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Synthesis of Fatty Acid Amides of Catechol Metabolites that Exhibit Antiobesity Properties

Bruno Almeida,[a, b, f] Jesús Joglar,*[c] María Jesús Luque Rojas,[d] Juan Manuel Decara,[d] Francisco Javier Bermúdez-Silva,[d] Manuel Macias-González,[e, f] Montserrat Fitó,[f, g] Miguel Romero-Cuevas,[d] Magí Farré,[a, h] María Isabel Covas,[f, g] Fernando Rodríguez de Fonseca,[d, f] and Rafael de la Torre*[a, b, f]

In memory of Prof. Dr. José Manuel Concellón (Universidad de Oviedo)

A series of fatty acid amides of 3,4-methylenedioxymethamphetamine (MDMA) catechol metabolites were synthesized in order to evaluate their biological activities. Upon administration, all synthesized compounds resulted in negative modulation of food intake in rats. The most active compounds have affinity for the CB_1 receptor and/or PPAR-α; part of their biological activity may be caused by these double interactions.

Introduction

The discovery of endocannabinoids, including the fatty acid amide anandamide (arachidonylethanolamide, AEA), paved the way for lipidomic discoveries within the growing family of endogenous fatty acid amides.[1] Fatty acid amides can be formed from metabolites of xenobiotics such as acetaminophen, or from endogenous substrates like dopamine (Figure 1). For example, it has been proposed that the analgesic effects of acetaminophen are derived from the compound known as AM404, which results from the conjugation of *p*-aminophenol with arachidonic acid and its subsequent interaction with the endocannabinoid system.[2–4]

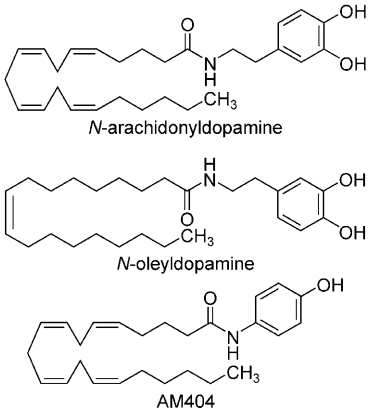

Figure 1. Fatty acid amides of endogenous or exogenous substrates with cannabinoid activity.

It is well known that cannabinoid agonists modulate food intake, among other physiological effects, and that the inhibition of cannabinoid type 1 receptors causes an appetite decrease, leading to a corresponding decrease in weight.[5] It was also discovered that, although the endogenous fatty acid amide oleylethanolamide inhibits food intake, it does not interact with cannabinoid type 1 (CB_1) or 2 (CB_2) receptors, but rather through the peroxisome proliferator-activated nuclear receptor-α (PPAR-α).[6]

[a] *B. Almeida, Dr. M. Farré, Dr. R. de la Torre*
Human Pharmacology and Clinical Neurosciences Research Group
Neuropsychopharmacology Research Program
IMIM-Hospital del Mar Research Institute
Dr. Aiguader 88, Barcelona 08003 (Spain)
Fax: (+34) 933160467
E-mail: rtorre@imim.es

[b] *B. Almeida, Dr. R. de la Torre*
Universitat Pompeu Fabra, CEXS-UPF
Dr. Aiguader 88, Barcelona 08003 (Spain)

[c] *Dr. J. Joglar*
Departamento de Química Biológica y Modelización Molecular
Instituto de Química Avanzada de Cataluña (IQAC-CSIC)
Jordi Girona 18-26, 08034 Barcelona (Spain)
E-mail: joglar@iqac.csic.es

[d] *M. J. Luque Rojas, J. M. Decara, F. J. Bermúdez-Silva, M. Romero-Cuevas, Dr. F. Rodríguez de Fonseca*
Fundación IMABIS, Hospital Carlos Haya de Málaga
Avda. Carlos Haya 82, Pabellón de Gobierno, 29010 Málaga (Spain)

[e] *Dr. M. Macias-González*
Hospital Clínico Virgen de la Victoria
Campus Universitario Teatinos, 29010 Málaga (Spain)

[f] *B. Almeida, Dr. M. Macias-González, Dr. M. Fitó, Dr. M. I. Covas, Dr. F. Rodríguez de Fonseca, Dr. R. de la Torre*
CIBEROBN, Instituto de Salud Carlos III, Madrid (Spain)

[g] *Dr. M. Fitó, Dr. M. I. Covas*
Oxidative Stress and Nutrition Research Group
Inflammation and Cardiovascular Research Program
IMIM-Hospital del Mar, Dr. Aiguader 88, Barcelona 08003 (Spain)

[h] *Dr. M. Farré*
Universitat Autònoma de Barcelona, UDIMAS-UAB
Dr. Aiguader 88, Barcelona 08003 (Spain)

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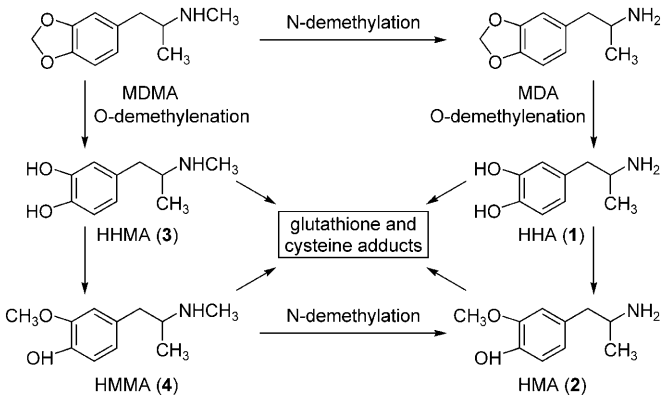
Herein we present the synthesis and biological activity of fatty acid amides of several metabolites of 3,4-methylenedioxymethamphetamine (MDMA). MDMA is a widely abused, recreational drug that produces effects commonly associated with stimulants and hallucinogens.[7,8] It is believed that long term use can cause neurodegeneration in the serotonergic neurotransmission system, both in animals and humans.[9]

MDMA has two main metabolic pathways: 1) O-demethylenation, followed by methylation and/or glucuronide/sulfate conjugation; and 2) N-dealkylation. The latter of these MDMA pathways gives rise to 3,4-methylenedioxyamphetamine (MDA). The parent compound and MDA are further O-demethylated to 3,4-dihydroxymethamphetamine (HHMA, **3**) and 3,4-dihydroxyamphetamine (HHA, **1**), respectively. Both HHMA and HHA are subsequently O-methylated to 4-hydroxy-3-methoxymethamphetamine (HMMA, **4**) and 4-hydroxy-3-methoxyamphetamine (HMA, **2**), respectively. These metabolites are mainly present in plasma and urine as their glucuronide or sulfate conjugates.[10,11] As catechols, HHA and HHMA are highly redox active and may form adducts with glutathione, after autooxidation to their corresponding quinones. (Scheme 1).[12]



Scheme 1. MDMA metabolic distribution.

It must be noted that body disposition of MDMA is subjected to selective enantiomeric metabolism.[13] Although the methods reported herein would allow for the preparation of enantiomerically pure compounds, it was considered unnecessary for this preliminary study of the pharmacological activity of the synthesized compounds. However, work in our research group is ongoing toward production of the enantiomeric MDMA metabolites, which are crucial for ongoing and future studies of the differential metabolism of MDMA enantiomers and their role in the neurotoxicity and other pharmacological effects of MDMA. Catechol metabolites of MDMA are analogues of dopamine, and can be renamed as α-methyldopamine for HHA, or as *N*-methyl-α-methyldopamine for HHMA. Fatty acid amides of dopamine (*N*-oleyldopamine, OLDA and *N*-arachidonyldopamine, NADA) have been found in the brain and other tissues, and their biosynthesis has recently been reported.[14] OLDA and NADA act with varying potency on the (CB_1) receptor and on the transient receptor potential vanilloid subtype 1 (TRPV1). As such, they are considered putative endocannabinoids and endovanilloids that play a role in the regulation of pain and inflammation.[15–18]

Some existing data supports the interaction between MDMA and the cannabinoid system. In clinical practice, MDMA and cannabis consumption are strongly associated. It has been postulated that the connection does not occur randomly, or because of drug availability, but through modulation of pharmacological effects. It has already been established in animal models that some in vivo effects of MDMA, such as long term rewarding process, hyperthermia, and mood alteration, among others, are associated with endocannabinoid system interactions.[19–22] Recent results in humans have shown that cannabinoids regulate cardiovascular activity and body temperature when combined with MDMA.[23] By analogy, based on what is known of the fatty acid derivatives of dopamine and the possibility that xenobiotic metabolites may form fatty acid derivatives with relevant biological activities, it is postulated that fatty acid acyl derivatives of several catecholic metabolites of MDMA (HHA and HHMA) and their *O*-methyl derivatives (HMA and HMMA) could display biological activities of interest. If their in vivo formation is ever demonstrated, it may significantly improve our understanding of MDMA pharmacology. Additionally, these amides may display a potential therapeutic profile and could become useful pharmacological tools.

In this context, the oleic and arachidonic fatty acid amides of MDMA metabolites HHA, HMA, and HHMA were synthesized as racemic mixtures in order to evaluate their biological activities (Figure 2).

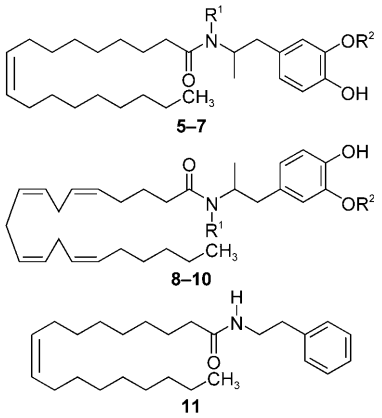
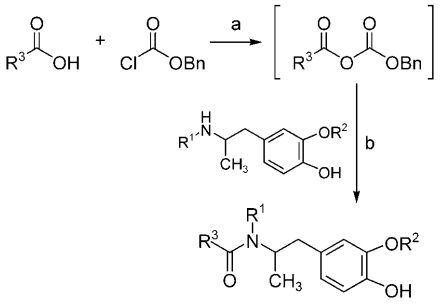


Figure 2. Proposed structures of fatty acid amides. $R^1 = R^2 = H$, *N*-(1-(3,4-dihydroxyphenyl)propan-2-yl)oleamide (**5**); *N*-(1-(3,4-dihydroxyphenyl)propan-2-yl)arachidonamide (**8**); $R^1 = H$, $R^2 = Me$, *N*-(1-(4-hydroxy-3-methoxyphenyl)-propan-2-yl)oleamide (**6**); *N*-(1-(4-hydroxy-3-methoxyphenyl)propan-2-yl)arachidonamide (**9**); $R^1 = Me$, $R^2 = H$, *N*-(1-(3,4-dihydroxyphenyl)propan-2-yl)-*N*-methyloleamide (**7**); *N*-(1-(3,4-dihydroxyphenyl)propan-2-yl)-*N*-methylarachidonamide (**10**); *N*-phenethyloleamide (**11**).

Results and Discussion

Chemistry

MDMA metabolites were synthesized according to previously described methodology (Scheme 2).[24] Next, fatty acid amides were synthesized by a one-pot method: the fatty acid carbox-



Scheme 2. General procedure for the synthesis of fatty acid amides of MDMA metabolites: a) CH_3CN, Et_3N, 4 °C, 1 h; b) DMF, Et_3N, 25 °C, 24 h.

ylic group was activated with benzyl chloroformate, and this intermediate was subsequently treated with MDMA metabolites **1**–**3**. (Scheme 2). Compounds **7** and **10** were obtained as a mixture of *cis* and *trans* isomers and were identified by NMR (see Supporting Information).

Biology

Feeding experiments

The acute food intake effect of all compounds was tested in 24 h food-deprived rats. The amide of phenylethylamine with oleic acid (**11**) was synthesized as a theoretical negative control due to the lack of a catechol group. All compounds induced a significant decrease in food intake (Figure 3). Compounds **5** and **8** were most active and displayed an acute food intake modulation profile and potency similar to that of oleyl ethanolamide (OEA, a known PPAR-α selective endogenous agonist), although OEA activity lasts longer.[6,25] The food intake modulation profile is also compatible with other PPAR-α ligands.[26]

HHA derivatives are more potent than HMA and HHMA derivatives, thus we concluded that *meta*-O-methylation of the catechol ring and N-methylation of the amide group are deleterious with respect to food intake activity. Compound **11** showed only a minor increase in food intake at the 1 mg kg^{-1} dose. This is indicative of the importance of the catechol group as a pharmacophore. To confirm that the negative modulation of food intake was not caused by an anesthetic effect of the compounds, we tested **5** in an open field test using the same doses and established that there was no effect on animal locomotion (see Supporting Information).

A study of the affinity of this compound series for the CB_1 receptor, as well as efficacy toward PPAR-α, was performed to further evaluate the biological activity of the synthesized compounds. The CB_1 receptor binding test evaluated the capacity of these compounds to displace [^{3}H]SR141716 in rat cerebellum homogenate. Compounds **5**–**8**, which exhibited greatest activity in the food ingestion test, were evaluated. Only compounds **5**, **6,** and **8** had affinity for the CB_1 receptor (Figure 4, Table 1) (see Supporting Information). This indicates that N-methylation is deleterious for the CB_1 receptor compound affinity, corresponding to the decrease in activity observed for compound **7** in the food intake test. These results demonstrate that at least some of the biological activity of the compounds may be attributed to interaction with CB_1. Calculated pK_i values of our compounds are similar to that of endogenous anandamide, using the same labeled CB_1 ligand ([^{3}H]SR141716)

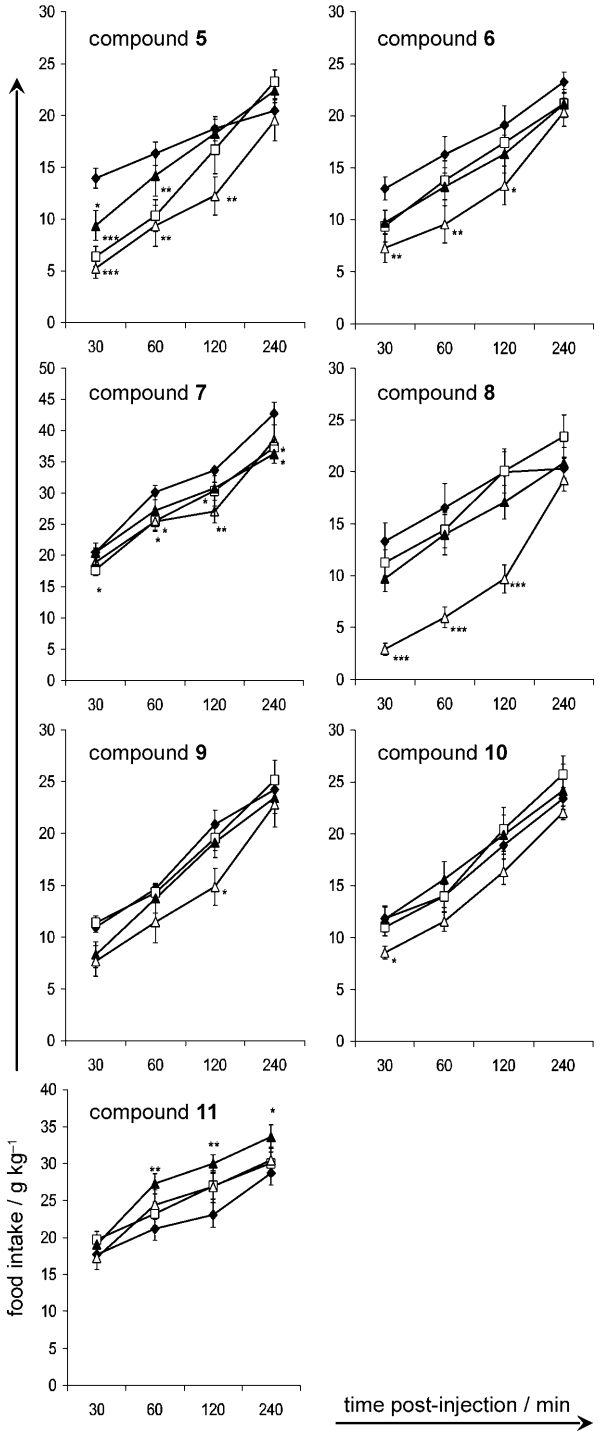


Figure 3. Relative food intake (g food per kg animal weight) by male Wistar rats deprived of food for 24 h. Food was weighed at 30, 60, 120, and 240 min after injection of vehicle (◆), test compounds **5**–**10** at 0.5 (□), 1 (▲), and 5 (△) mg kg^{-1}, or compound **11** at 0.3 (□), 1 (▲), and 3 (△) mg kg^{-1}. Results are shown as the mean ± SEM of a group of eight animals; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (ANOVA).

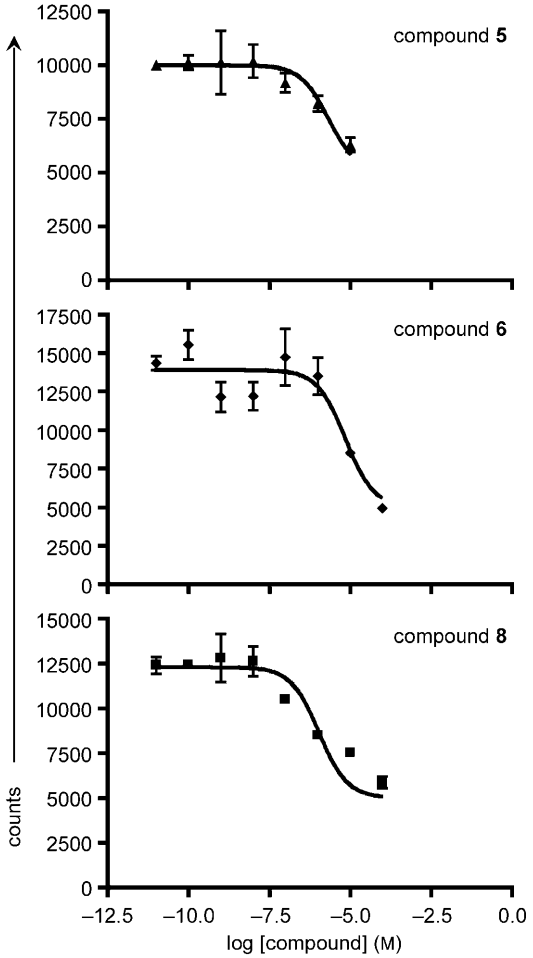


Figure 4. CB_1 binding assay for compounds **5** (▲), **6** (◆), and **8** (■).

Table 1. Pharmacological properties of fatty acid amides.

Compound	CB_1 K_i [M]	CB_1 pK_i	PPAR-α EC_{50} [nM][a]
SR141716	3.64×10^{-10}	9.44	–
anandamide	1.7×10^{-7}[b]	6.77	>10000
WIN55212-2	1.11×10^{-8}[b]	7.95	–
5	3.65×10^{-7}	6.44	698±102
6	1.44×10^{-6}	5.84	1022±206
8	2.02×10^{-7}	6.69	>10000
11	–	–	1879±384
oleic acid	–	–	218±150
OEA	–	–	148±29
GW7647	–	–	65±1

[a] Values calculated with GraphPad Prism 4; results for PPAR-α are the mean ±SEM of three experiments. [b] Values from reference [27].

and similar experimental conditions, but lower than those of synthetic ligands WIN55212-2 and SR141716.[27]

We also tested the efficacy of compounds **5**, **6**, **8**, and **11** for PPAR-α by the transient transfection and luciferase reporter assay. We used OEA and AEA as positive and negative controls, respectively. Compounds **5**, **6**, and **11** exhibited an EC_{50} in the micromolar range, while compound **8** showed no activity (Table 1). The activity displayed by compound **11** could indicate that the catechol moiety is not essential for PPAR activity. The tested compounds displayed lower PPAR-α efficacy than OEA and GW7647 (a synthetic PPAR-α ligand), although their activities were similar to those observed in other examples of PPAR-α ligands and food intake modulators, as described by Cano et *al.*[26]

It is worth noting that compound **8**, which displayed one of the highest levels of food intake inhibition, did not exhibit any activity toward PPAR-α. In this particular case, the food intake activity may be due entirely to CB_1 receptor interaction. On the other hand, modifications to the fatty acid moiety may alter the pharmacodynamics of these compounds.

We tested compounds **5**–**8** as potential fatty acid amide hydrolase (FAAH) inhibitors using Wistar rat cerebral cortex and tritiated anandamide. Degradation of the labeled anandamide by the FAAH present in the cortex homogenate was monitored by beta scintillation. None of the tested compounds showed any FAAH inhibitory activity (see Supporting Information).

Because some fatty acid amides are ligands for not only cannabinoid receptors but also for the TRPV1 and the PPAR-α receptors, we questioned the extent to which activities observed for our compounds were modulated by TRPV1 receptors. In order to study the hypothetical participation of TRPV1 receptors in the observed food intake modulation, a TRPV1 antagonist, capsazepine,[28] at 2.5 mg kg^{-1}, was co-administered with the most active compounds (**5**, **6**, and **8**) at 5 mg kg^{-1} doses. Capsazepine and fatty acid amides, either separately or in combination, were tested on groups of animals. Capsazepine was unable to significantly modify the effects on food intake elicited by the tested compounds (see Supporting Information), which likely indicates that TRPV1 receptors do not contribute to the observed biological effects.

Conclusions

The newly synthesized compounds presented herein have potential as food intake inhibitors, as all compounds effected a decrease in food intake to some extent. A number of these displayed affinity for the CB_1 receptor, similar to the endocannabinoid anandamide, and some exhibited activity against PPAR-α. N-methylation and catechol O-methylation were shown to be deleterious modifications for food intake modulation. In contrast, the catechol group is essential for retaining this activity. Observed pharmacodynamics of the biologically active compounds could be explained by CB_1, PPAR-α, or a dual CB_1/PPAR-α receptor interaction. Nevertheless, further studies are necessary to elucidate the precise mechanism of action of these compounds.

Experimental Section

Chemistry

MDMA metabolites HHA, HMA and HHMA were synthesized as described.[24] All reagents and solvents were commercially available and were used without further purification unless specifically indicated. A Nicolet Avatar 360 IR spectrophotometer was employed, as well as a Sigma 3K30 ultracentrifuge. ^{1}H and ^{13}C NMR analyses

were carried out with Varian Anova 500 and Varian Mercury 400 spectrometers. Progress of all reactions was monitored by TLC on aluminum sheets pre-coated with silica gel 60 (HF-254, Merck), film thickness 0.25 mm. Elemental analyses (C, H, N) were performed on a Thermo Finnigan Elemental Analyzer Flash 1112 Series and were within ±0.3% of theoretical values. MS data were recorded using a LCT Premier (Waters, Milford, MA, USA) ESI-TOF instrument equipped with a 4 GHz time-to-digital converter (TDC).

General synthesis of fatty acid amides

In a round-bottomed flask at 4 °C under nitrogen atmosphere, oleic or arachidonic acid (4.5 mmol), CH_3CN (30 mL), Et_3N (863 μL, 6.2 mmol), and benzyl chloroformate (771 μL, 5.4 mmol) were added and stirred for 1 h. CH_3CN was removed under reduced pressure, and the dried residue was redissolved in DMF (30 mL). Et_3N (640 μL, 4.6 mmol) and phenylethylamine or the corresponding MDMA metabolite (4.9 mmol) were added to the DMF solution under a nitrogen atmosphere at 4 °C. After this addition, the cold bath was removed, and the mixture stirred for 24 h at 25 °C. The reaction was monitored by TLC until no starting materials remained. The DMF was removed under reduced pressure, and the product was purified by flash chromatography using 20% ethyl acetate/hexane. Yields are given for isolated products and were not optimized.

***N*-(1-(3,4-dihydroxyphenyl)propan-2-yl)oleamide (5).** Compound **5** was obtained as a pale-yellow oil (306 mg, 43% yield). R_f (Hex/EtOAc, 4:1) = 0.09; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.86 (t, J = 6.95 Hz, 3H), 1.11 (d, J = 6.58 Hz, 3H), 1.19–1.34 (m, 20H), 1.50–1.60 (m, 2H), 1.96–2.03 (m, 4H), 2.12 (t, J = 7.78 Hz, 2H), 2.57 (A of an ABX syst., J = 7.08, 13.65 Hz, 1H), 2.66 (B of an ABX syst., J = 6.55, 13.70 Hz, 1H), 4.16–4.28 (X of an ABX syst., m, 1H), 5.28–5.39 (m, 2H), 5.53 (d, J = 8.28 Hz, 1H), 6.51 (dd, J = 1.40, 8.00, 1H), 6.75 (s, 1H), 6.76 ppm (d, J = 8.0 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 14.34, 20.37, 22.92, 26.03, 27.41, 27.46, 29.34, 29.44, 29.56, 29.77, 29.95, 30.00, 32.14, 37.20, 42.26, 46.91, 114.97, 115.90, 121.34, 129.90, 129.96, 130.22, 143.51, 144.51, 174.11 ppm; IR (KBr): $\tilde{\nu}$ = 3363, 3266, 2920, 1638, 1555, 1465, 1032, 814 cm^{-1}; MS $[M+H]^+$ calcd for $C_{27}H_{45}NO_3$: 432.3478, found: 432.3490; Anal. calcd for $C_{27}H_{45}NO_3$: C 75.13, H 10.51, N 3.24, found: C 74.92, H 10.33, N 3.20.

***N*-(1-(4-hydroxy-3-methoxyphenyl)propan-2-yl)oleamide (6).** Compound **6** was obtained as a pale-yellow oil (258 mg, 49%) yield. R_f (Hex/EtOAc, 4:1) = 0.26; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.87 (t, J = 6.58 Hz, 3H), 1.09 (d, J = 6.61 Hz, 3H), 1.20–1.37 (m, 20H), 1.51–1.62 (m, 2H), 1.95–2.05 (m, 4H), 2.10 (t, J = 7.48, 7.48 Hz, 2H), 2.60 (A of an ABX syst., J = 7.34, 13.61 Hz, 1H), 2.76 (B of an ABX syst., J = 5.70, 13.59 Hz, 1H), 3.86 (s, 3H), 4.18–4.29 (X of an ABX syst., m, 1H), 5.24–5.41 (m, 3H), 6.63 (dd, J = 1.28, 7.95 Hz, 2H), 6.69 (d, J = 1.39 Hz, 1H), 6.82 ppm (d, J = 7.97 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 14.36, 20.24, 22.92, 26.02, 27.40, 27.45, 29.36, 29.45, 29.51, 29.55, 29.75, 29.95, 29.99, 32.13, 37.25, 42.44, 46.25, 56.12, 111.87, 114.32, 122.34, 129.97, 130.04, 130.21, 144.45, 146.71, 172.72 ppm; IR (KBr): $\tilde{\nu}$ = 3418, 3293, 2922, 1639, 1556, 1465, 1030, 844 cm^{-1}; MS $[M+H]^+$ calcd for $C_{28}H_{47}NO_3$: 446.3634, found: 446.3545; Anal. calcd for $C_{28}H_{47}NO_3$: C 75.46, H 10.63, N 3.14, found: C 75.28, H 10.65, N 2.89.

***N*-(1-(3,4-dihydroxyphenyl)propan-2-yl)-*N*-methyloleamide (7).** Compound **7** was obtained as a pale-yellow oil (142 mg, 45% yield), and was a mixture of *cis* and *trans* isomers. R_f (Hex/EtOAc, 4:1) = 0.29; ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 0.85 (t, J = 6.87 Hz, 3H), 0.95 (*trans*, d, J = 6.80 Hz, 3H), 1.12 (*cis*, d, J = 6.50 Hz, 3H), 1.19–1.42 (m, 20H), 1.36–1.42 (*trans*, m, 2H), 1.80–1.87 (*cis*, m, 2H) 1.94–2.00 (m, 4H), 2.06–2.09 (*cis*, m, 2H), 2.11–2.19 (*trans*, m, 2H), 2.46–2.55 (m, 2H), 2.66 (*cis*, s, 3H), 2.74 (*trans*, s, 3H), 3.95–4.06 (*cis*, m, 1H), 4.64–4.75 (*trans*, s, 1H), 5.28–5.36 (m, 2H), 6.36–6.41 (m, 1H), 6.52–6.54 (m, 1H), 6.56–6.70 ppm (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 14.36, 14.41, 15.57, 19.50, 21.29, 22.92, 25.34, 25.52, 27.12, 27.45, 29.36, 29.40, 29.47, 29.55, 29.76, 29.96, 30.01, 32.14, 33.24, 34.32, 39.68, 40.00, 55.42, 60.69, 114.94, 115.62, 115.98, 120.66, 120.80, 130.01, 130.16, 143.43, 143.66, 143.65, 144.39, 144.76, 171.53, 174.76, 175.21 ppm; IR (KBr): $\tilde{\nu}$ = 3354, 3227, 2926, 1597, 1514, 1454, 1283, 815 cm^{-1}; MS $[M+H]^+$ calcd for $C_{28}H_{47}NO_3$: 446.3634, found: 446.3537; Anal. calcd for $C_{28}H_{47}NO_3$: C 75.46, H 10.63, N 3.14, found: C 75.25, H 10.48, N 3.07.

***N*-(1-(3,4-dihydroxyphenyl)propan-2-yl)arachidonamide (8).** Compound **8** was obtained as a pale-yellow oil (82 mg, 39% yield). R_f (Hex/EtOAc, 4:1) = 0.14; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.90 (t, J = 6.93 Hz, 3H), 1.13 (d, J = 6.62 Hz, 3H), 1.24–1.42 (m, 6H), 1.59–1.74 (m, 2H), 2.03–2.11 (m, 4H), 2.17 (t, 7.73 Hz, 2H), 2.59 (A of an ABX syst., J = 7.26, 13.69 Hz, 1H), 2.70 (B of an ABX syst., J = 6.48, 13.68 Hz, 1H), 2.77–2.87 (m, 6H), 4.19–4.29 (X of an ABX syst., m, 1H), 5.30–5.46 (m, 8H), 5.53 (d, J = 8.38 Hz, 1H), 6.54 (dd, J = 1.90, 8.02 Hz, 1H), 6.78 (s, 1H), 6.78 ppm (d, J = 10.89, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 14.30, 20.34, 22.80, 25.81, 25.85, 26.75, 27.44, 29.54, 31.74, 36.49, 42.26, 47.03, 115.04, 115.93, 121.39, 127.73, 128.05, 128.31, 128.52, 128.85, 129.04, 129.17, 129.94, 130.76, 143.47, 144.48, 173.82 ppm; IR (KBr): $\tilde{\nu}$ = 3381, 2926, 1651, 1516, 1448, 1281 960, 813 cm^{-1}; MS $[M+H]^+$ calcd for $C_{29}H_{43}NO_3$: 454.3321, found: 454.3221; Anal. calcd for $C_{29}H_{43}NO_3$: C 76.78, H 9.55, N 3.09, found: C 76.59, H 9.78, N 3.21.

***N*-(1-(4-hydroxy-3-methoxyphenyl)propan-2-yl)arachidonamide (9).** Compound **9** was obtained as a pale-yellow oil (114 mg, 40% yield). R_f (Hex/EtOAc, 4:1) = 0.40; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.87 (t, J = 6.60 Hz, 3H), 1.09 (d, J = 6.63 Hz, 3H), 1.21–1.40 (m, 6H), 1.61–1.76 (m, 2H), 1.99–2.15 (m, 6H), 2.59 (A of an ABX syst., J = 7.48, 13.59 Hz, 1H) 2.73–2.86 (m, 7H, B of an ABX syst. + 6H), 3.85 (s, 3H), 4.15–4.27 (X of an ABX syst., m, 1H), 5.27–5.43 (m, 9H), 6.62 (dd, J = 1.50, 7.95 Hz, 1H), 6.68 (d, J = 1.40 Hz, 1H), 6.81 ppm (d, J = 7.98 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 14.32, 20.18, 22.81, 25.80, 25.85, 26.86, 27.44, 29.55, 31.74, 36.52, 42.44, 46.33, 56.11, 111.89, 114.36, 122.33, 127.72, 128.06, 128.37, 128.44, 128.81, 128.95, 129.31, 130.01, 130.74, 144.47, 146.73, 172.45 ppm; IR (KBr): $\tilde{\nu}$ = 3389, 3264, 2924, 1638, 1553, 1273, 1033, 815 cm^{-1}; MS $[M+H]^+$ calcd for $C_{30}H_{45}NO_3$: 468.3478, found: 468.3459; Anal. calcd for $C_{30}H_{45}NO_3$: C 77.04, H 9.70, N 2.99, found: C 76.89, H 9.78, N 3.11.

***N*-(1-(3,4-dihydroxyphenyl)propan-2-yl)-*N*-methylarachidonamide (10).** Compound **10** was obtained as a pale-yellow oil (137 mg, 42% yield) and was a mixture of *cis* and *trans* isomers. R_f (Hex/EtOAc, 4:1) = 0.43; ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 0.87 (t, J = 6.60 Hz, 3H), 0.95 (*trans*, d, J = 7.00 Hz, 3H), 1.11 (*cis*, d, J = 6.5 Hz, 3H) 1.20–1.34 (m, 6H), 1.43–1.50 (*trans*, m, 2H), 1.83–1.94 (*cis*, m, 2H), 1.98–2.03 (m, 4H), 2.08–2.37 (m, 4H), 2.65 (*cis*, s, 3H), 2.73 (*trans*, s, 3H), 2.74–2.82 (m, 6H), 3.95–4.01 (*cis*, m, 1H), 4.65–4.72 (*trans*, m, 1H), 5.25–5.38 (m, 8H), 6.36–6.40 (m, 1H), 6.52–6.54 (m, 1H), 6.57–6.61 ppm (m, 1H); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 17.57, 19.53, 22.81, 25.06, 25.20, 25.85, 26.83, 26.87, 27.13, 27.45, 29.35, 29.56, 31.74, 32.56, 33.65, 39.68, 39.97, 49.95, 55.37, 60.70, 114.97, 115.57, 115.99, 120.69, 120.83, 127.74, 127.77, 128.07, 128.13, 128.34, 128.39, 128.42, 128.52, 128.81, 129.81, 129.00, 129.32, 129.35, 130.05, 130.08, 130.73, 143.42, 143.67, 144.38, 144.67, 144.74, 174.42, 174.92 ppm; IR (KBr): $\tilde{\nu}$ = 3534, 3272, 2928, 1603, 1518, 1447, 1282, 1117, 712 cm^{-1}; MS $[M+H]^+$ calcd for

$C_{30}H_{45}NO_3$: 468.3478; found 468.3469; Anal. calcd for $C_{30}H_{45}NO_3$: C 77.04, H 9.70, N 2.99, found: C 76.88, H 9.75, N 2.72.

***N*-phenethyloleamide (11).** Compound **11** was obtained as a white solid (105 mg, 95% yield). R_f (Hex/EtOAc, 4:1) = 0.31; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.87 (t, J = 6.93 Hz, 3H), 1.21–1.37 (m, 20H), 1.53–1.63 (m, 2H), 1.96–2.05 (m, 4H), 2.11 (t, J = 7.60 Hz, 2H), 2.81 (t, J = 6.94 Hz, 2H), 3.49–3.53 (m, 2H), 5.30–5.37 (m, 2H), 5.53 (br s, 1H), 7.18–7.33 ppm (m, 5H).); ^{13}C NMR (101 MHz, $CDCl_3$): δ = 14.38, 22.93, 25.99, 27.41, 27.46, 29.38, 29.48, 29.51, 29.56, 29.77, 29.95, 30.01, 32.14, 35.93, 37.05, 40.75, 126.72, 128.84, 128.99, 129.97, 130.22, 139.15, 173.47 ppm; IR (KBr): $\tilde{\nu}$ = 3309, 2920, 1640, 1559, 1446. 1085, 698 cm^{-1}; MS $[M+H]^+$ calcd for $C_{26}H_{43}NO$: 386.3423; found 386.3347; Anal. calcd for $C_{26}H_{43}NO$: C 80.98, H 11.24, N 3.63, found: C 80.75, H 11.31, N 3.58.

In vivo experiments

All in vivo experiments were carried out using Wistar male rats within a 200–450 g weight range. Animals were housed in individual cages in a room with controlled temperature (23 °C) and relative humidity (50%), as well as a 12 h/12 h light and dark cycle. Water and food were available ad libitum except in specific experimental proceedings. The animals were handled twice in the days prior to the experimental sessions. Compounds were dissolved in a mixture of DMSO/Tween-60/saline (5:5:90 *v/v/v*) and administered intraperitoneally. The experiments performed in this study are in compliance with the Spanish Real Decreto 1201/2005, October 21, 2005 (BOE number 252) concerning the protection of experimental animals, as well as with the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Feeding experiments

Feeding experiments were carried out using animals that had been deprived of food for 24 h but permitted free access to water. Thirty minutes post-injection, a weighed amount of food was placed in the cage. The food was weighed again at 30, 60, 120, and 240 min from test initiation. All feeding experiments were performed with groups of eight animals (n = 8). In capsazepine experiments, capsazepine was injected five minutes prior to the compound being tested.

Open field test

This test measures the natural conflict of the animal between the tendency to explore and the avoidance reaction to protect itself. All rats were handled for a period of 15 min over two days to minimize injection stress. They were acclimated to the testing room for 30 min before commencing behavioral testing. The animal was injected with the compound and placed on the center of a 40 cm × 40 cm table with 30 cm walls. The total displacement and the time spent by the animal on the corners or in the center of the table were registered using a video tracking system (Smart® Panlab, Barcelona, Spain).

In vitro experiments

Rat cerebellum and cortex membranes homogenization

The rats were anesthetized with Dolethal® (pentobarbital) and sacrificed by decapitation. The cortex and cerebellum were extracted and stored separately in dry ice. The cerebellum and cortex were homogenized with 50 mM Tris buffer (pH 7.4) using an Ultra-Turrax®. The homogenate was centrifuged at 25000 g for 15 min at 4 °C. The supernatant was discarded and the pellet was washed again with 5 mL of buffer, then centrifuged. The supernatant was again discarded, and the protein concentration of the pellet was measured using the Bradford test.

Binding assay

The binding assay was performed using the labeled CB_1 antagonist [^{3}H]SR141716; 450 μL buffer A (50 mM Tris, pH 7.4, with 0.5% bovine serum albumin (BSA)), 100–200 μg of rat brain membranes (cerebellum), the diluted compound, and tracer [^{3}H]SR141716 were added to each tube. The mixture was incubated while shaking at 37 °C for 60 min, and the reaction was stopped with 1 mL buffer A. The resulting mixture was centrifuged at 5000 rpm for 5 min. The supernatant was discarded, then the pellet was washed with 1 mL buffer A and centrifuged, and the supernatant was again discarded. Scintillation liquid was added, and the samples were read using a beta scintillator (Liquid scintillation analyzer, Tri-Carb 2100 TR, PACKARD, Packard Biosciences). All compounds were diluted in buffer B (50 mM Tris, pH 7.4, with 0.5% bovine serum albumin (BSA) and 0.3% DMSO) in 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} mol concentrations. All experiments were performed in triplicate.

PPAR-α affinity assay

Biology: The compounds reported in this study were first evaluated using an in vivo reporter gene assay in order to assess which compounds could induce interaction between PPAR-α and co-activator SRC-1 in MCF-7 cells.

Drugs: Oleylethanolamide (OEA), GW7647, anandamide (AEA) and oleic acid were purchased from Tocris Biosciences (Cookson Ltd. Bristol, UK). For in vitro cell culture experiments, all compounds were dissolved and diluted in DMSO (Sigma–Aldrich, Spain).

DNA constructs: Full-length cDNA for human PPAR-α, human RXRα, and human SRC-1 were subcloned into the T7/SV40 promoter-driven pSG5 expression vector (Qiagen). These constructs were used for viral promoter-driven overexpression of their respective proteins in mammalian cells.

Reporter gene constructs: Four copies of the human CPTI gene DR1-type RE (core sequence GTA GGG AAA AGG TCA) were individually fused with the thymidine kinase (tk) minimal promoter controlling the firefly luciferase reporter gene.

Transient transfection and luciferase reporter assays[29–31]

MCF-7 human breast cancer cells were seeded into 6-well plates (200000 cells per well) and grown overnight in phenol red-free DMEM, supplemented with 5% charcoal-stripped fetal bovine serum. Liposomes containing plasmid DNA were formed by incubating 1 μg of an expression vector for wild-type PPAR-α, RXRα, or SRC-1 and 1 μg of reporter plasmid with 10 μg of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP, Roche) in a total volume of 100 μL for 15 min at room temperature. After dilution with 900 μL phenol red-free DMEM, the liposomes were added to the cells. Phenol red-free DMEM, supplemented with 500 μL 15% charcoal-stripped fetal bovine serum was added 4 h after transfection. At this time, cells were treated for 16 h with solvent (DMSO) and various concentrations (10^{-9}, 10^{-8},

10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) of OEA, GW7647, AEA, and oleic acid, as well as the various compounds under evaluation as previously indicated. The cells were lysed 16 h after onset of stimulation using reporter gene lysis buffer (Roche). Both the transient transfection assay and the constant light signal luciferase reporter gene assay were performed as recommended by the suppliers (Roche and Roche Diagnostics, respectively). Stimulation of normalized luciferase activity was calculated in comparison with solvent-induced cells that did not overexpress protein. Data were used to calculate the EC_{50} value for each compound.

FAAH inhibition assay

440 μL of buffer A (50 mM Tris, pH 7.4, with 0.5% bovine serum albumin (BSA)), 100–200 μg of rat brain membranes (cortex), the tested compounds (at 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} and 10^{-11} M), 0.025 μCi [^{3}H]anandamide, and 10 μmol anandamide were added to each tube. After 60 min at 37 °C incubation with shaking, chloroform was added (1 mL). The tubes were shaken and afterward centrifuged at 3000 rpm for 5 min; 0.25 mL of the organic phase of each tube was transferred to a vial with scintillation liquid and analyzed in a beta scintillator (Liquid scintillation analyzer, Tri-Carb 2100 TR, Packard Biosciences). All the products were diluted in buffer B (50 mM Tris pH 7.4 with 0.5% bovine serum albumin (BSA) and 0.3% DMSO). All data points were performed in triplicate.

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