groups, as well as the placement of various groups (e.g., alkyl, halogens, etc.) on the pyridyl moiety. Next, we decided to test the effects of fluoro- groups on the inhibition at both

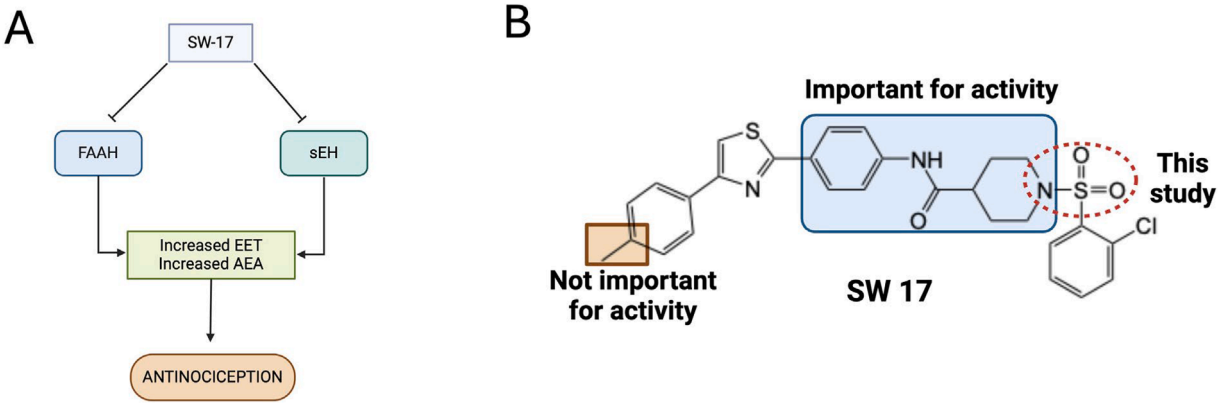
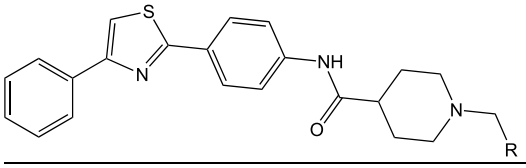
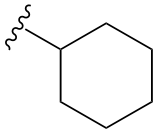
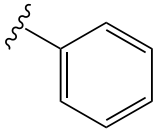
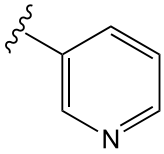
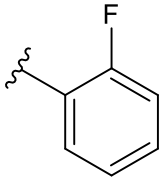
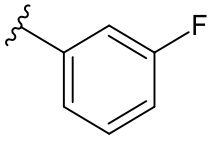
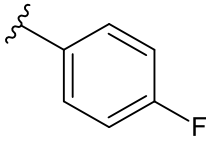
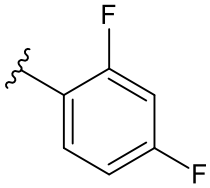
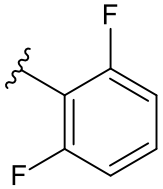


Fig. 1. A) General schematic of dual FAAH/sEH inhibition pathways. B) Schematic of relevant groups of our dual FAAH/sEH inhibitor.

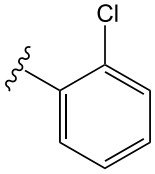
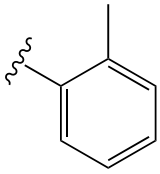
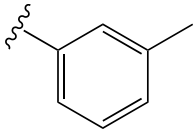
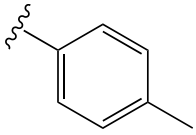
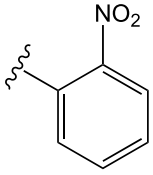
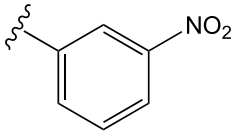
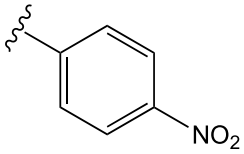
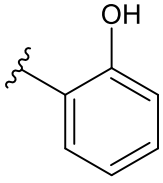
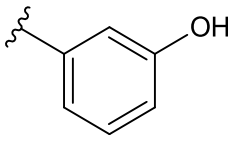
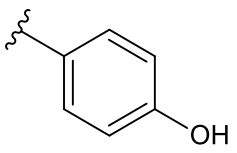
Table 1
Fatty acid amide hydrolase (FAAH) and soluble epoxide hydrolase (sEH) inhibitory activities.



Compound	R	hFAAH IC_{50} (nM)[a]	hsEH IC_{50} (nM)[a]	msEH IC_{50} (nM)[a]	rsEH IC_{50} (nM)[a]
URB 597	–	2.7	–	–	–
TPPU	–	–	0.7	1.9	3.1
SW-17	–	9.8	2.5	1670.3	3.9
3a		37.6	1154.5	–	–
3b		11.1	222.7	–	–
3c		16.9	38.8	>10,000	12.2
3d		168.3	1177.6	–	–
3e		73.2	376.7	–	–
3f		48.8	10.7	158.2	11.7
3 g		230.8	87.7	>10,000	44.1
3h		6.7	154.3	–	–

(*continued on next page*)

Table 1 (*continued*)

Compound	R	hFAAH IC_{50} (nM)[a]	hsEH IC_{50} (nM)[a]	msEH IC_{50} (nM)[a]	rsEH IC_{50} (nM)[a]
3i		165.3	2042.1	1682.5	205.0
3j		114.5	163.1	–	–
3k		75.3	301.1	>10,000	57.1
3l		209.0	525.2	–	–
3m		242.6	100.8	–	–
3n		286.5	255.9	–	–
3o		3.1	1722.3	–	–
3p		60.9	136.3	–	–
3q		9.8	224.5	–	–
3r		23.2	281.2	–	–

[a] Reported IC_{50} values are the average of three replicates. The assay as performed here has a standard error between 10 and 20% suggesting that differences of two-fold or greater are significant.

enzymes. Placement of the fluoro- group at the ortho- and meta- positions, analogs **3d** and **3e**, respectively, led to the lower potencies at both FAAH and sEH enzymes, but we were able to improve the potency for sEH enzyme (IC_{50} = 10.7 nM), with placing a fluoro- group at the para-position, analog **3f**. Since this analog had a moderate potency for FAAH (IC_{50} = 48.8), we decided to screen it in mouse and rat sEH inhibition assays. It also showed to be less active in mouse inhibition assay with IC_{50} value of 158.2 nM, while it was a strong inhibitor in rat sEH inhibition assay with the IC_{50} value of 11.7 nM. The introduction of two fluoro- groups to the ring (2,4- disubstitutions), analog 3 g showed moderate potencies at human FAAH, rat and human sEH and a complete loss of activity for mouse sEH. 2,6-disubstituted fluoro analog 3 h, showed an excellent potency for FAAH (IC_{50} = 6.7 nM), but just moderate activity at human sEH with IC_{50} value of 154.3 nM. We were particularly interested to see how will the 2-chlorophenyl-analog **3i** perform in the inhibition assays. This is the most structurally similar compound to our lead compound SW-17, i.e. it has a methylene group in place of the sulfonyl group. We observed significant decrease in activity in all inhibition assays and abandoned further SAR exploration of chloro-groups. Our previous SAR on the right side with the benzothiazole dual FAAH/sEH inhibitors showed that methyl groups are well tolerated on the left side of the molecule and contribute to high inhibition potencies at both enzymes. Thus, we synthesized analogs **3j, 3k** and **3l** with the methyl groups placed on the phenyl ring at the ortho-, meta- and para- position, respectively. These analogs possess moderate inhibition of both FAAH and sEH, with the *meta*-analog **3k** being the most potent in this series of analogs. Next, we decided to test the effects of the nitro- group on the inhibition by placing this strong polar electron withdrawing group into the *ortho*-, *meta*-, and *para*- positions on the phenyl ring, analogs 3 m, **3n** and **3o**, respectively. Adding a nitro group did not improve the potencies in the *ortho* and *meta* positions, but surprisingly we observed a strong inhibition at FAAH with the *para*-analog, **3o**, with the IC_{50} value of 3.1 nM, performing better even than the lead compound SW-17. However, this group significantly affected the inhibition potency of the sEH enzyme. Finally, we tested the effects of the polar hydroxy group on the inhibition potencies with the analogs **3p, 3q** and **3r**. Placing the hydroxy group at the ortho-position led to decrease in the potencies at sEH, but we were able to observe excellent inhibition potencies for FAAH with the *meta*-analog, **3q** and the para-analog **3r**. Since none of the 18 synthesized analogs were better than our lead compound SW-17, we decided to turn our attention to SW-17 and perform more in vitro and in vivo tests. We first evaluated the SW-17 in mouse and rat sEH inhibition assays (previously we just reported the human FAAH and human sEH inhibition potencies) and noticed that SW-17 is a strong inhibitor of rat sEH (IC_{50} = 3.9 nM) and is a weak inhibitor in mouse sEH.

Plasma stability assay

As part of our pharmacokinetic studies, we performed the plasma stability assay. Enzymes in plasma represent a significant part of the drugs metabolism and testing new drugs for stability in plasma is an important step in drug discovery process. In addition, there is known species difference in plasma enzymes, thus we decided to test our lead compound SW-17 in both human and rat plasm stability assays before we proceeded with the in vivo evaluation. As summarized in Table 2, SW-17 showed stability of 410 min in human plasma, outperforming the standard, FDA-approved drug Warfarin, which is known for its stability in plasma. The data show that species differences are significant, and SW-17 showed only half-life of ~30 min in rat plasma, which is lower than the standard Warfarin. Nevertheless, we concluded that the stability of SW-17 in plasma is good enough to continue with the in vivo testing.

Table 2
In vitro half-lives in human and rat plasma.

Compound	Species	Half-life (min)
SW-17	Human plasma	410
Warfarin	Human plasma	304
SW-17	Rat plasma	31.1
Warfarin	Rat plasma	159

In vivo assessment

Fig. 2A shows the time spent exhibiting face-washing behavior over a 45 min period following injection of dilute formalin into the perinasal area. Fig. 2B shows the area under the curve over the same period for each group. A one-way analysis of variance revealed that pain behaviors in female rats pre-treated with the traditional anti-migraine drug sumatriptan, SW-17, or vehicle differed [$F(3, 480) = 3.04$, $p = 0.028$]. A Tukey post-hoc test revealed that rats receiving 1 mg/kg sumatriptan exhibited less face-washing behavior over the 45 min period compared to animals receiving vehicle ($p < 0.05$). There were no differences between rats treated with vehicle or any dose of SW-17 ($p > 0.05$).

Discussion

Our SAR study revealed that strong FAAH or sEH inhibitors can be obtained without a sulfonamide moiety. For example, placement of the nitro group in the *para*-position of the phenyl ring (analog **3o**) with the IC_{50} value of 3.1 nM in the FAAH inhibition assay, or placement of the fluoro-group in the *para*-position (analog **3f**) with the IC_{50} values of 10.7 nM and 11.7 nM, in human and rat sEH inhibition assays, respectively. These novel compounds that have inhibitor activity for at least one of these two enzymes can be further investigated as potential therapeutics in other diseases such as cardiovascular, depression, Alzheimer's disease, etc. [19–21]. However, we were not able to obtain the required balance to inhibit both enzymes simultaneously with the replacement of sulfonyl group with the methylene group, except for the 3-pyridil analog (analog **3c**), which might provide strong dual inhibition for future SAR explorations.

Previous studies have demonstrated that inhibition of FAAH and sEH using their respective inhibitors produces synergy and enhances antinociception than inhibiting either enzyme alone [10]. This is particularly important as exploiting more than one pharmacological target may increase efficacy and increase potency, thereby limiting the potential for side effects associated with higher doses or multiple administrations. Using one compound to simultaneously inhibit both FAAH and sEH may avoid drug-drug interactions, simplify the administration of the therapeutic, and dramatically simplify the registration procedure of the combined therapies. This concept of polypharmacology (i.e., affecting multiple targets simultaneously) has been used before to explore pain treatments, even in the context of FAAH and sEH [22].

Engaging trigeminal systems and activating sensory neurons using dilute formalin in the perinasal area produces reliable face-washing behavior in rats [23,24]. However, pre-treatment of SW-17 did not inhibit face-washing behavior indicating that SW-17 may only be efficacious under certain conditions. It is unclear as to why dual inhibition of FAAH and sEH may only produce antinociception against hind paw inflammatory pain [12] and not orofacial pain. Both injection types produce acute inflammatory pain that resolves within an hour. One potential reason for this difference could be the engagement of two different anatomical systems. A hind paw injection of formalin engages sensory neurons in the hind paw that transmit pain via neurons originating in dorsal root ganglia [25]. A perinasal injection of formalin

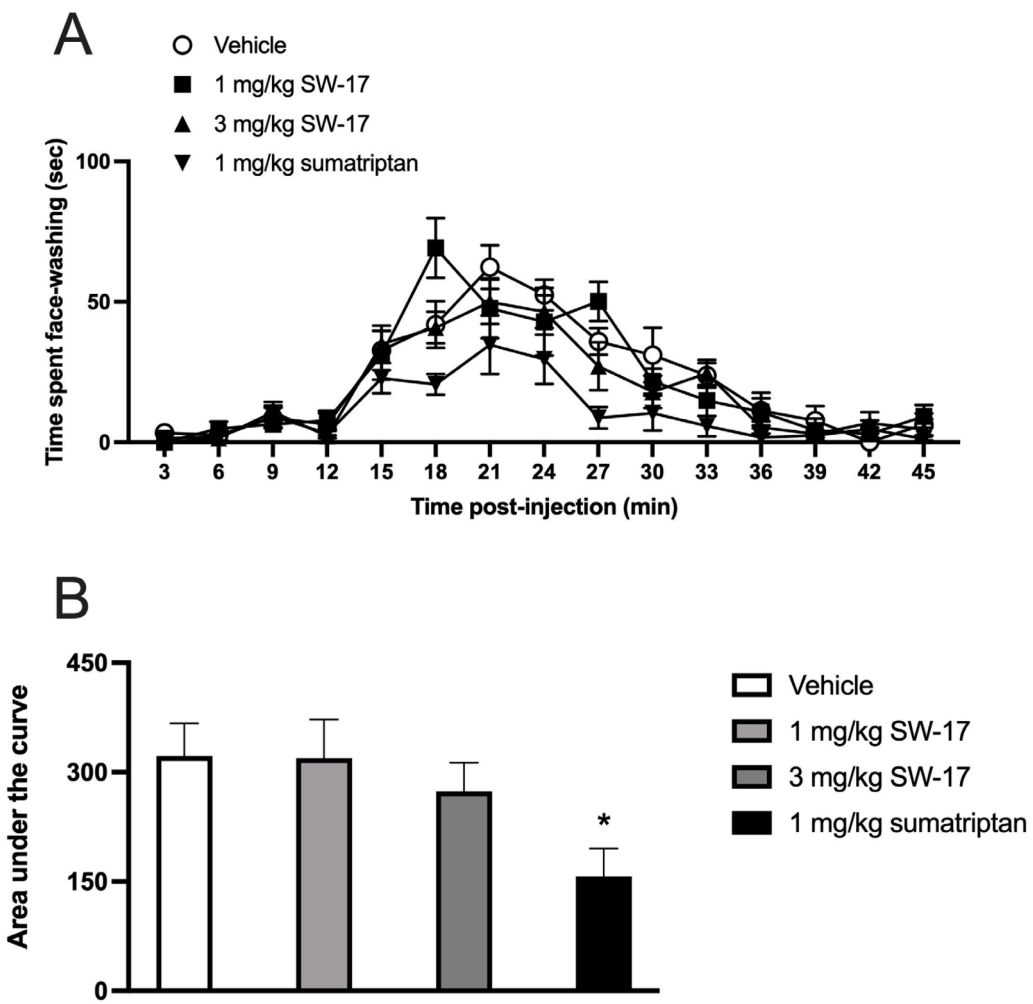


Fig. 2. Antinociceptive effects of SW-17 and sumatriptan on formalin-induced orofacial pain in female rats. A) The time spent exhibiting face-washing behavior following formalin injection is represented. B) The area under the curve of Fig. 2A is calculated and analyzed. * indicates $p < 0.05$ from vehicle. $n = 9$/group.

transmits pain via neurons originating in trigeminal ganglia [26]. Dorsal root ganglia and trigeminal ganglia vary in their genetic makeup and their ability to connect to the central nervous system to relay pain [27]. Other studies have shown that despite many similarities, several genes are uniquely expressed in either dorsal root or trigeminal ganglia [28]. Thus, it is possible that inhibition of FAAH and sEH at the level of the trigeminal ganglion is not sufficient to alleviate pain; however, this same inhibition at the level of the dorsal root ganglion is sufficient.

Although SW-17 did not produce antinociception in our experiments, sumatriptan inhibited the time spent face-washing following orofacial administration of formalin. We are not the first group to demonstrate that sumatriptan inhibits pain induced by orofacial formalin. Others have demonstrated that doses ranging from 0.5 to 5 mg/kg inhibit formalin-induced orofacial pain [29]. Sumatriptan is a serotonin (5-HT) 1B/1D receptor agonist. One theory as to why sumatriptan is effective is due to its actions on 5-$HT_{1B/1D}$ and 5-HT_{1F} receptors present in the trigeminal ganglion [30]. Sumatriptan may also inhibit the release of the pro-inflammatory calcitonin-gene related peptide (CGRP) from nerves to inhibit pain [30]. Thus, given the multi-modal effects of sumatriptan on trigeminal pain, one potential explanation could be due to the lack of direct serotonergic effects of SW-17.

There are multiple mechanisms by which dual inhibitors may produce antinociception [12]. Inhibition of sEH increases the concentration of epoxyeicosatrienoic acids (EETs) in pain-relevant areas of the nervous system [19]. These EETs have anti-inflammatory effects via inhibition of nuclear factor kappa B (NF-κB) signaling [31], which may be preventing many of the inflammation-induced pain behaviors induced by the hind paw injection of formalin. Similarly, inhibition of FAAH increases the concentration of endogenous cannabinoids. These cannabinoids produce antinociception by directly activating cannabinoid type 1 and type 2 receptors within the nervous system. While the results of this study cannot identify the relative contributions of these mechanisms to the behavioral changes produced by SW-17, it is likely that a combination of the inhibition of NF-κB signaling along with activation of cannabinoid receptors produce antinociception after acute inflammation of the hind paw, but not the orofacial region, in rats.

In sum, these results indicate that dual FAAH/sEH inhibitors are stable in human and rat plasma but may not inhibit pain resulting from orofacial formalin injection. Future in vitro studies will continue to optimize dual FAAH/sEH inhibitors for potency, efficacy, and solubility. Future in vivo studies will evaluate the extent to which dual FAAH/sEH inhibitors produce antinociception against other types of trigeminal pain (e.g., trigeminal neuralgia) and whether repeated administration of our inhibitors produces deleterious side effects such as tolerance or physical dependence.

CRediT authorship contribution statement

Daniel Carr: Writing – original draft, Methodology, Investigation. **Christopher Chin:** Writing – original draft, Methodology, Investigation. **Tiffany Chacon:** Methodology, Investigation. **Monijeh Khoja Herawi:** Methodology, Investigation. **Michael Gonzalez:** Methodology,

Investigation. **Ryan West:** Methodology, Investigation. **Christophe Morisseau:** Methodology, Investigation. **Bruce D. Hammock:** Methodology, Investigation. **Stevan Pecic:** Writing – original draft, Methodology, Investigation. **Ram Kandasamy:** Writing – original draft, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bbadva.2024.100119.

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