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Biochemical Pharmacology 65 (2003) 1473–1481
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Novel selective and metabolically stable inhibitors of anandamide cellular uptake

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Received 13 December 2002; accepted 13 February 2003

Abstract

Novel aromatic analogues of *N*-oleoylethanolamine and *N*-arachidonoylethanolamine (anandamide, AEA) were synthesized and, based on the capability of similar compounds to interact with proteins of the endocannabinoid and endovanilloid signaling systems, were tested on: (i) cannabinoid CB_1 and CB_2 receptors; (ii) vanilloid VR1 receptors; (iii) anandamide cellular uptake (ACU); and (iv) the fatty acid amide hydrolase (FAAH). The (*R*)- and, particularly, the (*S*)-1′-(4-hydroxybenzyl) derivatives of *N*-oleoylethanolamine and AEA (OMDM-1, OMDM-2, OMDM-3, and OMDM-4) inhibited to a varied extent ACU in RBL-2H3 cells (K_i ranging between 2.4 and 17.7 μM), the oleoyl analogues (OMDM-1 and OMDM-2, K_i 2.4 and 3.0 μM, respectively) being 6- to 7-fold more potent than the arachidonoyl analogues (OMDM-3 and OMDM-4). These four compounds exhibited: (i) poor affinity for either CB_1 ($K_i \geq 5$ μM) or CB_2 ($K_i > 10$ μM) receptors in rat brain and spleen membranes, respectively; (ii) almost no activity at vanilloid receptors in the intracellular calcium assay carried out with intact cells over-expressing the human VR1 ($EC_{50} \geq 10$ μM); and (iii) no activity as inhibitors of FAAH in N18TG2 cell membranes ($K_i > 50$ μM). The oleoyl- and arachidonoyl-*N*′-(4-hydroxy-3-methoxybenzyl)hydrazines (OMDM-5 and OMDM-6), inhibited ACU (K_i 4.8 and 7.0 μM, respectively), and were more potent as VR1 agonists (EC_{50} 75 and 50 nM, respectively), weakly active as CB_1 receptor ligands (K_i 4.9 and 3.2 μM, respectively), and inactive as CB_2 ligands ($K_i > 5$ μM) as well as on FAAH ($K_i \geq 40$ μM). In conclusion, we report two novel potent and selective inhibitors of ACU (OMDM-1 and OMDM-2) and one "hybrid" agonist of CB_1 and VR1 receptors (OMDM-6). Unlike other compounds of the same type, OMDM-1, OMDM-2, and OMDM-6 were very stable to enzymatic hydrolysis by rat brain homogenates.

Keywords: Endocannabinoid; Cannabinoid; Receptor; Transport; VR1; FAAH

1. Introduction

Endocannabinoids are by definition endogenous compounds capable of binding to, and functionally activating, the two cannabinoid CB_1 and CB_2 receptors for marijuana's active principle Δ^9-tetrahydrocannabinol [1,2]. Four prototypical endocannabinoids have been described to date: AEA (anandamide) [3], 2-arachidonoylglycerol (2-AG) [4,5], 2-arachidonylglyceryl ether (2-AGE, noladin ether) [6], and *O*-arachidonoylethanolamine (virodhamine) [7]. A function as local mediators has been proposed for the endocannabinoids [1], which requires for these substances the presence of specific biosynthetic and metabolic

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Abbreviations: AEA, *N*-arachidonoylethanolamine; 2-AG, 2-arachidonoylglycerol; 2-AGE, 2-arachidonylglyceryl ether; OEA, *N*-oleoylethanolamine; ACU, anandamide cellular uptake; FAAH, fatty acid amide hydrolase; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 1; VR1, vanilloid receptor type 1; OLDA, *N*-oleoyldopamine; NADA, *N*-arachidonoyldopamine; NATA, *N*-arachidonoyltyramine; arvanil, *N*′-(4-hydroxy-3-methoxybenzyl)arachidonoylamine; olvanil, *N*′-(4-hydroxy-3-methoxybenzyl)oleoylamine; HEK-293 cells, human embryonic kidney-293 cells; RBL-2H3 cells, rat basophilic leukemia-2H3 cells.

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doi:10.1016/S0006-2952(03)00109-6

mechanisms subject to regulation during physiological and pathological conditions. Although it is now clear that AEA and 2-AG are produced ''on demand'' from the enzymatic hydrolysis of (phospho)-glyceride precursors, the proteins mediating these processes have not been fully characterized yet ([8] for a recent review). Nothing is known yet on the biosynthetic mechanisms of 2-AGE and virodhamine.

Endocannabinoid inactivation is instead better understood. AEA and 2-AG are inactivated *via* a three-step mechanism including: (i) cellular uptake from the extracellular matrix, which appears to occur *via* diffusion through the cell membrane, facilitated by one or more transporters (see [9] for a recent review); (ii) enzymatic hydrolysis, which is catalyzed by FAAH [10,11] and, in the case of 2-AG, also by monoacylglycerol lipases [12,13]; (iii) re-esterification of the hydrolysis products into membrane phospholipids. 2-AGE, which cannot be hydrolyzed enzymatically, is taken up by cells *via* the same mechanism as AEA and 2-AG and is partly metabolized *via* direct esterification into membrane phospholipids [14], a process used to inactivate also part of 2-AG [15]. Nothing is yet known on the inactivation of virodhamine.

Unlike the enzymes for AEA and 2-AG hydrolysis, the putative endocannabinoid transporter has not been cloned yet, and only indirect evidence points to its existence [9]. It was suggested that this protein facilitates endocannabinoid diffusion through the cell membrane *via* a saturable, energy-independent and selective mechanism, which, by being dependent on the gradient of solute concentrations across the cell membrane, relies on the rate of intracellular hydrolysis [16]. Accordingly, several inhibitors of ACU are also FAAH inhibitors [17] and *vice versa* [16]. However, nitric oxide donors were found to significantly enhance the rate of ACU, but not the activity of FAAH [18,19], and substances capable of inhibiting AEA and 2-AG cellular uptake without influencing FAAH have also been developed [20,21], including AEA analogues with variously derivatized phenyl groups instead of the ethanolamine ''head''. Furthermore, 2-AGE, which cannot be hydrolyzed by FAAH, is still rapidly taken up by cells [14], thus suggesting that ACU is mediated by a mechanism not relying uniquely on FAAH.

Another molecular target for AEA, considered by some as part of the endocannabinoid system [22], is VR1, a capsaicin-sensitive, non-selective cation channel acting as a molecular transducer for inflammatory and thermal pain in sensory neurons, and expressed also in the brain [23,24]. It has been observed that several substances acting as ACU inhibitors, and in particular the capsaicin-like *N*-acylvanillylamines, are also potent VR1 agonists [20,21,25–28]. In particular, *N*′-(4-hydroxy-3-methoxybenzyl)arachidonoylamine (arvanil) is one of the most potent ACU inhibitors and VR1 agonists described to date [26]. Another aromatic AEA analogue, and widely used ACU inhibitor, *N*-arachidonoyl-(4-hydroxyphenyl)amine (AM404) [29,30], activates VR1 at concentrations lower than those required to inhibit AEA transport into the cell [26,27,31]. These compounds behave as ''hybrid'' agonists of VR1 receptors and of cannabinoid CB_1 receptors, the latter of which they activate either directly or ''indirectly'', i.e. by enhancing the endogenous levels of AEA and 2-AG through inhibition of their inactivation [29]. On the other hand, two other AEA aromatic congeners, VDM11 and VDM13, have been developed with inhibitory activity on ACU comparable to AM404 and negligible activity at VR1 [26]. One compound, UCM-707, was found to inhibit ACU at sub-micromolar concentrations and to be inactive on VR1 [32]. However, VDM11 and AM404 are not very stable to enzymatic hydrolysis by rat brain homogenates,[1] while the metabolic stability of UCM-707 and its effect on FAAH have not been investigated.

With the aim of obtaining novel metabolically stable ACU inhibitors selective vs. both VR1/cannabinoid receptors and FAAH, in the present study we have synthesized novel aromatic analogues (Fig. 1) of AEA and of its congener, *N*-oleoylethanolamine (OEA), which inhibits AEA uptake [33]). We report the finding of two novel selective ACU inhibitors and of one potential ''hybrid'' agonist of CB_1 and VR1 receptors.

2. Materials and methods

2.1. Synthesis of compounds

Amides OMDM-1 to OMDM-4 (Fig. 1) were synthesized in 74–82% yields by treatment of (*S*)- or (*R*)-tyrosinol with oleic or arachidonic acid using 1-hydroxybenzotriazole (HOBt)–*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as the carboxylate activator. Briefly, to a solution of fatty acid in DMF at 0°, HOBt (1.05 Eq.) and EDC (1.05 Eq.) were added under stirring. After 15 min at 0° and 0.5 hr at room temperature, (*S*)- or (*R*)-tyrosinol hydrochloride (1 Eq.) and Et_3N (1 Eq.) were added and the mixture was stirred at room temperature overnight. The mixture was diluted with brine and extracted with AcOEt. The organic phase was washed with 10% citric acid solution, saturated $NaHCO_3$, and brine, dried (Na_2SO_4), and evaporated under reduced pressure. The residue was purified on silica gel by using CH_2Cl_2/AcOEt = 6/4 as eluent. OMDM-1 ($C_{27}H_{45}NO_3$, 431.7) was characterized as follows: m.p. 88–89°; $[\alpha]_D$ −13° ($CHCl_3$, *c* 1.0); IR (KBr) 3432, 3325, 2928, 2856, 1651, 1515, 1234, 1201 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.87 (3H, t, $J = 6.5$ Hz), 1.26 (20H, m), 1.52–1.56 (2H, m), 1.96–2.00 (4H, m), 2.12 (2H, t, $J = 7.6$ Hz), 2.65–2.78 (2H, m), 3.12 (1H, br s), 3.47 (1H, dd, $J = 11.1$, 5.1 Hz), 3.56 (1H, dd, $J = 11.1$, 4.0 Hz), 4.07 (1H, m), 5.26–5.37 (2H, m), 6.38 (1H, d, $J = 8.4$ Hz), 6.70 (2H, d, $J = 8.4$ Hz), 6.97 (2H, d, $J = 8.4$ Hz), 8.05 (1H, br s);

[1] T. Bisogno and V. Di Marzo, unpublished data.

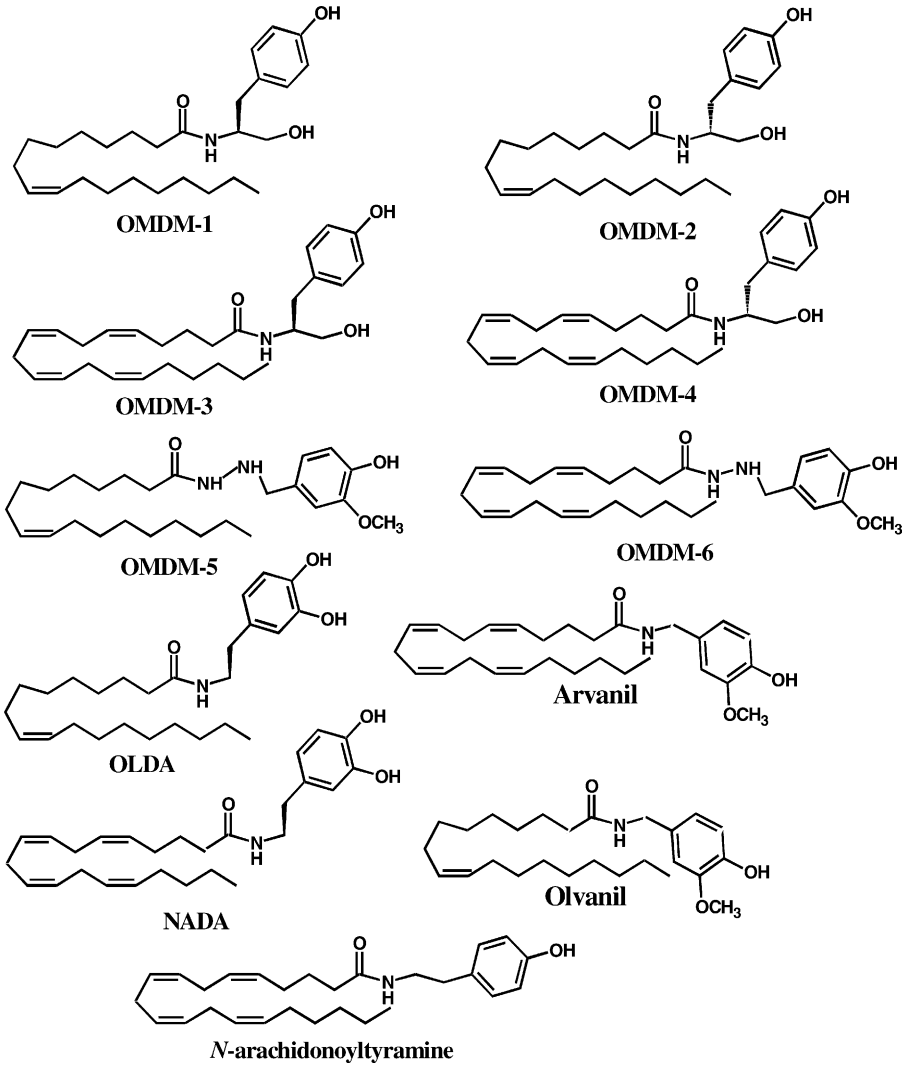

Fig. 1. Chemical structures of the six novel compounds described in this study and of *N*-oleoyldopamine (OLDA), *N*-arachidonoyltyramine (NATA), *N*-arachidonoyldopamine (NADA), olvanil, and arvanil, shown as a comparison.

^{13}C NMR (75 MHz, $CDCl_3$) δ 14.09, 22.66, 25.72, 27.17, 27.21, 29.15, 29.30, 29.50, 29.74, 31.87, 36.15, 36.77, 52.73, 52.82, 63.63, 115.34, 128.46, 129.55, 129.81, 129.97, 155.05, 174.19. OMDM-2 was characterized as follows: m.p. 88–89°; $[\alpha]_D$ +13° ($CHCl_3$, *c* 1.0); other data were identical to OMDM-1. OMDM-3 ($C_{29}H_{43}NO_3$, 453.7, wax) was characterized as follows: $[\alpha]_D$ −17° ($CHCl_3$, *c* 1.0); IR ($CHCl_3$) 3433, 3331, 2930, 2870, 1656, 1615, 1515, 1456, 1261 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, $J = 6.9$ Hz), 1.25–1.37 (6H, m), 1.64 (2H, quintet, $J = 7.5$ Hz), 2.04 (4H, q, $J = 6.9$ Hz), 2.15 (2H, t, $J = 7.6$ Hz), 2.71–2.83 (8H, m), 3.49 (1H, m), 3.52 (1H, dd, $J = 11.1$, 4.9 Hz), 3.61 (1H, dd, $J = 11.1$, 3.3 Hz), 4.12 (1H, m), 5.26–5.41 (8H, m), 5.98 (1H, d, $J = 7.8$ Hz), 6.71 (2H, d, $J = 8.4$ Hz), 6.97 (2H, d, $J = 8.4$ Hz), 7.65 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.07, 22.54, 25.47, 25.60, 26.53, 27.18, 29.27, 31.46, 36.12, 36.21, 52.96, 63.76, 115.44, 127.30, 127.62, 127.91, 128.07, 128.42, 128.49, 128.67, 129.94, 130.32, 154.91, 173.90. OMDM-4 was characterized as follows: $[\alpha]_D$ +18° ($CHCl_3$, *c* 1.0); other data were identical to OMDM-1. Acyl hydrazines, OMDM-5 and OMDM-6 (Fig. 1), were prepared in 43 and 48% overall yields, respectively, by reduction with Et_3SiH/CF_3CO_2H [32] of the corresponding acyl hydrazones, in turn obtained by condensation between vanillin hydrazone [34] and oleic or arachidonic acid using the isobutyl chloroformate method [35]. In detail, to a solution of fatty acid in dry CH_2Cl_2 at 0° under N_2, Et_3N (1.4 Eq.) and *i*-BuOCOCl (1.2 Eq.) were added. The mixture was stirred for 1 hr at 0°. A solution of vanillin hydrazone (1.5 Eq.) and Et_3N (1.2 Eq.) in dry DMF was then added and the mixture was stirred for 4 hr at room temperature. The mixture was diluted with brine and extracted with AcOEt. The organic phase was washed twice with brine, dried ($NaSO_4$), and evaporated under reduced pressure. To a solution of the crude acyl hydrazone in dry CH_2Cl_2 at 0°, CF_3CO_2H (20 Eq.) and Et_3SiH (3 Eq.) were added. The solution was stirred for

4 hr at 0°, brought to pH ~8 with 10% $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic phase was washed with brine, dried (Na_2SO_4), and evaporated under reduced pressure. The residue was purified on silica gel using CH_2Cl_2/AcOEt = 1/1 as eluent. OMDM-5 ($C_{26}H_{44}N_2O_3$, 432.6) was characterized as follows: m.p. 78–81°; IR (KBr) 3544, 3440, 2929, 2856, 1667, 1613, 1516, 1465, 1272, 1223 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.87 (3H, br t), 1.26–1.28 (20H, m), 1.59–1.63 (2H, m), 1.97–2.04 (4H, m), 2.09 (2H, t, $J = 7.5$ Hz), 3.85 (3H, s), 3.88 (2H, s), 5.30–5.34 (2H, m), 5.88 (2H, br s), 6.79 (1H, dd, $J = 8.4$, 1.8 Hz), 6.83 (1H, d, $J = 8.4$ Hz), 6.87 (1H, d, $J = 1.8$ Hz), 7.03 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.10, 22.66, 25.52, 27.14, 27.20, 29.19, 29.29, 29.49, 29.67, 31.87, 34.68, 55.74, 55.82, 111.31, 114.16, 121.83, 129.12, 129.49, 129.82, 145.00, 146.41, 172.43. OMDM-6 ($C_{28}H_{42}N_2O_3$, 454.7, wax) was characterized as follows: IR ($CHCl_3$) 3544, 3439, 2930, 2858, 1668, 1612, 1515, 1463, 1272, 1234 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88 (3H, t, $J = 6.8$ Hz), 1.25–1.37 (6H, m), 1.70 (2H, quintet, $J = 7.5$ Hz), 2.01–2.13 (6H, m), 2.76–2.84 (6H, m), 3.85 (3H, s), 3.88 (2H, s), 5.29–5.41 (8H, m), 5.90 (2H, br s), 6.79 (1H, dd, $J = 1.8$, 8.1 Hz), 6.84 (1H, d, $J = 8.1$ Hz), 6.86 (1H, d, $J = 1.8$ Hz), 6.99 (1H, br s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.06, 22.54, 25.30, 25.61, 26.57, 27.19, 29.28, 31.47, 33.98, 55.73, 55.83, 111.27, 114.15, 121.80, 127.31, 127.62, 127.87, 128.08, 128.41, 128.65, 128.75, 129.10, 130.31, 144.99, 146.40. VDM11, *N*-arachidonoyltyramine (NATA), *N*-arachidonoyldopamine (NADA), *N*-oleoyldopamine (OLDA), arvanil, *N*′-(4-hydroxy-3-methoxybenzyl)oleoylamine (olvanil), and AM404 were synthesized in 70–80% yields as described previously [26]. All compounds were purified to homogeneity (>99%) and characterized on the basis of their ^1H and ^{13}C NMR and IR spectra and elemental analyses.

2.2. Cells

Rat basophilic leukemia-2H3 cells (RBL-2H3 cells) and mouse N18TG2 neuroblastoma cells were purchased from DSMZ and grown according to the specifications of the manufacturer [36,37].

2.3. Cytosolic calcium concentration ($[Ca^{2+}]_i$) assay

Human embryonic kidney-293 cells (HEK-293) overexpressing the human VR1 receptor were a kind gift from John Davis (SmithKline Beecham). Cells were grown as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% fetal calf serum, and 0.2 mM glutamine, and maintained under 95%/5% O_2/CO_2 at 37°. The effect of the substances on $[Ca^{2+}]_i$ was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+}. One day prior to experiments, cells were transferred into 6-well dishes coated with poly-L-lysine (Sigma) and grown in the culture medium mentioned earlier. On the day of the experiment the cells (50,000–60,000 per well) were loaded for 2 hr at 25° with 4 μM Fluo-3 methylester (Molecular Probes) in DMSO containing 0.04% Pluoronic. After the loading, cells were washed with Tyrode's pH 7.4, trypsinized, resuspended in Tyrode's, and transferred to the cuvette of the fluorescence detector (Perkin-Elmer LS50B) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25° ($\lambda_{EX} = 488$ nm, $\lambda_{EM} = 540$ nm) before and after the addition of the test compounds at various concentrations. Data are expressed as the concentration exerting a half-maximal effect (EC_{50}). The efficacy of the effect was determined by comparing it to the analogous effect observed with 4 μM ionomycin.

2.4. ACU assay

The effect of compounds on the uptake of [^{14}C]AEA by intact RBL-2H3 cells was studied by using 5.0 μM (20,000 cpm) of [^{14}C]AEA as described previously [34]. Cells were incubated with [^{14}C]AEA for 5 min at 37°, in the presence or absence of varying concentrations of the inhibitors. Residual [^{14}C]AEA in the incubation media after extraction with $CHCl_3/CH_3OH$ 2:1 (by vol.), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the AEA that was taken up by cells. Previous studies [36] had shown that, after a 5 min incubation, the amount of [^{14}C]AEA disappeared from the medium of RBL-2H3 cells is found mostly (>90%) as unmetabolized [^{14}C]AEA in the cell extract. Non-specific binding of [^{14}C]AEA to cells and plastic dishes was determined in the presence of 100 μM AEA and was never higher than 30%. Data are expressed as the K_i, calculated using the Cheng–Prusoff equation from the concentration exerting 50% inhibition of AEA uptake (IC_{50}).

2.5. CB_1 receptor binding assay

Displacement assays for CB_1 receptors were carried out by using [^3H]SR141716A (0.4 nM, 55 Ci/mmol, Amersham) as the high-affinity ligand, and the filtration technique described previously [20], on membrane preparations (0.4 mg per tube) from frozen male CD rat brains (Charles River), and in the presence of 100 μM PMSF. Specific binding was calculated with 1 μM SR141716A and was 84.0%. Data are expressed as the K_i, calculated using the Cheng–Prusoff equation from the concentration exerting 50% inhibition of [^3H]SR141716A specific binding (IC_{50}).

2.6. CB_2 receptor binding assay

Displacement assays for CB_2 receptors were carried out by using [^3H]WIN55,212 (0.8 nM, 43 Ci/mmol, Amersham) as the high-affinity ligand, and the filtration technique described previously [20], on membrane preparations (0.05 mg per tube) from frozen male CD rat spleens

(Charles River). Specific binding was calculated with 1 μM SR144528 and was 69.4%.

2.7. FAAH assay

The effect of compounds on the enzymatic hydrolysis of AEA was studied as described previously [36], using membranes prepared from mouse neuroblastoma N18TG2 cells, incubated with the test compounds and [^{14}C]AEA (10 μM, 40,000 cpm) in 50 mM Tris–HCl, pH 9, for 30 min at 37°. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 vol. of $CHCl_3$/CH_3OH 2:1 (by vol.). Data are expressed as the K_i, calculated using the Cheng–Prusoff equation from the concentration exerting 50% inhibition of [^{14}C]AEA hydrolysis (IC_{50}).

2.8. Enzymatic hydrolysis of compounds

Whole homogenates of rat (CD male) brain in 50 mM Tris–HCl, pH 7.4 (1 mg protein) were incubated with 1 mg of each compound in 1 mL of 50 mM Tris–HCl, pH 7.4, for 30 min at 37°. After the incubation, the reaction was stopped by adding 2 mL $CHCl_3$/CH_3OH 2:1 (by vol.) and mixing. The organic phase was then purified by thin layer chromatography on analytical silica gel thin layer chromatography plates (Merck), using $CHCl_3$/CH_3OH/NH_4OH 85:15:1 (by vol.), in order to separate the starting compound from the long chain fatty acid (oleic or arachidonic acid) potentially produced from its hydrolysis. Spots were visualized by exposure to iodine vapors followed by charring with cerium sulfate. Intensity of bands was determined by densitometry scanning. Under these conditions, the R_f of OMDM-1, OMDM-2, and OMDM-6 and of long chain fatty acids were 0.62, 0.64, 0.86, and 0.34, respectively. Control incubations were carried out either with boiled homogenates or with [^{14}C]AEA as the substrate, under the same conditions.

3. Results

The effect of the novel compounds on the uptake of [^{14}C]AEA by intact RBL-2H3 cells, where ACU has been well characterized [36], is shown in Fig. 2. In these cells, ACU has been reported to exhibit an apparent K_m for AEA ranging from 9.3 to 33 μM [9,36]. All compounds inhibited the uptake, OMDM-1, OMDM-2, OMDM-5, and OMDM-6 being significantly more potent than OLDA, OMDM-3, and OMDM-4 (Table 1). The previously described NATA was also a relatively potent inhibitor (Table 1).

The novel compounds were able, to some extent, to displace [^{3}H]SR141716A from its specific CB_1 binding sites in rat brain membranes (Fig. 3). However, only OMDM-6 and OLDA exhibited some notable affinity for these binding sites, whereas OMDM-1 was almost inactive (Table 1). Using this same assay, the K_d for unlabeled SR141716A is 0.4 nM [20]. Since NADA, OLDA, arvanil, olvanil, and other aromatic AEA derivatives were previously found to be inactive on CB_2 receptors from rat spleen [20,21,28], the six novel compounds were tested in the same preparation only at two concentrations (1 and 10 μM). None of the compound did appreciably displace [^{3}H]WIN55,212 binding from rat

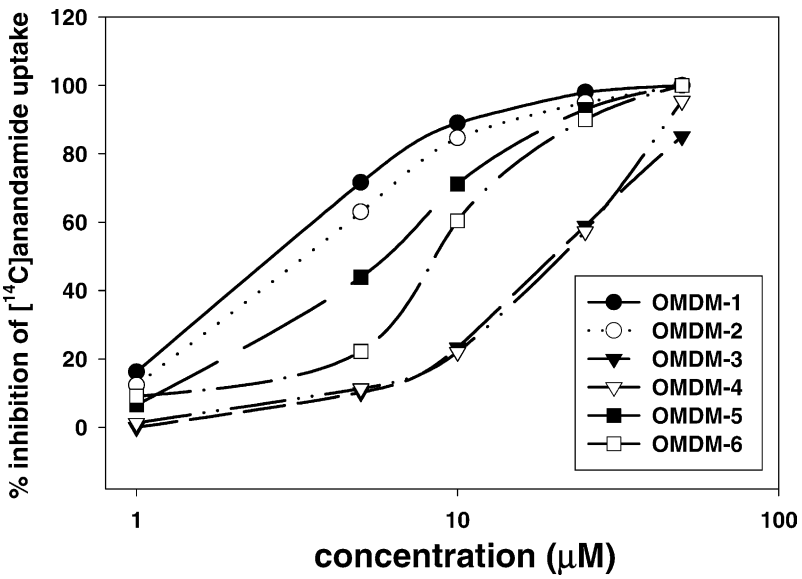

Fig. 2. Effect of the novel compounds on anandamide cellular uptake. The effect of increasing concentrations of the six compounds on the uptake of [^{14}C]anandamide by intact RBL-2H3 cells is shown. Data are means of three independent experiments carried out in duplicate. Standard deviations are not shown for the sake of clarity and were never higher than 15% of the means.

Table 1
Summary of the effects of the novel compounds on anandamide cellular uptake (ACU) in RBL-2H3 cells, the fatty acid amide hydrolase (FAAH) in N18TG2 cell membranes, on cannabinoid CB_1 receptors in rat brain membranes, and on human VR1 in HEK cells

	ACU (K_i, μM)	FAAH (K_i, μM)	CB_1 (K_i, μM)	VR1 (EC_{50}, μM)	VR1 (maximum effect)
OMDM-1	2.4 ± 0.7	>50	12.1 ± 1.3	>10	16.0 ± 2.0
OMDM-2	3.0 ± 0.6	>50	5.1 ± 0.3	10.0 ± 0.5	31.0 ± 3.2
OMDM-3	16.6 ± 0.9	>50	6.1 ± 0.4	>10	15.0 ± 2.2
OMDM-4	17.7 ± 1.5	>50	4.9 ± 0.2	>10	27.5 ± 2.8
OLDA	14.1 ± 2.5	>25	1.6 ± 0.4	0.036 ± 0.009	62.1 ± 5.5
NADA	17.3 ± 7.3[a]	22.0 ± 5.0[b]	0.5 ± 0.2	0.063 ± 0.011	73.1 ± 6.6
NATA	7.4 ± 2.6	11.0 ± 2.1	0.6[c]	>10	18.0 ± 3.7
OMDM-5	4.8 ± 0.5	>50	4.9 ± 0.3	0.075 ± 0.006	67.3 ± 3.5
OMDM-6	7.0 ± 0.6	40.1 ± 4.2	3.2 ± 0.2	0.050 ± 0.004	72.0 ± 3.1
Olvanil	7.3 ± 1.6[a]	>50[d]	10.1 ± 0.5	0.0005 ± 0.0003[a]	67.2 ± 9.1[a]
Arvanil	2.9 ± 0.6[a]	32.0 ± 5.0[d]	2.6 ± 0.6	0.0005 ± 0.0002[a]	75.4 ± 4.7[a]

The effect on VR1 is expressed as both EC_{50} (potency) and as the effect at 10 μM concentration (as percent of the effect of 4 μM ionomycin) (efficacy). Data are means ± SD of three independent experiments carried out in duplicate. Little, if any, displacement of [^{3}H]WIN55,212 from rat spleen membranes by any of the six novel compounds (tested at either 1 or 10 μM) was found (K_i > 10 μM, data not shown). Abbreviations used: OLDA, *N*-oleoyldopamine; NADA, *N*-arachidonoyldopamine; NATA, *N*-arachidonoyltyramine; arvanil, *N*′-(4-hydroxy-3-methoxybenzyl)arachidonoylamine; olvanil, *N*′-(4-hydroxy-3-methoxybenzyl)oleoylamine.

[a] Data were calculated from the corresponding IC_{50} from Ref. [26].
[b] Data were calculated from the corresponding IC_{50} from Ref. [41].
[c] Data were calculated from the corresponding IC_{50} from Ref. [44].
[d] Data were calculated from the corresponding IC_{50} from Ref. [20].

spleen membranes, except for OMDM-5 and OMDM-6 (67.5 ± 2.1 and 66.9 ± 2.4% displacement at 10 μM, means ± SEM, N = 3) (data not shown).

When the novel compounds were tested on [^{14}C]AEA hydrolysis by N18TG2 cell membranes, which express high levels of FAAH with an apparent K_m for AEA of 15 μM [37], none was found to exert an appreciable inhibitory activity, except for OMDM-6 at very high concentrations (Table 1). The previously described NATA was instead a good inhibitor (Table 1).

Of the novel compounds tested on VR1-mediated increase of $[Ca^{2+}]_i$ in HEK cells over-expressing with the human VR1, only OMDM-5 and OMDM-6 exerted agonist activity (Fig. 4), with potency (EC_{50}) and efficacy (maximal effect expressed as percent of the effect of ionomycin) similar to that of the prototypical VR1 agonist, capsaicin (Table 1). The two compounds were inactive in HEK cells that had not been transfected with human VR1 (not shown).

Finally, when VDM11, AM404, OMDM-1, OMDM-2, and OMDM-6 were incubated with rat brain whole

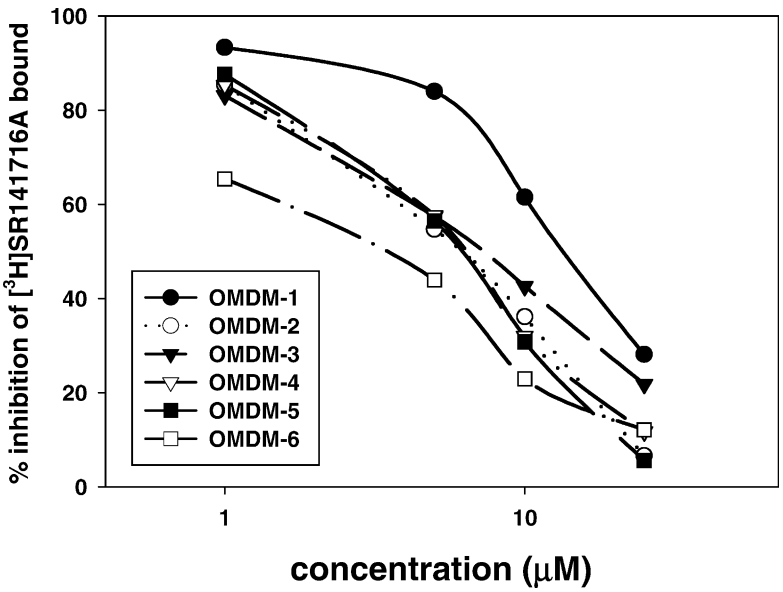

Fig. 3. Effect of the novel compounds on cannabinoid CB_1 receptors. The effect of increasing concentrations of the six compounds on the amount of [^{3}H]SR141716A bound to rat brain membranes is shown. Data are means of three independent experiments in duplicate. Standard deviations are not shown for the sake of clarity and were never higher than 15% of the means.

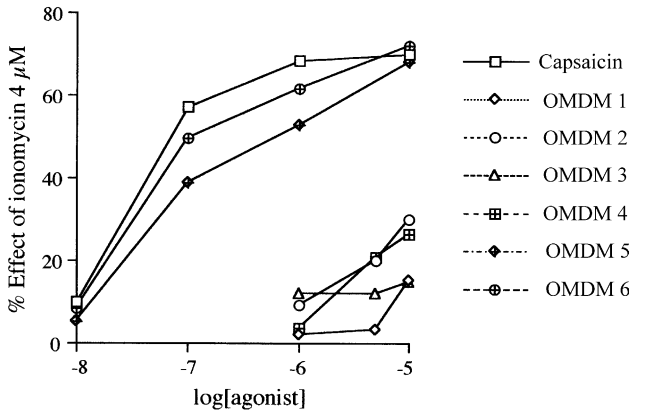

Fig. 4. Effect of the novel compounds on VR1 receptors. The effect of increasing concentrations of the six compounds on the intracellular calcium concentration in HEK cells over-expressing the human VR1 is shown and expressed as percent of the effect of ionomycin (4 μM). Data are means of three independent experiments carried out in duplicate. Standard deviations are not shown for the sake of clarity and were never higher than 15% of the means.

homogenates under conditions sufficient to hydrolyze about 40% of [^{14}C]AEA, hydrolysis to the corresponding fatty acids could be observed only with the former two compounds (i.e. 15.8 ± 1.2, 18.3 ± 1.3, <5, <5, and <5% of the starting compound was hydrolyzed, respectively, means ± SEM, N = 3).

4. Discussion

In this study, we synthesized novel analogues of AEA and OEA and tested them on some of the best characterized proteins of the endocannabinoid system, with the aim of developing new potent, selective, and metabolically stable inhibitors of ACU. When deciding what types of modifications had to be introduced into the chemical structure of AEA and OEA, several previous observations needed to be taken into account. First, the most potent ACU inhibitors developed to date have been obtained by introducing into AEA an aromatic group instead of the ethanolamine "head" (see [38] for a recent review). This is possibly due to strong interaction with the AMT binding site *via* aromatic stacking [17]. Secondly, it must be remembered that, to date, the major cause of the lack of selectivity of ACU inhibitors has been their capability of interacting with either VR1 receptors or FAAH, or both [38]. Therefore, changes previously shown to diminish the affinity of aromatic long chain fatty acid amides for these two proteins, such as modification of their phenol or amide moieties, respectively, had to be introduced. For example, alkylation of the amide nitrogen in arvanil produced an ACU inhibitor selective vs. both FAAH and VR1, O-2093, which, however, might interact also with an as yet unknown brain receptor [21,22]. Changes on the phenol moiety, on the other hand, resulted in two ACU inhibitors, VDM11 and VDM13 [26], which are almost inactive at VR1 and FAAH but, in the case of the former compound at least, not very stable to enzymatic hydrolysis.

These observations prompted the synthesis of the (*R*)- and (*S*)-1′-(4-hydroxybenzyl) analogues of OEA (OMDM-1 and OMDM-2) and AEA (OMDM-3 and OMDM-4), and of the oleoyl- and arachidonoyl-*N*′-(4-hydroxy-3-methoxybenzyl)hydrazines (OMDM-5 and OMDM-6). In fact, alkylation of the 1′-carbon atom, as in (*R*)-methanandamide [39], or modification of the amide region, as in "retro-anandamide", dramatically enhance the metabolic stability of AEA (see [40] for review). Furthermore, the synthesis of OMDM-1, OMDM-2, OMDM-3, and OMDM-4 was also suggested by the previous findings that: (i) the chemically related NADA [41,42], also inhibits ACU and is stable to enzymatic hydrolysis, although it potently activates VR1 (Table 1); and (ii) elimination of the 3-hydroxy group in NADA, as in NATA, abolishes activity at VR1 (see below)[2]. The synthesis of OMDM-5 and OMDM-6, on the other hand, was also prompted by the fact that two closely related compounds, arvanil and olvanil, are more stable to enzymatic hydrolysis than AEA and OEA, and are potent, although non-selective, inhibitors of ACU [20,26].

We found that, in the 1′-(4-hydroxybenzyl) series, only the derivatives of OEA potently inhibited ACU (K_i < 10 μM). Furthermore, the 1′-(*S*)-enantiomers (OMDM-1 and OMDM-3) were slightly (although not significantly) more potent than the 1′-(*R*)-enantiomers (OMDM-2 and OMDM-4), as previously shown also for the 1′-methyl analogues of AEA (methanandamides) [39,40]. Conversely, none of the four compounds exhibited high affinity for CB_1 receptors, although it is interesting to note that, as previously reported again for the methanandamides [39,40], and opposite to the situation observed here with ACU, the 1′-(*S*)-enantiomers were weaker ligands than the 1′-(*R*)-enantiomers. Finally, very little, if any, activity at CB_2 and VR1 receptors, or at FAAH, was found for the four compounds. Therefore, OMDM-1 and OMDM-2 can be considered among the most potent and selective inhibitors of ACU reported so far since they are: (i) more potent than VDM11 and VDM13, when these latter compounds are tested under the same conditions (K_i 9.0 and 9.7 μM, respectively, [26]), (ii) more selective than arvanil (K_i 2.9 μM, Table 1), and both more potent and more selective than AM404 (K_i 6.5), two compounds that are also VR1 agonists; and (iii) possibly at least as potent as UCM-707 (IC_{50} = 0.8 μM, [32]), which was originally tested under conditions (i.e. using a concentration of radiolabeled AEA substrate 50 times lower than the one used here) likely to yield lower IC_{50} values than those obtainable using our procedure, and was never assayed on FAAH. Moreover, compared to VDM11 and AM404, both OMDM-1 and OMDM-2 are extremely stable to enzymatic hydrolysis in rat brain whole homogenates, while the

[2] V. Di Marzo, unpublished data.

metabolic stability of UCM-707 has not been examined [32].

It is worthwhile noting how the chemical modifications introduced into OMDM-1 and OMDM-2 with respect to OLDA (i.e. a missing 3-hydroxy group in the phenol moiety and the presence of the 1′-hydroxymethyl group) confer to these compounds a more potent activity as ACU inhibitors, while abolishing the VR1 agonist activity and strongly decreasing the CB_1 ligand activity (Table 1). Also, notable is the fact that the presence of the 1′-hydroxymethyl group alone, disregardingly of its stereochemistry, reduces the potency at the AMT of the arachidonoyl analogue, NATA, which is significantly more potent than both OMDM-3 and OMDM-4 (Table 1). NATA, however, although inactive at VR1, significantly inhibits FAAH.

The fact that OMDM-1 and OMDM-2 inhibited the AMT and bound at CB_1 at low and medium micromolar concentrations, respectively, should not seen as poor selectivity for the putative AEA transporter vs. the receptors. In fact, the K_i of the two compounds in the two assays should be matched against the affinity constants of the putative AEA transporter and of CB_1 for their natural ligands (which are in the middle micromolar and nanomolar ranges of concentrations, respectively), as well as with the concentrations normally required to compounds to produce pharmacological actions *via* these two targets. Thus, a K_i of 5–10 μM is normally sufficient for ACU inhibitors to enhance AEA CB_1-mediated effects both *in vivo* and *in vitro*, but not to CB_1 ligands to cause CB_1-mediated effects.

The two hydrazides, OMDM-5 and OMDM-6, which can be considered close analogues of olvanil and arvanil, respectively, inhibited ACU with potency lower or comparable to that of these latter compounds and to VDM11 and AM404. However, OMDM-5 and OMDM-6 stimulated VR1 activity as efficaciously and potently as capsaicin, and, particularly OMDM-6, exhibited higher affinity for CB_1 receptors than the other five compounds examined in this study. Neither compound exhibited high affinity for CB_2 receptors in rat spleen membranes ($K_i > 5$ μM). Therefore, the introduction of a further NH group in the two *N*-acylvanillylamines appears to: (i) preserve or slightly improve the ACU inhibitory activity, (ii) decrease the VR1 agonist activity, and (iii) preserve or slightly enhance the CB_1 ligand activity. Like arvanil (see Table 1), OMDM-6 can be considered a direct "hybrid" agonist at VR1 and CB_1 receptors [20,28]. Although its capability to activate CB_1 receptors directly still needs to be assessed in functional assays (see [21], for example), OMDM-6, as evidenced previously for AM404 [29], is likely to enhance endocannabinoid levels by inhibiting endocannabinoid degradation, thereby leading to "indirect" activation of cannabinoid receptors. Furthermore, OMDM-6 appears to be more metabolically stable than both arvanil and AM404, and hence is likely to have a longer half-life *in vivo*.

In conclusion, we have reported here the development of OMDM-1 and OMDM-2, two novel selective, metabolically stable, and relatively potent inhibitors of ACU. The future use as pharmacological tools for the study of the patho-physiological role of endocannabinoids can be predicted for these two compounds, since they represent a further improvement over two previously developed and widely used inhibitors of ACU, AM404 and VDM11. Furthermore, we have described a further potential member of the family of "hybrid" CB_1/VR1 agonists [20,28], whose multiple potential therapeutic uses are being increasingly suggested by recent reports [43].

Acknowledgments

The authors are grateful to Dr. Tiziana Bisogno for her support during the enzymatic hydrolysis experiments and Dr. John Davis, SmithKline Beecham, for the gift of HEK cells over-expressing hVR1. Supported by MURST (Grant 3933 to V.D.M.).

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