Design and Synthesis of Peripherally Selective Endocannabinoid Enzyme Inhibitors for Ocular Indications

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Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial
fulfillment of the requirements for graduation from the
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Abstract

Peripherally selective compounds have the potential to stimulate endocannabinoid receptor activity, which has been observed to have positive physiological effects such as ocular wound healing and inflammation control. The activation of the cannabinoid 1 receptor via binding of the endogenous ligands, anandamide and 2-arachidonoylglycerol, has been indicated to elicit these effects. Both ligands are controlled by two hydrolase enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), which can be targeted for therapeutic inhibition. Sulfonamide derivatives of JZL195 containing carbamate functionalities in the southern region of the inhibitor compounds were produced using novel carbamate exchange reactions. Polar functionalities were introduced to increase their high topological surface area (TPSA) to prevent the crossing through the blood-brain barrier (BBB), which in turn prevents any potential adverse effects on the central nervous system. The potency of each compound was measured using inhibitor screening assay kits for FAAH and MAGL.

Introduction

The use of chemical weapons in warfare is an inhumane practice that has caused significant harm to both soldiers and civilians. One of the most notorious chemical agents during World War I was sulfur mustard gas. This blistering agent was first used by the German military in 1917 and its usage quickly spread to other countries (1). The use of chemical weapons, including sulfur mustard gas, is banned under international law and its use is considered a war crime. Despite the prohibition, the threat posed by this type of chemical weapon remains. For instance, Iran was reported to possess aerial bombs filled with mustard gas as recently as 2012 (2).

Sulfur mustard gas causes severe burns, blisters, and respiratory damage. The eye is more susceptible to this gas than any other part of the body due to its unique physiology. In fact, 90% of individuals exposed to sulfur mustard during WWI experienced damage to the conjunctiva (1). Given the persistent utilization of chemical ocular irritants, there is a need for a unified approach to treating eye injuries caused by different chemical irritants.

Background and Rationale

Receptors of the Endocannabinoid System

The endocannabinoid system is a crucial modulator of the autonomic nervous system and the immune system. It regulates and supports various physiological processes, such as inflammatory responses, pain control, gastrointestinal regulation, energetic homeostasis, and temperature control (3, 4). This system consists of transporters, cannabinoid receptors, endogenous cannabinoids (endocannabinoids), and enzymes that regulate endocannabinoid levels.

The cannabinoid receptors are ubiquitous in the human brain and have been identified as G-protein coupled receptors. The two main receptors identified in this biological system are the

cannabinoid 1 receptor (CB1) and the cannabinoid 2 receptor (CB2), which are encoded by the CNR1 and CNR2 genes respectively. These receptors were first discovered in an effort to identify the target responsible for the psychoactive effects of Δ9-tetrahydrocannabinol (THC) (5).

The CB1 receptor has been found to be present in abundant amounts at the anterior of the human eye. It is involved in corneal wound healing (6,7,8) and thus has received considerable attention as a potential therapeutic target. To stimulate this membrane receptor, the human body produces biomolecules known as endocannabinoids which interact with the receptor at its orthosteric site (9). While several endogenous ligands have been found to have activity on cannabinoid receptors, two endocannabinoids, namely N-arachidonoyl ethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), have been more extensively characterized than others.

N-arachidonoyl ethanolamine

AEA, commonly known as anandamide, is a neurotransmitter that has similar properties to THC, a psychoactive compound found in cannabis. It is a lipid neurotransmitter that is synthesized on demand by neurons and subsequently transported to its target receptors via protein carriers. This biomolecule is mainly localized around the brain and plays a role in pain modulation and anti-inflammation (10).

AEA is synthesized from glycerophospholipids through the transacylation phosphodiesterase pathway, which involves two enzymatic steps (11). The initial step is the formation of N-acyl phosphatidylethanolamine (NAPE) by transferring an acyl group from a glycerophospholipid to the amino group of phosphatidylethanolamines. A Ca^{2+}-dependent N-acyltransferase catalyzes this reaction. The second step is the hydrolysis of NAPE to release N-acylethanolamine, which is mediated by NAPE-hydrolyzing phospholipase D (11).

Alongside the ability of AEA to bind and activate CB1 and CB2 receptors, this neurotransmitter also interacts with the Transient Receptor Potential vanilloid receptor (TRPV1). Activation of the CB1 receptor by the release of endocannabinoids following injury has been shown to have a downregulatory effect on TRPV1-mediated inflammation, pain and corneal opacification (12,6). This receptor is activated by injury to the epithelium and the subsequent release of endocannabinoids; it is now frequently referred to as an endo-vanilloid due to the evidence showing an interplay between cannabinoid and vanilloid receptors. This inhibition is brought about through a protein-protein interaction between TRPV1 and CB1, resulting in decreased phosphorylation of TRPV1 (6). The GTP binding protein is activated by CB1, which leads to the dephosphorylation of TRPV1 and subsequent desensitization. This causes a reduction in TRPV1-induced increases in current flow and proinflammatory signaling events (6).

The modulation of TRPV1 receptor activity via prolonged activation leads to desensitization of these receptors. This is facilitated by the interaction between the cannabinoid and vanilloid receptors. Such an interaction holds promise as a therapeutic strategy for pain relief. This reduction in TRPV1 activity has been linked to the desensitization of the channel and an attenuated release of pro-inflammatory molecules, such as cytokines (12). This form of CB-1-induced suppression of TRPV1 activation has significant therapeutic potential, as it has the potential to alleviate pain, reduce inflammation, and minimize scarring caused by corneal injury. Nonetheless, the biological effects of its agonist, anandamide, are limited by its rapid enzyme-mediated hydrolytic degradation.

Fatty Acid Amide Hydrolase

The principal enzyme responsible for breaking down AEA has been identified to be the target enzyme fatty acid amide hydrolase (FAAH), a ~63 kDa serine hydrolase. FAAH is a

complex integral membrane enzyme that hydrolyzes anandamide into arachidonic acid (13), disallowing the activation of the cannabinoid and TRPV1 receptors. FAAH degrades AEA by breaking it down into arachidonic acid and ethanolamine (**Figure 1**), neither of which activate the cannabinoid receptors. Inhibition of FAAH, either genetically or pharmacologically, was shown to result in the elevation of AEA (14). **Scheme 1** illustrates the order of this enzymatic action (all schemes and figures presented in this project were produced for this study.)

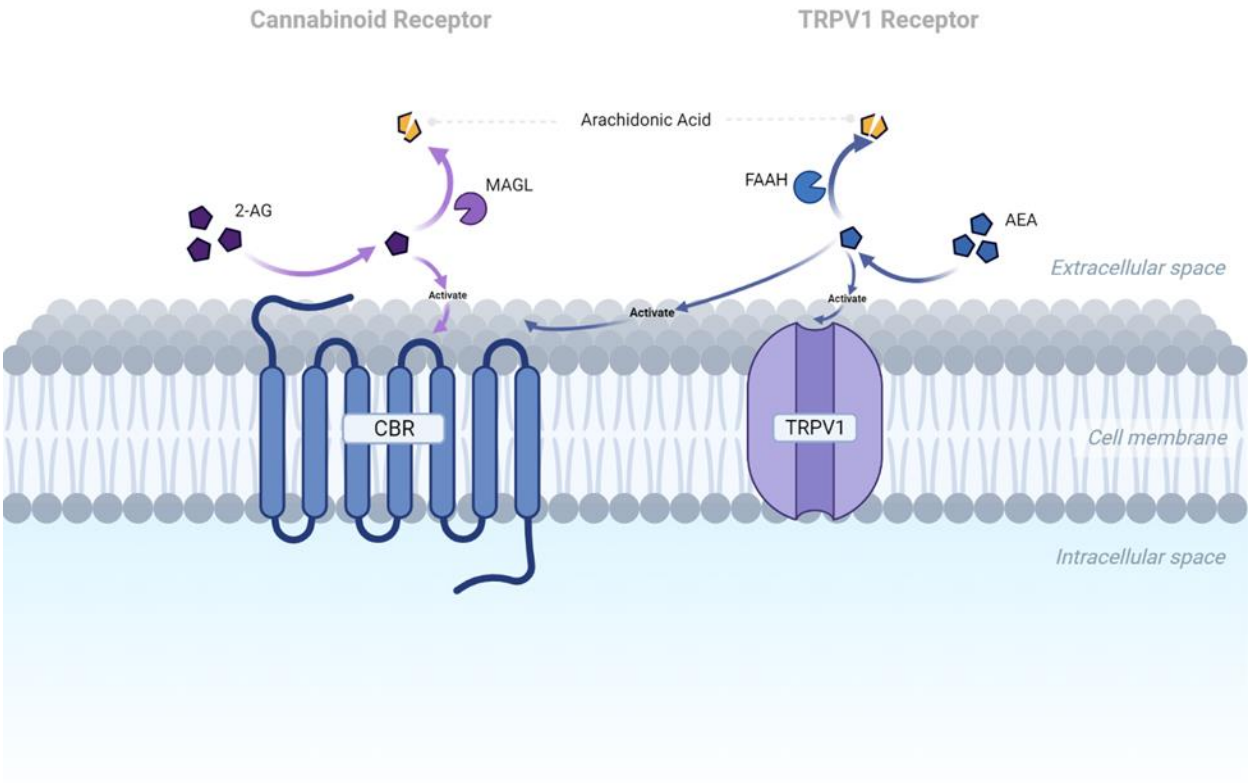
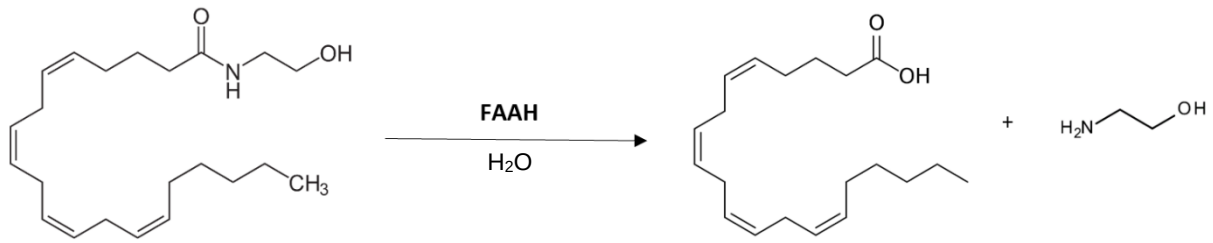


Figure 1. The Endocannabinoid System



Scheme 1. Hydrolysis of anandamide by FAAH into arachidonic acid and ethanolamine.

2-Arachidonoylglycerol

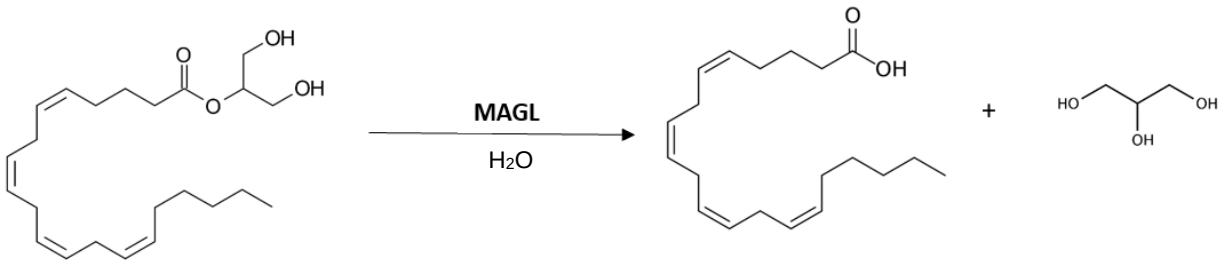
Another endogenous agonist of the CB1 receptor is 2-AG, which is an endocannabinoid present at relatively high concentrations in the central nervous system (15). As the most abundant endocannabinoid in the human body, it is present in quantities 170 times greater than anandamide (16) and plays a significant role in synaptic plasticity and the development of the central nervous system (7). This biomolecule is an ester synthesized from arachidonic acid-containing diacylglycerol (DAG) and is formed from omega-6-arachidonic acid and glycerol (17). Additionally, the primary enzyme responsible for synthesizing this endocannabinoid is Diacylglycerol lipase (DAGL). This lipid endocannabinoid binds to membrane receptors (i.e., AEA) and diffuses across the membrane of the cell in which it is synthesized. Activation of the endocannabinoid receptors via the binding of 2-AG is associated with the regulation of inflammation, pain, stress, and learning. However, just like AEA, it is hydrolyzed by a target enzyme.

Monoacylglycerol lipase

Monoacylglycerol lipase (MAGL), a ~33 kDa serine hydrolase, is considered the chief 2-AG hydrolyzing enzyme. It functions by breaking down 2-AG into arachidonic acid (the precursor of various inflammatory mediators, including prostaglandin) and glycerol (**Scheme 2**),

which in turn decreases the bioavailability of 2-AG and disallows its binding to the CB1 receptor (**Figure 1**.). There are several other serine hydrolases, one of which is FAAH, which function in the same way albeit to a lesser extent.

MAGL inhibitors have gained recognition as important therapeutic agents for various ailments, including nociception, anxiety, inflammation, and cancer. Inhibition of MAGL (using JZL 184) has been observed to increase levels of 2-AG eightfold (18).



Scheme 2. Hydrolysis of 2-Arachidonoylglycerol by FAAH into arachidonic acid and ethanolamine.

FAAH and MAGL Inhibition

While the exact mechanisms that trigger the production and secretion of AEA and 2-AG from both neuronal and non-neuronal cells remain unknown, it is known that the activity of both AEA and 2-AG is tightly controlled by FAAH and MAGL. Both FAAH and MAGL are members of the serine hydrolase superfamily of enzymes. FAAH is a membrane-bound homodimer enzyme with a parallel orientation, allowing it to recruit and cleave the endocannabinoid simultaneously. This enzyme is primarily responsible for hydrolyzing AEA and, to a lesser extent, 2-AG. MAGL is a serine hydrolase that carries out very similar functions. Nevertheless, this enzyme primarily hydrolyzes 2-AG, with AEA being hydrolyzed to a lesser extent.

The CB1 receptor is ubiquitous in the human body. As mentioned above, this receptor and its ligands are involved in many physiological processes including inflammation, pain sensation, stress and neuroprotection. In turn, activation of the endocannabinoid receptor by 2-AG and AEA has many therapeutic benefits. However, the rapid hydrolysis of the two endocannabinoids by FAAH and MAGL inhibits their binding to the CB1 receptors.

It has been found that AEA and 2-AG pathways interact, so expectedly, suppression of both FAAH and MAGL gives additive effects (19). This supports the rationale that such a dual inhibitor could be of significant therapeutic benefit compared to inhibiting each signaling pathway individually.

There is substantial evidence connecting the endocannabinoid system to efficient eye wound healing (6,7,8). However, there have been no studies investigating the use of FAAH, MAGL, or dual FAAH/MAGL inhibitors for this purpose. Therapeutic inhibition of FAAH and MAGL can result in the stimulation of CB1 receptor activity via an increase in 2-AG and AEA bioavailability in corneal epithelial cells, which in turn modulates pain and encourages eye wound healing.

Δ9-Tetrahydrocannabinol

CB1 and CB2 receptors are also activated by THC (**Figure 2**). THC is a psychoactive compound that activates several receptors in the endocannabinoid signaling system. This compound is known to have medicinal benefits for pain relief and accelerated healing (14). However, they also have undesirable side effects on cognitive function, motor coordination, mental health, and, most critically, tolerance. All of this limits their use as a therapeutic agent. It would be beneficial if there were a way to activate the endocannabinoid receptors without the

undesirable side effects. A potential solution is to indirectly activate the cannabinoid receptors by inhibiting the target enzymes FAAH and MAGL.

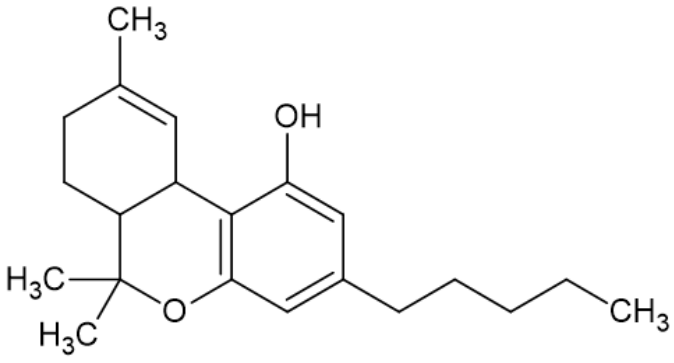


Figure 2. Δ^9-tetrahydrocannabinol

JZL195 as a Dual Inhibitor of FAAH and MAGL

Numerous studies have shown a correlation between the endocannabinoid system and enhanced ocular wound healing (6,20). This study aims to develop a solution that activates these endocannabinoid receptors, which can effectively modulate pain and inflammation while promoting the healing of ocular injuries (corneal epithelial cells). This can be done by synthesizing compounds that can achieve indirect activation of the cannabinoid receptors through the dual inhibition of FAAH and MAGL. The desired outcome would be increased levels of 2-AG and AEA within corneal epithelial cells.

JZL195 (**Figure 3.)** is a well-established and thoroughly characterized dual inhibitor of FAAH and MAGL, with IC50 values of 2 and 4 nM, respectively (19). Administration of this inhibitor has been shown to decrease FAAH and MAGL activity in a dose-dependent manner (19).

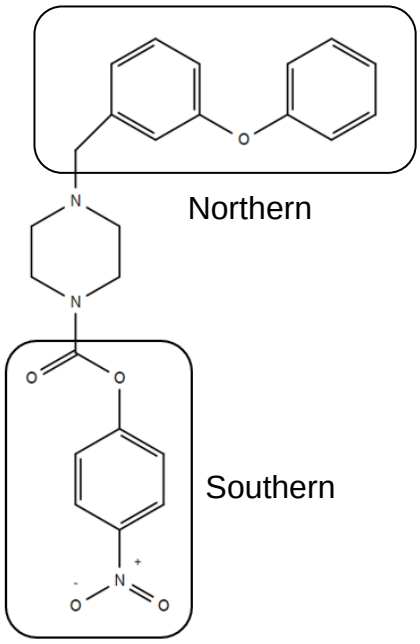


Figure 3. JZL 195 southern portion to be modified.

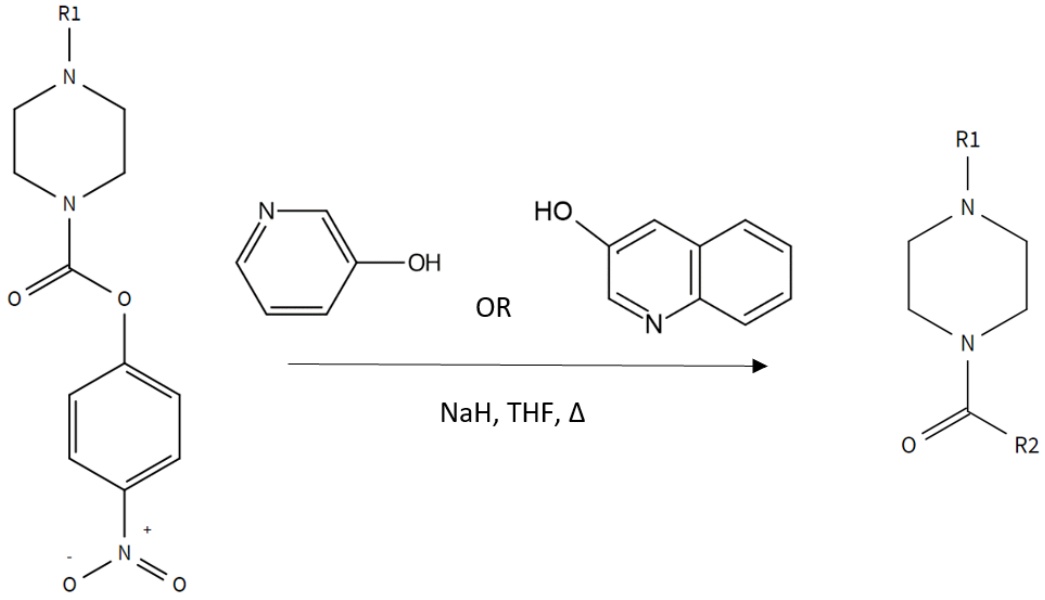
While the potential for JZL195 to be used in ocular in vivo assays is significant, its interaction with the central nervous system makes it highly undesirable to treat eye wounds. A more suitable compound would be one with higher peripheral selectivity, which would not cross the blood-brain barrier (BBB) and induce adverse effects on the central nervous system (CNS).

Compounds with polar functionalities would be desired as they would contribute to their high topological surface area (TPSA), preventing the crossing of the BBB and inducing any adverse effect on the central nervous system. It is expected that the active functionalities of the serine hydrolases will form an inhibitor-enzyme adduct.

Materials and Methods

Overall, ocular wound healing can be targeted by the activation of cannabinoid receptors via dual inhibition of FAAH and MAGL. This project synthesized four analogous compounds of

JZL195 (**Figure 3.**) using northern sulfonamide derivatives with carbamate functionalities at the southern portion of the JZL195. The inhibitors (Products 143, 153, 157 and 165) were synthesized using carbamate exchange reactions, as depicted in **Scheme 3.** In this reaction, a compound containing phenolic hydrogens, such as pyridine or quinoline, was deprotonated using sodium hydride. The mixture is then subjected to heating, which facilitates a nucleophilic acyl substitution reaction. This reaction results in the replacement of 4-nitrophenol with the pyridine or quinoline substrate.



Scheme 3. Carbamate exchange reaction

Product 143

A nucleophilic acyl substitution reaction was performed by reacting *N*-BOC-Piperazine (502 mg, 2.69 mmol) (107-1) with THF (20 ml), TEA (1.13 ml) and 4-Butylbenzenesulfonyl chloride (626 mg, 2.69 mmol) (107-2). This resulted in the formation of 1,1-Dimethylethyl 4-[(4-butylphenyl)sulfonyl]-1-piperazinecarboxylate (107-3), which was confirmed using thin layer

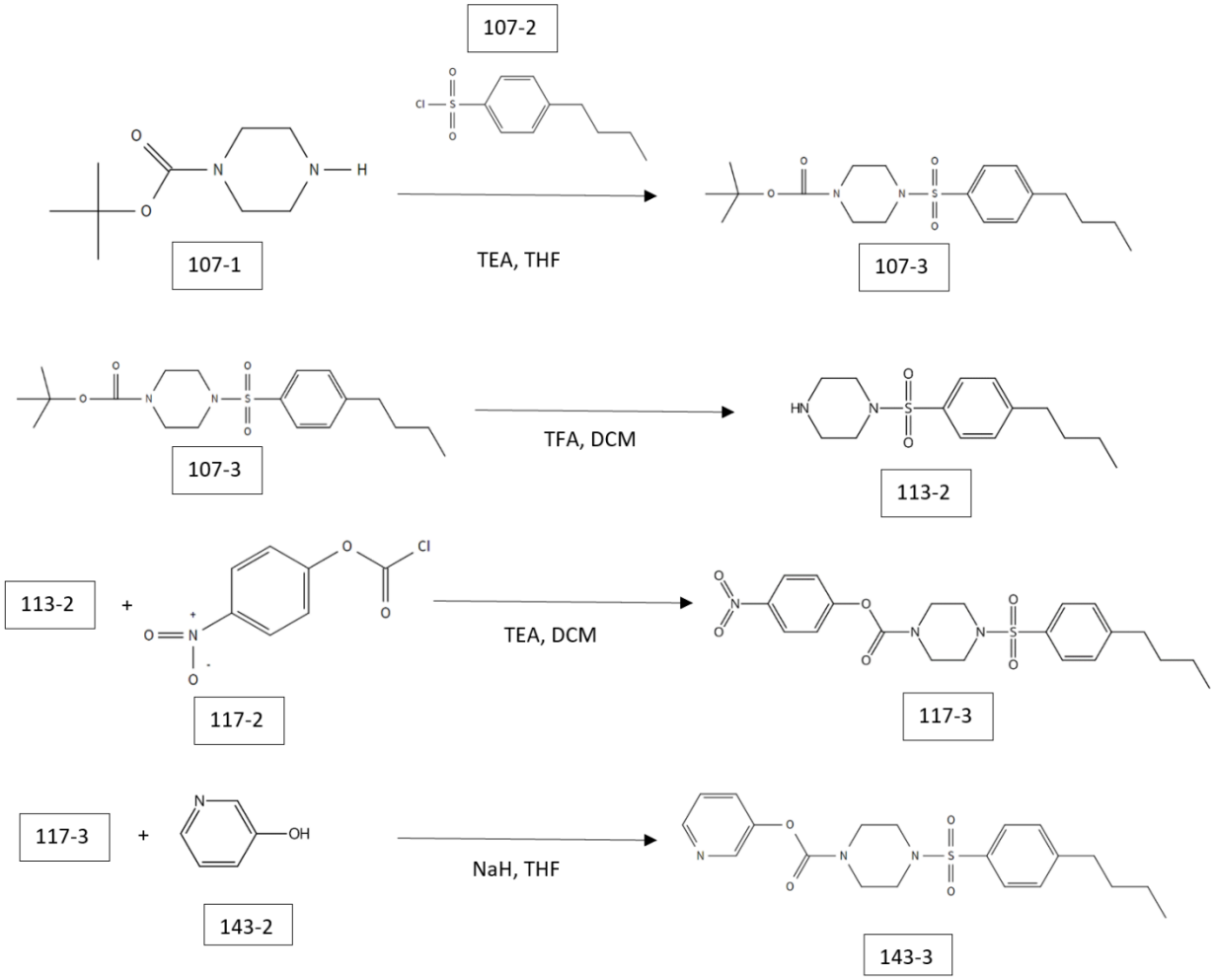
chromatography (TLC) after the product was worked up using hydrochloric acid (HCl), ethyl acetate and salt brine. In this reaction the hydrogen on the nitrogen atom at the core of the *N*-BOC-Piperazine was substituted with 4-Butylbenzenesulfonyl chloride.

Crude 107-3 was stirred with TFA (30% TFA, 70% DCM) overnight to remove the BOC group and the resulting compound, 1-[(4-Butylphenyl)sulfonyl]piperazine (113-2), which was concentrated in vacuo. The heterogeneous mixture was worked up using ethyl acetate (40 mL). sodium bicarbonate (20 mL), and brine (10 mL).

Crude 113-2 (0.362 mg, 1.28 mmol) was reacted with 4-nitrophenyl chloroformate (388 mg, 1.90 mmol) (Compound 117-2) and triethyl amine (0.28 ml, 3.8 mmol) in dichloromethane (20 mL) to form 4-Nitrophenyl 4-[(4-butylphenyl)sulfonyl]-1-piperazinecarboxylate (Compound 117-3). This reaction was stirred overnight, and the desired compound was confirmed using TLC. The product was washed with 2N HCl and brine, followed by which it was purified using Radial Preparative Layer Chromatography (RPLC). Mass Spectrometry (MS) and Nuclear Magnetic Resonance spectroscopy (NMR) were performed to confirm the desired compound.

The nitrobenzene group of 4-nitrophenol was replaced with 3-hydroxy pyridine, Compound 143-2 (153 mg, 1.6 mmol), using a nucleophilic acyl substitution reaction. This produced pyridin-3-yl 4-(4-butylbenzene-1-sulfonyl) piperazine-1-carboxylate, compound 143-3 (37 mg, 0.096 mmol). This was done by deprotonating the compound with sodium hydride (38.64 mg, 0.97 mmol) and reacting it with tetrahydrofuran (20 ml) (**Scheme 4**).

The reaction was worked up with bicarbonate (10 mL), ethyl acetate (30 mL), and water (10 mL). Once the aqueous was separated from this mixture, 10 mL brine was used, and the organic layer was collected and dried using MgSO4. An RPLC was then performed to purify it.

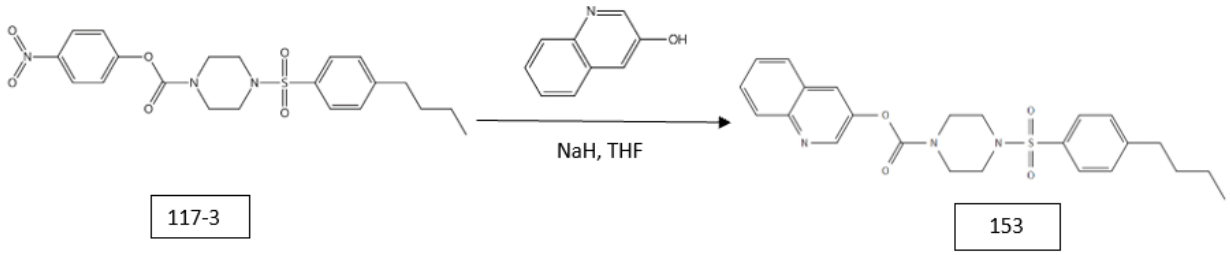


Scheme 4.

Product 153

Crude 117-3 (144 mg, 0.32 mmol) was reacted with sodium hydride (90.16 mg, 2.25 mmol), 3-hydroxyquinoline (467 mg, 3.22 mmol), and THF (20 mL). The reaction was left to reflux overnight, and the resulting product was worked up using 10 mL bicarbonate, 10 mL water, and 10 mL brine. The product was concentrated in vacuo, and RPLC was performed using 3 mL of dichloromethane (DCM) to dissolve the compound. Fraction 1 was determined to be the desired product. In this reaction, the nitrobenzene group of 117-3 was replaced with 3-

hydroxyquinoline to form quinolin-3-yl 4-(4-butylbenzenesulfonyl) piperazine-1-carboxylate (compound 153) (**Scheme 5**).



Scheme 5.

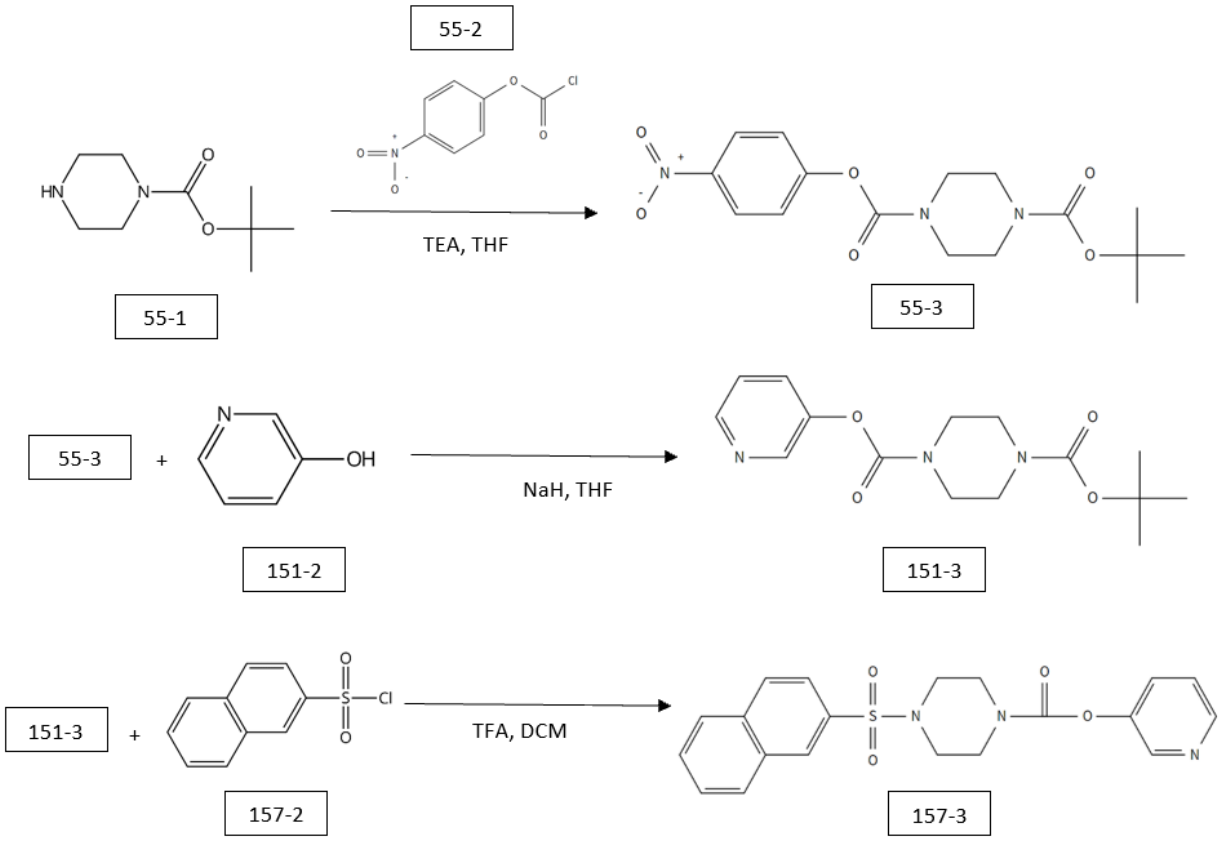
Product 157

N-BOC-Piperazine (1000 mg, 5.37 mmol) (Compound 55-1) was reacted with 4-nitrophenyl chloroformate (1190 mg, 5.91 mmol) (Compound 55-2), THF (20 ml), and triethylamine (TEA) (2.25 ml, 16.1 mmol). This was left to stir at room temperature for 8 hours before reacting it with THF (80 mL); this reaction was left to stir overnight and concentrated in vacuo. TLC was performed, and the compound was purified using RPLC. The resulting product was determined to be (4-{[4-(tert-butoxycarbonyl)piperazine-1-carbonyl]oxy}phenyl)(hydroxy)oxoammonium (Compound 55-3).

Crude 55-3 (90.4 mg, 0.31 mmol) was added to a 50 mL round bottom flask and combined with NaH (37 mg, 0.92 mmol), 3-hydroxy pyridine (146 mg, 1.53 mmol) (compound 151-2), and THF (20 mL). The reaction was stirred and allowed to reflux overnight. The product (compound 151-3) was diluted with 30 mL of ethyl acetate. It was then washed with 10 mL of sodium bicarbonate and 10 mL of brine. The organic layer was collected and dried using

$MgSO_4$, and the product was filtered. This was then concentrated in vacuo, after which it was dissolved in DCM in order to run an RPLC. In this reaction, the nitrobenzene group was replaced with 3-hydroxy pyridine to form tert-butyl pyridin-3-yl piperazine-1,4-dicarboxylate (151-3).

In the following reaction, the tert-butyl group was replaced with Naphthalene-2-sulfonyl chloride to form pyridin-3-yl 4-(naphthalene-2-sulfonyl)-piperazine-1-carboxylate (**Scheme 6**). Crude 151-3 (53 mg 0.26 mmol) was stirred with TFA (30% TFA, 70% DCM) overnight and concentrated in vacuo. This was then reacted with Naphthalene-2-sulfonyl chloride (58mg, 0.26 mmol; Compound 157-2), TEA (0.108 mL), and DCM (10 mL) and stirred overnight. The resulting product was concentrated in vacuo, then dissolved in DCM and purified via RPLC. LCMS confirmed that fraction 2 was the desired product.



Scheme 6.

Product 165

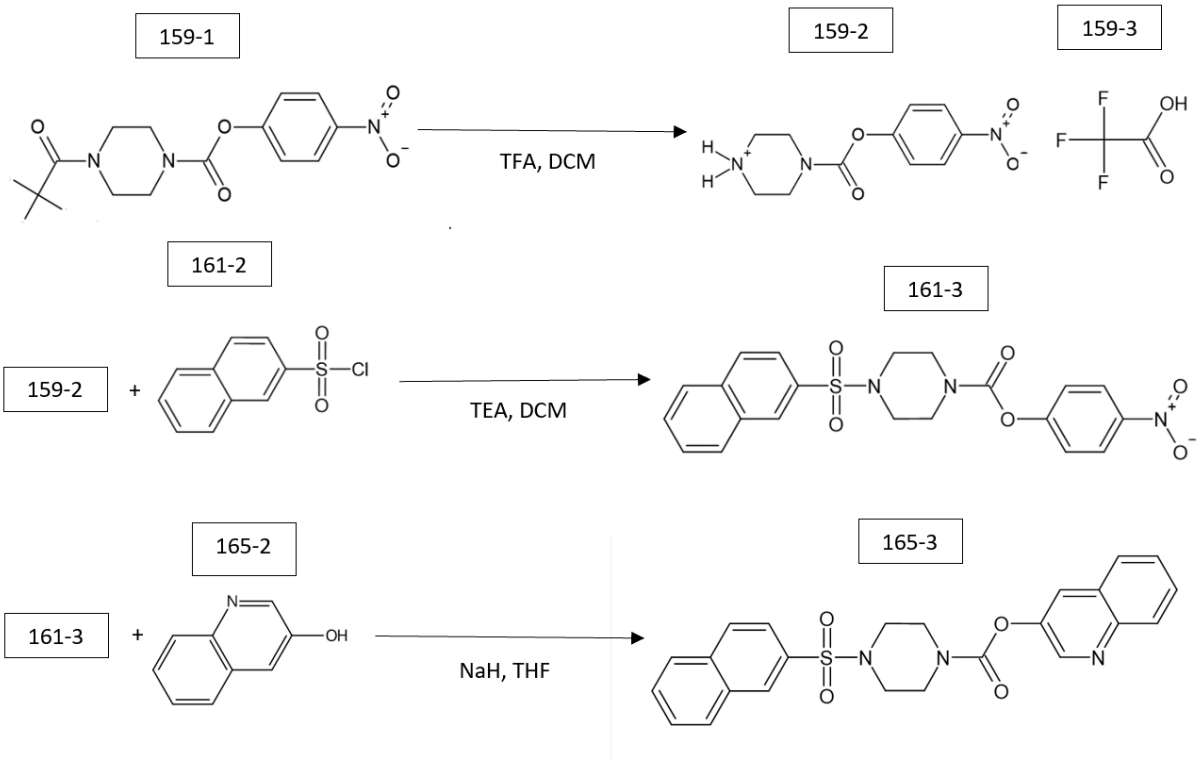
DCM (7 mL) and TFA (3 mL) were added to phenyl 4-(2,2-dimethylpropanoyl) piperazine-1-carboxylate (803 mg, 2.55 mmol) in a round bottom flask. The resulting product was concentrated in vacuo and determined to be 4-(phenoxycarbonyl)piperazin-1-ium and trifluoroacetic acid.

Crude 159-3 (1.28 mmol) was combined with naphthalene-2-sulfonyl chloride (289 mg, 1.28 mmol), TEA (0.530 ml, 3.83 mmol), and DCM (20 ml). This reaction was run overnight at room temperature and resulted in the formation of (4-{[4-(naphthalenesulfonyl)piperazine-1-

carbonyl]oxy}phenyl)(hydroxy)oxoammonium. The reaction was purified using RPLC, and fraction 2 was determined to be the desired product.

Crude 161 (120 mg, 0.27 mmol) was added to a 100 mL round bottom flask and reacted with THF (20 mL), Sodium Hydride (76.2 g, 1.91 mmol), and 3-Hydroxyquinoline (395 mg, 2.72 mmol). The reaction was left to reflux overnight at room temperature. 30 mL ethyl acetate, 10 mL bicarbonate, and 10 mL water were initially used to work up the reaction. The solution was filtered, and the inorganic layer was separated. 10 mL of brine was added to the organic layer, and the inorganic layer was separated. The reaction was dried using $MgSO_4$ and concentrated in vacuo using a rotary evaporator and vacuum pump.

DCM was used to dissolve the resulting compound, and RPLC was performed. Fraction 3 was determined to be the desired product using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS). In this reaction, the nitrobenzene group was replaced with 3-hydroxyquiniline, resulting in the formation of quinolin-3-yl 4-naphthalensulfonyl)piperazine-1-carboxylate (**Scheme 7**).



Scheme 7.

FAAH and MAGL assay

The following assays were performed using a Tecan Infinite M200pro plate reader. All compounds were evaluated against FAAH using a Fatty Acid Amide Hydrolase Inhibitor Screening Assay Kit (Cayman, Ann Arbor, MI) with JZL 195 serving as the control according to manufacturer protocol. The assay was performed on a 96 well flat bottom black polystyrene plate. Briefly, 170 µL assay buffer, 10 µL of FAAH (120 µl of human recombinant FAAH with 480 µL assay buffer), and 10 µL of one of the four compounds were added to three wells. This plate was incubated at 37°C for five minutes. Reactions were initiated using FAAH substrate (1.5 mL 400 M AMC Arachidonoyl amide) and left to incubate at 37°C for another thirty minutes. The plate was read at an excitation wavelength of 360 nm and an emission wavelength

of 460 nm. The percentage inhibition was calculated using the following formula followed by which the data was analyzed using GraphPad prism:

$$\% \text{ Inhibition} = \frac{\text{corrected 100\% inhibition activity} - \text{corrected inhibitor activity}}{\text{corrected 100\% initial activity}} \times 100$$

To evaluate the inhibitory activity of the synthesized compounds against MAGL, the Monoacylglycerol Lipase/MGL Inhibitor Screening Kit- Fluorometric (ab283388) from Abcam was used, utilizing JJKK-048 as the standard inhibitor. To prepare the MAGL enzyme for the assay, 8.8 ml of the MAGL enzyme 50X stock solution was mixed with 0.2 ml of 1X MAGL Assay Buffer. Then, the concentration of the MAGL substrate was adjusted to be used in this assay. A 50X solution was made from a 200X stock solution by adding 220 µl MAGL Assay Buffer to the provided vial. Additionally, the MAGL control inhibitor, JJKK-048, was included in the kit as a 2mM stock solution in DMSO. The stock was diluted at a 1:10 ration by adding 45 µM MAGL Assay Buffer to 5µL of the 2mM stock, resulting in a 0.2 mM working solution.

To assess the inhibitory activity of the synthesized compounds, 100% initial activity wells were prepared by adding 5 µl of 1X MAGL Assay Buffer and 90 µl of MAGL enzyme. Four wells were used to determine the background, which were filled with 95 µl of 1X MAGL Assay Buffer. Other wells were filled with 90 µl of MAGL enzyme. The contents of the wells were mixed through gentle pipetting and incubated for 30 minutes at 37°C.

Half log dilutions were made with a starting concentration of 30 mM to test the synthesized compounds. The positive control, JJKK-0048, was also subjected to the same dilutions with a starting concentration of 0.001 mM. Each compound, including the control, was tested in duplicate. The reaction was initiated by adding 5 µl of the substrates into their

respective wells on the plate. The positive control wells contained JJKK-0048 (2mM stock solution in DMSO) along with 90 µl of MAGL enzyme solution. Similarly, other wells were filled with 5 µl of each compound with their respective dilutions. The contents of the wells were mixed and then incubated for 40 minutes at 37°C. Finally, the absorbance was read at 450 nm after a 40-minute incubation period and data was analyzed in the same manner as the previous assay.

Results

Mass spectrometry (MS)

Liquid chromatography-mass spectrometry (LC-MS) was utilized in this study to characterize the desired organic chemical compounds that were synthesized. The resulting mass spectrum for compound 143 (**Figure 4**), revealed a well-defined molecular ion peak at 404.1 m/z, which corresponds to the theoretical molecular weight of the compound (403.5 amu). The spectrum also displayed several significant fragment ions, which were used to confirm the molecular structure of the compound. Similarly, the analysis of the mass spectrum of compound 153 (**Figure 5**), displayed a clear molecular ion peak at 454.1 m/z, aligning with the expected weight of 453.56 amu.

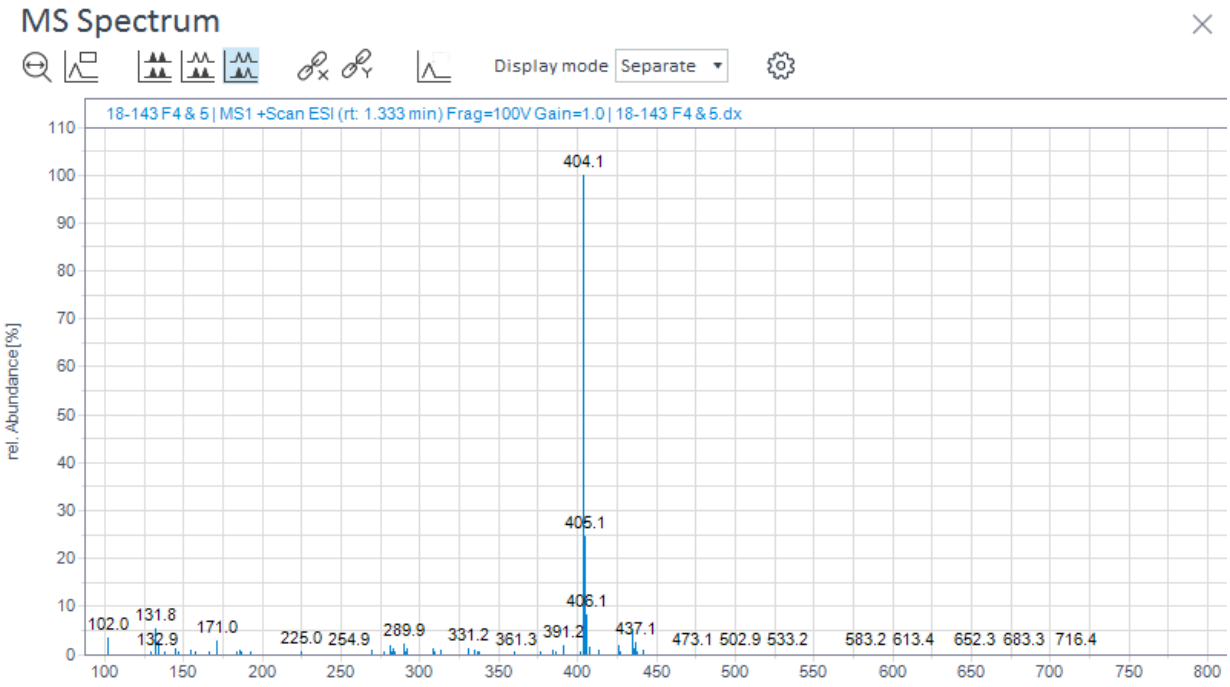


Figure 4. Mass spectrum of compound 143

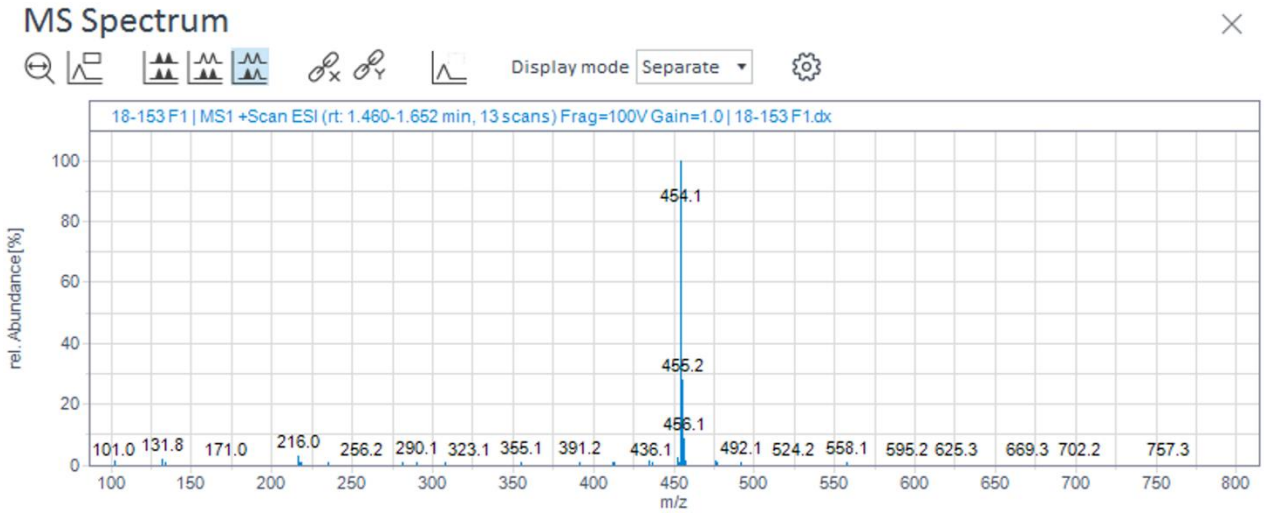


Figure 5. Mass spectrum of compound 153

Like the other compounds, the mass spectrum analysis of compound 157 (**Figure 6**) highlighted a sharp molecular ion peak at 398.1 m/z, which confirms the accuracy of its calculated molecular weight of 387.45 amu.

Finally, the mass spectrum of compound 165 (**Figure 7**), exhibits a prominent molecular ion peak at 448.1 m/z. This observation is consistent with the anticipated molecular weight of 447.51 amu. These findings allow us to verify the identification of the synthesized compound and establish a solid foundation for further testing to determine its efficacy and potential applications.

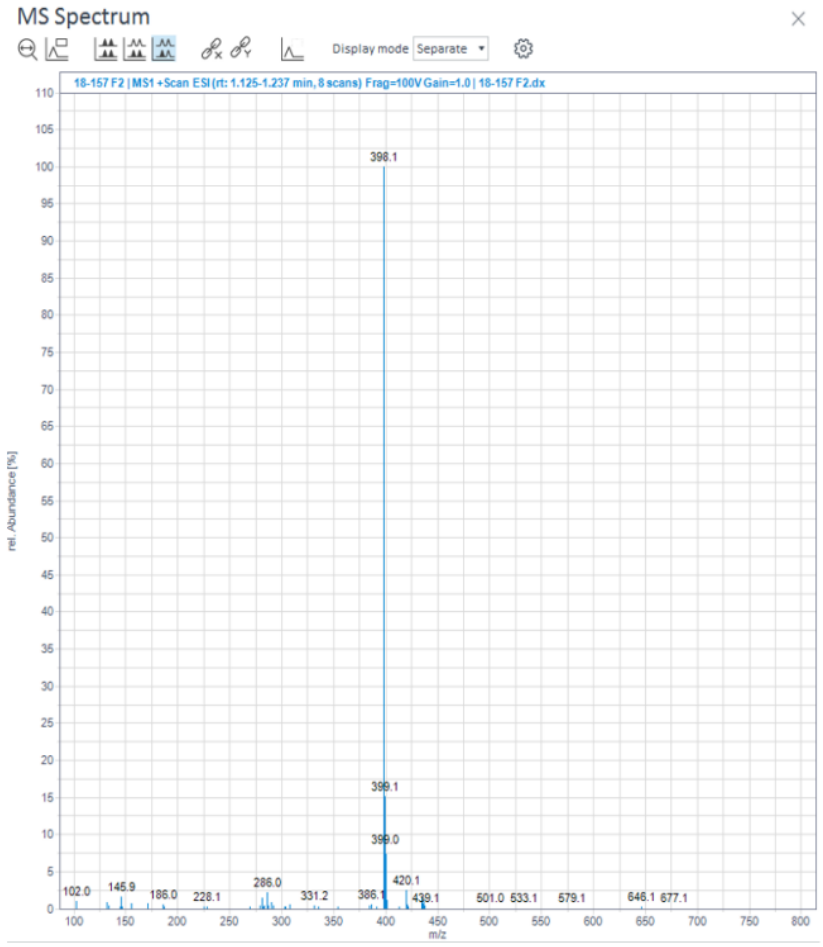


Figure 6. Mass spectrum of compound 157

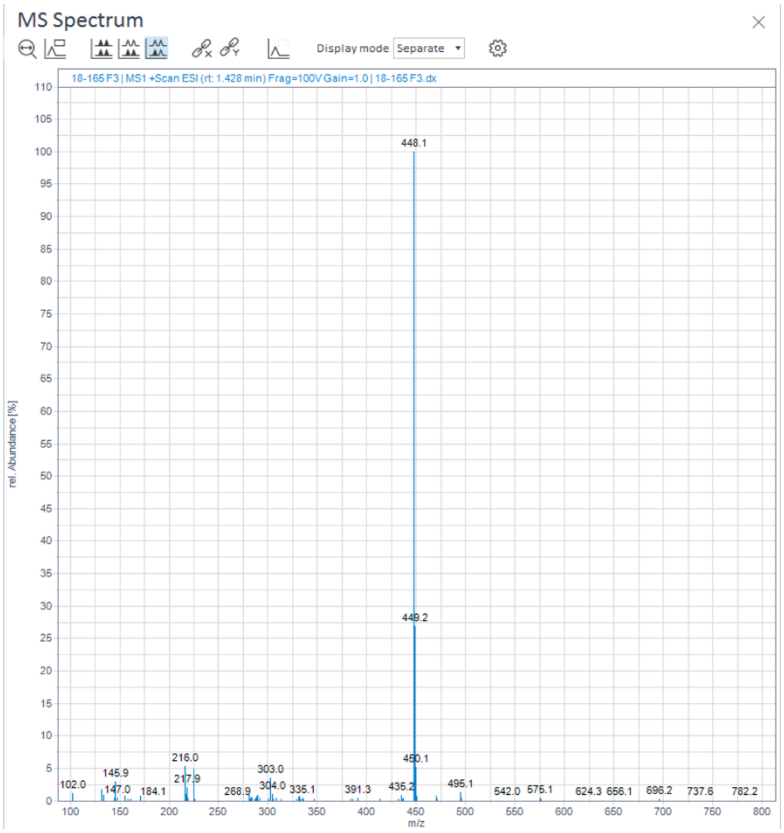


Figure 7. Mass spectrum of compound 165

Inhibitory Concentration (IC50) Curve

Spectrophotometric analysis was performed to quantify the fluorescent signal generated by 7-amino-4-methylcoumarin (AMC), with excitation wavelengths optimized at 360 nm and emission wavelength of 460 nm. This methodology provided a sensitive and reliable mean for the assessment of FAAH activity.

The graphs in **Figure 8**. show the inhibition of FAAH by the synthesized compounds as a function of the log concentration of the novel compound. The x-axis of each graph represents the concentration of the compound in μM, while the y-axis represents the percentage of inhibition of FAAH activity. The data indicates that increasing concentrations of the compounds result in a corresponding increase in the percentage of FAAH inhibition.

The IC50 value, which represents the concentration of a product required to inhibit 50% of FAAH activity, can be estimated from the curve. In the case of product 143 (**Figure 8a.**), the IC50 value appears to be around 0.0174 μM. Similarly, the IC50 of product 153 (**Figure 8b.**), 157 (**Figure 8c.**) and 165 (**Figure 8d.**) were determined to be 0.0348 μM, 0.0405 μM and 0.207 μM, respectively.

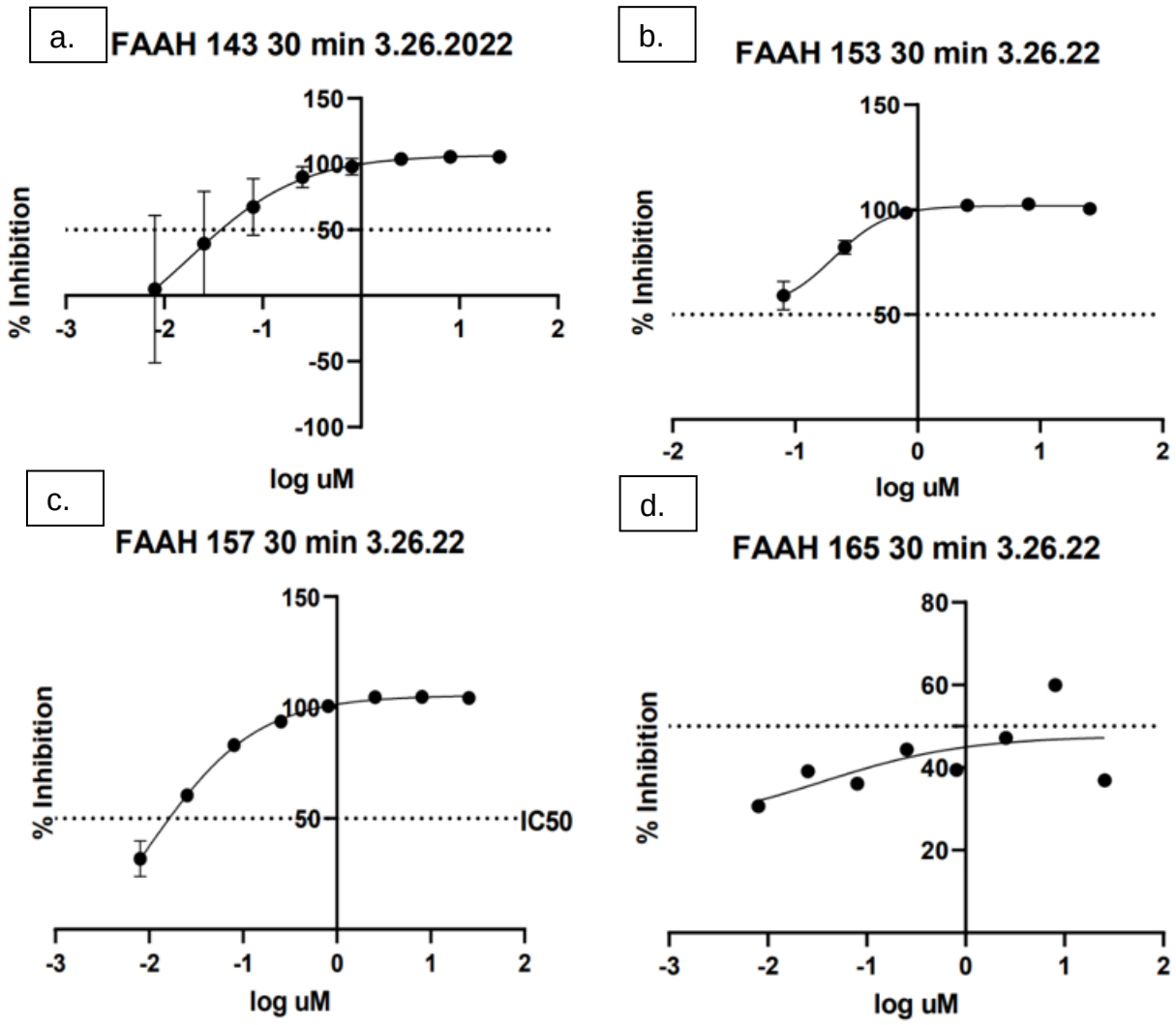


Figure 8. Inhibition of FAAH by compounds 143, 153, 157, 165

Similar to FAAH activity, MAGL inhibition was measured in a fluorescence assay. **Figure 9.** displays four different graphs, each showing the inhibition of human recombinant MAGL by the synthesized compounds as a function of the log concentration of the novel compound. The x-axis of each graph represents the concentration of the compound in μM, while the y-axis represents the percentage of inhibition of MAGL activity. Each graph shows a dose-response curve, with the percentage of inhibition increasing as the concentration of the compound increases.

The first graph (**Figure 9a**.) represents the inhibitory effect of compound 143, with an IC50 value of 1.53 μM. The second graph (**Figure 9b**.) represents the inhibitory effect of compound 153, with an IC50 value of 9.11 μM. The third graph (**Figure 9c.**) represents the inhibitory effect of compound 157, with an estimated IC50 value of <10 nM. Finally, the fourth graph (**Figure 9d.**) represents the inhibitory effect of compound 165, with an estimated IC50 value of 12.3 μM.

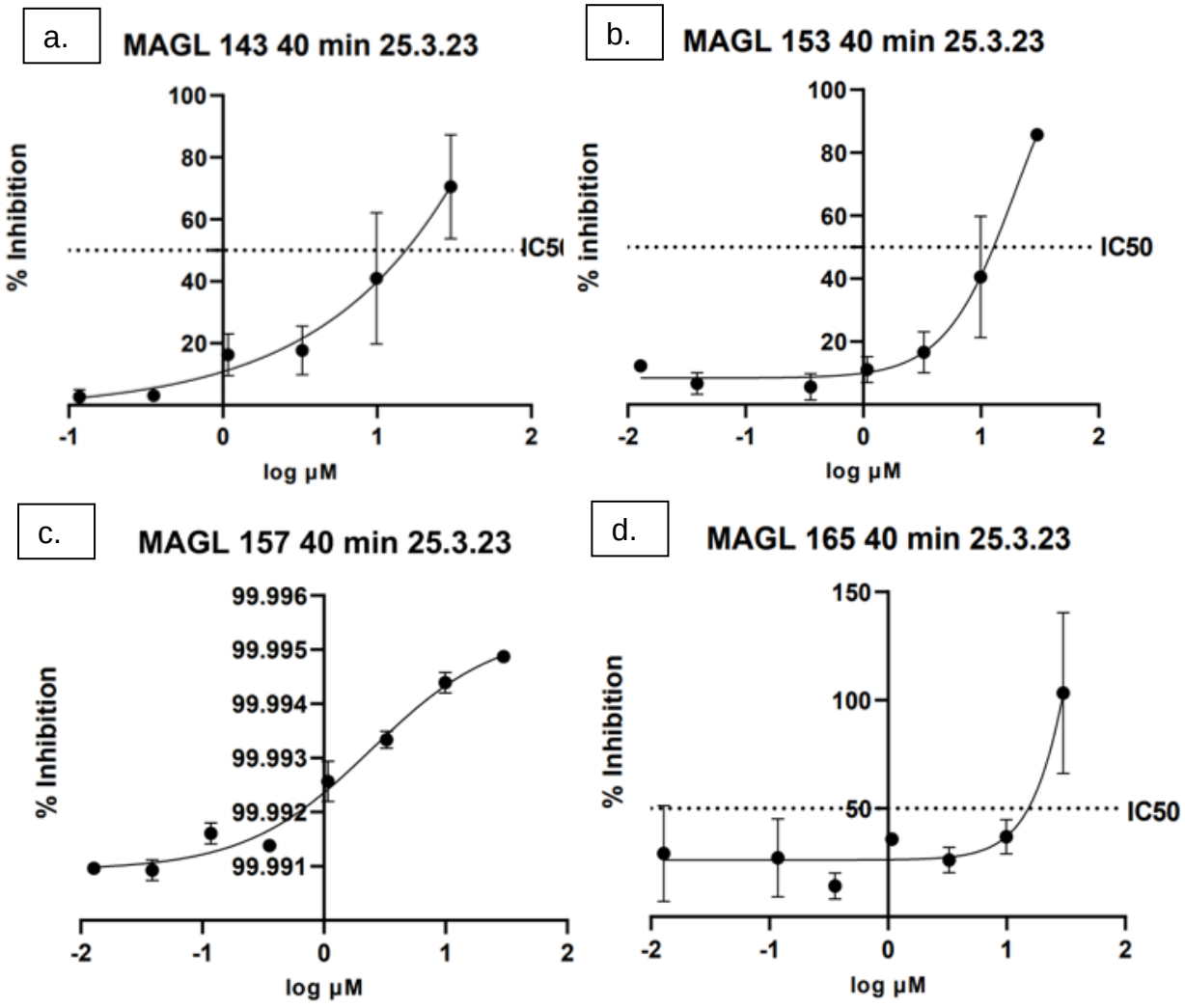


Figure 12. Inhibition of human recombinant MAGL by compounds 143, 153, 157, 165

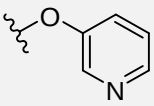
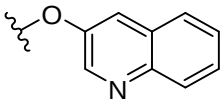
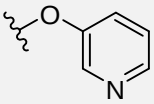
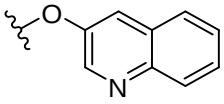
PRODUCT	SOUTHERN REGION	IC50 FAAH	IC50 MAGL
143		0.0174 µM	1.53 µM
153		0.0348 µM	9.11 µM
157		0.0405 µM	<10 nm
165		0.207 µM	12.3 µM

Table 1. IC50 values for each compound

Discussion

The potential therapeutic benefits of inhibiting FAAH and MAGL enzymes, which play a crucial role in regulating the endocannabinoid system, are substantial. This system has been shown to impact various physiological functions, including pain, inflammation, and wound healing. Notably, the anterior part of the human eye has a high abundance of CB1 receptors, which are activated by AEA and 2-AG. By inhibiting FAAH and MAGL enzymes, hydrolysis of AEA and 2-AG could be downregulated, allowing for the activation of CB1 receptors. This could, in turn, modulate pain, inflammation, and wound healing while avoiding opacification in the human eye.

Four novel compounds were synthesized for dual inhibition of FAAH and MAGL via carbamate exchange reaction. The southern portion of compounds with a similar structure to JZL 195 were synthesized and modified using carbamate exchange reaction. After synthesis, the

compounds were subject to LC-MS to verify identity. The utilization of LC–MS allowed for the separation and detection of compounds based on their unique molecular characteristics. The mass spectrometric data generated from this method provided crucial information for the definitive identification of the compounds and the determination of their molecular structures. These results were then used to evaluate the structure-activity relationships and assess the efficacy of the compounds in inhibiting FAAH and MAGL. The implementation of LC–MS in this study ensured the acquisition of accurate and reliable results, providing a solid foundation for further studies in the field of medicinal organic chemistry. The mass spectral analysis of the compounds was performed using a mass spectrometer equipped with an electrospray ionization (ESI) source.

Theoretical molecular weights of Products 143, 153, 157, and 165 were calculated as 403.5 amu, 453.56 amu, 387.45 amu, and 447.51 amu, respectively. The actual molecular weights of these products were obtained from mass spectrum analysis. A comparison was made between the obtained values and the theoretical values, and the products were progressed to the next steps of the study only if the two values were in agreement.

Once identities were verified, the inhibitory effect of the compounds against FAAH was measured using a fluorescence assay. FAAH has been demonstrated to efficiently hydrolyze AEA, releasing the fluorescent product, AMC. Thus, AMC can be used as a fluorescent probe to indirectly measure the activity of the enzymes. This allows this assay to be suitable for a high-throughput screening.

The IC50 value, which represents the concentration of the compound required to inhibit 50% of the target activity, is a crucial parameter in the assessment of compound efficacy. The data revealed that compound 143, containing a pyridine in its southern portion, exhibited the

strongest inhibitory activity against FAAH. Compound 153 showed the second-best inhibitory activity, followed by 157 and 165. All compounds demonstrated potent inhibitory activity against FAAH in a dose-responsive manner.

The lower data points for compound 143 had relatively large error bars, suggesting the need for further analysis. Likewise, additional testing will be necessary to determine lower data points for compound 153 to increase reliability of the curve. The data for compound 165 was found to be scattered and unstable, indicating that it may not be the most effective FAAH inhibitor. Compound 157 demonstrated the most promising curve, with data points varying across levels and minimal error bars, indicating its potential as a potent inhibitor of FAAH.

The Monoacylglycerol Lipase/MGL Inhibitor Screening Kit provided by Abcam is a kit designed to measure the inhibitory effect of test compounds on MAGL activity. MAGL is an enzyme that hydrolyzes monoacylglycerols to glycerol and fatty acids, and it plays a role in the regulation of endocannabinoid signaling. This kit provides a convenient approach to identifying inhibitors of human MAGL. In the assay, a MAGL substrate is cleaved by a human recombinant MAGL. This results in a fluorescence increase that is negated by the presence of a MAGL inhibitor. With the help of a fluorescent plate reader, this kit can screen up to 96 test compounds in a high-throughput format.

Among the compounds tested, product 157, which contains pyridine in the southern region, exhibited the highest potency with an IC50 value of less than 10 nm and inhibition of over 99% across all dose levels. This finding suggests that product 157 has a high affinity for the target enzyme and could be a promising candidate for further development as a therapeutic agent. Following product 157, product 165, 143, and 153 exhibited decreasing levels of potency in that order.

The analysis of the obtained graphs revealed that all compounds, other than compound 157, had data points both above and below the IC50 values, indicating their dose responsive nature. The graph obtained for product 157 exhibited the least error bars and a desirable sigmoid curve, indicating a high level of reliability and potency. This was further supported by the percentage inhibition of over 99% across all data points and an IC50 below 10nM, confirming the potency of the compound. The other compounds did not exhibit comparable levels of potency as good potency threshold is typically considered to have an IC50 value under 300 nm, which they did not meet.

The assays also revealed that quinoline-containing compounds, such as products 153 and 165, demonstrated interesting potency against FAAH but only minimal activity against MAGL. It was noted that these compounds appeared to lose activity over the course of the assay, potentially due to instability or reactivity issues. In contrast, pyridine-containing compounds were found to be well-tolerated and maintained activity throughout the duration of both assays. This suggests that pyridine may be a more favorable aromatic structure for the design of stable and potent enzyme inhibitors.

Despite all designed compounds being potent inhibitors for both enzymes, our results demonstrated that compound 157 showed the most potential as a dual inhibitor of FAAH and MAGL in a dose-responsive manner. Therefore, it could be advanced into further studies and characterization to determine its clinical potential. Similarly, it was observed that pyridine-containing compounds were better tolerated within assays.

Overall, the study provides valuable insights into the potential of enzyme inhibition as a promising therapeutic strategy for ocular wound healing. It also explored the structure-activity relationship of these compounds that could guide the development of more effective inhibitors in

the future. This provides a solid foundation for future studies aimed at optimizing the structure of these compounds in order to maximize their efficacy.

Current/Future research

While these findings are promising, it is essential to note that further research is needed to fully evaluate the potential of these compounds. Further optimization of these structures, along with the additional characterization of synthesized compounds, is currently in progress alongside other work with FAAH and MAGL using advanced Activity-Based Protein Profiling (ABPP).

These experiments tested the potency of the FAAH and MAGL inhibitors designed based on current knowledge regarding the molecular interactions in the endocannabinoid system. Additional testing of other compounds with different types of permanently charged groups at different positions (Northern, Central, and Southern) has been planned. After further characterization of compounds using enzymes, permeability, and pharmacokinetic assays, in vivo studies can begin.

In vivo studies will be required to determine the therapeutic effect of the compound on live models and evaluate its membrane permeability. Nonetheless, the results of this study provide a solid foundation for future research aimed at developing effective FAAH and MAGL inhibitors for therapeutic use.

Conclusion

This study involved the synthesis and analysis of four novel compounds for their inhibitory effects against FAAH and MAGL. The compounds were synthesized via carbamate exchange reaction for the potential modulation of pain and inflammation in the eye. The identity of the synthesized compounds was verified using LC-MS, followed by the analysis of their inhibitory effects using assays, which provided dose-response curves for each compound.

Compound 143 was observed to have the lowest IC50 for FAAH (0.0174 μM), and hence is the most potent inhibitor of this enzyme, while compound 157 was shown to have the lowest IC50 against MAGL (<10 nm). Nonetheless, based on the IC50 values and their respective curves, it was determined that compound 157 showed the most promising inhibitory activity against both FAAH and MAGL. These findings suggest that compound 157 may have potential for further development as a lead compound for drug development against FAAH and MAGL and may warrant further investigation.

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