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DUAL INHIBITION OF ALPHA/BETA HYDROLASE DOMAIN 6 AND FATTY ACID AMIDE HYDROLASE INCREASES ENDOCANNABINOID LEVELS IN NEURONS

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Running title: Dual inhibition of ABHD6 and FAAH in neurons

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Agonists at cannabinoid receptors, such as the phytocannabinoid Δ^9-tetrahydrocannabinol, exert a remarkable array of therapeutic effects, but are also associated with undesirable psychoactive side effects. Conversely, targeting enzymes that hydrolyze endocannabinoids (eCBs) allows for more precise fine-tuning of cannabinoid receptor signaling, thus providing therapeutic relief with reduced side effects. Here we report the development and characterization of an inhibitor of eCB hydrolysis, UCM710, which augments both AEA and 2-AG levels in neurons. This compound displays a unique pharmacological profile in that it inhibits fatty acid amide hydrolase (FAAH) and alpha/beta hydrolase domain 6 (ABHD6), but not monoacylglycerol lipase (MAGL). Thus, UCM710 represents a novel tool to delineate the therapeutic potential of compounds that manipulate a subset of enzymes that control eCB signaling.

N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are lipid transmitters that act as endogenous ligands for the cannabinoid receptors CB_1 and CB_2. In the central nervous system, these eCBs are produced by neurons and glial cells in response to specific stimuli, and are rapidly inactivated by cellular uptake and enzymatic hydrolysis (*1*). The eCB signaling system regulates a wide array of physiological processes, including cell differentiation and viability, neurotransmission, and immune cell activation and migration. As such, the enzymes and receptors belonging to the eCB signaling system represent attractive therapeutic targets.

Unlike classical neurotransmitter systems in which signaling diversity is principally accomplished *via* multiple receptor subtypes for a single endogenous ligand, the eCB signaling system utilizes multiple endogenous ligands, two of which, AEA and 2-AG, have been studied in detail (*2-4*). Enzymes that produce and inactivate these eCBs are tightly, and for the most part independently, regulated (*5*). Thus, targeting these enzymes allows for the discrete manipulation of eCB signaling, laying the groundwork for focused therapeutic approaches. However, the respective roles of AEA and 2-AG in modulating neuronal and glial cell functions are still being delineated.

With regard to eCB inactivation, AEA is mainly hydrolyzed by FAAH, and accordingly selective FAAH inhibition increases AEA levels in neurons without affecting 2-AG levels (*6*). *In vivo* FAAH inhibition recapitulates a narrow subset of the behavioral effects produced by CB_1 agonists (*7*). 2-AG is hydrolyzed by at least two enzymes: MAGL (*8*) and ABHD6 (*9*). Selective MAGL inhibition increases 2-AG levels in neurons without affecting AEA levels (*8, 10*). *In vivo* MAGL inhibition recapitulates most of the behavioral effects induced by CB_1 agonists (a phenotype that is largely independent from that of FAAH inhibition), suggesting a functional segregation between AEA and 2-AG signaling

pathways *in vivo* (*11*). Concomitant inhibition of FAAH, MAGL and ABHD6 *in vivo* increases the levels of both AEA and 2-AG in brain, and recapitulates the complete behavioral phenotype induced by direct CB_1 agonists (*12*). Selective inhibition of ABHD6 *in vivo* has so far not been reported. Together, these results suggest that some cellular functions are controlled by AEA, some are controlled by 2-AG, and some are controlled by both of these eCBs. With these concepts in mind, we sought to develop novel inhibitors of eCB hydrolysis in order to expand the available repertoire of pharmacological tools for the exploration and exploitation of eCB signaling. Here we describe one such compound, UCM710 (compound **12**), which is a dual inhibitor of FAAH and ABHD6.

Experimental Procedures

Synthesis of compounds: Infrared (IR) spectra were determined on a Shimadzu-8300 infrared spectrophotometer. Optical rotation was measured using a Perkin-Elmer 781 polarimeter. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 3000-AM instrument. Chemical shifts (δ) are expressed in parts *per* million relative to internal tetramethylsilane; coupling constants (*J*) are in hertz. Mass spectrometry (MS) was carried out on a Bruker LC-Esquire in electrospray mode (ESI). Thin-layer chromatography (TLC) was run on Merck silica gel 60 F-254 plates. For normal pressure chromatography, Merck silica gel type 60 (size 70-230) was used. Starting materials used were high-grade commercial products from Aldrich, Acros or Fluka. Arachidonic, oleic, palmitoleic, γ-linolenic and linoleic acids (≥99% purity) were purchased from Sigma.

Spectroscopic data of all described compounds were consistent with the proposed structures. Satisfactory HPLC chromatograms were also obtained for the final compounds (**1-12**) and elemental analyses (C, H, N) for these compounds were obtained on a LECO CHNS-932 apparatus at the UCM's analysis services and were within 0.5% of the theoretical values, confirming a purity of at least 95% for all tested compounds.

Compounds **1-12** were synthesized according to the method previously described (*13*). Briefly, to a stirred solution of 1 equivalent (100 mg) of carboxylic acid in dry dichloromethane (0.82 ml/mmol) and the appropiate alcohol (5 equiv) in dry dichloromethane (0.27 ml/mmol) in ice bath under argon, a solution of dicyclohexylcarbodiimide (DCC, 1 equiv) and catalytic amounts of *N,N*-dimethyl-4-aminopyridine (DMAP, 0.068 equiv) in dry dichloromethane (1.9 ml/mmol) was added dropwise. The mixture was stirred for 5 min at this temperature and then removed from the cooling bath and stirred at room temperature (3-6 h) until no further evolution was observed by TLC. The dicyclohexylurea was filtered off, and the filtrate washed with saturated $NaHCO_3$. The organic extracts were dried over anhydrous Na_2SO_4. Then, the solvent was evaporated under reduced pressure and the product purified by column chromatography on silica gel using the appropriate eluent.

As a representative example, the complete characterization of compound **12** (UCM710) is given. (±)-Oxiran-2-ylmethyl (9*Z*)-hexadec-9-enoate (**12**). Yield: 45%. Chromatography: hexane/chloroform, 2:8. Rf: 0.5 (chloroform). IR ($CHCl_3$, cm^{-1}): 1551, 1745, 2854, 2928. ^1H-NMR (200 MHz, $CDCl_3$, δ): 0.89 (t, J = 6.3 Hz, 3H); 1.21-1.38 (m, 16H); 1.59-1.68 (m, 2H); 1.97-2.02 (m, 4H); 2.35 (t, J = 7.1 Hz, 2H); 2.64 (dd, J = 4.9; 2.4 Hz, 1H); 2.85 (dd, J = 5.0; 4.2 Hz, 1H); 3.15-3.25 (m, 1H); 3.90 (dd, J = 12.3; 6.3 Hz, 1H); 4.41 (dd, J = 12.3; 3.2 Hz, 1H); 5.25-5.36 (m, 2H). ^{13}C-NMR (50 MHz, $CDCl_3$, δ): 14.1, 22.6, 24.9, 27.1, 27.2, 28.9, 29.0 (2C), 29.2, 29.7, 29.8, 31.8, 34.1, 44.7, 49.3, 64.8, 129.7, 130.0, 173.4. Elemental analysis calcd. for $C_{19}H_{34}O_3$: C 73.50, H 11.04; found: C 73.63, H 11.18.

Cell Culture: COS-7 cells were expanded in DMEM (HyClone Cat. # SH30243.01) supplemented with HEPES (10 mM), $NaHCO_3$ (10 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and FBS (10%, HyClone Cat. # SH30071.03). Cells (~ 90% confluent in 10 cm dishes) were transfected with expression vectors (mFAAH, mMAGL, or mABHD6 in pcDNA3 plasmids, OpenBioSystems, 3 µg *per* dish) by using Lipofectamine™ 2000. After 4-6 hrs, the media was changed and the cells were incubated for an additional 30-36 hrs in DMEM + FBS (10%) before use for experiments. The enzymatic activity of mock-transfected COS-7 cell homogenates was 4.0 nmol 2-AG/mg/min,

whereas there was no detectable AEA hydrolysis in these homogenates. The enzymatic activities measured in mMAGL-transfected and mABHD6-transfected COS-7 cell homogenates were 68 and 5.2 nmol 2-AG/mg/min, respectively. The enzymatic activity for mFAAH-transfected COS-7 cell homogenates was 0.8 nmol AEA/mg/min. Mouse neurons in primary culture were prepared as described (*14*), according to the guidelines of the Institutional Animal Care and Use Committee of the University of Washington. Briefly, one-day-old mouse brains (C57BL/6) were collected, and their meninges and cerebellum removed. The remaining brain tissue was chopped, the cells dissociated and plated in 10 cm dishes (BD Falcon) coated with poly-D-lysine (0.1 mg/ml) at 5.0 X 10^5 cells/ml of Neurobasal Medium (Gibco Cat. # 21103-049) supplemented with B-27 (2%), Glutamax (1%), penicillin (100 U/ml), and streptomycin (100 µg/ml). Neurons were tested after 7-8 days in culture.

[^{3}H]-2-AG and [^{3}H]-AEA hydrolysis in cell homogenates and intact cells: Cell homogenates were prepared as previously described (*15*), and added to silanized glass tubes containing 100 µl of Tris-HCl buffer (100 mM, pH 7.4) supplemented with fatty acid-free BSA (0.1%), [^{3}H]-2-AG (~1 nM, 40 Ci/mmol, obtained from American Radiolabeled Chemicals, Inc.) or [^{3}H]-AEA (~1 nM, 60 Ci/mmol, obtained from American Radiolabeled Chemicals, Inc.), and inhibitors or vehicle (DMSO, 0.1%). Tubes containing this solution, but without cell homogenate, were used as control for non-enzymatic hydrolysis ("blank") and this value was systematically subtracted from values obtained with homogenates. Tubes were incubated for 10 min in a shaking water bath at 37°C. Reactions were stopped by adding ice-cold CH_3OH-$CHCl_3$ (1:1, 2 ml) and vortexing. The hydrophobic and hydrophilic phases were separated by centrifugation (800 x g, 10 min). One ml of the upper phase was recovered and mixed with Ecoscint (4 ml) for radioactivity determination by liquid scintillation. Note that linear enzymatic activity with a set amount of protein was systematically verified and chosen for each homogenate (Supplementary Figure 1), as well as for the intact cell experiments (Supplementary Figure 2). For intact cells, the following solution was prepared in a silanized glass vial and allowed to equilibrate at room temperature for 75 min: Hepes-Bicarbonate buffer (in mM): NaCl (120), KCl (5), $CaCl_2$ (2), $MgSO_4$ (1), NaH_2PO_4 (1), Glucose (10), $NaHCO_3$ (5), and HEPES (20), supplemented with BSA (0.15%) and [^{3}H]-2-AG or [^{3}H]-AEA (~1 nM). Cells grown in 12-well plates were pre-treated with inhibitors or vehicle for 30 min by adding 0.1 ml to each well. Cells were then incubated with either [^{3}H]-AEA (0.1 ml *per* well, ~130,000 dpm) or [^{3}H]-2-AG solution (0.1 ml *per* well, ~90,000 dpm) for the indicated amount of time (with gentle shaking in a water bath at 37^{o}C). The entire media (~1 ml) was recovered in a silanized glass tube, ice-cold CH_3OH (2 x 1 ml) was added to the cells, and the resulting lysate pooled with the media. $CHCl_3$ was added such that there was a final ratio of 1:2:2 for Media:CH_3OH:$CHCl_3$. The amount of [^{3}H]-ethanolamine or [^{3}H]-glycerol present in the hydrophilic phase was quantified by liquid scintillation as described above.

eCB levels: Neurons grown in 10 cm dishes (one dish *per* condition) were pretreated for 30 min with inhibitors (10x) or vehicle (DMSO, 0.1%) by adding 1 ml directly to the media (37˚C, shaking water bath). To stimulate the neurons, one ml of glutamate (100 µM) and carbachol (1 mM) was added for an additional 2.5 min (in the continued presence of the inhibitor) (*16*). The reaction was stopped by collecting the media and adding 5 ml of ice-cold CH_3OH. 2-AG and AEA were extracted, purified, and their levels determined as described (*17*). Briefly, the cell media and homogenate were added to $CHCl_3$ containing d_5-2-AG (150 pmol) and d_4-AEA (50 pmol) for Folch extraction. The organic phase was recovered and dried under N_2, and AEA and 2-AG partially purified by solid-phase chromatography columns (silica), eluting them with ethylacetate/acetone (1:1, 2 ml). Eluates were dried under N_2, derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, SupelCo) and analyzed by CI-GC-MS (Varian CP-8400 Autosampler, CP3800 Varian GC, Varian Saturn 2000 mass spectrometer). Under basal conditions, the measured levels of AEA and 2-AG were 1.8 and 20 fmol/100,000 neurons, respectively.

Radioligand binding: Competition of compounds against [^{3}H]CP-55,940 binding at untagged, full-length, mouse CB_1 and CB_2 receptors was performed as described (*18*). Briefly, mouse

astrocytoma cells, DBT cells, stably expressing either CB_1 or CB_2 receptors were homogenized and P2 membrane fractions isolated. [^{3}H]CP-55,940 (1-2 nM) ± 1 μM CP-55,940 were incubated for 1 hr with 100 μg membrane fraction *per* reaction. Rapid filtration over GF/B glass fiber filters stopped reactions.

Results

To develop novel inhibitors of eCB hydrolysis, we designed and synthesized a series of esters that were based on the 2-AG scaffold, but with its glycerol moiety replaced by different oxygenated heterocycles (except **7,** which contains a Michael acceptor) (*13*). These derivatives conserved the arachidonic acid moiety (**1-8**) or other relevant fatty acid moieties ranging from one to three unsaturations (**9-12**). These compounds were tested for their ability to inhibit AEA and 2-AG hydrolysis in neuron homogenates, with the goal of identifying compounds with high potency against these two enzymatic activities. We found that all 12 compounds inhibited both AEA and 2-AG hydrolysis in neuron homogenates with IC_{50} values between 0.2 to 32 μM (Table 1).

In neurons, AEA hydrolysis is mediated by FAAH, and 2-AG hydrolysis is mediated by MAGL and ABHD6 (*10*). To determine the selectivity of these 12 compounds at these enzymes, we tested their ability to inhibit eCB hydrolysis in homogenates prepared from COS-7 cells heterologously expressing FAAH, MAGL or ABHD6. Table 1 summarizes the IC_{50} values for compounds **1-12**. Remarkably, three of the compounds that we tested (**7**, **11** and **12**) inhibited FAAH and ABHD6 activities, without significantly affecting MAGL activity.

We focused the next set of experiments on compound **12**, as it exhibited the highest potency against FAAH and ABHD6, as well as the highest selectivity *versus* MAGL. Specifically, compound **12** dose-dependently inhibited AEA hydrolysis by FAAH and 2-AG hydrolysis by ABHD6 (IC_{50} = 4.0 μM and 2.4 μM, respectively), and had no effect on 2-AG hydrolysis by MAGL (Figure 1A). Furthermore, this compound was tested for its ability to interact with CB_1 and CB_2 receptors, and we found that it does not compete for [^{3}H]-CP55940 binding at either receptor when tested at concentrations up to 30 μM (data not shown). Thus, **12** targets FAAH and ABHD6, but not MAGL, CB_1 or CB_2.

Compound **12** efficiently inhibits eCB hydrolysis in neuron homogenates, but this does not necessarily mean that it is efficacious in intact cells. To address this question, we measured eCB hydrolysis by intact neurons in primary culture in the presence of **12**, and found that it inhibits AEA and 2-AG hydrolysis in intact neurons by 60% and 30%, respectively (Figure 1B). Thus, **12** is a dual inhibitor of FAAH and ABHD6 that retains its efficacy in intact neurons, although not fully inhibiting these activities.

Inhibition of eCB hydrolysis in intact neurons typically only increases the *stimulated* accumulation of AEA and 2-AG, not the basal level of these eCBs (*10*). To assess the effect of **12** on eCB accumulation in neurons in primary culture, AEA and 2-AG levels under both basal and stimulated conditions were quantified by chemical ionization gas chromatography - mass spectrometry (CI-GC-MS). The stimulation protocol consisted of treatment with glutamate (100 μM) and carbachol (1 mM) for 2.5 min, which increases eCB production in these cells (*16*). Compound **12** had no effect on the basal levels of AEA or 2-AG in neurons, but both of these eCBs were significantly increased under stimulated conditions (Figure 1C,D), suggesting that inhibition of ABHD6 and FAAH is sufficient to increase the stimulated accumulation of 2-AG and AEA in intact neurons, even when MAGL activity is preserved.

Discussion

Considering the plethora of medicinal effects produced by drugs activating cannabinoid receptors, there is growing interest in understanding the therapeutic potential of compounds targeting specific enzymes of the eCB signaling system to develop treatments that lack the typical side effects associated with cannabinoid agonists. Enzymes that regulate the levels of eCBs constitute attractive targets because they represent control points within the eCB signaling system. For example, *in vivo* inhibition of FAAH causes a selective elevation of AEA levels in brain, which results in significant reductions in pain, anxiety and depression, while not inducing other common cannabinoid

behavioral effects such as body temperature reduction and catalepsy (*7*). On the other hand, *in vivo* inhibition of MAGL causes a robust elevation of 2-AG levels in brain, but the therapeutic efficacy of targeting this enzyme is limited by the observation that chronic MAGL inhibition results in a significant desensitization of CB_1 receptors in the brain (*5*). Since MAGL is the dominant 2-AG hydrolase in the adult brain (*9*), it is perhaps not surprising that inhibition of this enzyme has a profound stimulatory effect on the eCB signaling system *in vivo*, resulting in an "over-flow" of 2-AG that causes compensatory adaptations similar to what is seen with chronic administration of direct CB_1 receptor agonists (*19*). In support of this view, it's worth noting that 2-AG is a potent full agonist at cannabinoid receptors, whereas AEA is a partial agonist, and 2-AG is much more abundant in the brain than AEA (*20, 21*). Thus it is important to assess the therapeutic potential of targeting other 2-AG hydrolases, such as ABHD6, to determine whether a moderate reduction of 2-AG hydrolysis represents the key to operating within the "therapeutic window" of enhanced 2-AG signaling. Indeed, since ABHD6 has a different expression pattern than MAGL (*10, 22*), and also exhibits less intrinsic enzymatic activity than MAGL (*9*), ABHD6 inhibition is likely to lead to a differential activation profile of CB_1 receptors in brain compared to MAGL inhibition. Furthermore, considering that some of the functions of the eCB signaling system are mediated by AEA and 2-AG working together, concomitant inhibition of FAAH and ABHD6 has the potential to provide unique therapeutic benefits.

We now report the development of a series of structurally similar "substrate-mimicking" inhibitors of eCB hydrolysis. The most potent inhibitor of the series, compound **12**, as well as two additional compounds, **7** and **11**, display a unique pharmacological profile in that they inhibit FAAH and ABHD6, but not MAGL. This raises the question of how these compounds manage to attain such selectivity. Having been developed as substrate-mimicking inhibitors, these compounds presumably exert their effects by interacting directly with the active sites of these enzymes, rather than by modulating their enzymatic activity *via* an allosteric site (*23*). Thus, our results suggest that competitive inhibitors that contain a 2-(acryloyloxy)ethyl head group (compound **7**) or an oxiran-2-yl head group (compounds **11** and **12**) will not fit into the active site of MAGL. When considering the differences between the active site of MAGL and the active sites of FAAH and ABHD6, a striking feature is the unique cap domain that covers the MAGL active site, confirmed by the analysis of the crystal structure of human MAGL (*24*). This cap domain is involved in substrate recognition and is crucial for mediating the necessary interaction between this soluble protein and the lipophilic environments of its substrates. While the crystal structure of ABHD6 remains unsolved, this enzyme is predicted to be an integral membrane protein with its active site facing the cytosol, similar to FAAH (*25*). Furthermore, based on what is known about the amino acid sequences and putative structures of FAAH and ABHD6, neither of these enzymes is believed to contain a cap domain analogous to the one found close to the MAGL active site. On the other hand, there are also key differences between the active sites of FAAH and ABHD6 (such as the amino acid residues that make up their catalytic triads and the surrounding residues that form the binding pocket) (*26*). Regardless of these considerations, compounds that manage to inhibit FAAH and ABHD6 while sparing MAGL represent useful chemical tools to probe the roles of these enzymes in regulating the eCB signaling system.

Stimulation of neurons in primary culture with glutamate and carbachol does not elevate eCB levels unless the stimulation is combined with inhibitors of eCB hydrolysis, suggesting that in the absence of any inhibitor the stimulated production of eCBs is controlled by this hydrolytic enzymatic activity. Inhibition of MAGL supports this point, since 2-AG levels are much higher in stimulated neurons when MAGL inhibitors are present (*10*). Accordingly, MAGL is known to play an important role in regulating 2-AG levels in neurons (*8*), but it has been less clear whether it is possible to significantly increase the stimulated accumulation of 2-AG in these cells by targeting a 2-AG hydrolase other than MAGL (*10*). Here we report that compound **12** increases the stimulated accumulation of both AEA and 2-AG in intact neurons, demonstrating that it is indeed possible to

augment 2-AG levels in intact neurons in this manner, even when MAGL is fully active. This result lends further credibility to the notion that ABHD6 represents a promising target for the fine-tuned modulation of eCB levels in the nervous system.

In summary, we have described a novel inhibitor of eCB hydrolysis, UCM710 (compound **12**), with efficacy at controlling AEA and 2-AG levels in intact neurons by blocking FAAH and ABHD6 activities, while preserving MAGL activity. In addition to specific inhibitors for each of these three enzymes, inhibitors of various combinations of the different eCB-hydrolyzing enzymes have been reported as well, but MAGL has consistently been one of the targets of these less-selective compounds. Thus, UCM710 represents the first reported compound that inhibits FAAH and ABHD6 without affecting MAGL. We believe that this addition to the pharmacological repertoire provides a valuable tool to assist researchers in the further elucidation of the pathophysiological role of the eCB signaling system, as well as to guide the development of a new generation of therapeutically useful compounds that would selectively target specific aspects of the eCB signaling system.

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Footnotes

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The abbreviations used are: ABHD6, alpha/beta hydrolase domain 6; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; eCB, endocannabinoid; AEA, N-arachidonoylethanolamine; 2-AG, 2-arachidonoylglycerol; CI-GC-MS, chemical ionization gas chromatography-mass spectrometry.

Figure Legends

Figure 1: Compound **12** inhibits FAAH and ABHD6, and augments AEA and 2-AG levels in intact neurons

A) [^{3}H]-AEA hydrolysis in homogenates prepared from COS-7 cells transfected with FAAH is inhibited by **12** in a dose-dependent manner; similarly, [^{3}H]-2-AG hydrolysis in homogenates prepared from COS-7 cells transfected with ABHD6 is inhibited by **12** in a dose-dependent manner; however, [^{3}H]-2-AG hydrolysis in homogenates prepared from COS-7 cells transfected with MAGL is not inhibited by **12**. B) [^{3}H]-AEA hydrolysis and [^{3}H]-2-AG hydrolysis by intact neurons in primary culture are both inhibited by treatment with a maximally effective concentration of **12** (30 μM). ** $p<0.01$, *** $p<0.001$ Unpaired t test (compared to respective vehicle treated controls). C) Levels of endogenously-produced AEA in intact neurons in primary culture after treatment with a maximally effective concentration of **12** (30 μM) or its vehicle (DMSO); AEA levels were significantly increased by **12** treatment only when the neurons were exposed to stimulating conditions (right). D) Levels of endogenously-produced 2-AG in intact neurons in primary culture after treatment with a maximally effective concentration of **12** (30 μM) or its vehicle (DMSO); 2-AG levels were significantly increased by **12** treatment only when the neurons were exposed to stimulating conditions (right). Data are mean ± s.e.m. of three separate experiments. * $p<0.05$, ** $p<0.01$ One-way ANOVA followed by Dunnett post-test (compared to Basal DMSO condition).

Table 1: [^{3}H]-2-AG and [^{3}H]-AEA hydrolysis competed by compounds 1-12

Compounds **1-12** were tested for their potency at inhibiting [^{3}H]-AEA and [^{3}H]-2-AG hydrolysis in homogenates prepared from primary neurons, and also in homogenates prepared from COS-7 cells heterologously expressing FAAH, MAGL or ABHD6. Compounds were serially diluted and spiked into homogenates before measuring enzymatic hydrolysis in order to obtain dose-response curves for each compound. Data values reflect three separate dose-response experiments, each performed in duplicate. N/A means IC_{50} > 1 mM.

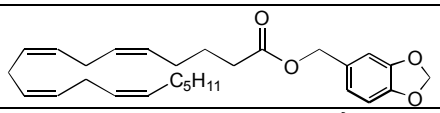
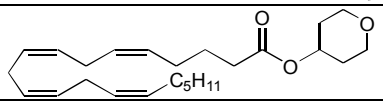
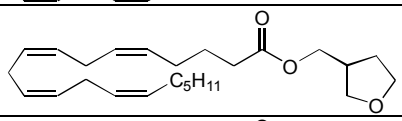
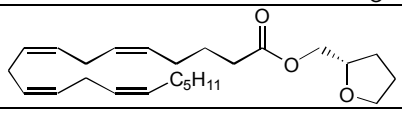
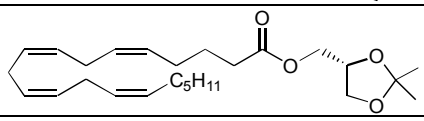
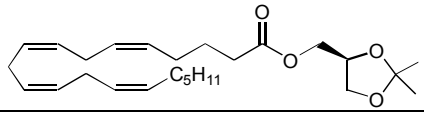
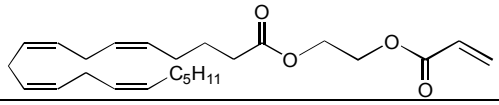
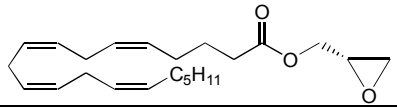
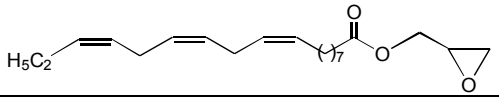
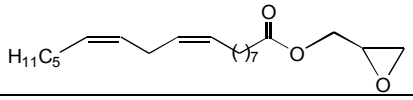
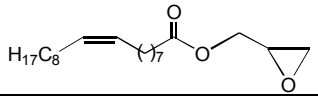
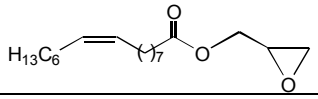
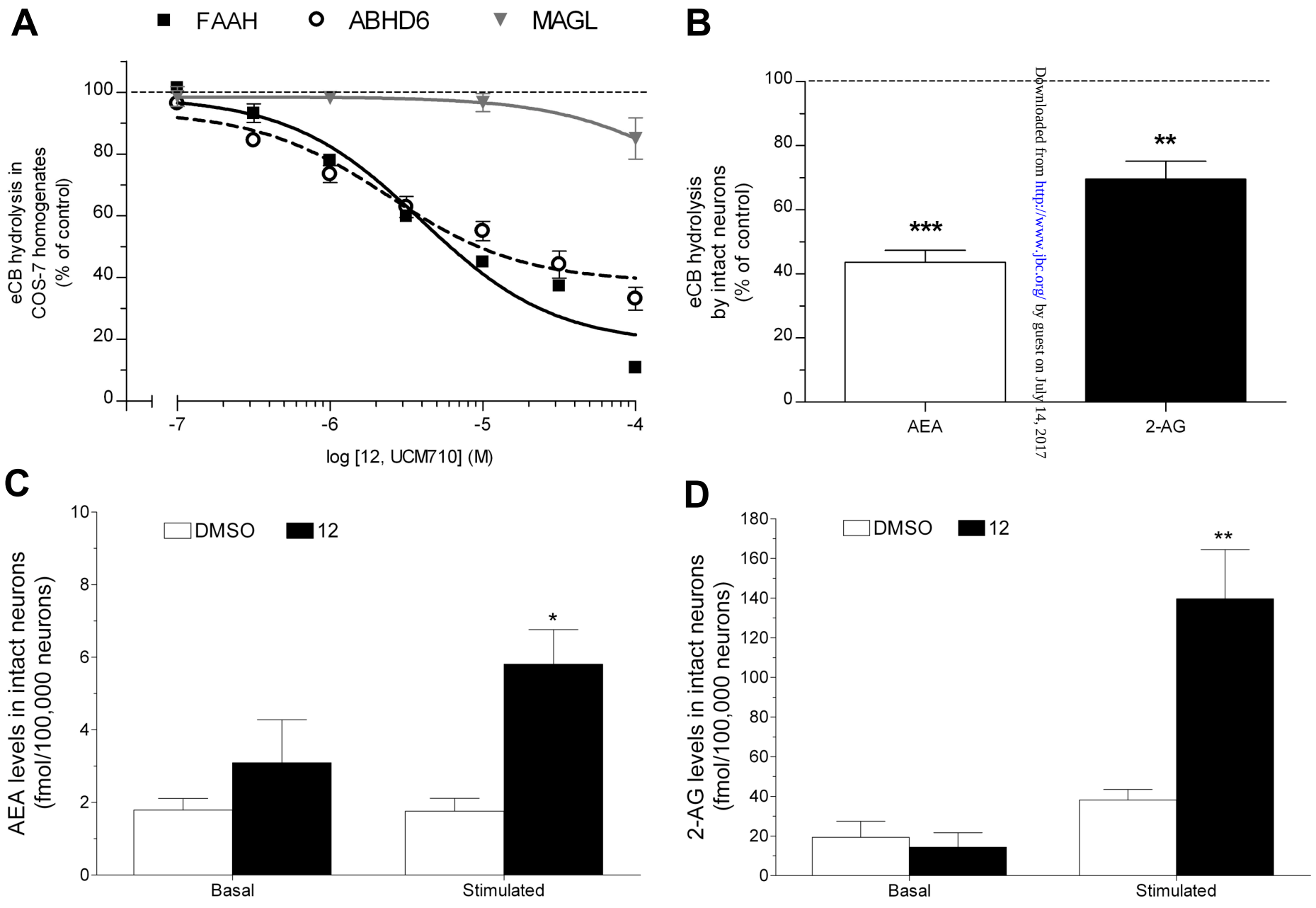
Compound	Structure	Inhibition of 2-AG hydrolysis in neurons (IC_{50}, μM)	Inhibition of AEA hydrolysis in neurons (IC_{50}, μM)	Inhibition of ABHD6-mediated 2-AG hydrolysis (IC_{50}, μM)	Inhibition of MAGL-mediated 2-AG hydrolysis (IC_{50}, μM)	Inhibition of FAAH-mediated AEA hydrolysis (IC_{50}, μM)
1		27.0	1.0	8.5	5.5	3.9
2		5.5	1.6	4.0	5.7	12.0
3		7.7	4.5	54.0	60.0	5.0
4		4.9	0.7	24.2	26.6	8.6
5		11.3	5.3	24.9	87.4	4.5
6		19.2	7.1	63.8	90.2	87.0
7		31.7	0.9	9.8	N/A	54.8
8		4.9	3.2	45.0	40.7	45.2
9		1.7	0.3	0.8	270	0.9
10		2.5	0.2	2.5	120	3.1
11		14.6	1.9	130	N/A	200
12 (UCM710)		1.2	0.4	2.4	N/A	4.0

Figure 1



Dual inhibition of alpha/beta hydrolase domain 6 and fatty acid amide hydrolase increases endocannabinoid levels in neurons

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