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Development of the first potent and specific inhibitors of endocannabinoid biosynthesis

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Abstract

Enzymes for the biosynthesis and degradation of the endocannabinoid 2-arachidonoyl glycerol (2-AG) have been cloned and are the *sn*-1-selective-diacylglycerol lipases α and β (DAGLα and β) and the monoacylglycerol lipase (MAGL), respectively. Here, we used membranes from COS cells over-expressing recombinant human DAGLα to screen new synthetic substances as DAGLα inhibitors, and cytosolic fractions from wild-type COS cells to look for MAGL inhibitors. DAGLα and MAGL activities were assessed by using *sn*-1-[^{14}C]-oleoyl-2-arachidonoyl-glycerol and 2-[^{3}H]-arachidonoylglycerol as substrates, respectively. We screened known compounds as well as new phosphonate derivatives of oleic acid and fluoro-phosphinoyl esters of different length. Apart from the general lipase inhibitor tetrahydrolipstatin (orlistat®) (IC_{50} ~ 60 nM), the most potent inhibitors of DAGLα were O-3640 [octadec-9-enoic acid-1-(fluoro-methyl-phosphoryloxymethyl)-propylester] (IC_{50} = 500 nM), and O-3841 [octadec-9-enoic acid 1-methoxymethyl-2-(fluoro-methyl-phosphinoyloxy)-ethyl ester] (IC_{50} = 160 nM). Apart from being almost inactive on MAGL, these two compounds showed high selectivity over rat liver triacylglycerol lipase, rat *N*-acylphosphatidyl-ethanolamine-selective phospholipase D (involved in anandamide biosynthesis), rat fatty acid amide hydrolase and human recombinant cannabinoid CB_1 and CB_2 receptors. Methylarachidonoyl-fluorophosphonate and the novel compound UP-101 [O-ethyl-*O*-*p*-nitrophenyl oleylphosphonate] inhibited both DAGLα and MAGL with similar potencies (IC_{50} = 0.8–0.1 and 3.7–3.2 μM, respectively). Thus, we

Abbreviations: AA-5-HT, arachidonoyl-5-hydroxytryptamine; 2-AG, 2-Arachidonoyl-glycerol; AMT, anandamide membrane transporter; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; [^{3}H]CP-55,940, [^{3}H]-(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol; DAGLα, *sn*-1-selective-diacylglycerol lipase α; DAGLβ, *sn*-1-selective-diacylglycerol lipase β; DAGs, diacylglycerols; DAST, diethylamino-sulfur trifluoride; DCC, *N,N′*-Dicycloexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DSE, depolarization-induced suppression of excitatory neurotransmission; DSI, depolarization-induced suppression of inhibitory neurotransmission; EDCI, N-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride; FAAH, fatty acid amide hydrolase; MAFP, methylarachidonoyl-fluorophosphonate; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acylphosphatidyl-ethanolamine-selective phospholipase D; O-3453, octadec-9-enoic acid-2-(methoxy-fluoro-phosphinoyl)-1-octadec-9-enoyloxymethyl-ethyl ester; O-3640, octadec-9-enoic acid-1-(fluoro-methyl-phosphoryloxymethyl)-propylester; O-3696, octadec-9-enoic acid-1-(fluoro-methoxy-phosphinoylmethyl)-hexyl ester; O-3841, octadec-9-enoic acid 1-methoxymethyl-2-(fluoro-methyl-phosphinoyloxy)-ethyl ester; OMDM-1, 4-hydroxybenzil-N-oleoyl-ethanolamide; PA, phosphatidic acid; PI, phosphoinositides; PI-PLC, PI-selective phospholipase C; RHC80267, 1,6-bis-(cyclohexyloximino-carbonylamino)-hexane; solketal, 2,2-dimethyl-[1,3]dioxolan-4-yl-methanol; TEP, triethyl-phosphite; THL, tetrahydrolipstatin (orlistat®); UP99, *O,O*-diethyl oleylphosphonate; UP100, *O*-Ethyl oleylphosphonate; UP-101, O-ethyl-*O*-*p*-nitro-phenyl oleylphosphonate; UP104, *O*-ethyl-*O*-oleyl oleylphosphonate; VDM-11, 2-methyl-4-hydroxybenzyl-*N*-arachidonoyl-ethanolamine

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report the first potent and specific inhibitors of the biosynthesis of 2-AG that may be used as pharmacological tools to investigate the biological role of this endocannabinoid.

Keywords: Inhibitor; Diacylglycerol; Cannabinoid; 2-Arachidonoylglycerol; Lipase

1. Introduction

2-Arachidonoyl-glycerol (2-AG), an intermediate in phosphoglyceride and glyceride metabolism, acts as endogenous ligand for both CB_1 and CB_2 cannabinoid receptors [1,2], i.e., as an endocannabinoid [3]. 2-AG and anandamide [4] are the best investigated endocannabinoids, but 2-AG was recognized as the most abundant and selective member of this class of mediators. Its involvement as a neuromodulator in both short- and long-term synaptic plasticity, as well as in diseases of central and peripheral organs has been proposed [5–7]. Several stimuli were shown to lead to the formation of 2-AG in intact neuronal and non-neuronal cells. In most cases, the diacylglycerols (DAGs) serving as direct precursors for 2-AG biosynthesis can be produced, depending on the type of stimulus, from the hydrolysis of either phosphoinositides (PI), catalyzed by a PI-selective phospholipase C (PI-PLC), or phosphatidic acid (PA), catalyzed by a PA phosphohydrolase (see [7,8] for reviews). Two *sn*-1-DAG lipases (DAGLα and DAGLβ) have been cloned, characterized and proposed to be responsible for the conversion of DAGs into 2-AG [9]. Like with the enzyme involved in anandamide biosynthesis [10], the two DAGLs are not selective for 2-arachidonate-containing DAGs. When transfected into COS-7 cells, both proteins localize to the plasma membrane, are stimulated by Ca^{2+} and GSH and are optimally active at pH = 7. While DAGLα is more abundant in the adult brain, DAGLβ seems to be predominantly expressed in the developing brain. Both enzymes, however, undergo a shift in their neuronal localization during development, passing from axons to dendrites [9]. This reflects the two major physiological roles proposed for 2-AG via axonal cannabinoid CB_1 receptors in the brain, i.e., as an autocrine signal involved in axonal sprouting, and a retrograde messenger in synaptic plasticity, respectively [6,11].

Although fatty acid amide hydrolase (FAAH) [12], the enzyme responsible of the hydrolysis of anandamide, catalyzes 2-AG hydrolysis [7,8], a monoacylglycerol lipase (MAGL) inactive on anandamide has been cloned and suggested to act as a major 2-AG degrading enzyme [13]. MAGL is co-distributed with CB_1 receptors throughout the adult central nervous system, mostly with a pre-synaptic localization [14], thus supporting a function for 2-AG as a retrograde messenger in short-term forms of synaptic plasticity known as depolarization-induced suppression of excitatory (DSE) or inhibitory (DSI) neurotransmission [6,11]. Such a function was conclusively demonstrated by studies carried out in the rat midbrain first [6] and more recently in the hippocampus [15,16].

Since 2-AG metabolic enzymes have been identified only recently, no potent and selective inhibitor for these proteins has been developed so far. As both DAGLs and MAGL are members of the large lipase-3 family of Ser hydrolases, one expects that inhibitors of one enzyme might exhibit activity also on the other. Several new compounds were screened as possible MAGL inhibitors, but they either lacked selectivity over FAAH, as in the case of α-Methyl-1-AG, O-2203 and O-2204 [17] or, even when selective vs. FAAH and other enzymes, they have not been tested on DAGLs, as in the case of arachidonylmaleimide [18], some trifluoromethylketone derivatives [19], and URB602 and URB754 [15]. To date, only non-specific inhibitors have been shown to inhibit the formation of 2-AG through the DAGL, i.e., RHC80267 (1,6-bis-(cyclohexyloximino-carbonylamino)-hexane), and the lipase inhibitor tetrahydrolipstatin (orlistat®), which inhibits the two enzymes at concentrations lower than those required to inhibit other lipases [9]. These compounds, while useful, have never been tested on the enzyme mostly responsible for anandamide biosynthesis, the *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD) [10]. The aim of this study was to design new compounds in order to identify potent as well as specific inhibitors for DAGLs and/or MAGL that may be used as new pharmacological tools to investigate further the biological role of 2-AG.

2. Materials and methods

2.1. Synthesis

O,O-diethyl oleylphosphonate (UP99) was obtained from oleyl bromide (Sigma-Aldrich) by reaction with NaI and triethylphosphite (TEP, 5 mol. equiv.) at 100–110 °C for 24 h. ^{1}H NMR ($CDCl_3$) δ 5.48–5.21 (m, 2 H), 4.18–4.00 (m, 4 H), 2.10 (m, 4 H), 1.80 (t, 2 H), 1.35–1.20 (m, 30 H), 0.96 (t, 3 H).

O-Ethyl oleylphosphonate (UP100) was obtained from UP 99 by reaction with KOH (13 mol. equiv.) at 100–110 °C for 24 h, followed by acidification with H_2SO_4 and extraction with EtOAc. ^{1}H NMR ($CDCl_3$) δ 5.48–5.21 (m, 2 H), 4.18–4.00 (m, 2 H), 2.10 (m, 4 H), 2.00 (s, 1 H), 1.80 (t, 2 H), 1.35–1.20 (m, 30 H), 0.96 (t, 3 H).

O-Ethyl-*O*-*p*-nitrophenyl oleylphosphonate (UP101) was obtained from UP100 by reaction (toluene, 65 °C, 12 h) with *p*-nitrophenol (1.1 mol. equiv.), DCC (*N,N′*-Dicycloexylcarbodiimide, 8 eq) and DMAP (4-dimethylaminopyridine 0.5 eq). ^{1}H NMR ($CDCl_3$) δ 8.30–8.10 (dd, 2 H), 6.90–7.20 (dd, 2 H), 5.48–5.21 (m, 2 H), 4.18–4.00 (m, 2 H), 2.10 (m, 4 H), 2.00 (s, 1 H), 1.80 (t, 2 H), 1.35–1.20 (m, 30 H), 0.96 (t, 3 H).

O-Ethyl-*O*-oleyl oleylphosphonate (UP104) was obtained by reacting oleyl alcohol and ethyl oleylphosphonyl chloride (from UP100 and oxalyl chloride) overnight at room temperature. ^{1}H NMR ($CDCl_3$) δ 5.48–5.21 (m, 4 H), 4.23–3.90 (m, 4 H), 2.10 (m, 8 H), 1.80 (t, 2 H), 1.39–1.16 (m, 51 H), 0.96 (t, 6 H). All compounds were purified by gravity column chromatography on silica gel, eluted with either Hexane/EtOAc (UP99, UP101 and UP104, 6:4, 9:1 and 8:2 by vol., respectively) or EtOAc/MeOH (UP100, 5/5 by vol.) O-3453 was synthesized from the commercially available 2,2-dimethyl-[1,3]dioxolan-4-yl-methanol (solketal), which was converted to the bromo compound (CBr_4, Ph_3P, CH_2Cl_2), which, after treatment with a large excess of trimethylphosphite (reflux), gave the corresponding phosphonate derivative. The acetal group was then cleaved (ethereal HCl, THF) and the hydroxyl groups were esterified with oleic acid [(N-(3-dimetylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride

(EDCI), 4-dimethylaminopyridine (DMAP), CH_2Cl_2)] to give the diester. Further treatment with NaI and acetone followed by treatment with (diethylamino)sulfur trifluoride (DAST) formed the target compound (10% overall) using our published procedure [20]. O-3640 was synthesized from 1-bromo-2-butanol, which was condensed with oleic acid as before to give the bromo ester. Treatment of this ester with trimethylphosphite gave the desired phosphinoyloxymethyl derivative, which on subsequent treatment with NaI, acetone and then DAST yielded the target compound (7% overall). O-3696 was prepared from commercially available dimethyl-2-oxoheptylphosphonate. The ketone on reduction ($NaBH_4$, methanol) formed the alcohol (89%), which on condensation with oleic acid, followed by treatment with NaI, acetone and then DAST, as before, provided the target compound (32% overall). O-3841 was prepared from 3-methoxy-1,2-propanediol. The primary alcohol was protected (triisopropylsilyl chloride, imidazole, THF, 26%) and the secondary alcohol was condensed with oleic acid as before to give the oleate ester (98%). The silyl protecting group was converted to the bromide (Ph_3P, Br_2, CH_2Cl_2, 82%) and then treated with dimethyl methylphosphonate (sealed tube, 130 °C, 3d, 56%) to give the corresponding phosphinoyloxy compound. Subsequent treatment with NaI, acetone and DAST (43%) gave the target compound (5% overall). All "O-compounds" were purified by Flash Chromatography (silica gel) eluting with 50% $CH_2Cl_2/CHCl_3$. The structure and purity of each new compound was confirmed by ^{1}HNMR and mass spectra and the final compounds were characterized by ^{1}H-NMR, ^{19}F-NMR, mass spectra and elemental analyses. All NMRs were taken in $CDCl_3$.

O-3453: ^{1}HNMR δ 5.35 (m, 5H), 4.25 (dt, 1H), 4.14 (dd, 1H), 3.89 (d, ^{3}H), 2.33 (m, 6H), 2.00 (m, 8H), 1.58 (m, 6H), 1.30 (m, 38H), 0.89 (t, J=6.5 Hz, 6H); ^{19}FNMR δ-61.18 (2d, J=1068 Hz, 1F). Anal. Calcd for $C_{40}H_{74}O_5FP{\cdot}0.1\ CHCl_3$: C, 69.11; H, 10.72. Found: C, 68.96; H, 10.87.

O-3640: ^{1}HNMR δ 5.35 (m, 2H), 5.03 (m, 1H), 2.33 (t, J=7.7 Hz, 2H), 2.00 (m, 4H), 1.66 (dd, ^{3}H), 1.61 (m, 4H), 1.28 (m, 20H), 0.93 (t, J=6.5 Hz, ^{3}H), 0.88 (t, J=6.5 Hz, ^{3}H); ^{19}FNMR δ-57.68 (2d, J=1054 Hz, 1F); m/z $(M+1-98)^+$, 337. Anal. Calcd for $C^3_2H_{44}O_4FP{\cdot}0.3\ H_2O$: C, 62.79; H, 10.22. Found: C, 62.92; H, 10.20.

O-3696: ^{1}HNMR δ 5.34 (m, 2H), 5.32 (m, 1H), 3.88 (d, ^{3}H), 2.30 (m, 4H), 1.99 (m, 4H), 1.64 (m, 4H), 1.28 (m, 28H), 0.89 (t, J=6.5 Hz, ^{3}H), 0.88 (t, J=6.5 Hz, ^{3}H); ^{19}FNMR δ-61.51 (2d, J=1075 Hz, 1F); m/z $(M+1)^+$, 477. Anal. Calcd for $C_{26}H_{50}O_4FP{\cdot}0.8\ H_2O$: C, 63.59; H, 10.59. Found: C, 63.55; H, 10.52.

O-3841: ^{1}HNMR δ 5.35 (m, 2H), 5.33 (m, 1H), 4.35 (m, 2H), 3.53 (m, 2H), 3.37 (s, ^{3}H), 2.35 (t, J=7.7 Hz, 2H), 2.00 (m, 4H), 1.64 (dd, ^{3}H), 1.63 (m, 2H), 1.26 (m, 20H), 0.89 (t, J=6.5 Hz, ^{3}H); ^{19}FNMR δ-57.8 (2d, J=1054 Hz, 1F); m/z $(M+1)^+$, 451; $(M+1-98)^+$, 353. Anal. Calcd for $C^3_2H_{44}O_5FP{\cdot}0.3\ H_2O$: C, 60.59; H, 9.86. Found: C, 60.28; H, 9.82.

2.2. Binding assays

For CB_1 and CB_2 binding assays we used membranes from HEK-293 cells transfected with either the human CB_1 or CB_2 receptor (Perkin Elmer, Italia) incubated with increasing concentration of compounds and [^{3}H]CP-55,940 ([^{3}H]-(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol) as the high affinity ligand, as described by the manufacturer.

2.3. Fatty acid amide hydrolase assays

The effect of compounds on the enzymatic hydrolysis of [^{14}C]anandamide was studied as previously reported [21] by using membranes prepared from rat brain incubated with increasing concentrations of compounds in 50 mM Tris–HCl, pH 9, for 30 min at 37 °C. [^{14}C]Ethanolamine produced from [^{14}C] anandamide hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3$/CH_3OH (1:1 by volume).

2.4. DAGL assays

DAGL activity was detected as previously reported [9] by using membrane preparations (100 μg proteins) obtained from COS cells overexpressing human DAGLα, and 1-[^{14}C]oleoyl-2-arachidonoyl-glycerol (1.0 mCi/mmol, 25 μM, synthesized as reported [9]) or *sn*-1-stearoyl-2-[^{14}C]-arachidonoyl-glycerol (Amersham Biosciences, 56.0 mCi/mmol) as substrates and increasing concentrations of compounds in Tris–HCl buffer, pH 7 for 15 min. After the incubation, lipids were extracted with 2 volumes of $CHCl_3/CH_3OH$ 2:1 (by vol). The extracts, lyophilised under vacuum, were purified by using TLC on silica on polypropylene plates eluted in $CHCl_3/CH_3OH/NH_4OH$ (85:15:0.1, by vol). Bands corresponding to either [^{14}C]oleic acid (when using 1-[^{14}C]oleoyl-2-arachidonoyl-glycerol as substrate) or [^{14}C]2-arachidonoyl-glycerol (when using *sn*-1-stearoyl-2-[^{14}C]-arachidonoyl-glycerol as substrate) were cut and their radioactivity measured with a β-counter.

2.5. MAGL assays

MAGL activity was measured as reported [9], using the cytosol derived from the 10,000 g fraction of homogenates from wild-type COS cells and synthetic 2-[^{3}H]arachidonoyl-glycerol (1.0 mCi/mmol, 25 mM) as substrate. Previous studies indicated that this fraction exhibits the highest MAGL activity and that COS cells express high amounts of MAGL mRNA (T. Bisogno and V. Di Marzo, unpublished observations). 100 μg of proteins were used for each assay and increasing concentrations of compounds in Tris–HCl buffer, pH=7 for 20 min. After the incubation, lipids were extracted, purified and quantified as above. Bands corresponding to [^{14}C]arachidonic acid were cut and their radioactivity measured with a β-counter.

2.6. Triacylglycerol lipase assays

For triacylglycerol lipase assays, 100 μg of proteins (from the debris-free homogenate of rat liver) were incubated with synthetic 1,2,3-[^{14}C]oleoyl-glycerol (NEN 100.0 mCi/mmol, 25 μM) and increasing concentrations of compounds in Tris–HCl buffer, pH 7 for 20 min. After the incubation, lipids were extracted as above. TLC bands corresponding to [^{14}C]oleic acid were cut and their radioactivity measured with a β-counter.

2.7. NAPE-PLD assays

The effect of compounds of the enzymatic hydrolysis of NAPE was performed by using the procedure described by Okamoto et al. [10] with slight modifications. Membrane fractions obtained from confluent HEK-293 transiently transfected with NAPE-PLD were incubated with 100μM Phosphatidylethanolamine-N-[^{3}H]-arachidonoyl (ARC, Inc., USA 200 Ci/mmol) and increasing concentrations of compounds for 60 min at 37 °C. After the incubation, lipids were extracted with 2 volumes of $CHCl_3/CH_3OH$ 2:1 (by vol). The extracts, lyophilised under vacuum, were purified by using TLC on silica on polypropylene plates eluted in the organic phase from a mixture of isooctane/ethylacetate/water/acetic acid 50/110/100/20 (by vol.). Bands corresponding to [^{3}H]anandamide or [^{3}H]arachidonic acid were cut and their radioactivity was measured with a β-counter.

2.8. 2-AG formation in intact cells

Confluent N18TG2, C6 and RBL2-H3 cells were stimulated for 20 min at 37 °C with ionomycin with or without THL (1 μM), O-3640 (5 μM) and O-3841 (1 μM). After stimulation, cells plus medium were extracted with $CHCl_3/CH_3OH$ 2:1 (by vol). Each extract was purified by open bed chromatography and 2-AG was quantified by LC-MS as previously reported [9].

3. Results

3.1. Effects of compounds on human DAGLα

The possible inhibitory effect of compounds on DAGL was tested in COS cells over-expressing human DAGLα, the most abundant of the two DAGLs in the adult brain [9]. Wild-type (i.e., non-transfected) COS cells also exhibited DAGL activity but this was significantly lower that the activity of cells

Table 1
Effects of previously developed endocannabinoid enzyme inhibitors on DAGLα, MAGL and NAPE-PLD

	DAGLα human recombinant (IC_{50} μM)	MAGL COS cell cytosol (IC_{50} μM)	NAPE-PLD rat recombinant (IC_{50} μM)
VDM-11	>25	>25	>25
AA-5-HT	>25	>25	>25
OMDM-1	>25	>25	>25
MAFP	0.8±0.1	0.1±0.02	>25
THL	0.06±0.02	>25	10±1.3

n.t.=not tested. MAFP: methylarachidonoylfluorophosphonate, AA-5-HT: arachidonoyl-5-hydroxytryptamine, VDM-11: 2-methyl-4-hydroxybenzyl-N-arachidonoyl-ethanolamine, OMDM-1: 4-hydroxybenzil-N-oleoyl-ethanolamide, THL: tetrahydrolipstatin. Data are means±S.E. of $n=3$ determinations.

transfected with human DAGLα cDNA (143±30 vs. 870±40 pmol mg protein^{-1} min^{-1}), and was subtracted from the latter when calculating the IC_{50} values of the inhibitors. As reported in Table 1 and Fig. 2A, THL and methylarachidonoyl fluorophosphonate (MAFP) exhibited an IC_{50} in the nM range (IC_{50} 0.06±0.02 and 0.8±0.1 μM, respectively). Of the UP compounds (Table 2), only UP-101 exhibited moderate inhibitory action on DAGLα (IC_{50} 3.7±0.2 μM), whereas the others were inactive at all concentrations tested (UP-99) or up to 25 μM (UP-100 and UP-104). Under the same conditions, O-3453 and O-3696 were also almost inactive on DAGLα, while O-3640 and O-3841 exhibited high potency as DAGLα inhibitors with IC_{50} values of 0.5±0.13 μM and 0.16±0.12 μM, respectively (Table 2 and Fig. 2B and C). THL and O-3640 were also tested in the presence of increasing concentrations of substrate. Both compounds modified both the apparent K_m and the apparent V_{max} of DAGLα (apparent K_m values changed from 157±20 to 63.5±10 and 98±19 μM, while apparent V_{max} changed from 33±4 to 14.2±2 and 16.7±3 nmol min^{-1} mg prot^{-1} in presence of vehicle, O-3640 or THL, respectively).

3.2. Effects of compounds on MAGL

The effect of the compounds on MAGL was studied in cytoplasmatic fractions of COS cells by using [^{3}H]2-AG as substrate. None of the compounds exhibited any significant inhibitory activity up to 25 μM (Tables 1 and 2), except for UP-101 and MAFP (IC_{50}=3.2±0.17 μM, and 0.1±0.02 μM, respectively).

3.3. Binding affinity for human recombinant CB_1 and CB_2

Radioligand competition binding studies were performed by using membranes from HEK-293 cells transfected with human CB_1 and CB_2 receptors. These data are summarized in Table 2. Of the new synthetic compounds only UP-101 showed some affinity for cannabinoid receptors (IC_{50} 5±0.2 μM and 10±0.14 μM for CB_1 and CB_2 receptors, respectively).

3.4. Activity on rat liver triacylglycerol lipase

The possible inhibitory effect on rat liver triacylglycerol lipase was investigated only for those new synthetic compounds showing inhibitory activity on DAGL. UP-101 and O-3841 were inactive, while O-3640 exhibited a weak inhibitory action on triacylglycerol hydrolysis with an IC_{50} of 10±0.3 μM.

3.5. Effect of compounds on rat brain FAAH

The effect of new compounds of FAAH was studied by using membrane preparations form rat brain. None of the compounds exerted any significant inhibition of anandamide hydrolysis up to a 25 μM concentration (Table 2), except for UP-101 and O-3640 (IC_{50} 0.18±0.14 μM and 8.0±0.32 μM, respectively).

3.6. Effect of compounds on rat recombinant NAPE-PLD

The possible inhibitory effect on the recently cloned NAPE-PLD responsible for AEA formation was investigated: (1) for those new synthetic derivatives showing inhibitory activity on DAGLα; and (2) for selective inhibitors of FAAH or the putative anandamide membrane transporter (AMT) never tested in this enzymatic assay. None of the compounds tested exerted any significant inhibitory action up to a 25 μM concentration, except for THL, which exhibited a weak inhibitory action on NAPE-PLD enzymatic activity with an IC_{50} of 10±1.3 μM.

Table 2
Screening of the eight new compounds on enzymes of the endocannabinoid system and other enzymes

	DAGLα human recombinant (IC_{50} μM)	MAGL COS cell cytosol (IC_{50} μM)	TAGL rat liver (IC_{50} μM)	FAAH rat brain membranes (IC_{50} μM)	CB_1 human recombinant (IC_{50} μM)	CB_2 human recombinant (IC_{50} μM)	NAPE-PLD rat recombinant (IC_{50} μM)
UP-99	>25	>25	n.t.	>25	>25	>25	n.t.
UP-100	>25	>25	n.t.	>25	>25	>25	n.t.
UP-101	3.7±0.2	3.2±0.17	25	0.18±0.14	5±0.2	10±0.14	n.t.
UP-104	25±0.19	>25	n.t.	>25	25±0.9	>25	n.t.
O-3453	>25	>25	n.t.	n.t.	n.t.	n.t.	n.t.
O-3696	25±0.21	>25	n.t.	n.t.	n.t.	n.t.	n.t.
O-3841	0.160±0.12	>25	>25	>25	>25	>25	>25
O-3640	0.500±0.13	25±1.1	10±0.3	8±0.32	25±0.07	>25	>25

n.t.=not tested. Data are means±S.E. of $n=3$ determinations.

3.7. Experiments in intact cells

The effect of THL (1 μM), O-3640 (5 μM) and O-3841 (1 μM) on 2-AG formation stimulated by ionomycin, was studied in intact N18TG2 cells were the enzymatic formation of 2-AG has been previously reported [9]. As reported in Fig. 2D, THL decreased the ionomycin-induced formation of 2-AG in intact cells (15.6±1.9% of the maximum effect observed with ionomycin 3 μM). O-3640 and O-3841 exerted no significant inhibition of 2-AG levels stimulated by ionomycin. Similar results were obtained in C6 and RBL2-H3 intact cells (data not shown).

4. Discussion

We commenced by testing previously reported inhibitors of FAAH or the putative anandamide membrane transporter (AMT) on DAGLα and MAGL enzymatic activity. We also investigated if THL, the only potent inhibitor of 2-AG biosynthesis known at the onset of this study [9], could also was able to inhibit anandamide biosynthesis through the NAPE-PLD. The first finding of our study was that, with the exception of MAFP, which potently inhibited both DAGLα and MAGL, and THL, which inhibited potently only DAGLα, all the previously described inhibitors tested were inactive on all the proteins of the endocannabinoid system examined (Table 1). In particular, the AMT inhibitor VDM-11, which was recently reported to inhibit NAPE-PLD activity in mouse brain [22] and MAGL from rat cerebellum [23], was found to be inactive on these two enzymes up to a 25 μM concentration. The reasons for these discrepancies might reside in the different experimental protocols used in the three studies. In the case of NAPE-PLD, as we used HEK-293 cells transfected with the enzyme, it is possible that the its overexpression minimizes the hydrolyzing activity of other phospholipase-like enzymes that may recognize *N*-arachidonoyl-phosphatidylethanolamine as substrate and be inhibited by VDM-11 [10]. Regarding the lack of activity of VDM-11 on MAGL, we used a concentration of radiolabelled substrate that is much nearer to the apparent K_m of the enzyme, and it is possible that these conditions, although more physiologically significant, are more restrictive, particularly for competitive inhibitors for which higher concentrations are needed to overcome the binding of the substrate to the enzyme. Regarding MAFP, the data obtained on MAGL are in agreement with the ability of the compound to inhibit [^{3}H]2-AG hydrolysis in RBL-2H3 and N18TG2 cells [24] and rat cerebellum [25]. Finally, regarding the weak activity of THL on the NAPE-PLD this may be regarded as surprising since the active site of this enzyme, involving two histidine residues, is quite different from that of the lipases that are normally inhibited by THL [10]. However, it is likely that at high μM concentrations, such as those shown to be necessary for THL to inhibit the NAPE-PLD, the inhibitor does not directly interact with the enzyme active site, but it rather modifies the enzyme activity via unselective interactions with the membrane.

The observation that MAPF inhibited both MAGL and DAGLα suggested that inhibitors targeting these two enzymes might be developed from phosphonate-like compounds. Therefore, we synthesized eight new compounds and tested them for their inhibitory effect on MAGL and DAGLα, as well as against other proteins of the endogenous cannabinoid system (Table 2). The first strategy of synthesis led to three compounds (Fig. 1), UP-99, UP-101 and UP-104, all ester derivatives of the

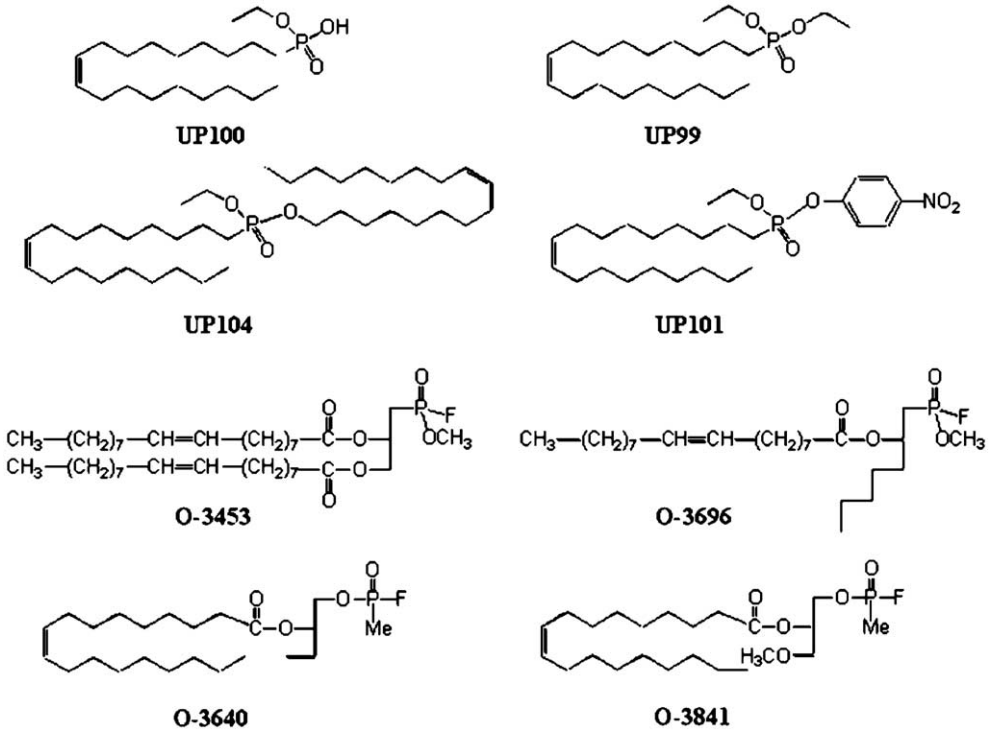

Fig. 1. Chemical structures of the eight new synthetic compounds tested in this study.

phosphonic acid UP-100, where the two acidic hydroxyl are replaced by a -OC_2H_5, O-$C_{18}H_{35}$ and *p*-NO_2PheO-groups, respectively. The second strategy was to modify the chemical structure of MAPF in order to obtain fluoro-phosphonate derivatives whose conformation and size would be similar to the di- or mono-acylglycerols, i.e., the physiological substrates of DAGLα and MAGL, respectively. Thus, the polar *O*-methyl fluorophosphonate moiety of MAFP was implanted as its fluorophosphate version into a dioleoylglyceryl- or into a 3-deoxy-3-butyl-2-oleylgliceryl template, obtaining O-3453 and O-3696, respectively (Fig. 1). Finally, the fluorophosphonates O-3640 and O-3841 are MAFP analogues, where the arachidonyl moiety is replaced by a 2-oleylglyceryl residue variously substituted at the *sn*-3 carbon. Among the compounds tested on DAGLα, O-3640 and O-3841 acted as potent inhibitors with IC_{50} values of 500 and 160 nM, respectively (Fig. 2B and C). In particular, these two new compounds, although less potent than THL (Fig. 2A), showed high selectivity for DAGLα, as they did not strongly bind to human cannabinoid CB_1 and CB_2 receptors nor did they inhibit rat liver triacylglycerol lipase, rat FAAH and the rat NAPE-PLD (Table 2). THL and O-3640 reduced both the apparent K_m and the apparent V_{max} of DAGLα. However, our evaluation of apparent K_m and V_{max} values might be biased by the fact that our assays were performed with membrane-bound enzyme preparations and poorly water-soluble substrates, thus preventing us from drawing any definitive conclusion on the mechanism of action of either THL or O-3640 on DAGLα. It should be emphasized that previous studies showed that both phosphonates and THL are competitive/covalent inhibitors for other lipases, and it is difficult to imagine that they behave in a different way with a protein like DAGLα, which belongs to the same class of enzymes [9]. Of the UP series of compounds, instead, only UP-101 inhibited DAGLα with moderate potency, but this compound, like MAFP, also inhibited the MAGL (IC_{50} = 3.7 ± 0.2 and 3.2 ± 0.17 μM, respectively) as well as FAAH (Table 2).

Next, the compounds exhibiting selective inhibitory activity on DAGLα vs. MAGL were assessed for their capability to inhibit ionomycin-induced de novo formation of 2-AG in intact cells, a process that we have previously reported to be mediated by DAGLα [9] (Fig. 2D). Neither O-3640 nor O-3841, exerted any significant reduction of 2-AG formation stimulated by ionomycin. By contrast, THL, like the less potent DAGL inhibitor RHC80267 [26], exerted a strong inhibition of ionomycin-induced formation of 2-AG. A possible explanation for these data is that, although quite potent and selective, O-3640 and O-3841 might not be able to penetrate the cell membrane, which might limit the applicability of these two compounds as pharmacological tools in intact cell experiments. However, this explanation would imply that, like RHC80267, THL is instead cell-membrane permeable, a possibility that

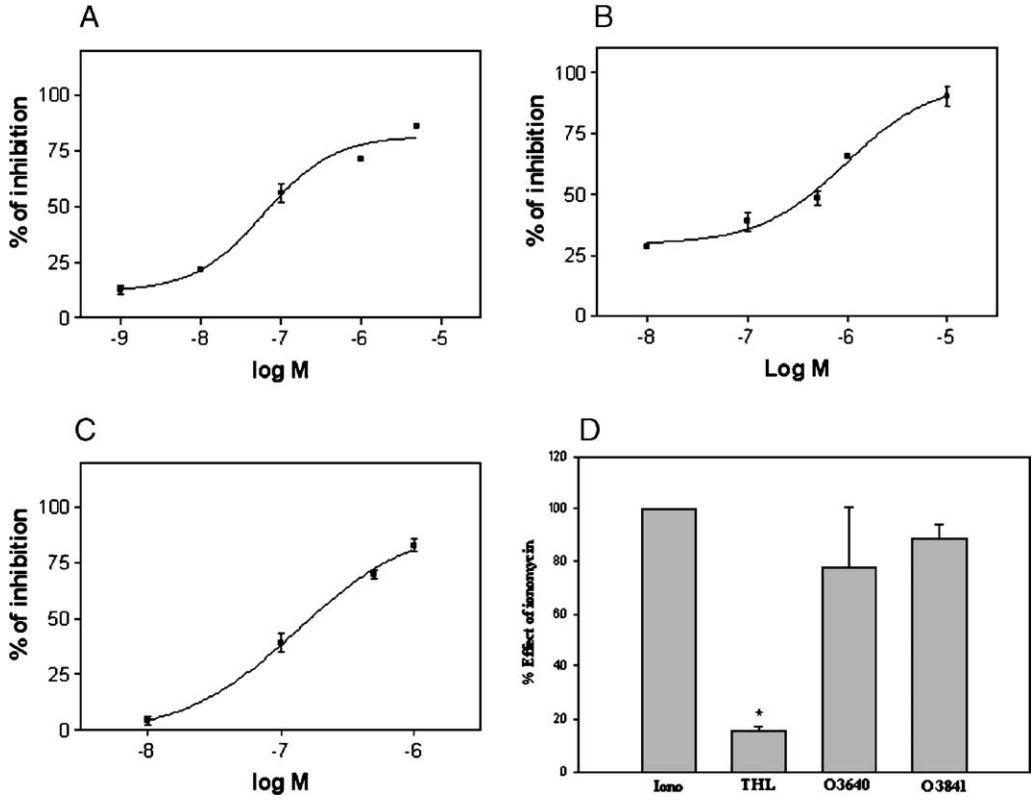

Fig. 2. Dose–response curves for inhibition of DAGLα activity by tetrahydrolipstatin (THL, orlistat®) (A), O-3640 (B) and O-3841 (C). The effect of increasing concentrations of drugs are expressed as percentage of inhibition, graph and IC_{50} obtained by using GraphPad Prism4®. (D) Ionomycin-induced formation of 2-AG in intact N18TG2 cells. The effects of THL (1 μM), O-3640 (5 μM) and O-3841 (1 μM) are reported as percentage of the maximum effect observed with ionomycin 3 μM. Similar results were obtained in rat basophilic leukemia RBL-2H3 and rat glioma C6 cells (not shown). Data are means ± S.E. of $n = 3$ determinations. Means were compared by ANOVA followed by the Bonferroni's test. *$P < 0.01$.

might be seen in contrast with the earlier observations showing that this compound cannot be absorbed from the gastrointestinal mucosa, nor cross the brain–blood barrier [27]. As we did not directly assess the penetration of O-3640, O-3841 and THL into the cells used in our studies, alternative explanations might also exist, particularly as we observed that O-3841, when stereotaxically injected into the rat ventrolateral periaqueductal grey, was instead found to effectively reduce 2-AG-mediated supraspinal effects on nociception [28]. It is possible that also other pharmacokinetic factors, such as the occurrence of intracellular degradation or the sequestration by subcellular organelles and/or intracellular binding proteins, account for the different efficacy of these two phosphonate compounds in different cell types. At any rate, O-3841 is likely to be very useful as a pharmacological tool to block 2-AG biosynthesis also in electrophysiology studies, where the compound can be injected directly into neurons thereby inhibiting typical endocannabinoid-mediated neuromodulatory events, as recently shown for example by Bacci and collaborators [29].

In conclusion, the present study led to the identification of O-3640 and O-3841 as the first two specific inhibitors of the last step of the formation of 2-AG. Though less potent than THL, these compounds showed excellent selectivity for DAGLα over all the other proteins of the endocannabinoid system tested in this study, and qualify as chemical tools to selectively modulate the formation of 2-AG with respect to anandamide.

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