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Dual Inhibition of α/β-Hydrolase Domain 6 and Fatty Acid Amide Hydrolase Increases Endocannabinoid Levels in Neurons*[S]

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Agonists at cannabinoid receptors, such as the phytocannabinoid Δ⁹-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 *N*-arachidonoylethanolamine and 2-arachidonoylglycerol levels in neurons. This compound displays a unique pharmacological profile in that it inhibits fatty acid amide hydrolase and α/β-hydrolase domain 6 but not monoacylglycerol lipase. 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)[2] 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 endocannabinoids (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 hydrolyzed mainly 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: monoacylglycerol lipase (MAGL) (8) and α/β-hydrolase domain 6 (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 of 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 cell 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 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.

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[2] The abbreviations used are: AEA, *N*-arachidonoylethanolamine; 2-AG, 2-arachidonoylglycerol; CB_1 and CB_2, cannabinoid receptor types 1 and 2, respectively; eCB, endocannabinoid; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; ABHD6, α/β-hydrolase domain 6; m, mouse.

EXPERIMENTAL PROCEDURES

Synthesis of Compounds—IR spectra were determined on a Shimadzu 8300 infrared spectrophotometer. Optical rotation was measured using a PerkinElmer Life Sciences 781 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 3000-AM instrument. Chemical shifts (δ) are expressed in parts/million relative to internal tetramethylsilane; coupling constants (*J*) are in hertz. MS was carried out on a Bruker LC-Esquire spectrometer in electrospray mode. TLC was performed on Merck Silica Gel 60 F_{254} plates. For normal pressure chromatography, Merck Silica Gel 60 (size 70–230) was used. The 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 (carbon, hydrogen, and nitrogen) for these compounds were obtained on a LECO CHNS-932 apparatus (analysis services of the Universidad Complutense de Madrid) 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 described previously (13). Briefly, to a stirred solution of 1 eq (100 mg) of carboxylic acid in dry dichloromethane (0.82 ml/mmol) and the appropriate alcohol (5 eq) in dry dichloromethane (0.27 ml/mmol) in an ice bath under argon was added dropwise a solution of dicyclohexylcarbodiimide (1 eq) and catalytic amounts of *N*,*N*-dimethyl-4-aminopyridine (0.068 eq) in dry dichloromethane (1.9 ml/mmol). 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 was washed with saturated $NaHCO_3$. The organic extracts were dried over anhydrous Na_2SO_4. The solvent was then evaporated under reduced pressure, and the product was 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, 2:8 hexane/chloroform; R_F, 0.5 (chloroform); IR ($CHCl_3$, cm^{-1}): 1551, 1745, 2854, and 2928; ^{1}H NMR (200 MHz, $CDCl_3$, δ): 0.89 (t, J = 6.3 Hz, 3*H*), 1.21–1.38 (m, 16*H*), 1.59–1.68 (m, 2*H*), 1.97–2.02 (m, 4*H*), 2.35 (t, J = 7.1 Hz, 2*H*), 2.64 (dd, J = 4.9 and 2.4 Hz, 1*H*), 2.85 (dd, J = 5.0 and 4.2 Hz, 1*H*), 3.15–3.25 (m, 1*H*), 3.90 (dd, J = 12.3 and 6.3 Hz, 1*H*), 4.41 (dd, J = 12.3 and 3.2 Hz, 1*H*), and 5.25–5.36 (m, 2*H*); ^{13}C NMR (50 MHz, $CDCl_3$, δ): 14.1, 22.6, 24.9, 27.1, 27.2, 28.9, 29.0 (2*C*), 29.2, 29.7, 29.8, 31.8, 34.1, 44.7, 49.3, 64.8, 129.7, 130.0, and 173.4; elemental analysis calculated for $C_{19}H_{34}O_3$: C 73.50, H 11.04, and found: C 73.63, H 11.18.

Cell Culture—COS-7 cells were expanded in DMEM (HyClone catalog no. SH30243.01) supplemented with HEPES (10 mM), $NaHCO_3$ (10 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and FBS (10%; HyClone catalog no. SH30071.03). Cells (~90% confluent in 10-cm dishes) were transfected with expression vectors (mouse (m) FAAH, mMAGL, or mABHD6 in pcDNA3 plasmids (3 μg/dish), Open BioSystems) using Lipofectamine™ 2000. After 4–6 h, the medium was changed, and the cells were incubated for an additional 30–36 h in DMEM + FBS (10%) before use in experiments. The enzymatic activity of mock-transfected COS-7 cell homogenates was 4.0 nmol of 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 of 2-AG/mg/min, respectively. The enzymatic activity in mFAAH-transfected COS-7 cell homogenates was 0.8 nmol of 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, 1-day-old mouse brains (C57BL/6) were collected, and their meninges and cerebellums were removed. The remaining brain tissue was chopped, and the cells were dissociated and plated in 10-cm dishes (BD Falcon) coated with poly-D-lysine (0.1 mg/ml) at 5.0×10^5 cells/ml of Neurobasal Medium (Invitrogen catalog no. 21103-049) supplemented with B-27 (2%), GlutaMAX (1%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Neurons were tested after 7–8 days in culture.

2-[^{3}H]AG and [^{3}H]AEA Hydrolysis in Cell Homogenates and Intact Cells—Cell homogenates were prepared as described previously (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%), 2-[^{3}H]AG (~1 nM, 40 Ci/mmol; obtained from American Radiolabeled Chemicals) or [^{3}H]AEA (~1 nM, 60 Ci/mmol; obtained from American Radiolabeled Chemicals), and inhibitors or vehicle (dimethyl sulfoxide, 0.1%). Tubes containing this solution but without cell homogenate were used as controls 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 the addition of ice-cold $CH_3OH/CHCl_3$ (1:1, 2 ml) and vortexing. The hydrophobic and hydrophilic phases were separated by centrifugation (800 × *g*, 10 min). One ml of the upper phase was recovered and mixed with Ecoscint (4 ml) for determination of radioactivity by liquid scintillation. Linear enzymatic activity with a set amount of protein was systematically verified and chosen for each homogenate (supplemental Fig. 1), as well as for the intact cell experiments (supplemental Fig. 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 containing NaCl (120 mM), KCl (5 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1 mM), NaH_2PO_4 (1 mM), glucose (10 mM), $NaHCO_3$ (5 mM), and HEPES (20 mM) supplemented with BSA (0.15%) and 2-[^{3}H]AG or [^{3}H]AEA (~1 nM). Cells grown in 12-well plates were pretreated with inhibitors or vehicle for 30 min by the addition of 0.1 ml to each well. Cells were then incubated with either [^{3}H]AEA (0.1 ml/well, ~130,000 dpm) or 2-[^{3}H]AG solution (0.1 ml/well, ~90,000 dpm) for the indicated amount of time (with gentle shaking in a water bath at 37 °C). The entire medium (~1 ml) was recovered

in a silanized glass tube, ice-cold CH_3OH (2× 1 ml) was added to the cells, and the resulting lysate was pooled with the medium. $CHCl_3$ was added such that the final ratio was 1:2:2 medium/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/condition) were pretreated for 30 min with inhibitors (10×) or vehicle (dimethyl sulfoxide, 0.1%) by the addition of 1 ml directly to the medium (37 °C, shaking water bath). To stimulate the neurons, 1 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 medium and adding 5 ml of ice-cold CH_3OH. 2-AG and AEA were extracted and purified, and their levels were determined as described (17). Briefly, the cell medium 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 were partially purified by solid-phase chromatography columns (silica), eluting them with ethyl acetate/acetone (1:1, 2 ml). Eluates were dried under N_2, derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Supelco), and analyzed by chemical ionization GC-MS (Varian CP-8400 AutoSampler, Varian CP-3800 gas chromatograph, and 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 (delayed brain tumor cells) stably expressing either CB_1 or CB_2 receptors were homogenized, and P2 membrane fractions were isolated. [^{3}H]CP-55,940 (1–2 nM) with and without 1 μM CP-55,940 was incubated for 1 h with 100 μg of membrane fraction/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 compound **7**, which contains a Michael acceptor) (13). These derivatives conserved the arachidonic acid moiety (compounds **1–8**) or other relevant fatty acid moieties ranging from one to three unsaturations (compounds **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 and 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 for 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 and 2.4 μM, respectively) and had no effect on 2-AG hydrolysis by MAGL (Fig. 1*A*). Furthermore, this compound was tested for its ability to interact with CB_1 and CB_2 receptors, and we found that it did not compete for [^{3}H]CP-55,940 binding at either receptor when tested at concentrations up to 30 μM (data not shown). Thus, compound **12** targets FAAH and ABHD6 but not MAGL, CB_1, or CB_2.

Compound **12** efficiently inhibited 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 compound **12** and found that it inhibited AEA and 2-AG hydrolysis in intact neurons by 60 and 30%, respectively (Fig. 1*B*). Thus, compound **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 increases only the *stimulated* accumulation of AEA and 2-AG, not the basal level of these eCBs (10). To assess the effect of compound **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 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 (Fig. 1, *C* and *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.

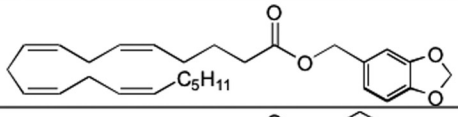
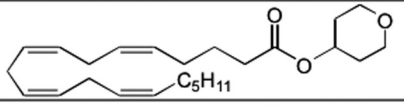
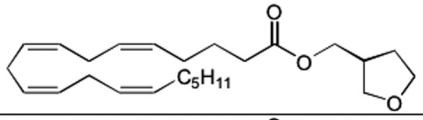
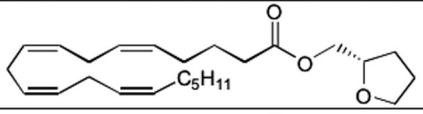
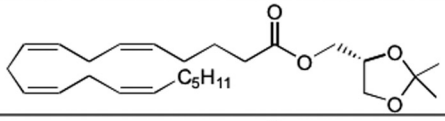
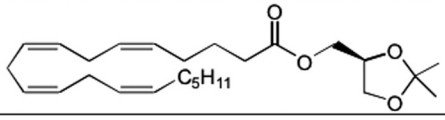
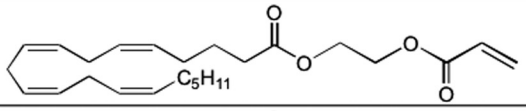
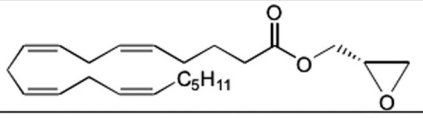
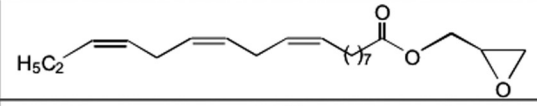
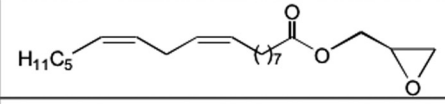
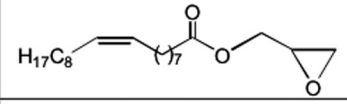
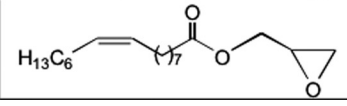
DISCUSSION

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). Because

TABLE 1

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

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

MAGL is the dominant 2-AG hydrolase in 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 "overflow" 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 is

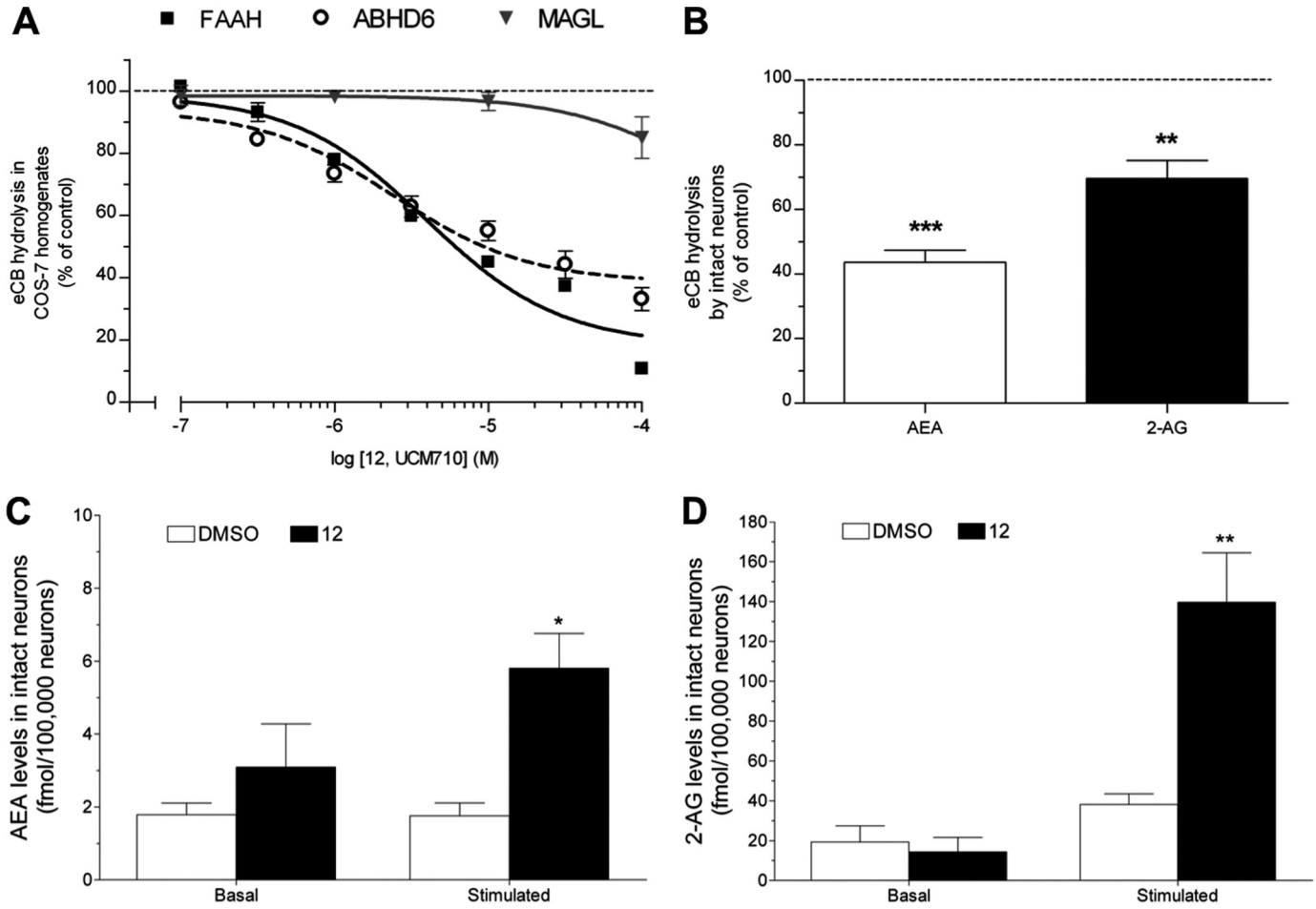


FIGURE 1. **Compound 12 inhibits FAAH and ABHD6 and augments AEA and 2-AG levels in intact neurons.** *A*, [^{3}H]AEA hydrolysis in homogenates prepared from COS-7 cells transfected with FAAH was inhibited by compound **12** in a dose-dependent manner. Similarly, 2-[^{3}H]AG hydrolysis in homogenates prepared from COS-7 cells transfected with ABHD6 was inhibited by compound **12** in a dose-dependent manner. However, 2-[^{3}H]AG hydrolysis in homogenates prepared from COS-7 cells transfected with MAGL was not inhibited by compound **12**. *B*, [^{3}H]AEA hydrolysis and 2-[^{3}H]AG hydrolysis by intact neurons in primary culture were both inhibited by treatment with a maximally effective concentration of compound **12** (30 μM). **, $p < 0.01$; ***, $p < 0.001$ (unpaired *t* test; compared with respective vehicle-treated controls). *C*, levels of endogenously produced AEA in intact neurons in primary culture after treatment with a maximally effective concentration of compound **12** (30 μM) or its vehicle (dimethyl sulfoxide (*DMSO*)). AEA levels were significantly increased by compound **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 compound **12** (30 μM) or its vehicle (dimethyl sulfoxide). 2-AG levels were significantly increased by compound **12** treatment only when the neurons were exposed to stimulating conditions (*right*). Data are means ± S.E. of three separate experiments. *, $p < 0.05$; **, $p < 0.01$ (one-way analysis of variance, followed by Dunnett's post-test; compared with basal dimethyl sulfoxide conditions).

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 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, because 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 with 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 have reported the development of a series of structurally similar "substrate-mimicking" inhibitors of eCB hydrolysis. The most potent inhibitor of the series, compound **12**, and 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 headgroup (compound **7**) or an oxiran-2-yl headgroup (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. Although 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 because 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 have reported 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 in 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 and 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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