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European Journal of Medicinal Chemistry 38 (2003) 403–412
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Design, synthesis and biological evaluation of new endocannabinoid transporter inhibitors

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Received 2 October 2002; received in revised form 17 December 2002; accepted 27 January 2003

Abstract

In the present work we describe the synthesis and the in vitro evaluation of a series of arachidonic acid derivatives of general structure **I** as endocannabinoid transporter inhibitors. In addition, we report the first in vivo studies of the most potent derivative (**4**, UCM707) within this series. The majority of compounds studied are highly potent ($IC_{50} = 24–0.8$ μM) and selective endocannabinoid uptake inhibitors with very low affinities for either the enzyme fatty acid amide hydrolase ($IC_{50} = 30–113$ μM) or for cannabinoid receptor subtype 1 (CB_1), cannabinoid receptor subtype 2 (CB_2) and vanilloid receptor subtype 1 (VR_1) ($K_i =$ 1000–10000 nM). Among them, (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(fur-3-ylmethyl)icosa-5,8,11,14-tetraenamide (UCM707) behaves as the most potent endocannabinoid transporter inhibitor described to date ($IC_{50} = 0.8$ μM) and exhibits improved potency for the anandamide transporter, high selectivity for CB_1 and VR_1 receptors, and modest selectivity for CB_2. In vivo it enhances the analgesia and hypokinetic effects induced by a subeffective dose of anandamide.

Keywords: Endocannabinoid transporter inhibitors; Structure-affinity relationship studies; Cannabinoids

1. Introduction

Cannabinoids are substances widely spread over a variety of organisms. Both plants and animals biosynthesise ligands able to bind cannabinoid receptors. (−)-Δ^9-Tetrahydrocannabinol (Δ^9-THC), the active principle of *C. sativa*, was described in the middle sixties for the first time [1]. But it has been only during the past decade that the presence of endogenous ligands for cannabinoid receptors in many animals, from the simplest leeches to the most evolutioned mammals as humans, was established [2].

These findings provided the adequate framework for the development of a growing number of studies aimed at elucidating the chemistry and the molecular bases of action of these compounds in order to rationalise the knowledge about their implication in a wide range of physiological and medical effects, something well known since ancient times, but only from an empirical point of view. This objective made necessary the development of synthetic agents able to bind and activate cannabinoid receptors in a more potent and selective way, compounds which very soon became a tool of primary importance to allow the characterisation of the endogenous cannabinoid system [3].

Abbreviations: AM404, *N*-(4-hydroxyphenyl)arachidonamide; ANT, anandamide transporter; BSA, bovine serum albumine; CB_1, cannabinoid receptor subtype 1; CB_2, cannabinoid receptor subtype 2; DCC, dicyclohexylcarbodiimide; DMAP, *N*,*N*-dimethyl-4-aminopyridine; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAAH, fatty acid amide hydrolase; FBS, fetal bovine serum; IC_{50}, compound concentration which cause a 50% of maximal attainable inhibition of the parameter under investigation; K_i, affinity constant; Max. Inh., maximum inhibition; PEI, poliethylenimine; RTX, resiniferatoxin; Δ^9-THC, (−)-Δ^9-tetrahydrocannabinol; UCM707, (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(fur-3-ylmethyl)icosa-5,8,11,14-tetraenamide; VR_1, vanilloid receptor subtype 1.

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0223-5234/03/$ - see front matter
doi:10.1016/S0223-5234(03)00045-X

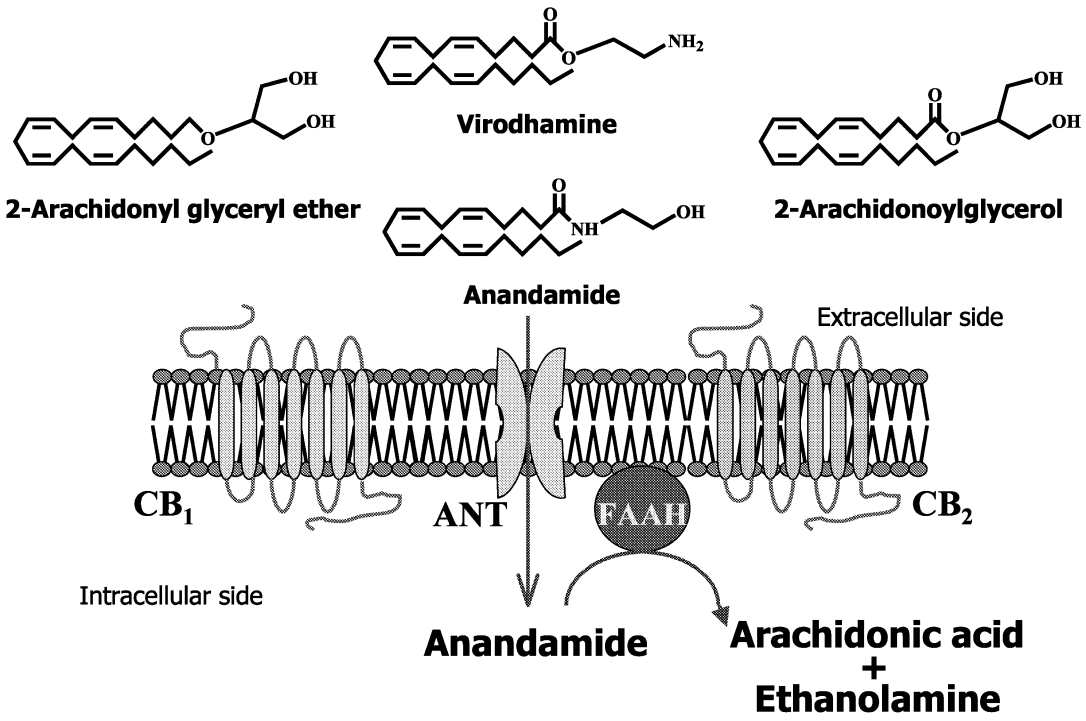


Fig. 1. Endogenous cannabinoid system.

The endogenous cannabinoid system (Fig. 1) mediates the effects attributed to cannabinoids. Thus, it includes the two cannabinoid receptors identified so far, the central cannabinoid receptor subtype 1 (CB_1) [4] and the peripheral cannabinoid receptor subtype 2 (CB_2) [5], both belonging to the G protein coupled receptor family, as well as the endogenous ligands anandamide [6], 2-arachidonoylglycerol [7], 2-arachidonyl glyceryl ether [8] and the recently reported virodhamine [9]. In addition, an inactivation system responsible for terminating with the biological activity induced by the endogenous ligands has been characterised. This termination system consists, in the case of anandamide, of a process of facilitated diffusion (anandamide transporter, ANT) [10] and an intracellular membrane bound enzyme termed fatty acid amide hydrolase or FAAH [11].

The endogenous cannabinoid system is involved in the regulation of a wide variety of central and peripheral processes [12–16] such as antinociception, brain development, retrograde neuronal communication, memory, appetite, psychomotor control, cardiovascular and immune regulation and cellular proliferation, among others. All these effects have attracted a renewed interest about the therapeutic applications of cannabinoids to a broad number of pathologies [17–21] including neurodegenerative diseases, glaucoma, pain relief, cancer and traumatic brain injury. In consequence, the proteins that constitute the endogenous cannabinoid system have become potential therapeutic targets for the treatment of diverse pathologies. Among them, the ANT is a promising pharmacological objective in the search of new agents able to regulate the endogenous cannabinoid system but deprived of undesirable side effects provoked by a direct activation of CB_1 cannabinoid receptors. In this respect, compounds shown to reduce the cellular accumulation of anandamide have been found to produce, in preliminary studies, promising results in animal models of pain and neurodegenerative diseases such as multiple sclerosis or Huntington's chorea [22,23].

However, the relative paucity of structure-affinity relationship studies reported so far as well as the lack of molecular characterisation of ANT has hampered the development of useful transporter inhibitors, and only a few have been described to date [24–26]. Among them, the *N*-(4-hydroxyphenyl)arachidonamide (AM404, Fig. 2), is the best studied [10,24]. However, recent data suggest that AM404 may act as a weak agonist at vanilloid VR_1 receptors [26–28], so there remains a need for the identification of more potent and selective ANT inhibitors.

In this line, we have designed and synthesised a new series of arachidonic acid derivatives of general structure **I** that have been characterised as highly potent and selective inhibitors of anandamide reuptake [29,30]. In

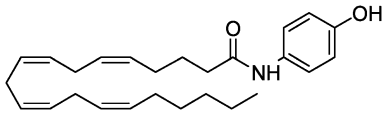


Fig. 2. Structure of AM404.

these derivatives we have analysed the effect of the replacement of ethanolamine moiety of anandamide with a fragment containing a five-membered ring with one heteroatom. Thus, we have evaluated the influence of several factors such as nature of the heteroatom, aromaticity, position of the arachidonic chain as well as distance between the heterocyclic moiety and this fatty acid chain. Also, we have compared amides with their isosteric corresponding esters.

As a result of our data, we have identified (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(fur-3-ylmethyl)icosa-5,8,11,14-tetraenamide (UCM707), as one of the most potent endocannabinoid transporter inhibitors described to date with an IC_{50} value of 0.8 μM and demonstrated that this inhibition can not be secondary to inhibition of anandamide metabolism. Additionally, the preliminary in vivo studies [31] show that UCM707 is able to potentiate the hypokinetic and antinociceptive effects of a subeffective dose of anandamide in a more specific way than previously described ANT inhibitors.

2. Results and discussion

2.1. Chemistry

The synthesis of the amides and esters of general structure **I** (**1**–**16**) listed in Table 1 is detailed in Fig. 3. These compounds were prepared from arachidonic acid by treatment of its acyl chloride with the appropriate amine or alcohol (method A) or by direct condensation between the arachidonic acid and the corresponding amine or alcohol in presence of dicyclohexylcarbodiimide (DCC) and catalytic amounts of *N*,*N*-dimethyl-4-aminopyridine (DMAP) (method B).

The synthesis of the noncommercial amines was carried out by direct reductive amination of the heterocyclic aldehydes [32] except for the (thien-3-ylmethyl)amine that was obtained by hydrogenation of its corresponding oxime precursor [33]. On the other hand, 2-(fur-2-yl)ethylamine, 2-(fur-3-yl)ethylamine and 2-(thien-3-yl)ethylamine were prepared by reduction of their nitrovinyl precursors previously obtained by condensation of aldehydes with nitromethane [34,35].

2.2. Biochemical in vitro assays

All synthesised compounds were assessed for their ability to inhibit [^{3}H]anandamide uptake in human lymphoma U937 cells, for their affinity for CB_1 and CB_2 cannabinoid receptors in radioligand binding assays using [^{3}H]WIN552122 in rat cerebellum membranes and [^{3}H]CP55940 in HEK293EBNA human CB_2 receptor transfected cells, respectively, as well as for their affinity for the vanilloid VR_1 receptor in radioligand binding assays using [^{3}H]resiniferatoxin ([^{3}H]RTX) in rat spinal cord membranes. Also, synthesised compounds were evaluated for their capacity to inhibit the FAAH catalysed metabolism of [^{3}H]anandamide using rat brain homogenate as enzyme source.

Most of compounds **I** display an excellent ability to inhibit anandamide reuptake with IC_{50} values in the low micromolar range (Table 1) as well as selectivity for the transporter, as deduced from their affinity constants for CB_1, CB_2 and VR_1 receptors ($K_i > 1000$–$10\,000$ nM) as well as for the enzyme FAAH, with IC_{50} values (30–110 μM) at least one order of magnitude greater than those observed for the ANT.

The separation between effects upon anandamide accumulation and anandamide hydrolysis are of some importance with respect to our understanding of the nature of the ANT. Although the ANT is usually described as a facilitated transport mechanism, based upon its saturability, temperature and inhibitor-sensitivity, it has been argued that these effects are consistent with the passive diffusion of a lipophilic compound with limited solubility in aqueous medium [36]. FAAH contributes to the uptake of extracellular anandamide by removing the accumulated compound (by metabolism to arachidonic acid), and thereby maintaining a high gradient across the cellular membrane [37]. These authors found that in the absence of FAAH, AM404 was a rather poor inhibitor of anandamide accumulation, which might indicate that the ability of AM404 to inhibit FAAH per se might be responsible for its proposed effects upon the ANT [37]. Such an explanation, however, is not valid for the present series of compounds, given that they are in general much better inhibitors of anandamide accumulation than of anandamide metabolism.

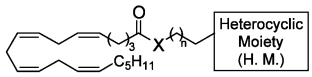
2.3. Structure-affinity relationship studies

As an exploration of the structure-affinity relationship between compounds of general structure **I** and ANT, we have examined the role of the heterocyclic ring, the X group as well as the increase in the distance between the arachidonic chain and the heterocyclic moiety. The most important points can be expressed in the following terms:

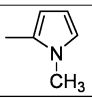
(i) With respect to the changes in the heterocyclic moiety, we have considered the position of the arachidonic acid chain, the influence of the heteroatom and also the aromaticity.

Regarding the position of the fatty acid chain, in general, for thienyl and furyl derivatives, we have observed that substitution in position 3 seems to be more favourable than in position 2 as show the IC_{50} values obtained for compound **3** vs its regioisomer **1** (14 vs 24 μM), **4** vs **2** (0.8 vs 5 μM) and **14** vs **13** (3.3 vs 12.8 μM). This effect is specially remarkable in the thienyl derivatives **10** and **8**, where the potent compound **10**

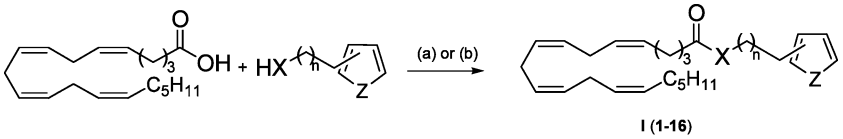
Table 1
Inhibition of ANT of compounds **I**



I (1-16)

Compound	H. M.	X	n	ANT Max. Inh. (%)[a]	ANT IC_{50} (μM)[b]
1		O	0	>85	24±14
2		NH	0	>85	5±2
3		O	0	>85	14±2
4		NH	0	>85	0.8±0.4
5		O	0	>85	18±7
6		NH	0	>85	8±2
7		O	0	>85	*c*
8		O	0	Inactive	
9		NH	0	>85	5.7±0.6
10		O	0	>85	3±2
11		NH	0	>85	5±2
12		NH	0	>85	5.0±0.7
13		NH	1	>85	12.8±0.4
14		NH	1	>85	3.3±0.9
15		NH	1	>85	19±3
16		NH	1	60±11	1.3±0.5
AM404				>85	4±2

[a]Inhibition of anandamide transport was determined using human lymphoma U937 cells and [^{3}H]anandamide. [b]Max. Inh. values are expressed as the percentage of inhibition produced by the highest dose of the compounds tested (50 μM). IC_{50} and Max. Inh. values were obtained from two to four independent experiments carried out in triplicate and are expressed as the mean ± standard error. [c]This compound presented a strange profile which did not fit the sigmoid dose–response, characteristic of the rest of the compounds.



X = O, NH; n = 0,1; Z = N-CH_3, O, S

Reagents: (a) Oxalyl chloride, DMF, CH_2Cl_2, rt; (b) DCC, DMAP, CH_2Cl_2, rt.

Fig. 3. Synthesis of compounds of general structure **I**.

($IC_{50} = 3$ μM) becomes inactive (compound **8**) with this change.

With respect to the nature of the heterocycle, furane ring seems to be the best among all the heterocycles analysed in its capacity to inhibit the transporter. For instance, compound **1** results more potent than its isoster **8** (IC_{50} (**1**) = 24 μM, whereas **8** is inactive), **4** more potent than **11** (0.8 vs 5 μM, respectively) and **13** more potent than **15** (12.8 vs 19 μM, respectively). In spite of this general trend, some examples indicate that the presence of the furane ring does not imply a remarkable increase in the affinity for the ANT as show the comparable IC_{50} values observed for compounds **2**, **9** and **12** (all of them ~5 μM). Also, one exception is observed in the furane derivative **3** (IC_{50} = 14 μM) which displays a lower potency to inhibit the ANT compared with its corresponding thienyl analogue **10** ($IC_{50} = 3$ μM).

Regarding aromaticity, it does not seem to be an essential factor for affinity, as show the equipotent IC_{50} values observed for compounds **1** vs **5** (24 and 18 μM) and **2** vs **6** (5 and 8 μM, respectively). However, when the arachidonic chain is attached to position C-3 (compounds **3** vs **7**), aromaticity becomes an important factor in the inhibitory trend observed for compound **7**, which does not fit the sigmoid dose–response typical of the rest of compounds. This unusual behaviour may reveal the existence of different mechanisms of interaction or recognition between the transporter and its substrates.

(ii) In the CO-X fragment, we have evaluated the effect of substitution of amide for its isosteric ester group. In general, carboxamides and carboxylates are capable of competing with [^{3}H]anandamide for transport. Comparison between furane derivatives suggests that the presence of the NH group enhances the affinity for the carrier, as shown from the IC_{50} values for analogues **6** vs **5** (with IC_{50} values of 8 and 18 μM, respectively), **2** vs **1** (IC_{50} (**2**) = 5 μM and IC_{50} (**1**) = 24 μM) and specially **4** vs **3** with an increase in the affinity of nearly 20-fold (IC_{50} (**4**) = 0.8 μM and IC_{50} (**3**) = 14 μM).

(iii) Increase in the distance between the arachidonic chain and the heterocyclic subunit (compounds with n = 1) produces in all cases important decreases in the inhibitory potency of the compounds. For instance, a 3-fold loss of affinity is observed for furane derivatives **14** vs **4** (3.3 vs 0.8 μM) and **13** vs **2** (12.8 vs 5 μM). A similar effect was obtained for thienyl derivatives with marked decreases in affinity (**9** vs **15**, with IC_{50} values of 5.7 and 19 μM, respectively) or in maximum inhibition (Max. Inh.) capacity (**11** vs **16**, with Max. Inh. values of >85 and 60%, respectively).

To sum up, among all the compounds described here, special attention should be paid to compound **4** (UCM707), which exhibits the best IC_{50} value and incorporates in its structure all the optimum features deduced from the structure-affinity relationship study herein reported. The excellent IC_{50} value of 0.8 μM exhibited by this compound and its selectivity for the ANT above the other analysed proteins (see Table 2): CB_1 (K_i = 4800 nM), VR_1 (K_i > 5000 nM) and FAAH ($IC_{50} = 30$ μM), makes it a valuable candidate for future and deeper pharmacological studies. In their recent article where strong arguments were raised to suggest that anandamide accumulation may be simply a process

Table 3
Potentiation by compound **4** (UCM707) of the hypokinetic and analgesic actions of a subeffective dose of anandamide

Parameters	+Anandamide [a]	+UCM707 [b]	+Both [a,b]
Ambulation [c]	−44.1%	+14.6%	−67.8% *
Inactivity [c]	+1.7%	−16.1%	+169.1% *
Antinociception [d]	+21.9%	+48.8%	+113.0% *

Values are expressed as percentages of change vs the corresponding controls.

[a] Used at a dose of 0.3 or 2 mg kg^{-1} for the open-field and the hot-plate tests, respectively.

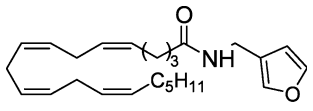
[b] Used at a dose of 0.5 or 1 mg kg^{-1} for the open-field and the hot-plate tests, respectively.

[c] Measured in the open-field test.

[d] Measured in the hot-plate test.

* $P < 0.05$.

Table 2
In vitro profile of compound **4** (UCM707)



4 (UCM707)

Comp.	ANT		FAAH			Receptor affinity	
	Max. Inh. (%)	IC_{50} (μM)	Max. Inh. (%)	IC_{50} (μM)	K_i CB_1 (nM)	K_i CB_2 (nM)	K_i VR_1 (nM)
4 (UCM707)	>85	0.8±0.4	100	30	4700±80	67±6	>5000

of passive rather than facilitated diffusion, Patricelli and Cravatt [36] pointed out that in addition to actions upon FAAH, anandamide analogues could 'simply saturate the membranes preventing accumulation'. The authors continued 'To date, no inhibitors with IC_{50} values are significantly lower than the K_M value measured for anandamide transport (1–50 μM) have been reported' [36]. Compound **4** would thus be the first such inhibitor. In addition, this compound, displays a moderate affinity only to CB_2 ($K_i = 67$ nM) which raises the possibility of selectively activating this receptor and, simultaneously, inhibiting the metabolism of endocannabinoids, with all the implicit attractive therapeutic applications.

2.4. In vivo studies of UCM707

In order to confirm this initial in vitro profile, we have already started the first in vivo studies aimed at examining the dose–response effects of UCM707 in tests of motor activity (open-field test) and antinociception (hot-plate test), as well as its capacity to enhance the hypokinetic and/or analgesic actions of a subeffective dose of anandamide (for details, see [31]). Our results indicated that UCM707 was mostly inactive when administered alone, but it was able to potentiate the action of a dose of anandamide that did not produce any effect by itself (see the more relevant results in Table 3).

In conclusion, in this study we have synthesised new arachidonic acid derivatives which act as potent inhibitors of anandamide uptake. Particularly, special attention should be paid to compound UCM707, which behaves in vitro as the most potent and selective inhibitor of the ANT described to date, and acts with great potency and selectivity in the in vivo tests of motor behaviour and analgesia.

This allows us to consider this compound as a promising tool when used alone or in combination with endocannabinoids for the treatment of a variety of disorders, where the elevation of the endocananbioid tone has been proposed to have therapeutic benefits. Furthermore, the presented data support the suggestion that the ANT system is a valid target for medicinal chemistry and may allow the development of new and alternative therapeutic strategies to deal with some of the most devastating neurodegenerative diseases still lacking satisfactory therapies.

3. Experimental

3.1. Chemistry

All the compounds were characterised according to their spectroscopic data. Infrared (IR) spectra were determined on a Perkin–Elmer 781 or Shimadzu-8300 IR spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian VXR-300S, Bruker Avance 300-AM or Bruker 200-AC instrument at room temperature (r.t.). Chemical shifts (δ) are expressed in parts per million relative to internal tetramethylsilane; coupling constants (J) are in hertz. Satisfactory elemental analyses were obtained for all the newly synthesised analogs and are within $\pm 0.4\%$ of the theoretical values. Thin-layer chromatography (TLC) was run on Merck silica gel 60 F-254 plates. For normal pressure chromatography, Merck silica gel type 60 (size 70–230) was used. Unless stated otherwise, starting materials used were high-grade commercial products from Aldrich, Acros, Fluka, Merck or Panreac except arachidonic acid (90% of purity) which was purchased from Sigma. Anhydrous *N*,*N*-dimethylformamide (DMF) was obtained by stirring over CaH_2 and vacuum distillation, methylene chloride was used fresh distillated over CaH_2.

3.1.1. General procedure for the synthesis of derivatives **1–16**

Method A. Arachidonyl chloride was synthesised by reaction of arachidonic acid (111.1 mg, 0.33 mmol) in methylene chloride (5 mL mmol^{-1}) with 2 equiv. of oxalyl chloride in the presence of 1 equiv. of dimethylformamide under argon atmosphere. After 1 h at r.t., the solvent was removed under vacuum and arachidonyl chloride was dissolved in methylene chloride (5 mL mmol^{-1}) and the appropriate alcohol or amine (ca. 3.3 mmol) was added in a methylene chloride solution (1.5 mL mmol^{-1}). The mixture was stirred (3–6 h) until no further evolution was observed by TLC (chloroform:methanol, 95:5) and then was washed with water and brine and dried over Na_2SO_4 or $MgSO_4$. Then, the solvent was evaporated under reduced pressure and the crude product purified by column chromatography on silicagel using the appropriate eluent.

Method B. To a stirred solution of 1 equiv. (0.33 mmol) of arachidonic acid in dry methylene chloride (1 mL mmol^{-1}) and the appropriate alcohol or amine (1.5 equiv.) in dry methylene chloride (1 mL mmol^{-1}) at -20 °C in an ice-bath under argon was added dropwise a solution of DCC (1 equiv.) and DMAP (0.068 equiv.) in dry methylene chloride (3 mL mmol^{-1} DCC). The mixture was stirred for 5 min at this temperature and then the cooling bath was removed and stirred at r.t. (3–6 h) until no further evolution was observed by TLC (chloroform:methanol, 95:5). The dicyclohexylurea was filtered off, the filtrate evaporated under reduced pressure and the obtained residue taken up in methylene chloride (20 mL mmol^{-1} of fatty acid). This resulting organic phase was washed with a cooled 0.5 M hydrochloric acid solution and brine and the organic extracts dried over Na_2SO_4 or $MgSO_4$. Then, the solvent was evaporated under reduced pressure and the product

purified by column chromatography on silicagel using the appropriate eluent.

The spectroscopic data of one representative compound is shown below. (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(Fur-3-ylmethyl)icosa-5,8,11,14-tetraenamide (**4**, UCM707). Method A (yield = 45%) and Method B (yield = 65%). $R_f = 0.43$ (hexane:ethyl acetate, 1:1). IR ($CHCl_3$, cm^{-1}): 3018, 2930, 2856, 1709, 1655, 1522, 1458, 1420, 1022. ^{1}H-NMR (200 MHz, $CDCl_3$-δ): 0.88 (t, 3H, J = 6.0 Hz), 1.22–1.35 (m, 6H), 1.73 (quint, 2H), 2.00–2.23 (m, 6H), 2.76–2.85 (m, 6H), 4.28 (d, 2H, J = 5.6 Hz), 5.32–5.47 (m, 8H), 5.58 (br s, 1H,), 6.35–6.36 (m, 1H), 7.37–7.38 (m, 2H).

Table of elemental analyses of compounds 1-16

Comp.	Formula	Calculated			Found		
		C	H	N	C	H	N
1	C25H36O3	78.08	9.44		78.01	9.34	
2	C25H37 NO2	78.28	9.72	3.65	78.17	9.77	3.71
3	C25H36O3	78.08	9.44		77.98	9.51	
4	C25H37 NO2	78.28	9.72	3.65	78.32	9.79	3.58
5	C25H40O3	77.27	10.38		77.43	10.42	
6	C25H41 NO2	77.47	10.66	3.61	77.38	10.57	3.65
7	C25H40O3	77.27	10.38		77.37	10.25	
8	C25H36O2S	74.95	9.06		74.85	9.11	
9	C25H37 NOS	75.13	9.26	3.50	75.09	9.31	3.47
10	C25H36O2S	74.95	9.06		75.01	8.99	
11	C25H37 NOS	75.13	9.26	3.50	75.07	9.35	3.57
12	C26H40N2O	78.74	10.16	7.06	78.69	10.09	6.97
13	C26H39NO2	78.54	9.89	3.52	78.59	10.00	3.58
14	C26H39NO2	78.54	9.89	3.52	78.48	9.80	3.46
15	C26H39NOS	75.49	9.50	3.38	75.58	9.41	3.45
16	C26H39NOS	75.49	9.50	3.38	75.57	9.55	3.31

3.2. Biochemical in vitro assays

3.2.1. Radioligand binding assays

3.2.1.1. CB_1 receptor. Receptor binding studies were performed according to the procedure of Houston et al. [38] with slight modifications. Briefly, rat cerebellar membranes were homogenised in 5 mL of ice-cold Tris buffer (50 mM Tris–HCl, pH 7.4 at 4 °C) and centrifuged at 48 000 × *g* and 4 °C for 10 min. The membrane pellet was washed twice by resuspension and centrifugation. The final pellet was resuspended in 20 vol. of incubation buffer (50 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM $MgCl_2$ and 5 mg mL^{-1} bovine serum albumine (BSA) fatty acid free, pH 7.4). Fractions of the final membrane suspension (~ 2 mg mL^{-1} of protein) were incubated at 30 °C for 90 min with 0.5 nM [^{3}H]-WIN552122 (180 Ci $mmol^{-1}$), in the presence or absence of several concentrations of the competing drug, in a final volume of 0.5 mL of assay buffer (50 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$ and 5 mg mL^{-1} BSA fatty acid free, pH 7.4). Non-specific binding was determined in the presence of 10 μM SR141716A. Silanized tubes were used throughout the procedure to minimise receptor binding loss due to tube adsorption. The reaction was terminated by rapid vacuum filtration with a Brandel Harvester apparatus through Whatman GF/C filters pre-soaked in Tris buffer (50 mM Tris–HCl, 1 mg mL^{-1} BSA, pH 7.4). The filters were washed three times with 5 mL of ice-cold buffer (50 mM Tris–HCl, 1 mg mL^{-1} BSA, pH 7.4, 4 °C) and bound radioactivity measured by placing filters in 4 mL of Ecolite scintillation cocktail followed by scintillation spectroscopy using a Packard 2500 TR liquid scintillation spectrometer.

3.2.1.2. CB_2 receptor. Receptor binding studies were performed according to the procedure of Griffin et al. [39] using membrane fractions of human CB_2 receptor transfected cells purchased from Receptor Biology, Inc. (Beltsville, MD).

HEK293EBNA membranes were resuspended in Tris buffer (50 mM Tris–HCl, 2.5 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM $MgCl_2$, 1 mg mL^{-1} BSA fatty acid free, pH 7.5).

Fractions of the final membrane suspension (~ 1.44 mg mL^{-1} of protein) were incubated at 30 °C for 90 min with 0.3 nM [^{3}H]-CP55940 (180 Ci $mmol^{-1}$), in the presence or absence of several concentrations of the competing drug, in a final volume of 0.2 mL of assay buffer (50 mM Tris–HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 1 mg mL^{-1} BSA fatty acid free, pH 7.5). Non-specific binding was determined in the presence of 5 μM CP55940. Silanized tubes were used throughout the experiment to minimise receptor binding loss due to tube adsorption. The reaction was terminated by rapid vacuum filtration with a Brandel Harvester apparatus through Whatman GF/C filters pre-soaked in 0.05% poliethylenimine (PEI). The filters were washed three times with 5 mL of ice-cold buffer (50 mM Tris–HCl, 1 mg mL^{-1} BSA, pH 7.4, 4 °C) and bound radioactivity was measured as described in Section 3.2.1.1.

3.2.1.3. VR_1 receptor. Receptor binding studies were performed according to the procedure of Szallasi et al. [40]. Briefly, rat spinal cord membranes were homogenised in HEPES buffer (10 mM HEPES, 5 mM KCl, 5.8 mM NaCl, 0.75 mM $CaCl_2$, 2 mM $MgCl_2$, 320 mM sucrose, pH 7.4) and centrifuged at 1000 × *g* at 4 °C for 10 min. The supernatant was removed and samples centrifuged again at 35 000 × *g* at 4 °C for 30 min. Finally, the membrane pellet was resuspended in 10 vol. of HEPES buffer (10 mM HEPES, 5 mM KCl, 5.8 mM NaCl, 0.75 mM $CaCl_2$, 2 mM $MgCl_2$, 320 mM sucrose, pH 7.4). Fractions of the final membrane suspension (~ 1 mg mL^{-1} of protein) were incubated at 37 °C for 60 min with 25 pM [^{3}H]RTX (48 Ci $mmol^{-1}$), in the presence or absence of several concentrations of the competing drug, in a final volume of 0.5 mL of assay buffer (10 mM HEPES, 5 mM KCl, 5.8 mM NaCl, 0.75 mM $CaCl_2$, 2 mM $MgCl_2$, 320 mM sucrose, 0.25 mg mL^{-1} BSA fatty acid free, pH 7.4). Non-specific binding was determined in the presence of 1 μM RTX. The reaction was stopped by chilling the assay mixture on an ice-cold water bath and then 100 μg of bovine α_1-acid glycoprotein in 50 μL of buffer were added to reduce non-specific binding. Bound and free [^{3}H]RTX were separated by pelleting the membranes in a Beckman TJ-6 centrifuge. After the supernatant was removed by aspiration and the pellet carefully dried, the tip of the eppendorf tube containing the pelleted membranes was cut off with a razor blade. Radioactivity retained by the samples was measured as described in Section 3.2.1.1.

For all binding experiments, competition–binding curves were analysed by using an iterative curve-fitting procedure GradPad (Prism) which provided IC_{50} values for test compounds. K_i values were determined by the method of Cheng and Prusoff [41].

3.2.2. Endocannabinoid transporter assay

Human lymphoma U937 cells, maintained at 37 °C and 5% CO_2 wet atmosphere, were grown in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) previously heat inactivated, 100 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2.5 mM sodium piruvate. For standard competition assays, 1 mL of U937 cells in RPMI 1640 culture medium (10^6 cells mL^{-1}) were preincubated at 37 °C during 10 min in the presence or absence of several concentrations of the tested inhibitors. Then, a mixture of [^{3}H]anandamide (0.45 nM) and cold anandamide at a final concentration of 100 nM was added and cells were incubated for 7 min. The reaction was stopped by rapid filtration over Whatman GF/C filters pre-soaked in 0.25% BSA. Filters were washed three times with 5 mL of ice-cold Krebs-HEPES buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 2.4 mM $MgSO_4$, 1 mM NaH_2PO_4, 20 mM $NaHCO_3$, 11.1 mM glucose, 3.98 μM Na_2EDTA, 110 mM ascorbic acid, 10 mM HepesNa and 1% BSA fatty acid free, pH 7.4) and bound radioactivity measured by embedding filters in 4 mL of Ecolite scintillation cocktail and counted using a Packard 2500 TR liquid scintillation spectrometer.

Non-specific reuptake was determined in the same experimental conditions at 4 °C. Silanized tubes were used throughout to minimise influence of effects of compound adsorption. Competition curves were analysed by using an iterative curve-fitting procedure GradPad (Prism) which provided IC_{50} values for test compounds.

3.2.3. FAAH inhibition assay

FAAH activity was assayed in rat brain homogenates as described by Omeir et al. [42] adapted to the tritiated substrate by Fowler et al. [43]. Briefly, test compounds or ethanol carrier (10 μL) were incubated at 37 °C for 10 min with diluted homogenates (165 μL) of rat brain (minus cerebellum, in 10 mM Tris–HCl + 1 mM EDTA, pH 7.6) and 25 μL of a mixture of 16 μM (i.e. 2 μM final assay concentration) non-radioactive anandamide containing trace amounts of [^{3}H]anandamide with the label in the ethanolamine side chain (30–60 Ci $mmol^{-1}$) and 1% w/v fatty acid free BSA. The reaction was terminated by putting the samples on an ice-bath followed by the addition of 0.4 mL chloroform–methanol (1:1 v/v), After vigorous vortexing aqueous and organic phases were separated by centrifugation, and the radioactivity (corresponding to the [^{3}H]ethanolamine produced as a result of the FAAH-catalysed breakdown of [^{3}H]anandamide) found in aliquots (200 μL) of the aqueous phase was determined by liquid scintillation counting with quench correction. pI_{50}, and hence IC_{50} values were determined as described previously [44].

3.3. In vivo assays for UCM707

3.3.1. Animals and treatments and sampling

Male Wistar rats were housed 2 weeks before the onset of the experiments in a room with controlled photoperiod (08:00–20:00 light) and temperature (23 ± 1 °C).

They had free access to standard food and water. Animals were used at about to months of age (250–350 g weight) in all experiments, which were always conducted according to European and local rules on the care of and research with experimental animals. In a first experiment, rats were injected i.p. with three different doses (0.1, 1.0 and 10 mg kg^{-1}) of UCM707 or with vehicle (Tween 80-saline, 1:16). Ten minutes later, animals were assessed in the open-field test or in the hot-plate test. In a second experiment, rats were divided into four groups and subjected to the following i.p. injections: (i) vehicle (Tween 80-saline, 1:16), (ii) a dose of UCM707, selected from the doses that did not produce any effects in the above experiment (0.5 mg kg^{-1} for the open-field test and 1 mg kg^{-1} for the hot-plate test), (iii) a subeffective dose of anandamide (0.3 mg kg^{-1} for the open-field test and 2 mg kg^{-1} for the hot-plate test), and (iv) the combination of both, UCM707 and anandamide, administered at the same time. Ten minutes later, animals were also assessed in the open-field test or in the hot-plate test.

3.3.2. Open-field test

Motor behaviour was analysed in an open-field test, whose characteristics have been previously described [45]. Ten minutes after drug administration, the animals were placed in the open-field and their behaviour was recorded for a period of 10 min, although only the last 5 min were scored (the first 5 min served as the period of habituation to the novel environment, which reduced the influence of emotional aspects). The following parameters were scored: (i) ambulation: number of sector crossing; (ii) exploratory activity: number of head entries into the square holes; (iii) frequency of stereotypic behaviours (rearing, self-grooming and shaking); and (iv) time spent in inactivity.

3.3.3. Hot-plate analysis

For the analysis of antinociception, we used the hot-plate procedure described by Girard et al. [46]. Rats were placed individually on a hot-plate maintained at 52 °C, and the latency to exhibit the first sign of pain (i.e., licking the hind paws or jumping) was measured for each rat. Animals not responding were removed after 30 s (cut-off time to avoid tissue damage).

3.3.4. Statistics

Data were assessed by the one-way analysis of variance followed by the Student–Newman–Keuls test.

Acknowledgements

This work has been supported by MCYT (BQU 2001-1459) and CICYT (PM99-0056). The authors would like to thank MECD and CAM for predoctoral grants to S.O.G. and E.L., respectively. C.J.F. and G.T. would like to thank the Swedish Science Research Council (Grant no. 12158. Medicine) and Konung Gustav V's and Drottning Victorias Foundation for financial support. The authors would also like to thank B.J. for her excellent technical assistance with the FAAH assays.

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