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Pharmacological Characterization of Hydrolysis-Resistant Analogs of Oleoylethanolamide with Potent Anorexiant Properties

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ABSTRACT

Oleoylethanolamide (OEA) is an endogenous lipid mediator that reduces food intake, promotes lipolysis, and decreases body weight gain in rodents by activating peroxisome proliferator-activated receptor-α (PPAR-α). The biological effects of OEA are terminated by two intracellular lipid hydrolase enzymes, fatty-acid amide hydrolase and *N*-acylethanolamine-hydrolyzing acid amidase. In the present study, we describe OEA analogs that resist enzymatic hydrolysis, activate PPAR-α with high potency in vitro, and persistently reduce feeding when administered in vivo either parenterally or orally. The most potent of these compounds, (*Z*)-(*R*)-9-octadecenamide,*N*-(2-hydroxyethyl,1-methyl) (KDS-5104), stimulates transcriptional activity of PPAR-α with a half-maximal effective concentration (EC_{50}) of 100 $\pm$ 21 nM (n = 11). Parenteral administration of KDS-5104 in rats produces persistent dose-dependent prolongation of feeding latency and postmeal interval (half-maximal effective dose, ED_{50} = 2.4 $\pm$ 1.8 mg kg^{-1} i.p.; n = 18), as well as increased and protracted tissue exposure compared with OEA. Oral administration of the compound also results in a significant tissue exposure and reduction of food intake in free-feeding rats. These results suggest that the endogenous high-affinity PPAR-α agonist OEA may provide a scaffold for the discovery of novel orally active PPAR-α ligands.

Oleoylethanolamide (OEA) is a naturally occurring lipid amide (Bachur et al., 1965) that may contribute in important ways to the regulation of feeding and energy balance (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Guzman et al., 2004; Lo Verme et al., 2005b). In the rat proximal small intestine, endogenous OEA levels decrease during fasting and increase upon refeeding, probably as a result of local alterations in OEA biosynthesis and/or hydrolysis (Rodríguez de Fonseca et al., 2001). These periprandial fluctuations in OEA mobilization may represent a previously undescribed signal that modulates between-meal satiety. Pharmacological studies have shown, indeed, that exogenous OEA produces profound anorexiant effects in rats and mice (Rodríguez de Fonseca et al., 2001; Nielsen et al., 2004; Proulx et al., 2005), which are due to selective prolongation of feeding latency and postmeal interval (PMI) (Gaetani et al., 2003; Oveisi et al., 2004). These effects are markedly different from and possibly complementary to those of other satiety signals, such as cholecystokinin and leptin, which inhibit feeding by accelerating meal termination (Woods et al., 2004; Broberger, 2005). The behavioral selectivity of OEA is further underscored by the fact that this lipid amide does not induce

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ABBREVIATIONS: OEA, oleoylethanolamide; PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; GW7647, propanoic acid, 2-[[4-[2-[[(cyclohexylamino)carbonyl](4-cyclohexylbutyl)amino]ethyl]phenyl]thio]-2-methyl; Wy-14643, acetic acid, [[4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]; MD, molecular dynamics; AUC, area under the curve; FAAH, fatty-acid amide hydrolase; URB597, carbamic acid, cyclohexyl-, 3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl ester; EtOAc, ethyl acetate; KDS-5104, (*Z*)-(*R*)-9-octadecenamide,*N*-(2-hydroxyethyl,1-methyl); LC, liquid chromatography; MS, mass spectrometry; EI, electron ionization; GW409544, L-phenylalanine, *N*-[(1*Z*)-1-methyl-3-oxo-3-phenyl-1-propenyl]-4-[3-(5-methyl-2-phenyl-4-oxazolyl)propyl]; PMI, postmeal interval; IR, infrared.

visceral malaise, anxiety-like behaviors, or stress hormone release (Rodríguez de Fonseca et al., 2001; Proulx et al., 2005).

The nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α) is a key regulator of lipid metabolism and energy balance in mammals (Desvergne and Wahli, 1999). Although OEA may bind to multiple receptors (Wang et al., 2005; Overton et al., 2006), three distinct lines of evidence indicate that PPAR-α mediates the satiety-inducing effects of this compound. First, OEA binds with high affinity to the purified ligand-binding domain (LBD) of mouse and human PPAR-α (K_D 37 and 40 nM, respectively) and activates with high-potency PPAR-α-driven transactivation in a heterologous expression system (EC_{50} = 120 nM) (Fu et al., 2003). Second, two structurally different synthetic PPAR-α agonists, the compounds GW7647 and Wy-14643, exert anorexiant effects that are due to a selective increase in feeding latency and thus are behaviorally identical to those produced by OEA (Fu et al., 2003). Third, mutant mice in which the PPAR-α gene has been deleted by homologous recombination (PPAR-$\alpha^{-/-}$ mice) do not respond to OEA or synthetic PPAR-α agonists, although they retain normal responses to two mechanistically different anorexiants, cholecystokinin-octapeptide and fenfluramine (Fu et al., 2003). Together, the findings outlined above suggest that endogenous OEA, produced in the proximal small intestine during feeding, regulates between-meal satiety by activating PPAR-α.

The biological deactivation of OEA is not fully understood but is likely to involve the enzymatic hydrolysis of this lipid amide to oleic acid and ethanolamine (Lo Verme et al., 2005b). Two structurally distinct OEA-hydrolyzing enzymes have been characterized: fatty-acid amide hydrolase (FAAH), an intracellular membrane-bound serine hydrolase (Désarnaud et al., 1995; Hillard et al., 1995; Ueda et al., 1995; Cravatt et al., 1996), and *N*-acylethanolamine-hydrolyzing acid amidase, a lysosomal cysteine hydrolase (Tsuboi et al., 2005; Ueda et al., 2005). In the present study, we set out to develop analogs of OEA that resist enzymatic hydrolysis while retaining agonist activity at PPAR-α. Such agents may not only be useful to characterize the pharmacological properties of OEA in live animals but might also provide a starting point for the development of novel antiobesity, antihyperlipidemic, and anti-inflammatory drugs (Willson et al., 2000).

Materials and Methods

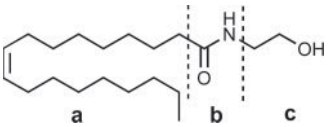
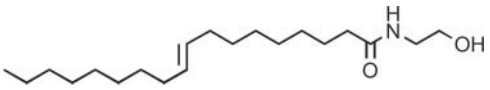
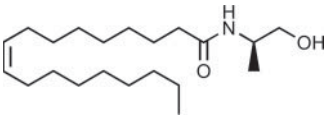
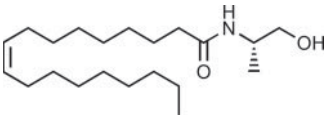
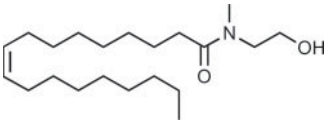
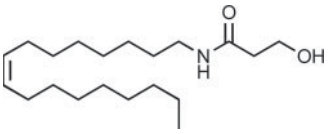
Animals. Adult male Wistar rats (250–300 g, 7 weeks old) and C57/BL6 mice (20–25 g, 8 weeks old) were housed in standard Plexiglas cages at room temperature. A 12-h light/dark cycle was set with the lights on at 4:45 AM. Water and standard chow pellets (Prolab RMH 2500; PMI Nutrition International, Brentwood, MO) were available ad libitum. All procedures met the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Drugs and Solvents. URB597 was synthesized as described previously (Mor et al., 2004). Solvents were obtained from Burdick and Jackson (Muskegon, MI), and all other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO).

Chemical Syntheses. The compounds prepared and tested in the present study are illustrated in Table 1. (*Z*)-9-Octadecenamide, *N*-(2-hydroxyethyl) (OEA, **1**) and (*E*)-9-octadecenamide,*N*-(2-hydroxylethyl) (**2**) were synthesized by the reaction of the respective fatty-acid chloride (Nu-Chek Prep, Elysian, MN) with a 10-fold excess of ethanolamine (Sigma-Aldrich Co.) in dichloromethane. The reaction was conducted at 0–4°C under stirring for 15 min, and the products were washed with water, dehydrated over sodium sulfate, filtered, and dried under reduced pressure. The crude product was purified by flash-chromatography on silica gel (EtOAc) to yield white solids (96% for **1** and 95% for **2**). (*Z*)-(*R*)-9-Octadecenamide,*N*-(2-hydroxyethyl,1-methyl) (**3**, KDS-5104), (*Z*)-(*S*)-9-octadecenamide,*N*-(2-hydroxyethyl, 1-methyl (**4**), and (*Z*)-9-octadecenamide,*N*-(2-hydroxyethyl),*N*-methyl (**5**) were synthesized by the stoichiometric reaction of oleoyl chloride with the respective amine: (*R*)-(−)-2-aminopropanol, (*S*)-(+)-2-aminopropanol, or *N*-methyl-*N*-ethanolamine (Sigma-Aldrich Co.) in the presence of triethylamine (Sigma-Aldrich Co.) (Lin et al., 1998). The reaction was conducted in dichloromethane at 0–4°C under stirring for 12 h. The solvent was removed under vacuum, and the mixture was dissolved in tetrahydrofuran/ethanol (1:1) and treated with an aqueous solution of 3 N KOH (2 Eq). The solution was kept at reflux for 30 min, after which the solvent was removed. The residue was dissolved in ethyl acetate and washed sequentially with water, sodium hydroxide (2 N), hydrochloric acid (2 N), and brine. The organic phase was dried over sodium sulfate, filtered, and concentrated under reduced pressure. Crude products were purified by flash-chromatography on silica gel (EtOAc) to yield 96% of white solid products (**3** and **4**) and 97% of an oily product (**5**). (*Z*)-3-Hydroxypropionamide,*N*-(8-heptadecenyl) (**6**) was synthesized in two steps. The first step consisted in the synthesis of (*Z*)-8-heptadecenylamine; oleoyl chloride was reacted with an aqueous solution of sodium azide in dichloromethane in phase-transfer conditions using tetrabutylammonium bromide as surfactant. The resulting acyl azide was converted to isocyanate through Curtius rearrangement in refluxing toluene (Pfister and Wymann, 1983). Subsequent treatment with 1 N NaOH in tetrahydrofuran at reflux afforded (*Z*)-8-heptadecenylamine. Reaction of this product with trimethylaluminium and β-propiolactone in dichloromethane (Lin et al., 1998) yielded (*Z*)-3-hydroxypropionamide,*N*-(8-heptadecenyl) (**6**). The reaction mixture was refluxed for 6 h, quenched with 50 ml of 1 N HCl, and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The product was purified by flash-chromatography on silica gel (EtOAc) to yield a solid (33%), with the following chemical-physical properties. The purity of all compounds was >98% by liquid chromatography/mass spectrometry (LC/MS). The syntheses of compounds **4** and **6** have never been reported before.

Chemical Analyses. Melting points were determined on a Buchi SMP-510 capillary melting point apparatus (Flawil, Switzerland) and were uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance 200 MHz spectrometer (Bruker, Newark, DE); chemical shifts are reported in parts per million relative to the central peak of the solvent. Coupling constants (*J* values) are given in Hertz. EI-MS spectra (70 eV) were taken on a Fisons Trio 1000 spectrometer (Fisons Instruments, Manchester, UK). Infrared spectra were obtained on a Nicolet Avatar 360 Fourier-Transfor-IR spectrometer (ThermoNicolet, Madison, WI); absorbance is reported in centimeter^{-1}. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA). Elemental analyses for C, H, and N were performed on a Carlo Erba analyzer and were within ±0.4% of theoretical values. Compound **1** presented the following chemical-physical properties: MS (EI) *m/z* 325 (M+); ^{1}H NMR ($CDCl_3$) δ: 5.84 (br s, 1H), 5.31 (m, 2H), 3.57 (m, 2H), 3.27 (m, 2H), 2.51 (br s, 1H), 2.17 (t, 2H), 2.01 (m, 4H), 1.59 (m, 2H), 1.31 (br d, 20H), 0.88 (t, 3H). Anal.($C_{20}H_{39}NO_2$) C, H, N. Compound **2**: M.p.: 82–84°C; MS (EI) *m/z* 325 (M+); IR (*film* cm^{-1}): 3398, 3299, 2919, 2849, 1640; ^{1}H NMR ($CDCl_3$) δ: 5.86 (br s, 1H), 5.38 (m, 2H) 3.73 (m, 2H), 3.42 (m, 2H), 2,20 (t, 2H), 1.95 (m, 4H), 1.82 (br s, 1H), 1.63 (m, 2H), 1.27 (br d, 20H), 0.88 (t, 3H). Anal.($C_{20}H_{39}NO_2$) C, H, N. Compound **3:** M.p.: 39–40°C; [a]$^{25}_D$ = +8.5; MS (EI) *m/z* 339 (M+); IR (*film* cm^{-1}): 3390, 3300, 2922, 2851, 1643; ^{1}H NMR ($CDCl_3$) δ: 5.87 (br d, 1H), 5.33 (m, 2H), 4.40 (br s,

TABLE 1
Chemical structures and pharmacological properties of various OEA analogs

Compound	Structure	PPAR-α Activation EC_{50}[a]	Inhibition of Feeding ED_{50}[b]
		nM	*mg kg^{-1} i.p.*
1		120 ± 16	9.2 ± 1.6
2		318 ± 65	9.2 ± 1.4
3 (KDS-5104)		100 ± 21	2.4 ± 1.8
4		333 ± 27	16.3 ± 2.5
5		>10,000	>20
6		>10,000	>20

[a] EC_{50} values (±S.E.M., n = 5–12) were assessed in gene activation assays.
[b] ED_{50} values (±S.E.M., n = 6–18) were assessed in feeding assay (using latency of feeding onset as variable).

1H), 4,06 (m, 1H), 3.67 (dd, J = 11.06 e 3.56 Hz, 1H), 3.52 (dd, J = 11.06 e 6.21 Hz, 1H), 2.19 (t, 2H), 1.99 (m, 4H), 1.62 (m, 2H), 1.27 (2br s, 20H), 1.17 (d, J = 6,76, 3H), 0.88 (t, 3H). Anal. ($C_{21}H_{41}NO_2$) C, H, N. Compound **4**: M.p.: 39–40°C; [a]^{25}d = −7.2; MS (EI) *m/z* 339 (M+); IR (*film* cm^{-1}): 3394, 3300, 2922, 2851, 1641; ^{1}H NMR ($CDCl_3$) δ: 5.80 (br d, 1H), 5.33 (m, 2H), 4.06 (m, 1H), 3.67 (dd, J = 11.06 e 3.56 Hz, 1H), 3.52 (dd, J = 11.06 e 6.21 Hz, 1H), 2.99 (br s, 1H), 2.19 (t, 2H), 1.99 (m, 4H), 1.62 (m, 2H), 1.27 (2br s, 20H), 1.17 (d, J = 6,76, 3H), 0.88 (t, 3H). Anal.($C_{21}H_{41}NO_2$) C, H, N. Compound **5**: MS (EI) *m/z* 339 (M+); IR (*film* cm^{-1}): 3388, 2924, 2854, 1626; ^{1}H NMR ($CDCl_3$) δ: 5.34 (m, 2H), 3.78 (t, 2H), 3.55 (br t, 2H), 3.37 (br s, 1H), 3.07 (bs, 3H) 2.35 (t, 2H), 2.03 (m, 4H), 1.63 (m, 2H), 1.29 (2br s, 20H), 0.88 (t, 3H). Anal.($C_{21}H_{41}NO_2$) C, H, N. Compound **6**: M.p.: 44–45°C, MS (EI) *m/z* 325 (M+); IR (*film* cm^{-1}): 3321, 3000, 2920, 2849, 1636; ^{1}H NMR ($CDCl_3$) δ: 5.85 (br m, 1H,), 5.35 (m, 2H), 3.88 (br t, 2H), 3.25 (br q, 2H) 2.81 (br m, 1H), 2,43 (br t, 2H), 2.02 (m, 4H), 1.50 (m, 2H), 1.28 (2br d, 20H), 0.89 (t, 3H). Anal.($C_{20}H_{39}NO_2$) C, H, N.

In Vitro Enzymatic Hydrolysis. We incubated test compounds (10 nmol) in 0.1 ml of Tris buffer, pH 8.2, containing mouse liver homogenate (0.7 mg of protein) and 0.05% bovine serum albumin (fatty acid-free; Sigma-Aldrich Co.) for 0 to 60 min at 37°C. Samples were subjected to protein precipitation with 0.25 ml of acetonitrile containing [$^2H_4$]OEA as an internal standard. We prepared [$^2H_4$]OEA by the reaction of oleoyl chloride with a 10-fold molar excess of [$^2H_4$]ethanolamine (Cambridge Isotope Laboratories, Andover, MA). The reaction was conducted in dichloromethane at 0–4°C for 15 min, with stirring. The product was washed with water, dehydrated over sodium sulfate, filtered, and dried under N_2. The samples were mixed and centrifuged, and the supernatants were transferred to a 96-well plate for LC tandem MS analyses. LC was carried out on a Waters 2790 Alliance system (Waters, Milford, MA). Separation was performed on a Synergi Polar-RP column (2 × 150 mm, 4 μ; Phenomenex, Torrance, CA) using an isocratic mobile phase of acetonitrile/water/formic acid (80:20:0.1 v/v) at a flow rate of 0.3 ml min^{-1} and a column temperature of 45°C. The LC system was interfaced with a Micromass Quattro Ultima tandem MS (Beverly, MA). The samples were analyzed using an electrospray probe in the positive ionization mode with cone voltage set at 40 V and capillary voltage at 3.2 kV. The source and desolvation temperatures were 130 and 500°C, respectively. The voltage of the collision-induced dissociation chamber was −20 eV. Multiple reaction monitoring was used for the detection of **1** (OEA) and **2** (*m/z* 326 > 62), **3** (KDS-5104), **4** and **5** (*m/z* 340 > 76), **6** (*m/z* 326 > 254), and [$^2H_4$]OEA (internal standard) (*m/z* 330 > 66).

In Vitro PPAR Activation. We used HeLa cells stably transfected with a luciferase reporter plasmid containing a hygromycin resistance gene. These cells were cotransfected with a plasmid containing the LBD of PPAR-α or PPAR-γ fused to the DNA-binding domain of the yeast regulatory protein GAL4 and a neomycin resistant gene under the control of human cytomegalovirus promoter (Fu et al., 2003). The cells were cultured in Dulbecco's modified Eagle's medium and supplemented with fetal bovine serum (10%), hygromycin, and G418. For transactivation assays, we seeded cells in six-well plates and incubated them for 7 h in Dulbecco's modified Eagle's medium containing an appropriate concentration of test compounds.

We used a luciferase reporter assay system (Promega, Madison, WI) and a MIX Microtiter plate luminometer (Dynex, Chantilly, VA) to determine luciferase activity in the cell lysates.

Pharmacokinetic Studies. For parenteral administration, we dissolved OEA and KDS-5104 in dimethyl sulfoxide/saline (70:30, v/v) and administered them (10 mg kg^{-1} i.p.) to free-feeding rats. After administrations, we anesthetized rats with halothane and collected tissues at various time points. Tissue samples were subjected to lipid extraction as reported previously (Giuffrida et al., 2000), and [$^2H_4$]OEA was added as an internal standard. OEA and KDS-5104 were separated on an 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Palo Alto, CA) equipped with an electrospray ionization interface. We used a XDB Eclipse C_{18} column (50 × 4.6 mm i.d., 1.8 μm; Zorbax; Agilent Technologies) eluted with a rapid gradient of methanol in water (from 85 to 90% methanol in 2.5 min) at a flow rate of 1.5 ml/min. Column temperature was kept at 40°C. MS detection was in the positive ionization mode, capillary voltage was set at 3 kV, and fragmentor voltage was varied from 120 to 140 V. N_2 was used as drying gas at a flow rate of 13 l/min and a temperature of 350°C. Nebulizer pressure was set at 60 p.s.i. We quantified the test compounds monitoring sodium adducts of the molecular ions $[M+Na]^+$ in the selected ion-monitoring mode. For oral administration, we suspended the drugs in water/carboxymethyl cellulose/Tween 80 (99.1:0.5:0.4, v/v) and administered them (100 mg kg^{-1}, gavage) to free-feeding rats. Rats were then anesthetized with halothane at various time points to collect tissues. Lipids were extracted and analyzed by LC/MS/MS as described under *In Vitro Enzymatic Hydrolysis.*

Behavioral Studies. Food intake and motor activity were recorded using a fully automated system (Technical and Scientific Equipment, Bad Homburg, Germany). The feeding station consists of a food container fixed to a sensor. The amount of food consumed was monitored every second, and the threshold for an eating episode was set at 0.5 g and >1 min. The motility system consists of 2 × 6 infrared light-barriers per cage disposed at right angles on the *x–y* axes to determine the animal's center of gravity and its displacement over time. Rats were habituated to test cages for 3 days prior to trials. We administered drugs parenterally (5, 10, and 20 mg kg^{-1} i.p.) in a vehicle of dimethyl sulfoxide/sterile saline (70:30, v/v) 15 min before food presentation or orally (100 mg kg^{-1}) in a vehicle of water/carboxymethyl cellulose/Tween 80 (99.1:0.5:0.4, v/v), 60 min before food presentation. Monitoring began at the onset of the dark phase (4:45 PM) and lasted 24 h. Recorded data were analyzed as food ingested per kilogram of body weight and motility counts on XY sensors. A detailed analysis of the meal patterns was performed adopting a minimal PMI of 10 min (Burton et al., 1981). The following meal parameters were analyzed (Reidelberger et al., 2001): latency of feeding onset (minutes), the time interval from trial inception to the first meal episode; first meal size (gram $kilogram^{-1}$), amount of food consumed during the first meal; first PMI (minute), the time interval between the end of the first meal and the beginning of the second meal; and second PMI, the time interval between the end of the second meal and the beginning of the third meal. ED_{50} values for feeding latency data were measured graphically.

Docking and Molecular Dynamics Simulations. Molecular modeling studies were performed with Sybyl 6.9 (Tripos Inc., St. Louis, MO) and with Autodock 3.0.5 (The Scripps Research Institute, La Jolla, CA). The three-dimensional structure of PPAR-α LBD was obtained from the Protein Data Bank [1K7L entry, chain C (Xu et al., 2001)], and was prepared for the docking experiments by deleting the cocrystallized agonist GW409544 and water molecules, adding polar hydrogens, checking tautomeric and protonation states, and calculating Kollman-united charges. The structure of KDS-5104 was built by the standard sketch options of Sybyl, and Gasteiger-Hückel charges were calculated. Docking experiments were performed within a region of space comprising the whole binding site starting from different conformations of KDS-5104; all of the rotatable bonds of the ligand were free to move, with the exception of the amide bond, which was fixed in the antiposition. A genetic algorithm-local search procedure (Morris et al., 1998) was applied, setting the maximal number of energy evaluations to 1,500,000 and the maximal number of generations to 300,000. For each docked conformation, the estimated free energy of binding was calculated by the standard Autodock scoring function. The conformations with binding energy lower than −7.00 kcal/mol were inspected to select those in which the three polar groups of KDS-5104 interacted with the protein. These conformations were inserted into the PPAR-α LBD, and the complexes were subjected to geometry optimization and molecular dynamics (MD) simulations. Energy minimization was performed with the MMFF94s force-field (Halgren, 1999) to an energy gradient of 0.05 kcal $(mol\ Å)^{-1}$. MD simulations were performed with the MMFF94 force-field (Halgren, 1996) for 300 ps at 310 Kelvins applying a time step of 1 fs. The average structure obtained from the last 200 ps of MD simulation was retrieved and submitted to geometry optimization. During energy minimization and MD simulations, only KDS-5104 and the amino acid side chains within 12 Å from the ligand were allowed to move.

Statistical Analyses. Biochemical data were analyzed by two-way ANOVA followed by Bonferroni's test as post hoc. Behavioral data were analyzed by one-way ANOVA followed by Dunnett's test. All statistical analyses were conducted using Prism software version 4.0 (GraphPad Software Inc., San Diego, CA), and differences were considered significant if $P < 0.05$.

Results

Synthesis of Hydrolysis-Resistant OEA Analogs. To develop hydrolysis-resistant analogs of OEA, we modified the chemical scaffold of this fatty-acid ethanolamide in three points (Table 1). Firstly, we replaced the oleic acid chain, which contains a *Z* double bond in the Δ^9 position, with an elaidic acid chain, in which the Δ^9 olefin has an *E* configuration (compound **2**). Secondly, we increased the steric bulk around the amide bond by introducing a methyl group either on the ethanolamine α-carbon (stereoisomers **3**-**4**) (Lin et al.,

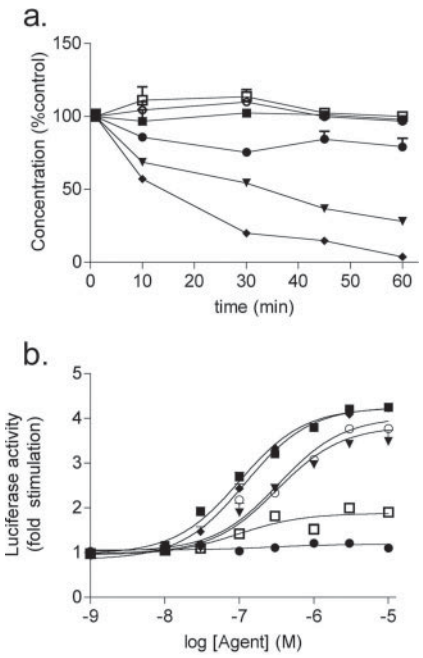


Fig. 1. a, time course of the hydrolysis of OEA and analogs **2** to **6** by mouse liver homogenates incubated at 37°C (n = 6). b, in vitro activation of the human PPAR-α LBD by OEA and its analogs (n = 5–12). Results are expressed as mean ± S.E.M. ♦, analog **1**, OEA; ▼, analog **2**; ■, analog **3**, KDS-5104; ○, analog **4**; □, analog **5**; ●, analog **6**.

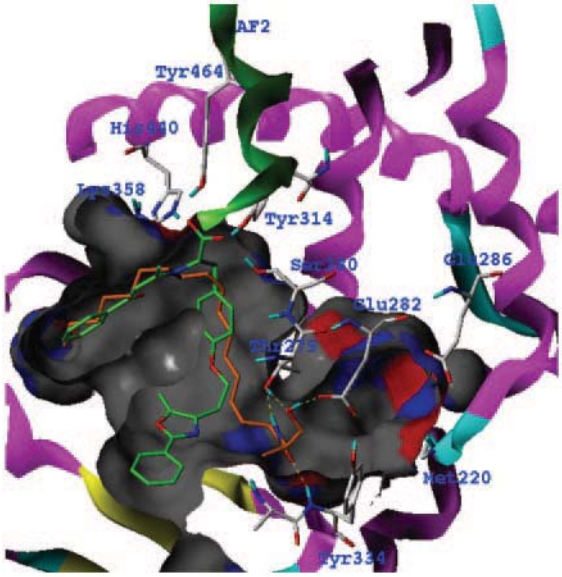


Fig. 2. Docking representation of one possible binding mode of KDS-5104 within the PPAR-α LBD. The agonist GW409544 is shown in green; The protein backbone is represented by ribbons (magenta, α-helix; yellow, β-sheet; cyan, other) with the activation function-2 domain (helix 12) in green; yellow dotted lines represent hydrogen bonds between KDS-5104 and the amino acids lining the LBD of PPAR-α.

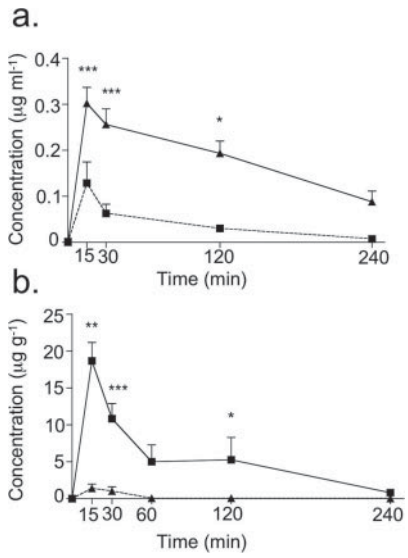


Fig. 3. Time course of OEA (dashed line) and KDS-5104 (full line) concentrations in plasma (a) and liver (b) after i.p. administration (10 mg kg^{-1}) in free-feeding rats (n = 3–4). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus OEA.

1998) or the amide nitrogen (**5**). Finally, we inverted the natural orientation of the carboxamide moiety to produce the reverse amide **6**. Chemical syntheses and analyses are described under *Materials and Methods*. We evaluated the ability of compounds **1** to **6** to resist degradation by mouse liver homogenates, which contain significant amounts of the OEA-hydrolyzing enzymes FAAH and *N*-acylethanolamine-hydrolyzing acid amidase (Désarnaud et al., 1995; Cravatt et al., 1996; Ueda et al., 1999; Tsuboi et al., 2005). As shown in Fig. 1a, incubation with liver homogenates resulted in the rapid degradation of OEA and its geometric isomer **2** but not the methyl-substituted derivatives **3** (KDS-5104), **4**, and **5**, or the reverse amide **6**. Median times to reach maximal hydrolysis ($t_{1/2}$) were 14 ± 3 min for OEA and 35 ± 2 min for compound **2** (n = 6). Incubation of liver homogenates with the highly selective FAAH inhibitor URB597 (Mor et al., 2004) (1 μM) decreased OEA hydrolysis by 85.9 ± 0.7% (n = 6), indicating that this reaction is predominantly catalyzed by FAAH.

Pharmacological Studies in Vitro. To determine whether compounds **2** to **6** activate PPAR-α, we used HeLa cells engineered to coexpress the PPAR-α LBD along with a luciferase reporter gene (Fu et al., 2003). HeLa cells are particularly well suited for this assay because they do not express significant levels of OEA-hydrolyzing activities (Fu et al., 2003). As reported previously (Fu et al., 2003), OEA activated PPAR-α with an EC_{50} value of 120 ± 16 nM (n = 12) (Fig. 1b; Table 1). KDS-5104 (**3**) was as potent as OEA (EC_{50} = 100 ± 21 nM, n = 11), whereas its stereoisomer **4** and the geometric OEA isomer **2** were approximately three times less potent than OEA (EC_{50} = 333 ± 27 and 318 ± 65 nM, respectively, n = 6) (Fig. 1b; Table 1). In contrast, the tertiary amide **5** and the reverse amide **6**, although resistant to catalytic hydrolysis (Fig. 1a), had little or no effect on PPAR-α activity at concentrations as high as 10 μM (n = 5) (Fig. 1b). Furthermore, as previously reported for OEA (Fu et al., 2003), KDS-5104 (10 μM) did not activate PPAR-γ in a HeLa cell reporter system (data not shown).

Molecular modeling studies suggest the existence of multiple potential binding modes of KDS-5104 onto the structure of the PPAR-α LBD. One such mode assigned the acyl chain of KDS-5104 to the same hydrophobic region occupied by synthetic agonists in crystals of the PPAR-α LBD (Xu et al., 2001; Cronet et al., 2001) (Fig. 2). For this solution, the hydroxyl group of KDS-5104 is close to the carboxyl group of Glu282, the amide CO interacts with the backbone NH of Tyr334, and the amide NH is hydrogen-bonded to the hydroxyl group of Thr279. Thr279 also donates a hydrogen bond to the hydroxyl group of KDS-5104 (Fig. 2). This binding mode is consistent with the small set of structure-activity relationship data obtained from OEA derivatives. In fact, it requires that both the amide NH and CO fragments and the ending OH group participate in the interaction in a concerted arrangement, which can explain the loss of activity observed for the *N*-methyl derivative **5** and the retroamide **6**. Moreover, whereas the position and interactions of compound **3** were stable during MD simulation, the less potent (*S*) enantiomer **4** could not maintain the same interactions and moved

TABLE 2
C_{max} (±S.E.M.) and AUC values for OEA and KDS-5104 (analog **3**) after parenteral administration (10 mg kg^{-1} i.p.)

	Plasma		Liver	
	C_{max}	AUC	C_{max}	AUC
	$\mu g\ ml^{-1}$	$\mu g\ min\ ml^{-1}$	$\mu g\ g^{-1}$ wet tissue	$\mu g\ min\ g^{-1}$
OEA	0.13 ± 0.05	8.95	1.44 ± 0.67	53.94
KDS-5104	0.30 ± 0.03	43.64	18.72 ± 2.51	1275.00

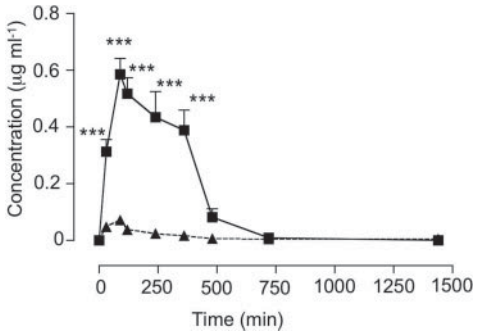


Fig. 4. Time course of OEA (dashed line) and KDS-5104 (full line) concentrations in plasma after oral administration (100 mg kg^{-1}) in free-feeding rats (n = 3–4). ***, $P < 0.001$ versus OEA.

away from the initial position (data not shown). However, in the absence of crystallization data, this binding mode must be considered tentative.

Initial Pharmacokinetic Assessment. The pharmacological effects of OEA are curtailed by a rapid clearance (Gaetani et al., 2003; Oveisi et al., 2004). To begin to characterize the pharmacokinetic properties of KDS-5104, we examined the distribution of this compound in plasma and liver following parenteral administration in rats. KDS-5104 (10 mg kg^{-1} i.p.) reached maximal plasma levels (C_{max}) 15 min after injection and was slowly cleared from the circulation. Its C_{max} in plasma was ~2-fold higher than that of OEA (10 mg kg^{-1} i.p.), and the area under the curve (AUC) was approximately 5 times greater (Fig. 3a; Table 2). An even more striking difference between the two compounds was observed in liver tissue, where the C_{max} of KDS-5104 was 13 times higher than that of OEA and its AUC was 23 times greater (Fig. 3b; Table 2). We next determined the distribution of KDS-5104 (100 mg kg^{-1}) after oral administration in free-feeding rats. The compound exhibited significantly higher C_{max} and AUC values in plasma and liver than did OEA (Fig. 4; Table 3).

Behavioral Studies. Because OEA inhibits food intake in rodents, we examined the effects of analogs **2** to **6** on feeding behavior after i.p. administration in free-feeding rats. Analysis of meal parameters revealed that OEA and KDS-5104 produced a dose-dependent increase in the latency of feeding onset, whereas the reverse amide **6** and the tertiary amide **5** were inactive (Fig. 5 and data not shown). The ED_{50} values for all compounds, assessed on feeding latency, are reported in Table 1. Two-way ANOVA analysis revealed a significant difference between OEA and KDS-5104 treatment ($P < 0.001$, $F_{1/68} = 15.58$) and a significant difference between 10 mg kg^{-1} doses of the two compounds ($P < 0.01$, Bonferroni's post hoc test). Moreover, KDS-5104, but not OEA, caused a significant prolongation of both first and second PMI, which is suggestive of a persistent satiety-inducing action (Fig. 5). Oral administration of KDS-5104 (100 mg kg^{-1}) resulted in a significant reduction in food intake in free-feeding rats, which lasted for 4 h following food presentation (Fig. 6, a and b). By contrast, no such effect was observed with the same oral dose of OEA (Fig. 6, a and b). Orally administered KDS-5104 or OEA exerted no significant effect on motor activity (Fig. 6, c and d).

Discussion

In the present study, we describe a new series of hydrolysis-resistant analogs of the endogenous PPAR-α ligand OEA, some of which display potent agonist activity at PPAR-α. Our synthetic approach focused on structural modifications of OEA that were expected to result in increased resistance to enzymatic hydrolysis based on previous studies with other lipid ethanolamides (Désarnaud et al., 1995; Khanolkar et al., 1996; Vandevoorde et al., 2003). We specifically modified three substructures of OEA—the fatty-acid chain, carboxamide group, and ethanolamine moiety—while leaving the general template of this natural ligand unaltered. As anticipated from our design, OEA analogs with increased steric bulk around the amide bond (compounds **3**–**5**) or inverted orientation of the carboxamide moiety (**6**), resisted hydrolysis by mouse liver homogenates. Among these compounds, the two C-α methyl stereoisomers **3** (KDS-5104) and **4** engaged PPAR-α with high potency in a reporter gene assay, whereas the tertiary amide **5** and the reverse amide **6** were inactive. It is noteworthy that the (*R*)-methyl isomer KDS-5104 was three times more potent at activating PPAR-α than was enantiomer **4** (EC_{50} 100 ± 21 and 333 ± 27 nM, respectively). We interpret these results to indicate that first the insertion of a methyl group on the C-α of ethanolamine protects OEA analogs from catalytic hydrolysis and second the presence of a free amide group in a precise orientation is essential for PPAR-α activation by this class of molecules.

Administration of OEA to live animals results in a variety of PPAR-α-mediated effects, which include prolongation of feeding latency (after i.p. injection) (Rodríguez de Fonseca et al., 2001) and PMI (after oral administration of gastroprotected capsules) (Oveisi et al., 2004), increased fatty-acid mobilization (Guzman et al., 2004), and reduced inflammation (after topical application) (Lo Verme et al., 2005a). However, many of these effects are rather short-lived, presumably on account of the liability of OEA to catalytic hydrolysis. The prolonged tissue exposure and duration of action of KDS-5104, which is resistant to catalytic hydrolysis in vitro, strongly supports this possibility. Furthermore, the in vivo anorexiant potency of KDS-5104 and indeed of all of the compounds tested in the present study is generally well correlated with their in vitro agonist potency at PPAR-α, providing further evidence for a role of this receptor in the biological actions of OEA.

We found that KDS-5104 reached significant concentra-

TABLE 3
C_{max} (±S.E.M.) and AUC values for OEA and KDS-5104 (analog **3**) after oral administration (100 mg kg^{-1})

	Plasma		Liver	
	C_{max}	AUC	C_{max}	AUC
	μg ml⁻¹	*μg min ml⁻¹*	*μg g⁻¹ wet tissue*	*μg min g⁻¹*
OEA	0.07 ± 0.01	18.42	0.16 ± 0.03	58.51
KDS-5104	0.58 ± 0.06	198.13	2.92 ± 0.79	674.12

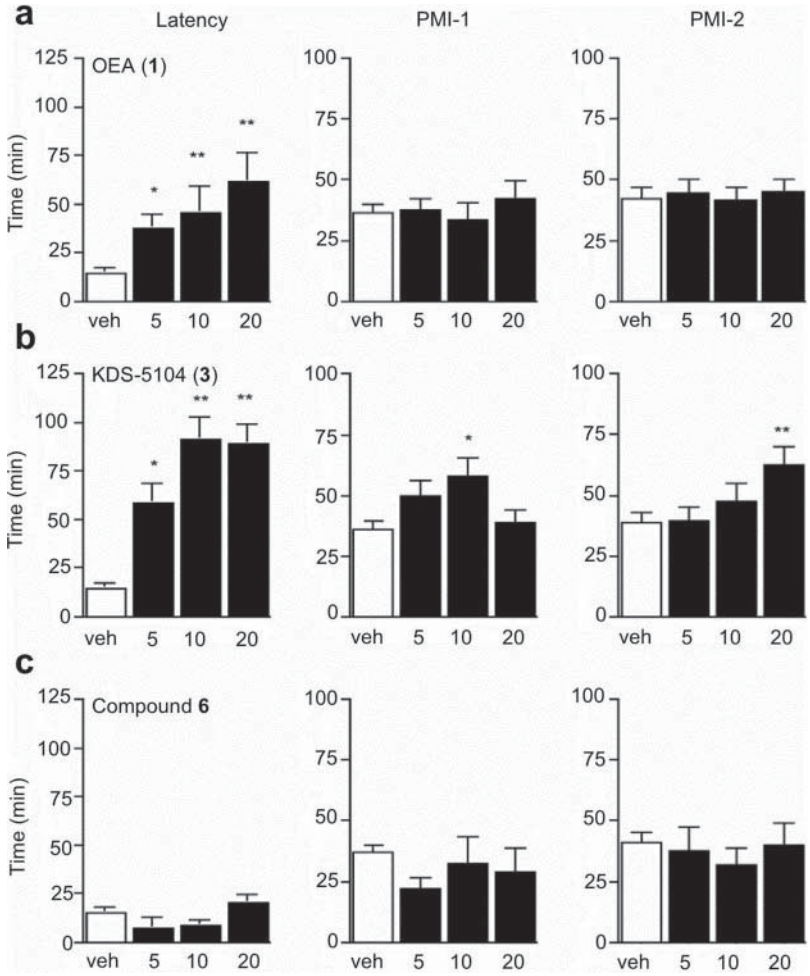


Fig. 5. a to c, effects of OEA (analog **1**) (a), KDS-5104 (analog **3**) (b), and compound **6** (c) on latency of feeding onset (left), PMI after the first meal (PMI-1, center), and PMI after the second meal (PMI-2, right). Compounds were administered by i.p. injection in free-feeding rats at doses of 5 to 20 mg kg^{-1} ($n = 6–18$). *, $P < 0.05$; and **, $P < 0.01$ versus vehicle.

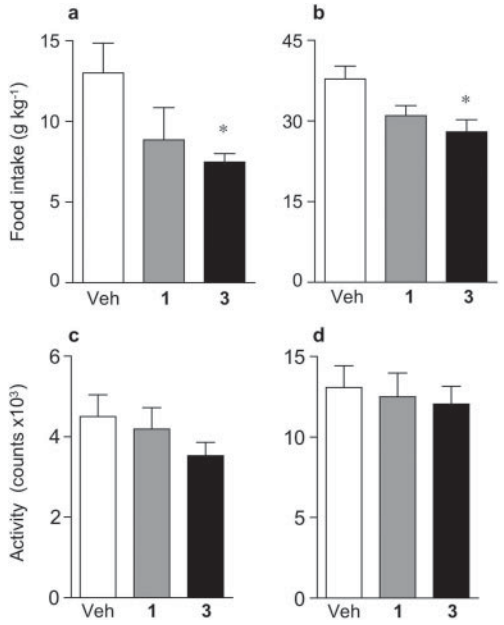


Fig. 6. a and b, effects of OEA (analog **1**) and KDS-5104 (analog **3**) on cumulative food intake 1 (a) and 4 h (b) after food presentation. Effects of OEA (analog 1) and KDS-5104 (analog 3) on locomotor activity 1 (c) and 4 h (d) after food presentation. Compounds were administered by gavage in free-feeding rats at a dose of 100 mg kg^{-1} ($n = 8$). *, $P < 0.05$ versus vehicle.

tions in plasma and liver and was pharmacologically active following oral administration in free-feeding rats. These results confirm that resistance to enzymatic hydrolysis is an important requisite for the design of orally active OEA analogs, which mimic the anorexiant actions of this endogenous PPAR-α agonist. Future studies will need to provide a more complete evaluation of the pharmacological properties of KDS-5104. It will be particularly important first to determine whether chronic administration of KDS-5104 exerts weight- and lipid-lowering effects similar to those of OEA (Rodríguez de Fonseca et al., 2001; Fu et al., 2003; Guzman et al., 2004; Lo Verme et al., 2005b) and second to characterize the impact of this compound on peroxisome proliferation, a prominent side effect of synthetic PPAR-α agonists in rodents (but not humans) (Klaunig et al., 2003).

In conclusion, our findings define a new class of potent orally active PPAR-α agonists designed on the scaffold of the endogenous ligand OEA. These agents provide a new set of molecular tools to investigate the pleiotropic functions of PPAR-α in feeding behavior, energy balance, and inflammation.

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References

Bachur NR, Masek K, Melmon KL, and Udenfriend S (1965) Fatty acid amides of ethanolamine in mammalian tissues. *J Biol Chem* **240:**1019–1024.

Broberger C (2005) Brain regulation of food intake and appetite: molecules and networks. *J Intern Med* **258:**301–327.

Burton MJ, Cooper SJ, and Popplewell DA (1981) The effect of fenfluramine on the microstructure of feeding and drinking in the rat. *Br J Pharmacol* **72:**621–633.

Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, and Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature (Lond)* **384:**83–87.

Cronet P, Petersen JF, Folmer R, Blomberg N, Sjoblom K, Karlsson U, Lindstedt EL, and Bamberg K (2001) Structure of the PPARalpha and -gamma ligand binding domain in complex with AZ 242; ligand selectivity and agonist activation in the PPAR family. *Structure (Camb)* **9:**699–706.

Désarnaud F, Cadas H, and Piomelli D (1995) Anandamide amidohydrolase activity in rat brain microsomes. Identification and partial characterization. *J Biol Chem* **270:**6030–6035.

Desvergne B and Wahli W (1999) Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* **20:**649–688.

Fu J, Gaetani S, Oveisi F, Lo Verme J, Serrano A, Rodriguez de Fonseca F, Rosengarth A, Luecke H, Di Giacomo B, Tarzia G, et al. (2003) Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha. *Nature (Lond)* **425:**90–93.

Gaetani S, Oveisi F, and Piomelli D (2003) Modulation of meal pattern in the rat by the anorexic lipid mediator oleoylethanolamide. *Neuropsychopharmacology* **28:** 1311–1316.

Giuffrida A, Rodríguez de Fonseca F, and Piomelli D (2000) Quantification of bioactive acylethanolamides in rat plasma by electrospray mass spectrometry. *Anal Biochem* **280:**87–93.

Guzman M, Lo Verme J, Fu J, Oveisi F, Blazquez C, and Piomelli D (2004) Oleoylethanolamide stimulates lipolysis by activating the nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR-α). *J Biol Chem* **279:**27849–27854.

Halgren T (1996) Merck molecular force field. Basis, form, scope, parametrization and performance of MMFF94. *J Comput Chem* **17:**490–519.

Halgren T (1999) MMFF VI. MMFF94s option for energy minimization studies. *J Comput Chem* **20:**720–729.

Hillard CJ, Wilkison DM, Edgemond WS, and Campbell WB (1995) Characterization of the kinetics and distribution of *N*-arachidonylethanolamine (anandamide) hydrolysis by rat brain. *Biochim Biophys Acta* **1257:**249–256.

Khanolkar AD, Abadji V, Lin S, Hill WA, Taha G, Abouzid K, Meng Z, Fan P, and Makriyannis A (1996) Head group analogs of arachidonylethanolamide, the endogenous cannabinoid ligand. *J Med Chem* **39:**4515–4519.

Klaunig JE, Babich MA, Baetcke KP, Cook JC, Corton JC, David RM, DeLuca JG, Lai DY, McKee RH, Peters JM, et al. (2003) PPARalpha agonist-induced rodent tumors: modes of action and human relevance. *Crit Rev Toxicol* **33:**655–780.

Lin S, Khanolkar AD, Fan P, Goutopoulos A, Qin C, Papahadjis D, and Makriyannis A (1998) Novel analogues of arachidonylethanolamide (anandamide): affinities for the CB1 and CB2 cannabinoid receptors and metabolic stability. *J Med Chem* **41:**5353–5361.

Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, and Piomelli D (2005a) The nuclear receptor peroxisome proliferator-activated receptor-α mediates the anti-inflammatory actions of palmitoylethanolamide. *Mol Pharmacol* **67:**15–19.

Lo Verme J, Gaetani S, Fu J, Oveisi F, Burton K, and Piomelli D (2005b) Regulation of food intake by oleoylethanolamide. *Cell Mol Life Sci* **62:**708–716.

Mor M, Rivara S, Lodola A, Plazzi PV, Tarzia G, Duranti A, Tontini A, Piersanti G, Kathuria S, and Piomelli D (2004) Cyclohexylcarbamic acid 3′- or 4′-substituted biphenyl-3-yl esters as fatty acid amide hydrolase inhibitors: synthesis, quantitative structure-activity relationships, and molecular modeling studies. *J Med Chem* **47:**4998–5008.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, and Olson AJ (1998) Automated docking using a lamarckian genetic algorithm and an empirical binding free energy function. *J Comput Chem* **19:**1639–1662.

Nielsen MJ, Petersen G, Astrup A, and Hansen HS (2004) Food intake is inhibited by oral oleoylethanolamide. *J Lipid Res* **45:**1027–1029.

Oveisi F, Gaetani S, Eng KT, and Piomelli D (2004) Oleoylethanolamide inhibits food intake in free-feeding rats after oral administration. *Pharmacol Res* **49:**461–466.

Overton HA, Babbs AJ, Doel SM, Fyfe MC, Gardner LS, Griffin G, Jackson HC, Procter MJ, Rasamison CM, Tang-Christensen M, et al. (2006) Deorphanization of a G protein-coupled receptor for oleoylethanolamide and its use in the discovery of small-molecule hypophagic agents. *Cell Metab* **3:**167–175.

Pfister J and Wymann W (1983) A useful variant of the Curtius reaction. *Synthesis* 38–40.

Proulx K, Cota D, Castaneda TR, Tschop MH, D'Alessio DA, Tso P, Woods SC, and Seeley RJ (2005) Mechanisms of oleoylethanolamide-induced changes in feeding behavior and motor activity. *Am J Physiol* **289:**R729–R737.

Reidelberger RD, Arnelo U, Granqvist L, and Permert J (2001) Comparative effects of amylin and cholecystokinin on food intake and gastric emptying in rats. *Am J Physiol* **280:**R605–R611.

Rodríguez de Fonseca F, Navarro M, Gómez R, Escuredo L, Nava F, Fu J, Murillo-Rodríguez E, Giuffrida A, LoVerme J, Gaetani S, et al. (2001) An anorexic lipid mediator regulated by feeding. *Nature (Lond)* **414:**209–212.

Tsuboi K, Sun YX, Okamoto Y, Araki N, Tonai T, and Ueda N (2005) Molecular characterization of *N*-acylethanolamine-hydrolyzing acid amidase, a novel member of the choloylglycine hydrolase family with structural and functional similarity to acid ceramidase. *J Biol Chem* **280:**11082–11092.

Ueda N, Kurahashi Y, Yamamoto S, and Tokunaga T (1995) Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide. *J Biol Chem* **270:**23823–23827.

Ueda N, Tsuboi K, and Lambert DM (2005) A second *N*-acylethanolamine hydrolase in mammalian tissues. *Neuropharmacology* **48:**1079–1085.

Ueda N, Yamanaka K, Terasawa Y, and Yamamoto S (1999) An acid amidase hydrolyzing anandamide as an endogenous ligand for cannabinoid receptors. *FEBS Lett* **454:**267–270.

Vandevoorde S, Tsuboi K, Ueda N, Jonsson KO, Fowler CJ, and Lambert DM (2003) Esters, retroesters, and a retroamide of palmitic acid: pool for the first selective inhibitors of *N*-palmitoylethanolamine-selective acid amidase. *J Med Chem* **46:** 4373–4376.

Wang X, Miyares RL, and Ahern GP (2005) Oleoylethanolamide excites vagal sensory neurones, induces visceral pain and reduces short-term food intake in mice via capsaicin receptor TRPV1. *J Physiol* (*Lond*) **564:**541–547.

Willson TM, Brown PJ, Sternbach DD, and Henke BR (2000) The PPARs: from orphan receptors to drug discovery. *J Med Chem* **43:**527–550.

Woods SC, Benoit SC, Clegg DJ, and Seeley RJ (2004) Clinical endocrinology and metabolism. Regulation of energy homeostasis by peripheral signals. *Best Pract Res Clin Endocrinol Metab* **18:**497–515.

Xu HE, Lambert MH, Montana VG, Plunket KD, Moore LB, Collins JL, Oplinger JA, Kliewer SA, Gampe RT Jr, McKee DD, et al. (2001) Structural determinants of ligand binding selectivity between the peroxisome proliferator-activated receptors. *Proc Natl Acad Sci USA* **98:**13919–13924.

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