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 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 ± 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 ± 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; Oveis 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

ABBREVIATIONS: OEA, oleoylethanolamide; PPAR, peroxisome proliferator-activated receptor; LBD, ligand-binding domain; GW7647, propanoic acid, 2-[[4-[[2-[[[cyclohexyloamino]carbonyl][4-cyclohexyl]butyl]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, carboxylic acid, cyclohexyl, 3-[[aminocarbonyl][1,1’-biphenyl]-3-y1 ester; EI, electron ionization; GW409544, l-phenylalanine, N-[[12]-1-methyl-3-oxo-3-phenyl-1-propenyl]-4-[3-(5-methyl-2-phenyl-4-oxazolyl)propyl]; PMI, postmeal interval; IR, infrared.

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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 (Kᵦ 37 and 40 nM, respectively) and activates with high-potency PPAR-α-driven transactivation in a heterologous expression system (EC₅₀ = 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-α⁻/⁻ mice) do not respond to OEA or synthetic PPAR-α agonists, although they retain normal responses to two mechanistically different anorexiant agents, 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 (Desvergne 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 (Tsuibo 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 C57BL6 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 provided 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 (Morgan 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-hydroxyethyl) (2) were synthesized by the reaction of the respective fatty-amide 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 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-heptadecenamide; 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 trimethylaluminum 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 CHCl₃. The organic layer was dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The product was purified by flash-chromatography on a silica gel column (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); 1H NMR spectra were recorded on a Bruker WM-300 spectrometer (Billerica, MA) with the following chemical shifts measured in CDCl₃: 300.13 (M∕z); 310.48 (M+ under CO₂); 320.65 (M+ under SO₃); 330.78 (M+ under CO₃). Optical rotary powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹. Optical rotatory powers were obtained on a PerkinElmer 241 polarimeter (PerkinElmer Life and Analytical Sciences, Boston, MA); absorbance is reported in centimeter⁻¹.
TABLE 1
Chemical structures and pharmacological properties of various OEA analogs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>PPAR-α Activation</th>
<th>Inhibition of Feeding</th>
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<tr>
<td></td>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>1</td>
<td><img src="image1" alt="Structure" /></td>
<td>120 ± 16</td>
<td>9.2 ± 1.6</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Structure" /></td>
<td>318 ± 65</td>
<td>9.2 ± 1.4</td>
</tr>
<tr>
<td>3 (KDS-5104)</td>
<td><img src="image3" alt="Structure" /></td>
<td>100 ± 21</td>
<td>2.4 ± 1.8</td>
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<tr>
<td>4</td>
<td><img src="image4" alt="Structure" /></td>
<td>333 ± 27</td>
<td>16.3 ± 2.5</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Structure" /></td>
<td>&gt;10,000</td>
<td>&gt;20</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Structure" /></td>
<td>&gt;10,000</td>
<td>&gt;20</td>
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*EC<sub>50</sub> values (±S.E.M., n = 5–12) were assessed in gene activation assays.

*ED<sub>50</sub> values (±S.E.M., n = 6–18) were assessed in feeding assay (using latency of feeding onset as variable).

In Vitro Enzymatic Hydrolysis. We incubated test compounds (10 nM) 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<sub>4</sub>]OEA as an internal standard. We prepared [2H<sub>4</sub>]OEA by the reaction of oleoyl chloride with a 10-fold molar excess of [2H<sub>4</sub>]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<sub>2</sub>. 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 Synergy Polar-RP column (2 × 150 mm, 4 μm; 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<sup>−1</sup> 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 2.3 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) and 3 (KDS-5104), 4 and 5 (m/z 340 > 76), 6 (m/z 326 > 254), and [2H<sub>4</sub>]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.

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 = 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<sub>21</sub>H<sub>41</sub>NO<sub>2</sub>) C, H, N.
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 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\(^{-1}\), 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.

**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 co-crystallized 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 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 selected to 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 \( \Delta^2 \) position, with an elaidic acid chain, in which the \( \Delta^3 \) 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 \( \alpha \)-carbon (stereoisomers 3–4) (Lin et al., 2001).

**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. \( \bullet \), analog 1; OEA; \( \bigcirc \), analog 2; \( \bigotimes \), analog 3; KDS-5104; \( \odot \), analog 4; \( \square \), analog 5; \( \bullet \), analog 6.
TABLE 2

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<tr>
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<th>Plasma C&lt;sub&gt;max&lt;/sub&gt; (±S.E.M.)</th>
<th>AUC µg min ml&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Liver C&lt;sub&gt;max&lt;/sub&gt; (µg g&lt;sup&gt;-1&lt;/sup&gt; wet tissue)</th>
<th>AUC µg min g&lt;sup&gt;-1&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>OEA</td>
<td>0.13 ± 0.05</td>
<td>8.95</td>
<td>1.44 ± 0.67</td>
<td>53.94</td>
</tr>
<tr>
<td>KDS-5104</td>
<td>0.30 ± 0.03</td>
<td>43.64</td>
<td>18.72 ± 2.51</td>
<td>1275.00</td>
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1998) or the amide nitrogen (5). Finally, we inverted the natural orientation of the carbamoyl 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<sub>1/2</sub>) 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<sub>50</sub> value of 120 ± 16 nM (n = 12) (Fig. 1b; Table 1). KDS-5104 (3) was as potent as OEA (EC<sub>50</sub> = 100 ± 21 nM, n = 11), whereas its stereoisomer 4 and the geometric OEA isomer 2 were approximately three times less potent than OEA (EC<sub>50</sub> = 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 Tyr334, and the amide NH is hydrogen-bonded to the backbone CO 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 hydroxyl 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.

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**Fig. 2.** Docking representation of one possible binding mode of KDS-5104 within the PPAR-α LBD. The agonist GW4009544 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.
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 (\(C_{\text{max}}\)) 15 min after injection and was slowly cleared from the circulation. Its \(C_{\text{max}}\) 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 \(C_{\text{max}}\) 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 \(C_{\text{max}}\) 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 (\(P < 0.001, F_{1,168} = 15.58\)) and a significant difference between 10 mg kg\(^{-1}\) doses of the two compounds (\(P < 0.01, \text{Bonferroni's post hoc test}\)). 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-\(\alpha\) ligand OEA, some of which display potent agonist activity at PPAR-\(\alpha\). 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; Vandervoorde 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-\(\alpha\) methyl stereoisomers 3 (KDS-5104) and 4 engaged PPAR-\(\alpha\) 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-\(\alpha\) than was enantiomer 4 (\(EC_{\text{50}} = 100 \pm 21\) and 333 \(\pm 27\) nM, respectively). We interpret these results to indicate that first the insertion of a methyl group on the C-\(\alpha\) 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-\(\alpha\) activation by this class of molecules.

Administration of OEA to live animals results in a variety of PPAR-\(\alpha\)-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-\(\alpha\), providing further evidence for a role of this receptor in the biological actions of OEA.

We found that KDS-5104 reached significant concentra-

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**TABLE 3**

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

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(C_{\text{max}})</td>
<td>(\mu g) ml(^{-1})</td>
</tr>
<tr>
<td>OEA</td>
<td>0.07 ± 0.01</td>
<td>18.42</td>
</tr>
<tr>
<td>KDS-5104</td>
<td>0.58 ± 0.06</td>
<td>198.13</td>
</tr>
</tbody>
</table>
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


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