A Structure/Activity Relationship Study on Arvanil, an Endocannabinoid and Vanilloid Hybrid

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ABSTRACT

Arvanil, a structural “hybrid” between the endogenous cannabinoind CB$_1$, receptor ligand anandamide and capsaicin, is a potent agonist for the capsaicin receptor VR$_1$ (vanilloid receptor type 1), inhibits the anandamide membrane transporter (AMT), and induces cannabimimetic responses in mice. Novel arvanil derivatives prepared by N-$\text{N}$-methylation, replacement of the amide with urea and thiourea moieties, and manipulation of the vanillyl group were evaluated for their ability to bind/activate CB$_1$ receptors, activate VR$_1$ receptors, inhibit the AMT and fatty acid amide hydrolase (FAAH), and produce cannabimimetic effects in mice. The compounds did not stimulate the CB$_1$ receptor. Methylation of the amide group decreased the activity at VR$_1$, AMT, and FAAH. On the aromatic ring, the substitution of the 3-methoxy group with a chlorine atom or the lack of the 4-hydroxy group decreased the activity on VR$_1$ and AMT, but not the affinity for CB$_1$ receptors, and increased the capability to inhibit FAAH. The urea or thiourea analogs retained activity at VR$_1$ and AMT but exhibited little affinity for CB$_1$ receptors. The urea analog was a potent FAAH inhibitor ($IC_{50}$ = 2.0 µM). A water-soluble analog of arvanil, O-2142, was as active on VR$_1$, much less active on AMT and CB$_1$, and more potent on FAAH. All compounds induced a response in the mouse “tetrad”, particularly those with $EC_{50}$ < 10 nM on VR$_1$. However, the most potent compound, $N^\text{N}$-$\text{di}$-(3-chloro-4-hydroxy)benzyl-arachidonamide (O-2093, $ED_{50}$ ~ 0.04 mg/kg), did not activate VR$_1$ or CB$_1$ receptors. Our findings suggest that VR$_1$ and/or as yet uncharacterized receptors produce cannabimimetic responses in mice in vivo.

Recent studies have revealed a certain overlap among the binding sites for fatty acid derivatives of the cannabinoid CB$_1$ receptor (for review, see Pertwee, 1997), the vanilloid receptor type 1 (VR$_1$) for capsaicin (Caterina et al., 1997; for review, see Szallasi and Blumberg, 1999), and the membrane transporter for the endocannabinoid arachidonoylthanolamide (AEA; Devane et al., 1992; for review, see Di Marzo et al., 2000a). In particular, it was shown (Di Marzo et al., 1998) that some long-chain derivatives of capsaicin, such as olvanil (Dray, 1992), weakly bind to and activate the CB$_1$ receptor and competitively inhibit the AEA membrane transporter (AMT), whereas AEA was found to act as a full, albeit weak, agonist of VR$_1$ receptors (Zygmunt et al., 1999; Smart et al., 2000). More recently, despite the fact that similar structural prerequisites are necessary for long-chain fatty acid derivatives to interact with both the human VR$_1$ and the AMT, selective VR$_1$ agonists and AMT competitive inhibitors could be developed (De Petrocellis et al., 2000). CB$_1$/VR$_1$ hybrid agonists were designed as possible analgesic, anti-inflammatory and antitumor agents (Melck et al., 1999; Di Marzo et al., 2000b, 2001b). For one of these hybrids, named arvanil (Fig. 1), the chemical modification of the fatty acid chain, and, particularly, the introduction of two methyl groups on the C-16 and of a bromine group instead of the methyl group on the C-20, led to a compound, O-1861 (Fig. 1), with nearly the same activity on VR$_1$ and CB$_1$ receptors and high potency in the mouse tetrad of behavioral tests of cannabimimetic activity in vivo (Di Marzo et al., 2001b). These tests consist of: 1) inhibition of spontaneous activity in an open field, 2) rectal hypothermia, 3) analgesia in the tail-flick test, and 4)
immobility on a ring. Although none of these behavioral assays taken alone is indicative of a particular class of compounds, a positive response in all four tests is considered to be diagnostic of cannabimimetic activity (Martin et al., 1991). However, it was found recently that capsaicin, which does not activate CB1 receptors (Di Marzo et al., 1998), can also elicit a response in the mouse tetrad tests and that this natural product can induce immobility and hypolocomotion in rats by acting on vanilloid receptors (Di Marzo et al., 2000b; 2001c). Hence, the possible interference from VR1 receptors in a cannabimimetic response in these tests deserves further investigation, particularly in view of the fact that AEA and some of its analogs (e.g., methanandamide; Zygmont et al., 1999; Ralevic et al., 2000) might activate both CB1 and VR1 receptors at similar concentrations (for review, see Di Marzo et al., 2001a).

No study so far has addressed the question of whether the chemical modification of the amide and aromatic moieties of arvanil leads to CB1/VR1 hybrid activators. Therefore, the activity of eight novel compounds, obtained from arvanil by modifying these two regions, was analyzed here on: 1) CB1 receptors (Hillard and Jarrahian, 2000b; 2001c). Hence, the possible interference from VR1 receptors in a cannabimimetic response in these tests deserves further investigation, particularly in view of the fact that AEA and some of its analogs (e.g., methanandamide; Zygmont et al., 1999; Ralevic et al., 2000) might activate both CB1 and VR1 receptors at similar concentrations (for review, see Di Marzo et al., 2001a).

Materials and Methods

Synthesis and Chemicals. Arachidonyl analogs O-1986, O-1988, and O-2094 were synthesized by treatment of the appropriate amines with the acid chloride of arachidonic acid as described by us previously (Dasse et al., 2000). The amines used for O-1988 and O-2094 were prepared by reductive amination procedures (Abdel-Magid et al., 1996) using 3-methoxy-4-hydroxybenzaldehyde/CH3NH2-HCl/methanol/NaCNBH4 for the former and 3-chloro-4-hydroxybenzaldehyde/ammonium acetate/NaCNBH4/methanol/mol.sieves 3Å for the latter. O-2093 was synthesized from the appropriate amine, which was formed as a by-product during the reductive amination of 3-chloro-4-hydroxybenzaldehyde described above. The urea analog O-1987 was prepared from arachidonic acid, in a one-pot reaction, via its isocyanate followed by treatment with 4-hydroxy-3-methoxybenzylamine HCl using our published procedure (Ng et al., 1999). The thiourea O-2095 was synthesized by treatment of vanillyl isothiocyanate with norarachidonylaniline (synthesized from arachidonyl isocyanate (Ng et al., 1999) and 2-trimethylsilylethanol/80°C/16 h/followed by deprotection with CF3COOH at 0°C). Similarly, the thiourea O-2109 was prepared using 3-chloro-4-hydroxybenzylisothiocyanate (prepared from 3-chloro-4-hydroxybenzylamine using the same procedure as described for vanillyl isothiocyanate). O-2142 was synthesized from arvanil and 4-morpholinobutyric acid in methylene chloride/N,N′-dicyclohexylcarbodiimide using our procedure (Razdan et al., 1976). All compounds were characterized on the basis of their [1H] nuclear magnetic resonance spectra (run on a Jeol Eclipse 300 MHz) and elemental analyses.

Guanosine 5′-O-(3′-[35S]thiotriophosphate) ([35S]GTPγS) was purchased from PerkinElmer Life Sciences (Boston, MA). [3H]CP55940 was purchased from PerkinElmer Life Sciences. GDP and GTPγS were purchased from Roche Molecular Biochemicals (Summerville, NJ). All other reagent grade chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Agonist-Stimulated [35S]GTPγS Binding Assays. The hippocampus of young adult rats, dissected on ice, was used for these assays since this brain area exhibits a more efficacious coupling of CB1 receptors to G-proteins than whole brain. Each hippocampus was homogenized with a Tissumizer (Tekmar, Cincinnati, OH) in cold membrane buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 0.2 mM EGTA, 100 mM NaCl, pH 7.7) and centrifuged at 42,000 g for 20 min at 4°C. Pellets were resuspended in membrane buffer, then centrifuged again at 42,000 g for 20 min at 4°C. Pellets from the second centrifugation were homogenized in membrane buffer and stored at −80°C. Frozen membranes were thawed and diluted in membrane buffer, homogenized, and preincubated for 10 min at 30°C in 0.004 units/ml adenosine deaminase (240 units/mg of protein; Sigma Chemical Co.) to remove endogenous adenosine, then assayed for protein content before addition to assay tubes. Assays were conducted at 30°C for 1 h in membrane buffer, including 10 μg of membrane protein with 0.1% (w/v) bovine serum albumin (BSA), 10 μM GDP, and 0.1 nM [35S]GTPγS in a final volume of 0.5 ml. Nonspecific binding was determined in the absence of agonists and in the presence of 30 μM unlabeled GTPγS. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold Tris-HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for [3S] in 4 ml of BudgetSolve scintillation fluid (Sigma-RBI, Natick, MA). Net agonist-stimulated [35S]GTPγS binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist). Data analyses (including agonist concentration effect and competition curves) were conducted by iterative nonlinear regression using Prism for Windows (GraphPad Software, San Diego, CA) to obtain EC50, Emax, and KI values. Significant stimulation of [35S]GTPγS binding was determined by analysis of variance followed by Dunnett’s test at the p < 0.05 level to compare each concentration of ligand with basal binding. Data are expressed as means ± S.E. of experiments performed in triplicate in membranes from at least three different hippocampi.

CB1 Receptor Binding Assays. All experiments were performed with whole brain membranes rather than hippocampal membranes, and preparation of these membranes was the same as for the hippocampal.
pocampus. Binding was initiated by the addition of 75 μg of whole brain membranes to siliconized tubes containing [3H]CP55940 (1 nM), competing ligand (concentrations from 0.001–30 μM), 0.5% (w/v) BSA, and a sufficient volume of buffer (membrane buffer minus sodium chloride) to bring the total volume to 0.5 ml. The addition of 2 μM unlabeled CP55940 was used to assess nonspecific binding. Membranes were then incubated at 30°C for 60 min. The reaction was terminated by addition of ice-cold wash buffer (50 mM Tris-HCl, 0.5% BSA, pH 7.4) followed by rapid filtration under vacuum through Whatman GF/B glass-fiber filters using a 96-well harvester (Brandell, Gaithersburg, MD). The tubes were washed twice with 2 ml of ice-cold wash buffer, and the filters were rinsed twice with 4 ml of wash buffer. Filters were placed into 7-ml plastic scintillation vials, and 5 ml of BudgetSolve scintillation fluid was added. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for [3H].

**Cytosolic Calcium Concentration ([Ca<sup>2+</sup>]i) Assay.** Over-expression of human VR<sub>1</sub> cDNA into human embryonic kidney (HEK293) cells was carried out as described previously (Hayes et al., 2000). Cells were grown as monolayers in minimum essential medium supplemented with nonessential amino acids, 10% fetal calf serum, and 0.2 mM glutamine and maintained under 95% O<sub>2</sub>/5% CO<sub>2</sub> at 37°C. The effect of the substances on [Ca<sup>2+</sup>]<sub>i</sub> was determined by using Fluo-3 (Molecular Probes, Eugene, OR), a selective intracellular fluorescent probe for Ca<sup>2+</sup> (De Petrocellis et al., 2000; Smart et al., 2000). Cells were transferred into six-well dishes coated with poly-L-lysine (Sigma) 1 day prior to experiments and grown in the culture medium mentioned above. On the day of the experiment, the cells (50–60,000/well) were loaded for 2 h at 25°C with 4 μM Fluo-3 methylester in dimethyl sulfoxide containing 0.04% Pluronic. After loading, cells were washed with Tyrode’s solution, pH = 7.4, trypsinized, resuspended in Tyrode’s solution, and transferred to the cuvette of the fluorescence detector (PerkinElmer LS50B) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25°C (λ<sub>ex</sub> = 488 nm, λ<sub>em</sub> = 540 nm) before and after the addition of the test compounds at various concentrations. Data are expressed as the concentration exerting a half-maximal effect (EC<sub>50</sub>). The efficacy of the effect was determined by comparing it to the analogous effect observed with 4 μM ionomycin.

**Anandamide Membrane Transport Assay.** The effect of compounds on the uptake of [3H]AEA by rat basophilic leukemia (RBL-2H3) cells was studied by using 3.6 μM (10,000 cpm) of [3H]AEA as described previously (Bisogno et al., 1997). Cells were incubated with [3H]AEA for 5 min at 37°C, in the presence or absence of varying concentrations of the inhibitors. Residual [3H]AEA in the incubation medium after extraction with CHCl<sub>3</sub>/CH<sub>3</sub>OH 2:1 (by volume), determined by scintillation counting of the aqueous phase, was used as a measure of the AEA that was taken up by cells (De Petrocellis et al., 2000). Previous studies (Bisogno et al., 1997) had shown that after a 5-min incubation the amount of [3H]AEA that disappeared from the medium of RBL-2H3 cells is found mostly (>90%) as unmetabolized [3H]AEA in the cell extract. Nonspecific binding of [3H]AEA to cells and plastic dishes was determined in the presence of 100 μM AEA and was never higher than 30%. Data are expressed as the concentration exerting 50% inhibition of AEA uptake (IC<sub>50</sub>) calculated by GraphPad.

**Fatty Acid Amide Hydrolase Assay.** The effect of compounds on the enzymatic hydrolysis of AEA was studied as described previously (Di Marzo et al., 2001b), using membranes prepared from frozen brains of CD rats (Charles River, France), incubated with the test compounds and [3H]AEA (9 μM) in 50 mM Tris-HCl, pH 9, for 30 min at 37°C. [14C]Ethanolamine produced from [3H]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of CHCl<sub>3</sub>/CH<sub>3</sub>OH 2:1 (by volume). Data are expressed as the concentration exerting 50% inhibition of AEA uptake (IC<sub>50</sub>) calculated by GraphPad.

**Pharmacological Effects in Mice.** Cannabinoids were dissolved in a 1:1:1 mixture of ethanol, Emulphor (North American Chemicals, Cranbury, NJ), and saline for i.v. administration. The analogs were administered to mice by tail-vein injection and evaluated for their ability to produce hypomotility, hypothermia, and antinociception. These pharmacological measures were determined in the same mouse at a time when maximal activity was present. To measure locomotor activity, mice were placed into individual photocell activity chambers (11 × 6.5 inches) 5 min after injection. Spontaneous activity was measured during the next 10-min period, and the number of interruptions of 16 photocell beams per chamber was recorded. Antinociception was determined using the tail-flick reaction time to a heat stimulus. Before vehicle or drug administration, the baseline latency period (2–3 s) was determined. Tail-flick latency was assayed once more 20 min after the injection, and the differences in control and test latencies were calculated. A 10-s maximum latency was used. Antinociception was expressed as %MPE as described below. As for hypothermia, rectal temperature was determined prior to vehicle or drug administration with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH) and a thermistor probe (model YSI 400; Markson LabSales Inc., Hillsboro, OR) inserted at a depth of 2 mm. Rectal temperature was measured again 30 min after the injection, and the difference between pre- and postinjection values was calculated.

**Data Analysis.** For production of hypomotility and hypothermia, the data were expressed as percentage of control activity and change in temperature, respectively. Antinociception was calculated as follows:

\[
\% \text{ MPE} = \left( \frac{\text{test latency} - \text{control latency}}{10s - \text{control latency}} \right) \times 100
\]

At least six animals were treated with each dose so that dose-response relationships could be determined for each analog. ED<sub>50</sub> values were determined from least-squares unweighted linear regression analysis of the log dose-response plots. Maximal effects for all compounds combined on spontaneous activity, temperature, antinociception, and catalepsy were, respectively, 90% inhibition, −5°C, 100% MPE, and 60% immobility. Thus, the ED<sub>50</sub> values indicate response levels of 45% inhibition, −2.5°C, 50% MPE, and 30% immobility.

**Results**

All compounds were found to exhibit low CB<sub>1</sub> receptor affinity and little efficacy for stimulating G-protein coupling (Table 1). The methylation of the amide in arvanil (O-1988) led to dramatic decreases in both the affinity and functional activity for CB<sub>1</sub> receptors. Deletion of the 3-hydroxy-group on the aromatic moiety (O-1986) resulted in similar decreases, although some affinity for CB<sub>1</sub> receptors was retained. Substitution of an m-chloro for the m-methoxy in arvanil led to O-2094, a compound that had reasonable CB<sub>1</sub> receptor affinity and stimulated GTPγS binding (E<sub>max</sub> = 15% stimulation (8–20), EC<sub>50</sub> = 131 nM (5–1900), reversed by 2 nM SR141716A) slightly less potently than arvanil. When a second 3-chloro-(4-hydroxy)benzyl group was substituted on the nitrogen, the resulting analog (O-2093) exerted a significant inhibition of GTPγS binding, which was not sensitive to the CB<sub>1</sub> antagonist SR141716A (2 nM, data not shown) or to the FAAH inhibitor phenylmethylsulfonyl fluoride (50 μM, data not shown).

Conversion of the amide group in arvanil to a urea in O-1987 decreased both CB<sub>1</sub> affinity and GTPγS binding activity. When the urea was changed to a thiourea (O-2095), CB<sub>1</sub> receptor affinity was reduced further. The introduction
of a chlorine atom in O-2095, which yielded O-2109, the thiourea analog of O-2094, increased the activity of this compound in the CB\textsubscript{1} binding assay but did not restore the activity in the GTP\textsubscript{S} binding assay (Table 1). Finally, introduction of a 4'-morpholinobutyryl group on the p-hydroxybenzyl group of arvanil, which yielded the water soluble compound O-2142, again did not greatly influence the affinity for CB\textsubscript{1} receptors. This might be the result of the hydrolysis of the ester bond during the binding assay (see also below). However, it should be noted that: 1) O-2142 was inactive in the GTP\textsubscript{S} binding assay, which is also performed with membrane preparations, and 2) a similar chemical modification in (R)-methanandamide decreased its affinity for CB\textsubscript{1} receptors about 20-fold (from 20–426 nM, data not shown).

All the novel compounds except one were quite potent and efficacious in the functional assay of VR\textsubscript{1} activity performed in this study (Table 1), where the capability of increasing the [Ca\textsuperscript{2+}]\textsubscript{i} was measured in HEK cells over-expressing the hVR\textsubscript{1} receptor (De Petrocellis et al., 2000). In agreement with previous studies carried out with capsaicin (Walpole et al., 1993a,b), the substitution of the m-methoxy group with a chlorine atom (O-2094) and the methylation of the amide group (O-1988) in arvanil decreased its potency at hVR\textsubscript{1} by 25–50-fold. Introduction of a second N-(3-chloro-4-hydroxy)-benzyl group (O-2093) abolished the activity at VR\textsubscript{1}. Introduction of an amide group (O-1987), and further substitution of the carbonyl for a C=S group (O-2095) did not alter arvanil efficacy/potency at VR\textsubscript{1}. The importance of the p-hydroxybenzyl group for the functional activation of these

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### TABLE 1

Evaluation of arvanil analogs at CB\textsubscript{1} receptors, VR\textsubscript{1} receptors, AEA transport, and FAAH*  

<table>
<thead>
<tr>
<th>Compound</th>
<th>CB\textsubscript{1} Affinity K\textsubscript{i} (nM)</th>
<th>GTP\textsubscript{S} Binding (E\textsubscript{max})</th>
<th>hVR\textsubscript{1} Potency (EC\textsubscript{50} (\mu\text{M}))</th>
<th>AEA Transport (IC\textsubscript{50} (\mu\text{M}))</th>
<th>FAAH (IC\textsubscript{50} (\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARVANIL</td>
<td>250 ± 2600\textsuperscript{b}</td>
<td>24\textsuperscript{b}</td>
<td>0.5 ± 0.2\textsuperscript{b}</td>
<td>3.6\textsuperscript{b}</td>
<td>32.0\textsuperscript{b}</td>
</tr>
<tr>
<td>O-1988</td>
<td>2829 ± 175</td>
<td>0</td>
<td>25.0 ± 3.9 (69.1 ± 5.2)</td>
<td>10.0 ± 2.1</td>
<td>&gt;50</td>
</tr>
<tr>
<td>O-1986</td>
<td>484 ± 17</td>
<td>0</td>
<td>63.0 ± 10.1 (68.0 ± 4.3)</td>
<td>27.3 ± 3.5</td>
<td>18.2 ± 2.8</td>
</tr>
<tr>
<td>O-2094</td>
<td>274 ± 19</td>
<td>15 (8 to 20)</td>
<td>10.0 ± 1.4 (63.5 ± 5.3)</td>
<td>19.0 ± 2.1</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>O-2093</td>
<td>1290 ± 140</td>
<td>−30 (−11.3 to −39.9)</td>
<td>&gt;50,000 (26.9 ± 4.2)</td>
<td>11.5 ± 1.3</td>
<td>&gt;50</td>
</tr>
<tr>
<td>O-2097</td>
<td>1718 ± 200</td>
<td>0</td>
<td>0.7 ± 0.2 (80.6 ± 8.3)</td>
<td>19.3 ± 3.3</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>O-2095</td>
<td>8626 ± 130</td>
<td>0</td>
<td>0.4 ± 0.1 (72.1 ± 4.8)</td>
<td>7.5 ± 1.2</td>
<td>&gt;50</td>
</tr>
<tr>
<td>O-2093</td>
<td>1801 ± 204</td>
<td>0</td>
<td>4.0 ± 1.1</td>
<td>3.8 ± 0.7</td>
<td>&gt;50</td>
</tr>
<tr>
<td>O-2109</td>
<td>483 ± 63</td>
<td>0</td>
<td>0.6 ± 0.2 (86.6 ± 8.2)</td>
<td>30.0 ± 4.3</td>
<td>9.1 ± 1.7</td>
</tr>
</tbody>
</table>

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* Affinity for CB\textsubscript{1} receptors was measured with the K\textsubscript{i} (nM) for the displacement of [\textsuperscript{3}H]CP55940 from whole rat brain membranes. Efficacy at these as well as other G-protein-coupled receptors was measured as the capability of stimulating the binding of [\textsuperscript{35}S]GTP\textsubscript{S} to rat hippocampal membranes. Potency (nM) and efficacy (maximal effect as percentage of the stimulation of the effect of 4 \(\mu\text{M}\) capsaicin) at human VR\textsubscript{1} receptors were measured as the capability of enhancing [Ca\textsuperscript{2+}]\textsubscript{i} in this study (Table 1), where the capability of increasing the [Ca\textsuperscript{2+}]\textsubscript{i} was measured in HEK cells over-expressing the hVR\textsubscript{1} receptor (De Petrocellis et al., 2000). In agreement with previous studies carried out with capsaicin (Walpole et al., 1993a,b), the substitution of the m-methoxy group with a chlorine atom (O-2094) and the methylation of the amide group (O-1988) in arvanil decreased its potency at hVR\textsubscript{1} by 25–50-fold. Introduction of a second N-(3-chloro-4-hydroxy)-benzyl group (O-2093) abolished the activity at VR\textsubscript{1}. Introduction of an amide group (O-1987), and further substitution of the carbonyl for a C=S group (O-2095) did not alter arvanil efficacy/potency at VR\textsubscript{1}. The importance of the p-hydroxybenzyl group for the functional activation of these...
receptors is underlined by the observation that O-1986 was more than 100-fold less potent than arvanil, whereas the role of the \( n \)-methoxy group in the correct interaction with VR\(_1\) was confirmed by the finding that O-2109 was 10-fold less potent than O-2095 (Table 1). Finally, and surprisingly, O-2142 was as potent as arvanil in inducing a VR\(_1\)-mediated increase of \([\text{Ca}^{2+}]_i\) in HEK-hVR\(_1\) cells. Since 1) this compound did not appear to be a good substrate for the AMT (see below), 2) AMT-mediated facilitated transport into HEK-hVR\(_1\) cells is important to observe high potency at hVR\(_1\) (Di Marzo et al., 2001a), and 3) the \( p \)-hydroxy group is fundamental for interaction with vanilloid receptors (see above and Walpole et al., 1993b), this finding suggests that O-2142 is hydrolyzed by cells prior to its interaction with VR\(_1\).

The order of potency of the novel compounds as AMT inhibitors (O-2109 > O-2095 > O-1988 > O-2093 > O-2094 = O1987 > O-1986 > O-2142) was slightly different from the order of potency at hVR\(_1\) (O-2095 ≥ O-2142 ≥ O-1987 > O-2109 > O-2094 > O-1988 > O-1986 > O-2093), although in most cases the differences between the activities of the compounds were not significant (Table 1). However, if one excludes O-2142, whose activity at VR\(_1\) might have been due to hydrolysis to arvanil, one of the compounds (O-1986) with the lowest activity on the AMT exhibited also low potency at VR\(_1\), whereas O-2095 and O-2109 were quite potent as both VR\(_1\) agonists and AMT inhibitors. In fact, the IC\(_{50}\) of the widely used AMT inhibitor, AM404 (Khanolkar and Makriyannis, 1999) was 8.1 ± 2.6 \( \mu \)M under our conditions. Another exception to the rule was O-2093, whose inhibitory activity on AEA uptake (IC\(_{50}\) = 11.5 ± 1.3 \( \mu \)M) was not surprising since the AMT, unlike VR\(_1\), has been shown to bind also to amides of arachidonic acids with very hindering aromatic groups (Jarrahian et al., 2000). As a consequence of these findings, only one AMT inhibitor (O-2093) with activity on VR\(_1\) much lower than that observed for AM404 in previous studies (EC\(_{50}\) = 26–32 nM; De Petrocellis et al., 2000) was found in this study. This suggests that a way of obtaining AMT inhibitors selective versus VR\(_1\) is to condense arachidonic acid with hindering aromatic moieties, as in the case of O-2093, VDM13, and, possibly, other previously described arachido-

### TABLE 2

Effect of arvanil derivatives in the mouse tetrad of behavioral tests

The ED\(_{50}\) (milligrams per kilogram, i.v.) dose for inhibition of sector crossings (spontaneous activity) in an open field, antinociception (delay in seconds in the tail-flick response), decrease of rectal temperature in degrees Celsius, and induction of time spent in immobility on a ring are shown. Data are means (and ranges) of \( n \geq 6 \) animals.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Spontaneous Activity</th>
<th>T.F.</th>
<th>Rectal Temperature</th>
<th>R.I.</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-1985</td>
<td>3.11 (1.54–6.29)</td>
<td>7.98 (5.17–12.34)</td>
<td>8.74 (5.61–13.60)</td>
<td>4.08 (2.99–5.56)</td>
<td>5.98</td>
</tr>
<tr>
<td>O-2094</td>
<td>0.05 (0.04–0.07)</td>
<td>0.49 (0.03–0.05)</td>
<td>0.49 (0.02–0.06)</td>
<td>0.49 (0.03–0.05)</td>
<td>0.49</td>
</tr>
<tr>
<td>O-2093</td>
<td>0.06 (0.04–0.08)</td>
<td>0.12 (0.08–0.17)</td>
<td>0.12 (0.03–0.20)</td>
<td>0.08 (0.06–0.12)</td>
<td>0.09</td>
</tr>
<tr>
<td>O-2095</td>
<td>0.02 (0.02–0.03)</td>
<td>0.08 (0.06–0.10)</td>
<td>0.08 (0.02–0.07)</td>
<td>0.08 (0.05–0.12)</td>
<td>0.05</td>
</tr>
<tr>
<td>O-2097</td>
<td>0.02 (0.02–0.03)</td>
<td>0.09 (0.06–0.12)</td>
<td>0.09 (0.02–0.07)</td>
<td>0.09 (0.10–0.23)</td>
<td>0.15</td>
</tr>
<tr>
<td>O-2099</td>
<td>0.20 (0.16–0.24)</td>
<td>0.19 (0.15–0.25)</td>
<td>0.19 (0.07–0.17)</td>
<td>0.19 (0.13–0.22)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

T. F., delay in seconds in the tail-flick response; R.I., induction of time spent in immobility on a ring.
nate derivatives (De Petrocellis et al., 2000; Jarrahian et al., 2000).

Unlike arvanil and its derivatives obtained through the modification of the aliphatic moiety (Melck et al., 1999; Di Marzo et al., 2001b), analogs obtained from the substitution of the \( m \)-methoxy group for a chlorine atom (O-2094) or from the introduction of an amide \( \alpha \) to the carbonyl (O-1987) are potent FAAH inhibitors. Also, elimination or derivatization of the \( \beta \)-hydroxy group, as in O-1986 and O-2142, respectively, slightly increases the affinity for FAAH. Given the esterase activity of FAAH (Goparaju et al., 1998), it is possible that the enzyme recognizes O-2142 as a better substrate due to the presence of the ester, rather than the amide, bond. Conversely, modification of one of those chemical moieties that were previously shown to confer to AEA derivatives the capability of interacting with the enzyme, e.g., the carbonyl group (Lang et al., 1999), as in O-2095 versus O-1987, abolished inhibitory activity (Table 1). Our data also indicate that the carbonyl group is such a necessary requisite for interaction with FAAH that its elimination in O-1987 cannot be compensated for by the presence of the \( m \)-chlorine atom in the vanillyl moiety (O-2109). Another important requisite is the presence of a secondary or primary amide (Lang et al., 1999; Boger et al., 2000), and in fact O-1988 and O-2093 were even less potent inhibitors of FAAH activity than arvanil (Table 1). Finally, the finding that O-1988, O-2095, and O-2096 are all much more potent as AMT than as FAAH inhibitors confirms that AEA transport into cells is not uniquely driven by FAAH activity (Day et al., 2001; Deutsch et al., 2001), because substances that inhibit this process without significantly affecting AEA hydrolysis can be found.

Of the eight novel compounds tested in this study, and with only one exception (O-2093), only those with a threshold potency at VR1 receptors of 10 nM exhibited very strong activity (average ED50 < 1 mg/kg) in the mouse tetrad of tests (Table 2). Usually, a positive response (inhibition of locomotor activity, induction of immobility, antinociception, and hypothermia) in all four tests is considered highly indicative of cannabimimetic activity (Martin et al., 1991). Yet, none of the arvanil analogs tested here bound with very high affinity to CB1 receptors or exhibited high efficacy in the GTPyS binding assay. Conversely, they displayed very high potency/efficacy at human VR1 receptors, although their EC50 values for VR1 activation did not appear to correlate linearly with the ED50 values observed in vivo. At any rate, the effect of O-2094 (either 1 or 3 mg/kg, i.v.) and of O-2093 (0.03, 0.056, and 0.1 mg/kg, i.v.) in the spontaneous activity, tail-flick, and rectal temperature tests, and of O-1988 (either 3 or 10 mg/kg, i.v.) in the spontaneous activity and tail-flick tests were not affected by a 10 min pretreatment of mice with SR141716A (3 mg/kg, i.v.) (Table 3 and data not shown).

**Discussion**

None of the novel arvanil analogs described here bound to the CB1 receptor with high affinity. However, some general conclusions emerge. Since O-2094 exhibited an affinity for CB1 receptors similar to that previously observed for arvanil, it is possible to conclude that the presence of an \( m \)-methoxy group in the latter molecule is not crucial for the functional interaction with the central cannabinoid receptor. Conversely, the derivatization of the amide in arvanil, as in O-1988 and O-2093, and the lack of the \( \beta \)-hydroxy group on the aromatic moiety, as in O-1986, led to dramatic changes in both the affinity for, and functional activity at, CB1 receptors. In fact, previous studies showed that a secondary amide group in AEA derivatives is fundamental for interaction with cannabinoid receptors (for reviews, see Khanolkar and Makriyannis, 1999; Martin et al., 1999). These findings are also in agreement with previous data showing that the carbonyl function in AEA is important for the interaction with CB1 binding site (Khanolkar and Makriyannis, 1999).

Although a certain overlap in the ligand recognition properties between CB1 and VR1 receptors can be observed, the chemical requisites for the optimal interaction of arvanil analogs with the binding sites within each receptor class are different. In particular, the \( \beta \)-hydroxy and \( m \)-methoxy groups on the vanillyl moiety are important for the interaction with VR1 but not so much with CB1 receptors. Conversely, a carbonyl function on C-1 and a methylene group on C-2 in arvanil are important to achieve high affinity for CB1 receptors but can be substituted for C=S and NH groups, respectively, without modifying the efficacy at VR1. On the other hand, it must be noted that the presence of a 20-carbon atom polyunsaturated chain (Melck et al., 1999; De Petrocellis et al., 2000) and a secondary amide group (this study) are important for an optimal interaction with both receptor classes.

Although the overlap between the AMT and VR1 ligand

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Spontaneous Activity (Beam Interruptions)</th>
<th>Tail-Flick Latency (%MPE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle + Compound</td>
<td>SR141716A + Compound</td>
</tr>
<tr>
<td>O-1988</td>
<td>0</td>
<td>1224 ± 94</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>766 ± 157</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>179 ± 34</td>
</tr>
<tr>
<td>O-2093</td>
<td>0</td>
<td>1288 ± 87</td>
</tr>
<tr>
<td>0.056</td>
<td>1198 ± 102</td>
<td>1461 ± 131</td>
</tr>
<tr>
<td>0.1</td>
<td>529 ± 110</td>
<td>71 ± 25</td>
</tr>
<tr>
<td>O-2094</td>
<td>0</td>
<td>1208 ± 35</td>
</tr>
<tr>
<td>0.1</td>
<td>1205 ± 93</td>
<td>2034 ± 182</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>83 ± 17</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>118 ± 16</td>
</tr>
</tbody>
</table>
recognition properties is supported to a great extent by the present findings, rather surprising data emerged here on the capability of some of the novel analogs to inhibit FAAH. In general, it can be concluded from our findings that, despite the relatively high potency of arvanil as an AMT inhibitor, the types of modifications of the amide and aromatic moieties made here on arvanil do not confer to this compound any further selectivity for the AMT versus VR₁ or FAAH.

The observation that: 1) capsaicin exhibits a certain albeit more limited activity in some of the tetrad tests (Di Marzo et al., 2000b), 2) arvanil analogs are very potent in this mouse model (Di Marzo et al., 2001b), and 3) an 18-atom carbon unsaturated capsaicin analog, livanil, inhibits locomotor activity in rats (Di Marzo et al., 2001c), might suggest that activation of VR₁ is also involved in inducing cannabinimic responses in these assays. This suggestion is strengthened by our present observation that the novel compounds with very high potency at hVR₁ are also the most potent in the mouse tetrad. Since AEA also activates VR₁ receptors with a potency that may depend on several regulatory factors (Di Marzo et al., 2001a), it is possible that these sites also participate in AEA actions in the mouse tetrad tests, actions that cannot be reversed by a CB₁ receptor antagonist (Adams et al., 1998). Another possible explanation is that non-CB₁, non-VR₁ cannabinoid receptors (for example, see Di Marzo et al., 2000c; Breivogel et al., 2001) are involved in the effects of arvanil-related compounds and, to some extent, of AEA in these four behavioral assays. In fact, several pharmacological actions of arvanil do not appear to be sensitive to effective doses of the CB₁ antagonist SR141716A or of the VR₁ antagonist capsazepine (Di Marzo et al., 2000b; V. Di Marzo, unpublished data). In support to this hypothesis, we have found here that: 1) the effects in some of the tetrad tests of three compounds with low, intermediate, and high potency, i.e., O-1988, O-2094, and O-2093, respectively, were not antagonized by SR141716A, and 2) O-2093 was one of the most potent compounds ever found in the mouse tetrad (average ED₅₀ ~ 0.04 mg/kg) and yet it exhibited very little affinity for CB₁ receptors and almost no potency/efficacy at hVR₁ receptors in vitro. The inhibitory effect by O-2093 of endocannabinoid uptake, with a possible subsequent increase of endogenous cannabinoid tonic activity in the tetrad tests, is unlikely to explain O-2093 high potency in vivo. In fact, other equipotent AMT and/or FAAH inhibitors in this study (e.g., O-1988 or O-2094) were active in the mouse tetrad only at 50- to 100-fold higher doses, and furthermore, a putative “indirect” activation of CB₁ receptors by these compounds would have been blocked by SR141716A. The behavioral effects of O-2093, therefore, might be mediated by non-CB₁, non-VR₁ sites of action specific for arvanil-like compounds, possibly via an inverse agonist effect on G-protein-coupled receptors, since O-2093 was found to inhibit GTP-γ-S binding to hippocampal membranes in a manner insensitive to SR141716A. Although the in vivo activity of O-2093 was not blocked by SR141716A, an effect for this compound as a prodrug at either site of action specific for arvanil-like compounds, possibly to explain O-2093 high potency in vivo. In fact, other equipo-

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References


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