N-acyl-dopamines: novel synthetic CB_1 cannabinoid-receptor ligands and inhibitors of anandamide inactivation with cannabimimetic activity *in vitro* and *in vivo*

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We reported previously that synthetic amides of polyunsaturated fatty acids with bioactive amines can result in substances that interact with proteins of the endogenous cannabinoid system (ECS). Here we synthesized a series of *N*-acyl-dopamines (NADAs) and studied their effects on the anandamide membrane transporter, the anandamide amidohydrolase (fatty acid amide hydrolase, FAAH) and the two cannabinoid receptor subtypes, CB_1 and CB_2. NADAs competitively inhibited FAAH from N18TG2 cells (IC_{50} = 19–100 μM), as well as the binding of the selective CB_1 receptor ligand, [^{3}H]SR141716A, to rat brain membranes (K_i = 250–3900 nM). The arachidonoyl (20:4 ω6), eicosapentaenoyl (20:5 ω3), docosapentaenoyl (22:5 ω3), α-linolenoyl (18:3 ω3) and pinolenoyl (5c,9c,12c 18:3 ω6) homologues were also found to inhibit the anandamide membrane transporter in RBL-2H3 basophilic leukaemia and C6 glioma cells (IC_{50} = 17.5–33 μM). NADAs did not inhibit the binding of the CB_1/CB_2 receptor ligand, [^{3}H]WIN55,212-2, to rat spleen membranes (K_i > 10 μM). *N*-arachidonyl-dopamine (AA-DA) exhibited 40-fold selectivity for CB_1 (K_i = 250 nM) over CB_2 receptors, and *N*-docosapentaenoyl-dopamine exhibited 4-fold selectivity for the anandamide transporter over FAAH. AA-DA (0.1–10 μM) did not displace D1 and D2 dopamine-receptor high-affinity ligands from rat brain membranes, thus suggesting that this compound has little affinity for these receptors. AA-DA was more potent and efficacious than anandamide as a CB_1 agonist, as assessed by measuring the stimulatory effect on intracellular Ca^{2+} mobilization in undifferentiated N18TG2 neuroblastoma cells. This effect of AA-DA was counteracted by the CB_1 antagonist SR141716A. AA-DA behaved as a CB_1 agonist *in vivo* by inducing hypothermia, hypo-locomotion, catalepsy and analgesia in mice (1–10 mg/kg). Finally, AA-DA potently inhibited (IC_{50} = 0.25 μM) the proliferation of human breast MCF-7 cancer cells, thus behaving like other CB_1 agonists. Also this effect was counteracted by SR141716A but not by the D2 antagonist haloperidol. We conclude that NADAs, and AA-DA in particular, may be novel and useful probes for the study of the ECS.

Key words: dopamine, endocannabinoid, FAAH, transporter, vanilloid.

INTRODUCTION

Research carried out during the last 6 years has led to understanding of the molecular mechanisms underlying the biosynthesis and inactivation of the endocannabinoids, i.e. the endogenous agonists of cannabinoid receptors [1–3]. In particular, hydrolytic enzymes for both anandamide (*N*-arachidonoyl-ethanolamine, AEA) [4] and 2-arachidonoyl-glycerol [5,6] have been identified [7–9] and, in the case of the 'fatty acid amide hydrolase' (FAAH) [10], cloned from several species (see [11] for a recent review). Furthermore, it has been possible to propose the existence of a selective, saturable and temperature-dependent membrane transporter for the accumulation of AEA into neuronal [12,13] and blood [14] cells (see [15] for a comprehensive review). These proteins, together with the endocannabinoids and the two cannabinoid-receptor subtypes characterized so far, the CB_1 [16] and CB_2 [17] receptors, constitute the endogenous cannabinoid system (ECS). Recently we have shown that synthetic amides of arachidonic acid and other long-chain unsaturated fatty acids with aromatic bioactive amines, such as 5-hydroxytryptamine (serotonin) and 4-hydroxy-benzylamine (vanillylamine), may interact with proteins of the ECS and yield potentially useful new molecules with which to investigate the physiological role of this signalling system. In particular, arachidonoyl-serotonin [18] is the only FAAH inhibitor developed so far that does not also bind to CB_1 receptors and does not inhibit cytosolic phospholipase A_2. Olvanil (*N*-oleoyl-4-hydroxy-3-methoxy-benzylamine) is a potent inhibitor of the AEA transporter [19], whereas arvanil (*N*-arachidonoyl-4-hydroxy-3-methoxy-benzylamine) is a 'hybrid' ligand of CB_1 and vanilloid VR1 receptors and an even more potent inhibitor of the transporter [20]. This compound is also a potent cannabimimetic compound in as much as it is 5-fold more active than AEA as an anti-proliferative agent in human breast cancer cells [20], and 100–500-fold more potent than AEA as an analgesic and vasodilator agent *in vivo* and *in vitro* (V. Di Marzo, G. Kunos, A. S. C. Rice and B. R. Martin, unpublished work). Finally, linvanil (*N*-α-linolenoyl-4-hydroxy-3-methoxy-benzylamine) is a selective inhibitor of the AEA transporter that is capable of enhancing significantly the effects of AEA in endothelial cells [21]. These observations suggest that long-chain fatty acids functionalized with aromatic bioactive amines [22] may represent a new source of biochemically and pharmacologically important compounds.

Abbreviations used: AEA, *N*-arachidonoyl-ethanolamine; FAAH, fatty acid amide hydrolase; ECS, endogenous cannabinoid system; NADA, *N*-acyl-dopamine; AA-DA, *N*-arachidonoyl-dopamine.

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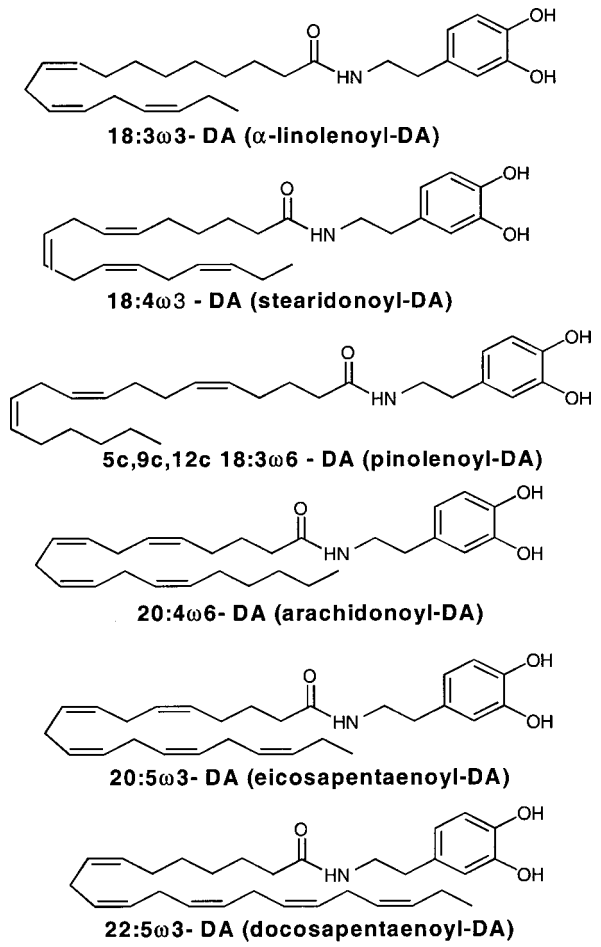

Figure 1 Chemical structures of the NADAs synthesized and examined in this study

DA, dopamine.

In the present study we have examined the interactions with proteins of the ECS of yet another series of compounds, the *N*-acyl-dopamines (NADAs; Figure 1). These compounds may be particularly interesting given the widely documented interactions between the ECS and the dopaminergic system. In fact, a picture is now emerging that suggests that activation of cannabinoid receptors may serve the purpose of either counteracting or reinforcing the pathological consequences of the over-activation of dopamine receptors [23,24]. We report that long-chain unsaturated NADAs inhibit the AEA transporter and FAAH to a variable extent and that they bind to and activate CB_1, but not CB_2, cannabinoid receptors. We propose that these compounds, and in particular *N*-arachidonoyl-dopamine (AA-DA), can be used as pharmacological tools for the study of the ECS.

EXPERIMENTAL

Synthesis of NADAs and other compounds

Synthesis of AA-DA and amides of other fatty acids with dopamine was performed according to a slight modification of a previous procedure [25]. Briefly, to a solution of fatty acid in acetonitrile at 0–4 °C, triethylamine (1.4:1 molar ratio) and isobuthylchloroformate (1.2:1 molar ratio) were added. After 30–40 min the mixture was diluted with water and extracted with ethyl acetate. The resulting mixed anhydride was treated with a solution of 3-hydroxytyramine hydrochloride (1.1:1 molar ratio) in dimethylformamide containing triethylamine (1.1:1 molar ratio) for 18 h at 0 °C. The mixture was diluted with water and extracted with ethyl acetate. The organic extracts were washed with water, dried over sodium sulphate and concentrated under vacuum. The crude products were purified on silica gel by using increasing concentrations of ethyl acetate in n-hexane. Yields ranged between 55 and 60 %. The purity of each compound, as checked by direct-phase HPLC on an analytical Spherisorb column (eluted with a linear 1-h gradient from 10 to 20 % propan-2-ol in n-hexane), was not less than 98 %, and analytical data (^{1}H- and ^{13}C-NMR, UV and mass spectra) were obtained that supported the assigned structures (V. Bezuglov, M. Bobrov, N. Greskaya, A. Gonchar, D. Melck, T. Bisogno, V. Di Marzo, D. Kuklev, J.-C. Rossi, J.-P. Vidal and T. Durand, unpublished work). AEA, arvanil and [^{14}C]AEA (5 mCi/mmol) were synthesized as described previously [4,14,20]. HU-210 was donated by Dr R. Mechoulam, The Hebrew University, Jerusalem, Israel, and SR141716A by Sanofi Recherche, Montpellier, France.

FAAH activity assays and hydrolysis of AA-DA by rat brain homogenates

N18TG2 and RBL-2H3 cells were cultured as described previously [8,14,19,20]. The effect of NADAs on the enzymic hydrolysis of [^{14}C]AEA (6 μM) was studied as described previously for *N*-acyl-vanillyl-amines [19,20] by using membranes prepared from these cells incubated with increasing concentrations of NADAs. Competitive inhibition experiments were carried out with AA-DA by using different concentrations of [^{14}C]AEA (1.2, 2.3, 5.7, 11.4 and 23 μM), and data interpreted by means of the Lineweaver–Burk equation [8]. AA-DA was also incubated with rat brain whole homogenates (2 mg of protein/ml) prepared in 50 mM Tris/HCl buffer, pH 7.4, for 30 min at 37 °C. After the incubation, the homogenates were extracted with 3 vols of chloroform/methanol (2:1, v/v), and the organic phase freeze-dried and analysed by TLC carried out on analytical silica plates (Merck) developed with chloroform/methanol/ammonia (95:5:1, by vol.). The formation of arachidonic acid from AA-DA was visualized as a band with an R_F of 0.4 after exposure to iodine vapours. Control incubations were carried out with boiled rat brain homogenates or with homogenates incubated with [^{14}C]AEA [19,20].

AEA transporter assays

The effect of NADAs on the accumulation of [^{14}C]AEA into RBL-2H3 cells was studied as described previously for *N*-acyl-vanillyl-amines [19,20] with a little modification consisting of incubating cells with 4 μM [^{14}C]AEA for only 5 min at 37 °C, in the presence or absence of varying concentrations of the inhibitors. [^{14}C]AEA found in the incubation media after extraction with chloroform/methanol (2:1, v/v), measured by scintillation counting, was added to that found in washes of cells with 0.1 % BSA in culture medium, and used as a measure of the AEA that was not taken up by cells. We applied the same protocol to C6 rat glioma cells, which also contain a characterized membrane transporter for AEA [15].

Receptor binding assays

Displacement assays for CB_1 receptors were carried out by using [^{3}H]SR141716A (0.4 nM, 55 Ci/mmol, Amersham) as the high-affinity ligand, and the filtration technique described previously

[26], on membrane preparations (0.4 mg/tube) from frozen male CD rat brains (Charles River, Wilmington, MA, U.S.A.), and in the presence of 100 μM PMSF. Specific binding was calculated with 1 μM SR141716A and was 84.0 %. The spleen from CD rats were used to prepare membranes (0.4 mg/tube) to carry out CB_2 binding assays by using [^{3}H]WIN55,212-2 (0.8 nM, 50.8 Ci/mmol, NEN-Dupont) as described in [27], in the presence of 100 μM PMSF. Specific binding was calculated with 1 μM HU-210 and was 75.0 %.

Binding assays for D1 and D2 dopamine receptors were performed by using membrane preparations (0.4 mg/tube) from frozen male CD rat brains in the presence of 100 μM PMSF. [^{3}H]SCH23390 (0.6 nM, 75.5 Ci/mmol) and [^{3}H]spiperone (3 nM, 16.5 Ci/mmol; both from NEN Life Science), respectively, were used as high-affinity ligands for D1 and D2 dopamine receptors. Specific binding was calculated with 1 μM (*R*)-SKF38393 and 1 μM spiperone (both from Sigma) for D1 and D2 binding assays, respectively, and was 43 and 96 %, respectively. Finally, rat spinal-cord membranes (0.1 mg/tube) and [^{3}H]resiniferatoxin (1 nM, 48 Ci/mmol, NEN-Dupont) were used for vanilloid VR1 receptor-binding assays, carried out according to the filtration procedure described previously [28]. Specific binding was calculated with 1 μM resiniferatoxin (Alexis Biochemicals) and was 45.0 %. In all cases, K_i values were calculated by applying the Cheng–Prusoff equation to the IC_{50} values (obtained by GraphPad) for the displacement of the bound radioligand by increasing concentrations of the test compounds.

Effect on intracellular Ca^{2+} concentration

These tests were carried out by using a procedure similar to that described previously for endothelial cells [29]. Subconfluent N18TG2 cells were loaded with fura-2 (Molecular Probes) as described in [29], before being transferred into a cuvette. Test substances at various concentrations were then added and variations in cell fluorescence ($R = F_{340}/F_{380}$) was measured with a fluorimeter (Perkin-Elmer LS50B). After each test substance 1 mM $CaCl_2$ was added to measure cell responsiveness. Experiments were carried out at 25 °C with cells under continuous stirring. SR141716A was added to cells 30 min before the test compounds. Data are expressed as percentages of the maximal response measured with 1 mM $CaCl_2$.

In vivo pharmacology

Female CBA mice (20–25 g body weight) were used. Before the experiments, animals were adapted to the laboratory conditions for 24 h, with free access to food and water. AA-DA was injected intraperitoneally in 0.2 ml of the vehicle containing Tween 20/physiological saline (1:19). Aliquots of AA-DA in ethanol were added to Tween 20, than ethanol was evaporated *in vacuo* and the residue was diluted by physiological saline. Vehicle alone was used as in control experiments. A dose of 10 mg/kg was used in almost all tests as the most active dose observed in previous reports for AEA [30]. The 'open field' was a circular arena with a diameter of 70 cm and a border of 10 cm in height. Radial and concentric lines on five internal and eight external sectors divided the field. Testing was conducted 5 min after drug administration. Animals were placed in the centre of the field and spontaneous activity was monitored for 10 min. The following elements of behaviour were registered: the number of sectors crossed, rearing and grooming. Before every test the field was cleaned with ethanol to eliminate specific smells. The ring test was performed as described by Pertwee [31]. Briefly, 5 min after administration, animals were settled on a wire ring, with a diameter of 5.5 cm, attached to a stand at a height of 16 cm. The time of immobility when only breathing movements could be observed was registered for a period of 5 min. Temperature was measured rectally with an electronic thermometer before, and 5 and 10 min after, drug administration. Analgesic effects were assessed on an electrically heated plate (50 $\pm$ 1 °C) with a diameter of 16 cm and a transparent border, and a height of 20 cm, as described previously [32]. Tests were conducted 5 min after AA-DA administration. The time interval was measured from the moment of placing the animal on the plate until the beginning of hind-paw licking or jumping. Animals were kept on the plate for not more than 60 s to prevent skin damage.

Cell-proliferation assays

The effect of AA-DA on the basal proliferation of MCF-7 cells was studied exactly as described previously for these cells [33,34]. Antagonists were added together with AA-DA every day at each change of medium. Cells were counted by means of a haemocytometer. AA-DA was not toxic to cells up to 10 μM, as assessed by the Trypan Blue method.

RESULTS

NADAs as inhibitors of AEA inactivation

The IC_{50} values for the inhibition by NADAs of [^{14}C]AEA hydrolysis by N18TG2 cell membranes are shown in Table 1. The most potent inhibitors were AA-DA (IC_{50} = 23 μM) and the pinolenoyl homologue (5c,9c,12c 18:3 ω6) (IC_{50} = 19 μM), whereas the least potent was the docosapentaenoyl analogue (IC_{50} = 100 μM). The data indicate that the presence of a C_{18}–C_{20} ω6 fatty acid chain in the NADA series allows a better interaction with the binding site of the hydrolytic enzyme, which in N18TG2 cells is FAAH [35]. In fact, the inhibitory action of AA-DA was shown to be due to a competitive mechanism, since at 25 μM the compound modified the apparent K_m (from 13 to 26.7 μM) but not the apparent V_{max} of the enzyme for [^{14}C]AEA (Figure 2). However, when incubated for 30 min at 37 °C with homogenates from rat brain, which also contains high levels of FAAH, AA-DA remained almost (> 95 %) unchanged, whereas [^{14}C]AEA was almost totally hydrolysed under the same conditions (results not shown). AA-DA also inhibited (IC_{50} = 22 μM) the hydrolysis of [^{14}C]AEA by RBL-2H3 cell membranes, where FAAH is also expressed abundantly [35].

Five of the NADAs were tested as inhibitors of the [^{14}C]AEA-facilitated transport into either RBL-2H3 or C6 cells (Table 1) and found to be active with IC_{50} values in the 17.5–33 μM range of concentrations. Particularly, the docosapentaenoyl (22:5 ω3) and the α-linolenoyl (18:3 ω3) homologues, found here to be very weak inhibitors of FAAH, were active in this assay.

NADAs as ligands of CB_1 cannabinoid receptors

All NADAs inhibited to some extent the binding of [^{3}H]SR141716A to rat brain membranes, which selectively contain CB_1 receptors, with K_i values ranging from 250 nM (for AA-DA) to 3.9 μM (for the α-linolenoyl analogue; Table 1). Under the same conditions AEA exhibited a K_i value of 0.8 μM, whereas the $C_{18:2}$, $C_{18:3}$ ω3, $C_{20:5}$ ω3 and $C_{22:5}$ ω3 *N*-acylethanolamines all exhibited K_i values higher than 25 μM, except for the $C_{20:5}$ ω3 homologue (K_i = 19.7 μM). The three dopamine analogues tested on the binding of [^{3}H]WIN55,212-2 to rat spleen membranes, which selectively contain CB_2 receptors, i.e. the 18:3 ω3, 5c,9c,12c 18:3 ω6 and 20:4 ω6 homologues, exhibited K_i values of 12.9, 11.3 and 12.0 μM, respectively, whereas AEA K_i was 2.4 μM.

Table 1 Effects of NADAs on anandamide enzymic hydrolysis and facilitated transport into cells, and on the binding of radiolabelled cannabinoid ligands to CB_1 and CB_2 receptor preparations

The effects of six NADAs, identified by their fatty acid chains, are shown. N.T., not tested. Data are means ± S.E.M. of three separate experiments carried out in duplicate. In parentheses are shown the K_i values for CB_1 receptor binding of some anandamide homologues.

	[^{14}C]AEA hydrolysis (N18TG2, IC_{50}, μM)	[^{14}C]AEA uptake (IC_{50}, μM)	[^{3}H]SR141716A binding (rat brain, K_i, μM)	[^{3}H]WIN55,212-2 binding (rat spleen, K_i, μM)
18:3 ω3	63.5 ± 7.1	24.0 ± 5.0*, 30.0 ± 4.3†	3.92 ± 0.37 (> 25)	12.9 ± 2.1
5c,9c,12c 18:3 ω6	19.0 ± 4.5	33.0 ± 7.2†	1.0 ± 0.4	11.3 ± 1.9
18:4 ω3	28.2 ± 2.9	N.T.	1.85 ± 0.35	N.T.
20:4 ω6	22.0 ± 5, 23.0 ± 3.7‡	21.5 ± 9.1*, 17.5 ± 1.7†	0.25 ± 0.13 (0.8)	12.0 ± 4.0
20:5 ω3	34.0 ± 3.9	20.3 ± 6.3†	1.41 ± 0.21 (19.8)	N.T.
22:5 ω3	100.0 ± 11.2	25.1 ± 5.1*, 27.2 ± 6.5†	0.7 ± 0.12 (> 25)	N.T.

* Tested in intact RBL-2H3 cells.
† Tested in intact C6 glioma cells.
‡ Tested in RBL-2H3 cell-membrane preparations.

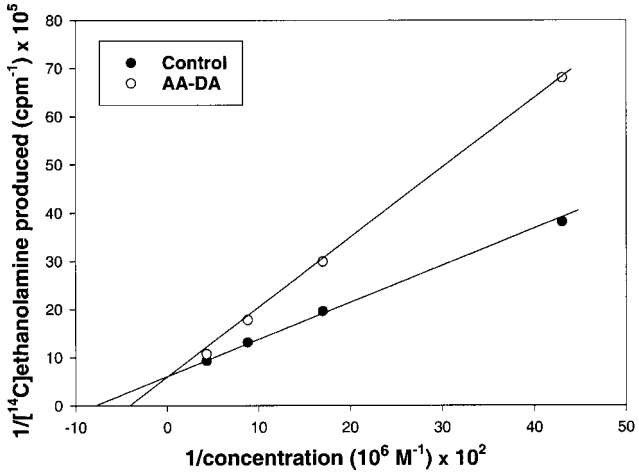

Figure 2 Lineweaver–Burk analysis of the FAAH activity in N18TG2 cell membranes in the absence or presence of 25 μM AA-DA and increasing concentrations (1.2, 2.3, 5.7, 11.4 and 23 μM) of [^{14}C]anandamide

Data obtained with the lowest concentration of substrate are not shown for the sake of clarity. Data are means of three experiments. S.E.M. values are not shown and were never higher than 10% of the means.

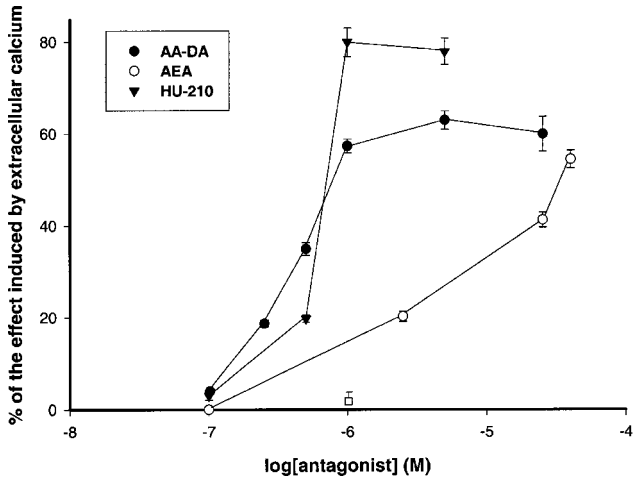

Figure 3 Effect of anandamide (AEA), AA-DA and HU-210 on intracellular Ca^{2+} in N18TG2 cells

The effects are expressed as a percentage of the maximal response obtained for each cell preparation, measured by adding 1 mM $CaCl_2$, which yielded an average R value of 1.01 ± 0.1 (mean ± S.E.M., $n = 13$). □, Denotes the effect of 1 μM AA-DA after a 30 min pre-incubation of cells with 0.5 μM SR141716A.

Assays carried out with [^{3}H]SR1417161A and rat brain membranes in the presence of the D2 dopamine antagonists spiperone and haloperidol did not cause any appreciable increase in the displacement of the radioligand by 0.05, 0.1, 0.5 or 1 μM AA-DA (results not shown), thus suggesting that these antagonists do not increase the availability of the unbound compound under the assay conditions used. Accordingly, AA-DA (0.1, 0.5, 1, 5 and 10 μM) did not significantly displace high-affinity selective radioligands for D1 or D2 dopamine receptors, i.e. [^{3}H]-SCH23390 and [^{3}H]spiperone, from rat brain membranes (results not shown).

Binding assays carried out with rat spinal cord membranes, and using [^{3}H]resiniferatoxin as a selective ligand of vanilloid receptors, revealed B_{max} and K_d values for this ligand of 262 ± 23 fmol/mg of protein and 0.96 ± 0.05 nM, respectively (means ± S.E.M., $n = 3$). AA-DA (0.5–25 μM) did not significantly displace [^{3}H]resiniferatoxin from spinal cord membranes, whereas the vanilloid–cannabinoid receptor 'hybrid' ligand, arvanil [20], did ($K_i = 1.0 \pm 0.2$ μM, mean ± S.E.M., $n = 3$).

AA-DA as an agonist at CB_1 receptors

Sugiura et al. [36] first described how activation of CB_1 receptors by synthetic or endogenous ligands leads to mobilization of Ca^{2+} from intracellular stores. In particular, they found that AEA is a weak and partial agonist when this functional response is measured in neuroblastoma × glioma NG105 × 18 and neuroblastoma N18TG2 cells [36]. We found that, in the absence of extracellular Ca^{2+}, AEA induced a rise in the intracellular Ca^{2+} concentration in N18TG2 cells with an estimated pEC_{50} of 4.5 ± 0.11, whereas AA-DA was about 45-fold more potent (estimated $pEC_{50} = 6.15 \pm 0.09$, means ± S.E.M., $n = 3$; Figure

Table 2 Pharmacological effects of AA-DA *in vivo*

Rectal temperature was assessed as the difference in temperature before and 10 min after AA-DA administration. In all tests control groups were treated with vehicle. Data are means ± S.E.M. of six experiments. $^*P < 0.01$ and $\dagger P < 0.05$ as compared with the respective controls by ANOVA.

Test	Vehicle	AA-DA (10 mg/kg)
Immobility time in the ring test (s)	34.75 ± 5.25	163.7 ± 34.09*
Decrease of rectal temperature (°C)	0 ± 0.22	1.78 ± 0.2†
Latency time on the hot plate (s)	17.3 ± 2.5	38.5 ± 14.1†

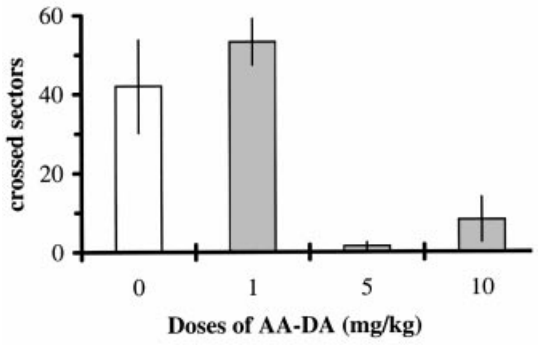

Figure 4 Dose-dependent inhibition of spontaneous activity of mice in the open field after administration of AA-DA or vehicle (0 mg/kg)

The numbers of sectors crossed were recorded for 10 min as described in the Experimental section. Data are means ± S.E.M. of six experiments. The effects of both 5 and 10 mg/kg AA-DA were significantly different from the vehicle ($P < 0.01$ and 0.05, respectively, by ANOVA).

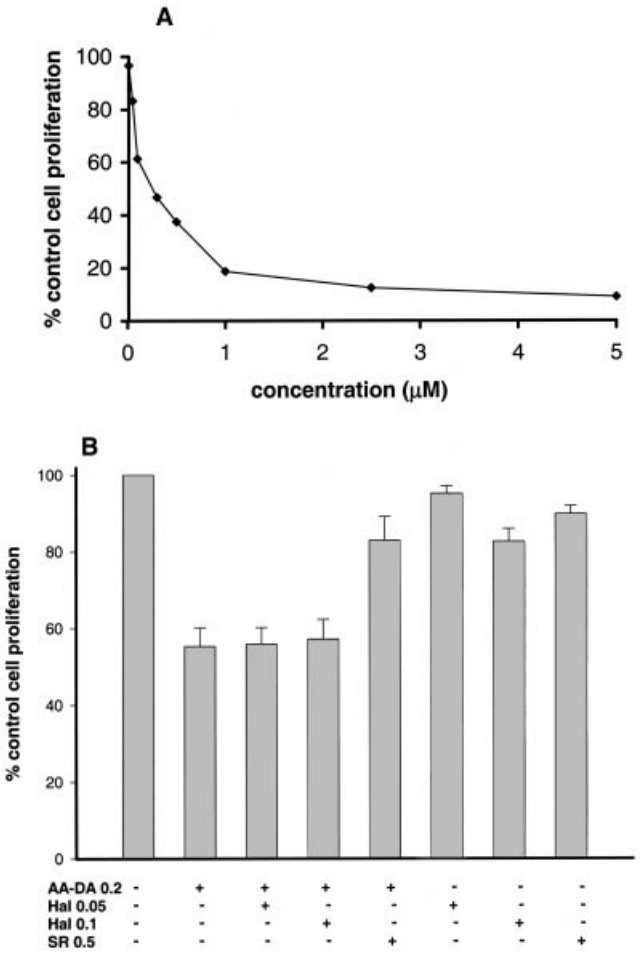

Figure 5 Anti-proliferative effects of AA-DA on MCF-7 cells

(**A**) Dose-dependent effect of AA-DA. (**B**) Effects of the D2 dopamine-receptor antagonist haloperidol (Hal) and the CB_1 cannabinoid-receptor antagonist SR141716A (SR) on the effect of AA-DA. In (**B**), numbers following abbreviations indicate concentrations in μM. AA-DA + 0.5 μM SR141716A was significantly different from AA-DA alone ($P < 0.01$ by ANOVA). In both (**A**) and (**B**) data are reported as the percentage of control-cell proliferation and are means ± S.E.M. of three separate experiments carried out in duplicate.

3). The AA-DA effect was erased by the CB_1 antagonist SR141716A (0.5 μM). The synthetic cannabinoid HU-210 (estimated $pEC_{50} = 6.20 \pm 0.07$) was more potent than AEA and was as potent as AA-DA in this test. As no FAAH inhibitor was introduced during the assay, enzymic hydrolysis of AEA, and to a lesser extent AA-DA (see above), may have reduced the potency of these two compounds, which exhibited similar maximal responses and behaved as partial agonists as compared with HU-210 (Figure 3).

AA-DA as a cannabimimetic substance *in vitro* and *in vivo*

In order to assess whether the capability of AA-DA to inhibit AEA degradation or bind to CB_1 receptors results in cannabimimetic actions *in vivo* or on intact cells, we tested the effect of this compound in the mouse 'tetrad' of tests predictive of Δ^9-tetrahydro-cannabinol-like activity [30], and on the proliferation of the human breast cancer MCF-7 cell line, where CB_1 agonists inhibit DNA synthesis and cell proliferation via CB_1 receptors [33,34,37]. In mice, AA-DA induced analgesia in the 'hot-plate' test, inhibition of spontaneous activity in an open field, decrease of rectal temperature and immobility on a ring (Table 2 and Figure 4). In MCF-7 cells, AA-DA potently inhibited MCF-7 cell proliferation ($EC_{50} = 0.25$ μM, Figure 5A), this effect being sensitive to SR141716A (0.5–1 μM) but not to the D2 antagonist haloperidol (0.05–0.1 μM, Figure 5B).

DISCUSSION

In this study we have shown that NADAs, and particularly AA-DA, can interact at several levels with proteins of the ECS and, subsequently, exert cannabimimetic actions *in vitro* and *in vivo*. First, we have shown that these compounds are good inhibitors of FAAH, provided that an ω6 fatty acid chain is present in the molecule, as in the cases of AA-DA and the pinolenoyl (5c,9c,12c 18:3 ω6) homologue. Within the ω3 series of NADAs, the 22:5 homologue was the weakest inhibitor, whereas the C_{20} and C_{18} homologues were more or less equally active. The 18:3 ω3 NADA homologue was less active than the $C_{18:4}$ ω3 homologue, thus suggesting that within the C_{18} series the presence of more polyolefinic double bonds strengthens the interaction with the enzyme. These findings are in agreement with what was found previously for the effect of AEA homologues on FAAH-catalysed AEA hydrolysis ([8,38], and [39] for a review). In fact, experiments carried out with AA-DA suggest that NADAs are competitive inhibitors of the enzyme and, therefore, are recognized by the catalytic site as potential substrates, as shown with most

of the AEA analogues studied so far [39]. However, the interaction of FAAH with AA-DA does not seem to lead to the hydrolysis of the latter compound since incubation of the compound with rat brain homogenates, which contain high amounts of the enzyme, caused the formation of negligible amounts of arachidonic acid when using assay conditions under which AEA is almost completely hydrolysed to this fatty acid. As hypothesized previously for arachidonoyl-serotonin [18], it is possible that the presence of either or both of the two hydroxy groups in the *para* and *meta* positions on the benzene ring stabilize the enzyme–substrate complex (through the formation of hydrogen bonds), thus increasing the energy barrier for the formation of the transition state. In support of a possible role played by phenolic hydroxy groups in the interaction with the catalytic site of FAAH is the observation that *ortho*- and *meta*-, but not *para*-, mono-hydroxy-substituted phenyl derivatives of AEA also competitively inhibit AEA hydrolysis by the enzyme [38], although these compounds are also efficiently hydrolysed. It is also possible that the presence of two methylenes between the amide and the aromatic group in AA-DA, as in arachidonoyl-serotonin, contributes to it not being efficiently hydrolysed by FAAH.

The five NADAs, including AA-DA, that were tested on the AEA transporter were found to act as inhibitors of this process. This finding is in agreement with previous data obtained with AEA homologues as well as with the observation that aromatic groups on the ethanolamine head of AEA confer on the molecule the capability of strongly interacting with the membrane transporter, provided that at least one *cis* double bond is present in the C_{18}–C_{20} fatty acid chain [15,20]. Indeed, it has been suggested that an active FAAH may contribute to making the AEA transporter more efficacious, since decreases in the intracellular concentration of AEA may enhance the facilitated diffusion of this compound into the cell. Accordingly, FAAH inhibitors have been found recently to inhibit in part AEA accumulation into RBL-2H3 cells [40]. However, although AA-DA was equally active on the transporter and on FAAH, its α-linolenoyl (18:3 ω3) and, particularly, docosapentaenoyl (22:5 ω3) homologues were significantly more active on the transporter than on FAAH, thus suggesting that inhibition of the enzyme contributes only to a limited extent to the inhibition of AEA-facilitated transport by NADAs. This suggestion is in agreement with the previous finding that *N*-acyl-vanillyl-amides and arachidonoyl-serotonin are selective inhibitors of the AEA transporter and of FAAH, respectively [18,20], and with the present observation that the pinolenoyl (5c,9c,12c 18:3 ω6) analogue is more active on FAAH than on the transporter.

All NADAs exhibited some affinity for CB_1 cannabinoid receptors, as determined in displacement assays carried out with rat brain membranes and the selective CB_1 ligand [^{3}H]SR141716A [26]. The most potent compound was AA-DA, in agreement with previous studies on structure/activity relationships carried out on AEA analogues and which showed that a ω6 C_{20} carbon chain with three or four polyolefinic double bonds is necessary for an optimal interaction with the CB_1 receptor [41]. Indeed, AA-DA was more potent than AEA. This finding suggests that the presence of the 3,4-dihydroxy-ethyl-phenyl chain increases considerably the affinity of AEA analogues for CB_1 receptors. Two other present observations support this hypothesis: (i) one of the NADAs tested here, the $C_{22:5}$ ω3 homologue, is a better CB_1 ligand than AEA, even though it lacks the terminal n-pentyl chain, which is considered to be an important part of the CB_1 receptor pharmacophore [39,41]; (ii) the ω3 *N*-acyl-ethanolamines tested here, which lack the 3,4-dihydroxy-ethyl-phenyl moiety, exhibit K_i values for [^{3}H]SR141716A displacement considerably higher than those of the corresponding dopamine derivatives. Previous studies (reviewed in [39,41]) had shown that phenyl derivatives of AEA with no more and, especially, no less than a methylene group between the amide and the aromatic function could be good ligands of CB_1 receptors. In particular, the K_i values reported for the 4-hydroxy-phenyl-, 4-hydroxy-benzyl- and 4-hydroxy-phenyl-ethyl-amides of arachidonic acid (the former of which is known as AM404) are all higher than that of AEA, and are 1.76, 0.217 and 0.6 μM, whereas *N*-(3-hydroxy-phenyl)-arachidonoyl-amine has a K_i of 1.56 μM [41]. Therefore, it is possible that, of the structural differences between AEA and AA-DA, the presence of both the *meta* and *para* hydroxy groups in the benzene ring is the most important feature in increasing the affinity of AEA analogues for CB_1 receptors. However, although our binding assays were carried out in the presence of an FAAH inhibitor, it is possible that the lower K_i value observed here for AA-DA as compared with AEA is due in part to the higher metabolic stability of the dopamine derivative (see above). The introduction of the derivatized aromatic group in AEA almost entirely suppressed its affinity for CB_2 receptors, in agreement with previous findings with the *N*-acyl-vanillyl-amides [20]. Thus AA-DA exhibits at least 40-fold selectivity for CB_1 over CB_2 receptors. It must be noted that the K_i values measured here for AEA in CB_1 and CB_2 binding assays are different from those reported by some authors (see [42] for review). Fluctuations of one order of magnitude in K_i values for cannabinoid ligands, depending on the animal species and strain, tissue, experimental procedure and radioligand used, have been pointed out previously [42].

We found that D2 dopamine-receptor antagonists do not significantly alter the capability of AA-DA of displacing [^{3}H]SR141716A from CB_1 receptors. This indicates that these antagonists do not increase the availability of unbound AA-DA when this compound is incubated with rat brain membranes (where dopamine receptors are expressed), under conditions similar to those used in dopamine-receptor-binding assays. This observation suggests that AA-DA does not bind to dopamine receptors at the concentrations used in these experiments. Accordingly, and more importantly, we did not observe any displacement by AA-DA (up to a 10 μM concentration) of either D1 or D2 high-affinity ligands from rat brain membranes. Three lines of evidence suggest strongly that AA-DA behaves instead as a CB_1 receptor agonist. (i) The compound was more potent than AEA, and as potent as the synthetic cannabinoid HU-210, in inducing an increase of intracellular Ca^{2+} in an undifferentiated neuroblastoma cell line, the N18TG2 cells. In these cells CB_1 receptors, but not voltage-operated Ca^{2+} channels, are expressed and mediate cannabinoid-induced mobilization of Ca^{2+} from intracellular stores [36], rather than the typical inhibition of voltage-operated Ca^{2+} channels [1,3]. The effect of AA-DA on intracellular Ca^{2+} was blocked by a low and selective concentration of the CB_1 receptor antagonist SR141716A. (ii) AA-DA exhibited pharmacological effects *in vivo* that are typical of CB_1 receptor agonists, i.e. it decreased body temperature, spontaneous activity and pain perception, and induced immobility in the ring test at doses similar to or lower than those required for AEA to induce the same effects [30]. (iii) AA-DA potently inhibited MCF-7 cell proliferation, a CB_1 receptor-mediated effect previously observed with AEA, 2-arachidonoyl-glycerol, the CB_1 receptor agonist HU-210, and the metabolically stable AEA analogue methanandamide [33,34]. Notably, human breast cancer cells contain low but detectable levels of D2 dopamine receptors [43] and respond to dopamine-receptor agonists by proliferating less rapidly [44], seemingly through a mechanism similar to that proposed for CB_1 agonists, i.e. inhibition of

prolactin mitogenic action. Whereas, in these cells, inhibition of cAMP levels by cannabinoids was shown to cause a suppression of prolactin-receptor expression [37], the same effect by dopamine-receptor agonists may cause inhibition of the release of endogenous prolactin. However, the AA-DA cytostatic effect was not influenced by haloperidol and it was sensitive instead to SR141716A. These observations, together with the binding data for AA-DA described above, strongly argue against the possibility that this compound acts directly at dopamine receptors. On the other hand, although AA-DA was not efficiently hydrolysed by rat brain homogenates (see above), we cannot rule out that, when administered *in vivo*, this compound acts as a dopamine 'pro-drug' in pharmacological tests that were not performed in this study.

The cannabimimetic effects of AA-DA could, in principle, be due also to the inhibition of the inactivation of endogenous AEA, with subsequent potentiation of a putative tonic effect of the endocannabinoid in the mouse tetrad of tests or in the MCF-7 proliferation assay. However, although substances that inhibit AEA inactivation, such as oleamide, oleoyl-ethanolamide and linoleoyl-ethanolamide, exert cannabimimetic effects in these tests [35,45,46], they do so at doses much higher than those necessary to observe the analogous actions with AEA. Therefore, it is very likely that the rather potent cannabimimetic effects observed in this study with AA-DA both *in vivo* and *in vitro*, at doses comparable with or lower than those necessary for AEA to exert the same actions, are due to direct activation of CB_1 receptors.

Finally, since other aromatic AEA analogues and AEA transporter inhibitors, such as arvanil and AM404, were found recently to activate VR1 vanilloid receptors, thereby inducing Ca^{2+}-influx into cells [47,48], we also tested AA-DA as a possible ligand of these receptors. AA-DA was at least 25-fold less active than arvanil in displacing the high-affinity vanilloid ligand [^{3}H]resiniferatoxin from rat spinal cord membranes, where VR1 is highly expressed [49]. Furthermore, AA-DA was also 100 times less active than arvanil in inducing Ca^{2+} influx into cells stably transfected with the human VR1 receptor (V. Di Marzo and L. De Petrocellis, unpublished work). This finding is in agreement with the previous observation [50] that the introduction of a further methylene group between the amide and phenol groups in capsaicin derivatives strongly reduces their affinity and efficacy at vanilloid receptors.

In conclusion, we have described the biochemical and pharmacological properties of a new class of AEA analogues, the NADAs, in particular regarding their interactions with proteins of the ECS. These properties confer on one of these compounds, AA-DA, strong cannabimimetic effects both *in vitro* and *in vivo*. This study has provided further information on the possible molecular basis for the interaction of AEA with proteins of the ECS, and has led to the development of new substances potentially useful as biochemical probes for the study of the physiological role of endocannabinoids.

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