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Development of the First Ultra-Potent "Capsaicinoid" Agonist at Transient Receptor Potential Vanilloid Type 1 (TRPV1) Channels and Its Therapeutic Potential

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ABSTRACT

Olvanil (*N*-9-*Z*-octadecenoyl-vanillamide) is an agonist of transient receptor potential vanilloid type 1 (TRPV1) channels that lack the pungency of capsaicin and was developed as an oral analgesic. Vanillamides are unmatched in terms of structural simplicity, straightforward synthesis, and safety compared with the more powerful TRPV1 agonists, like the structurally complex phorboid compound resiniferatoxin. We have modified the fatty acyl chain of olvanil to obtain ultra-potent analogs. The insertion of a hydroxyl group at C-12 yielded a compound named rinvanil, after ricinoleic acid, significantly less potent than olvanil (EC_{50} = 6 versus 0.7 nM), but more versatile in terms of structural modifications because of the presence of an additional functional group. Acetylation and phenylacetylation of rinvanil re-established and dramatically enhanced, respectively, its potency at hTRPV1. With a two-digit picomolar EC_{50} (90 pM), phenylacetylrinvanil (PhAR, IDN5890) is the most potent vanillamide ever described with potency comparable with that of resiniferatoxin (EC_{50}, 11 pM). Benzoyl- and phenylpropionylrinvanil were as potent and less potent than PhAR, respectively, whereas configurational inversion to *ent*-PhAR and cyclopropanation (but not hydrogenation or epoxidation) of the double bond were tolerated. Finally, iodination of the aromatic hydroxyl caused a dramatic switch in functional activity, generating compounds that behaved as TRPV1 antagonists rather than agonists. Since the potency of PhAR was maintained in rat dorsal root ganglion neurons and, particularly, in the rat urinary bladder, this compound was investigated in an in vivo rat model of urinary incontinence and proved as effective as resiniferatoxin at reducing bladder detrusor overactivity.

The "transient receptor potential" (TRP) channels are characterized by six *trans*-membrane domains and by permeability to several cations, including Ca^{2+}. Several members of this large family of plasma membrane channels function as sensors for physical stimuli such as temperature higher or lower than physiological, changes in osmotic pressure, and stretching. Of the vanilloid-type (TRPV) subfamily of TRP receptors (Gunthorpe et al., 2002), TRPV1 and TRPV4 respond to stimulation with natural products, with capsaicin and resiniferatoxin being the best known and most thoroughly studied natural TRPV1 agonists (Sterner and Szallasi, 1999) and 4α-phorbols being capable of activating TRPV4 (Nilius et al., 2004). Another common feature of TRPV1 and TRPV4 is their capability of being gated by endogenous ligands, which are distinct arachidonate derivatives in both cases (Di Marzo et al., 2002a; Nilius et al., 2004). It is now recognized that TRPV1 functions as a molecular

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ABBREVIATIONS: TRP, transient receptor potential; TRPV, vanilloid-type subfamily of TRP receptors; PhAR, phenylacetylrinvanil; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; ESI-MS, electrospray ionization-mass spectrometry; PPAA, propylphosphonic acid anhydride; MCPBA, *meta*-chloroperoxybenzoic acid; DIAD, diisopropylaxodicarboxylate; HEK, human embryonic kidney; AMT, anandamide membrane transporter; DRG, dorsal root ganglia; PBS, phosphate-buffered solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FAAH, fatty acid amide hydrolase.

integrator of nociceptive stimuli, including heat, protons, and plant toxins, and is most abundant in peripheral sensory fibers of the C type. Studies carried out with transgenic mice lacking a functional TRPV1 receptor have implicated this protein in the perception of thermal and inflammatory pain (Caterina et al., 2000; Davis et al., 2000). Other investigations showed that TRPV1 is also involved in inflammatory bowel disorders (Yiangou et al., 2001), neuropathic pain (Walker et al., 2003), and pathological cough (see for review, Chung and Chang, 2002). TRPV1 might also play an important role in physiological conditions, for example, by allowing the bladder to function correctly (Birder et al., 2002). The finding of TRPV1 in several brain nuclei (Mezey et al., 2000), as well as in keratinocytes (Inoue et al., 2002), epithelial cells (Birder et al., 2002), endothelial cells (Yamaji et al., 2003), mast cells (Stander et al., 2004), and astrocytes (Doly et al., 2004), widens considerably its biological importance, suggesting its involvement in the control of several physiological and pathological functions.

The advantages of targeting TRPV1 for therapeutic purposes are highlighted by the recent finding of a significant up-regulation of this protein in several pathological conditions, ranging from pruritus and inflammatory or diabetic neuropathic pain (Rashid et al., 2003; Luo et al., 2004; Stander et al., 2004) to fecal hyperactivity (Chan et al., 2003), vulvodynia (Tympanidis et al., 2004), and cancer of the cervix (Contassot et al., 2004). Indeed, nonpungent synthetic agonists capable of immediately desensitizing TRPV1, and even selectively deleting TRPV1-expressing nociceptors (Karai et al., 2004), can be used against inflammatory hyperalgesia, bladder hyperactivity, emesis, cancer growth, and neuronal excitotoxicity (Szallasi, 2002; Veldhuis et al., 2003). The respiratory side effects of most agonists, however, argue in favor of the use of selective TRPV1 antagonists. For many years, both basic and preclinical studies have relied on olvanil (Brand et al., 1987) as the prototypical nonpungent capsaicinoid with promising analgesic activity and on capsazepine, which remained the only known TRPV1 antagonist for over a decade (Bevan et al., 1992). On the other hand, the most potent TRPV1 agonist discovered so far, resiniferatoxin, is undergoing clinical trials for the treatment of urinary incontinence (Giannantoni et al., 2002; Szallasi and Fowler, 2002) and following the iodination of its vanillyl moiety, led to the most potent TRPV1 antagonist available to date, 5′-iodoresiniferatoxin (Wahl et al., 2001). We have previously reported that iodination of another TRPV1 agonist, nordihydrocapsaicin (Appendino et al., 2003), also yields a potent TRPV1 antagonist and have found that applying this chemical modification to other "capsaicinoids" transforms them into antagonists (G. Appendino and V. Di Marzo, unpublished data). Their potential of being rendered potent antagonists upon iodination of the aromatic ring represents one further reason to develop new potent TRPV1 agonists from long-chain vanillylamides.

In this study, we identified ultra-potent TRPV1 agonists from the progressive derivatization of the fatty acid chain of olvanil. We report the finding of the most potent capsaicinoid TRPV1 agonist ever discovered exhibiting high efficacy in a rat model of urinary incontinence and describe its structure activity relationships and its interactions with proteins of the endocannabinoid system.

Materials and Methods

Synthesis of Compounds

The synthetic procedure for the preparation of acylrinvanils and their iodinated analogs (Fig. 1) is schematized in Scheme 1. The experimental details and the characterization data for the key intermediates and final products were as follows.

Synthesis of *O*-Acylrinvanils. The exemplificative synthesis of phenylacetylrinvanil (PhAR, 1e) is as follows:

2′,2′,2′-Trichloroethylricinoleate: To a solution of ricinoleic acid (3 g, 10.07 mmol) in toluene (30 ml), 2,2,2-trichloroethanol (1.9 ml, 3.0 g, 20.14 mmol, 2 mol Eq), dicyclohexylcarbodiimide (DCC) (2.0 g, 10.07 mmol, 1 mol Eq), and 4-dimethylaminopyridine (DMAP) (1.23 g, 10.07 mmol, 1 mol Eq) were added. After stirring at room temperature for 18 h, the reaction was filtered and the filtrate evaporated. The residue was purified by gravity column chromatography (15 g silica gel, petroleum ether/ethyl acetate 9:1 as eluant) to afford 4.3 g (quantitative) of a colorless viscous oil. IR (KBr) cm^{-1}: 3049, 1758, 1651, 1377, 1098, 808, 721, 569. ^{1}H NMR (300 MHz): δ 5.53 (m, 1H), 5.41 (m, 1H), 4.73 (s, 2H), 3.60 (br t, J = 6.0 Hz, 1H), 2.44 (t, J = 7.4 Hz, 2H), 2.20 (t, J = 6.3 Hz, 2H), 2.03 (m, 2H), ca. 1.68 (m, 2 H), ca. 1.20 (br m, 20H), 0.87 (br t, J = 7.1 Hz, 3H).

12-Phenylacetyl-2′,2′,2′-trichloroethylricinoleate: To a solution of 2′,2′,2′-trichloroethylricinoleate (4.3 g, 10.7 mmol) in toluene (30 ml), phenylacetic acid (3.4 g, 25.2 mmol, 2.5 mol Eq), DCC (5.0 g, 25.2 mmol, 2.5 mol Eq), and DMAP (1.8 g, 15.0 mmol, 1.5 mol Eq) were added. After stirring at room temperature for 30 min, the reaction was worked up by filtration and evaporation. The residue was purified by gravity column chromatography on silica gel (35 g, petroleum ether/ethyl acetate 95:5 as eluant) to afford 5.4 g (quantitative) of a colorless syrup. IR (KBr) cm^{-1}: 3051, 1760, 1736, 1454, 1372, 1260, 1134, 1027. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25 (m, 5H), 5.41 (m, 1H), 5.29 (m, 1H), 4.86 (quint, J = 6.0 Hz, 1H), 4.73 (s, 2H), 3.58 (s, 2H), 2.45 (br t, J = 6.0 Hz, 2H), 2.26 (m, 2H), 1.96 (m, 2H), 1.68 (m, 2 H), 1.51 (m, 2H), ca. 1.29 (br m), ca. 1.21 (br m), 0.86 (br t, J = 7.1 Hz, 3H).

12-Phenylacetylricinoleic acid: To a stirred solution of 12-phenylacetyl-2′,2′,2′-trichloroethylricinoleate (4.6 g, 8.4 mmol) in acetic acid/methanol 1:1 (40 ml), activated zinc powder (4.6 g) was added. After stirring overnight at room temperature, the reaction was worked up by filtration over Celite and washing with sat. $NaHCO_3$. After evaporation of the solvent, the residue was purified by gravity column chromatography on silica gel (60 g, petroleum ether/ethyl acetate 95:5 as eluant) to afford 1.85 g (53%) of a colorless syrup. IR (KBr) cm^{-1}: 3300–2800 (broad), 1731, 1603, 1586, 1497, 1255, 1106, 964, 724. ^{1}H NMR (300 MHz): δ 7.25 (m, 5H), 5.41 (m, 1H), 5.26 (m, 1H), 4.86 (quint, J = 6.0 Hz, 1H), 3.58 (s, 2H), 2.34 (t, J = 6.0 Hz, 2H), 2.28 (br t, J = 6.7 Hz, 2H), 1.97 (m, 2H), 1.62 (m, 2 H), 1.51 (m, 2H), ca. 1.29 (br m), ca. 1.21 (br m), 0.86 (br t, J = 7.1 Hz, 3H). Electrospray ionization-mass spectrometry (ESI-MS): 439 $[M + Na]^+$ $[C_{26}H_{40}O_4 + Na]^+$.

Phenylacetylrinvanil (1e): To a solution of 12-phenylacetylricinoleic acid (1.85 g, 4.4 mmol) in dry dichloromethane (15 ml), vanillamine hydrochloride (835 mg, 4.4 mmol, 2 mol Eq), triethylamine (2.45 ml, 1.78 g, 17.6 mmol, 4 mol Eq), and propylphosphonic acid anhydride (PPAA) (50% in ethanol, 3.4 ml, 1.68 g, 5.28 mmol, 1.2 mol Eq) were added. The reaction was stirred at room temperature for 3 h and then worked up by evaporation. The residue was purified by gravity column chromatography (50 g of silica gel, petroleum ether/ethyl acetate 7:3) to afford 848 mg (35%) of a colorless syrup. $[\alpha]_D^{25}$: +3.2 (methanol, *c* 1.0), IR (KBr) cm^{-1}: 3300–2800 (broad), 3278, 1731, 1515, 1454, 1362, 1261, 1034, 798. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.26 (m, 5 H), 6.85 (d, J = 7.9 Hz, 1H), 6.80 (d, J = 2.0 Hz, 1H), 6.74 (dd, J = 7.9, 2.0 Hz, 1H), 5.69 (br s, 1H), 5.65 (br s, 1H), 5.41 (m, 1H), 5.27 (m, 1H), 4.85 (quint, J = 6.0 Hz, 1H), 4.34 (d, J = 5.8 Hz, 2H), 3.86 (s, 3H), 3.57 (s, 2H), 2.25 (m, 2H), 2.17 (t, J = 7.4 Hz), 1.95 (m, 2H), 1.63 (m, 2 H), 1.50 (m, 2H), 1.27 (br m), 1.20 (br m), 0.85 (br t,

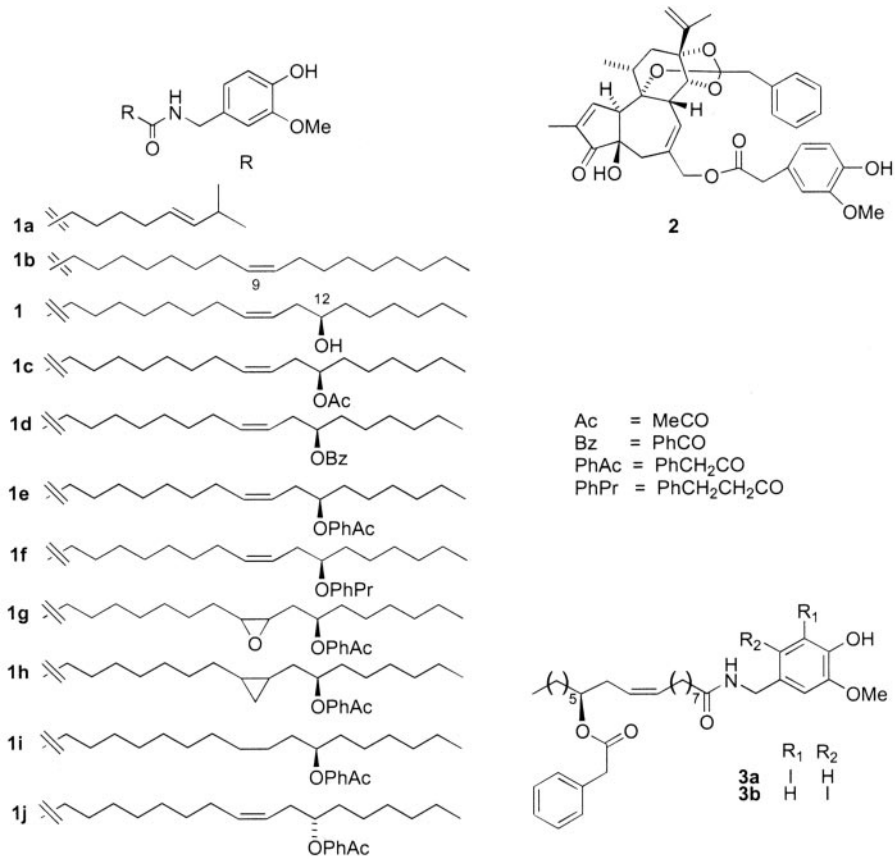


Fig. 1. Structure of capsaicin (CPS, 1a), olvanil (1b), rinvanil (1), resiniferatoxin (2), PhAR (1e), and the other compounds synthesized in this study.

J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.0 (s), 173.5 (s), 171.7 (s), 156.6 (s), 147.2 (s), 145.4 (s), 141.6 (d), 136.8 (s), 132.7 (s), 130.2 (d), 129. 5 (d), 129.3 (d), 128.5 (d), 128.3 (d), 127.5 (d), 127.0 (d), 126.2 (d), 124.1 (d), 120.6 (d), 114.9 (d), 110.9 (d), 106.1 (d), 74.6 (d), 56.3 (q), 43.8 (t), 43.4 (t), 41.9 (t), 36.6 (t), 31.8 (t), 29.1 (t), 27.2 (t), 25.9 (t), 25.3 (t), 22.6 (t), 13.9 (q). ESI-MS: 574 $[M + Na]^+$ $[C_{34}H_{49}NO_5 + Na]^+$.

Chemical Modification of Phenylacetylrinvanil

Epoxyphenylacetylrinvanil (1g): To a solution of phenylacetylrinvanil (400 mg, 0.72 mmol) in dichloromethane (10 ml), *meta*-chloroperoxybenzoic acid (MCPBA) (75%, 318 mg, 1.8 mmol, 2.5 mol Eq) was added. After stirring at room temperature overnight, the reaction was worked up by dilution and washed with 5% $Na_2S_2O_3$ and 5% NaOH. After removal of the solvent, the residue was purified by gravity column chromatography (10 g silica gel, petroleum ether/ethyl acetate 8:2 as eluant) to afford 151 mg (35%) of 1g as a colorless syrup. IR (KBr) cm^{-1}: 3150, 1732, 1654, 1513, 1454, 1271, 1125, 1033, 723. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.26 (m, 5H), 6.87 (d, J = 7.9 Hz, 1H), 6.82 (br s, 1H), 6.78 (br d, J = 7.9 Hz, 1H), 5.69 (br s, 1H), 5.64 (br s, 1H), 5.05 (quint, J = 6.0 Hz, 1H), 4.37 (d, J = 5.8 Hz, 2H), 3.89 (s, 3H), 3.66 (m, 2H), 2.90 (m, 1H), 2.84 (m, 1H), 2.20 (t, J = 7.4 Hz, 2H), ca. 1.76 (m), 1.44 (m), 1.27 (br m), 1.20 (br m), 0.88 (br t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.0 (s), 171.4 (s), 146.8 (s), 145.2 (s), 134.2 (s), 130.4 (s), 129.3 (d), 128.6 (d), 128.3 (d), 127.3 (d), 127.2 (d), 120.9 (d), 114.5 (d), 110.8 (d), 73.0 (d), 57.0, 56.4 (d), 56.0 (t), 53.6, 53.0 (d), 43.6 (t), 41.8 (t), 36.8 (t), 31.7 (t), 29.4 (t), 29.2 (t), 27.5 (t), 25.8 (t), 25.3 (t), 22.6 (t), 14.2 (q). ESI-MS: 590 $[M + Na]^+$ $[C_{33}H_{47}NO_6 + Na]^+$.

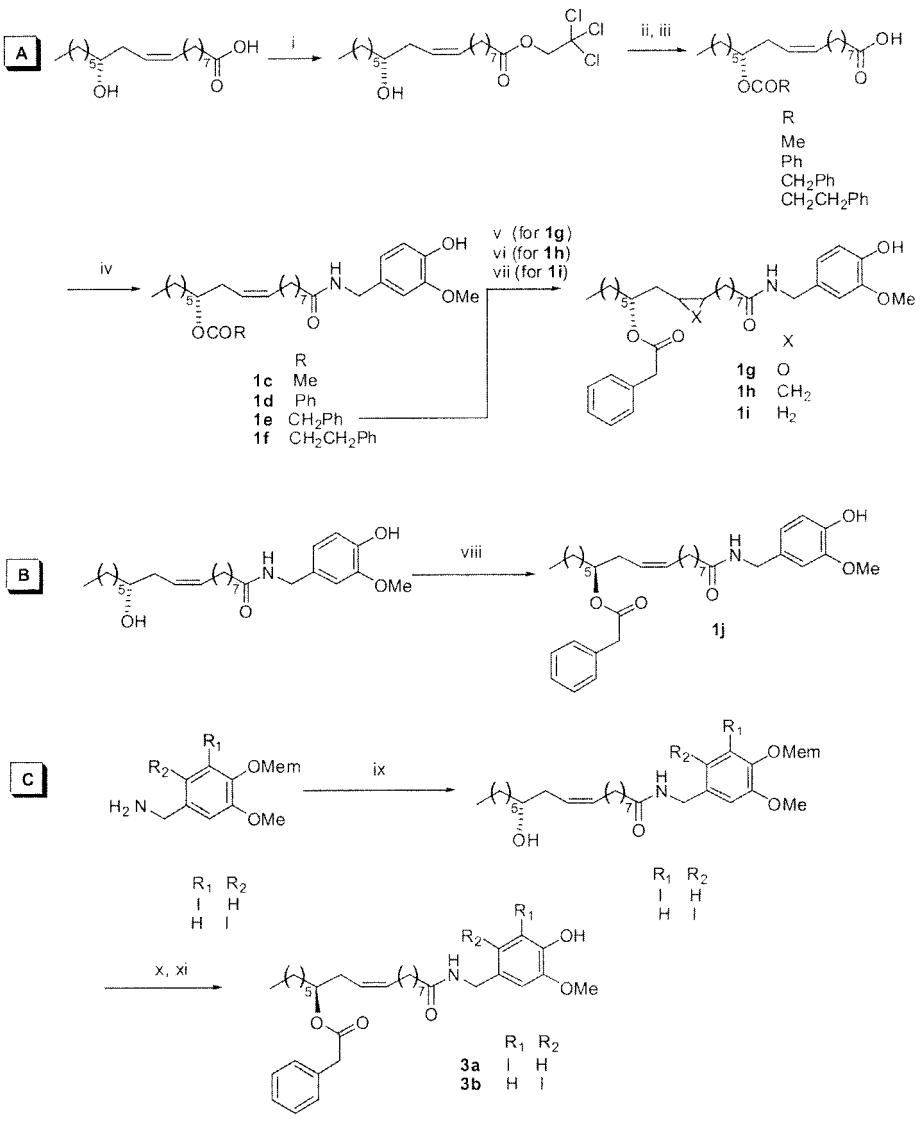
Methylenphenylacetylrinvanil (1h): To a solution of phenylacetylrinvanil (380 mg, 0.69 mmol) in dry toluene (29 ml), diethylzinc (1.0 M in hexanes, 10.35 mmol, 10.35 ml, 15 mol Eq) and diiodomethane (0.837 ml, 2.78 g, 10.35 mmol, 15 mol Eq) were added. The solution was stirred at 65°C for 7 h and then worked up by cooling to 0°C and the addition of 2 N H_2SO_4. The mixture was extracted with ethyl acetate and the organic phase was washed with sat. $NaHCO_3$ and brine. After removal of the solvent, the residue was purified by gravity column chromatography (15 ml silica gel, petroleum ether/ethyl acetate 6:4 as eluant) to afford 149 mg (40%) of 1h as a colorless syrup. IR (KBr) cm^{-1}: 3150, 1731, 1651, 1515, 1274, 1157, 1033, 818, 721. ^{1}H NMR (300 MHz): δ 7.26 (m, 5H), 6.86 (d, J = 7.9 Hz, 1H), 6.80 (d, J = 2.0 Hz, 1H), 6.74 (dd, J = 7.9, 2.0 Hz, 1H), 5.70 (br s, 1H), 5.65 (br s, 1H), 4.95 (quint, J = 6.0 Hz, 1H), 4.33 (d, J = 5.8 Hz, 2H), 3.86 (s, 3H), 3.60 (s, 2H), 2.17 (t, J = 7.4 Hz), 1.63–1.52 (m, 6H), 1.27 (br m), 0.84 (br t, J = 7.1 Hz, 3H), 0.58 (m, 3H), −0.30 (m, 1H). ESI-MS: 586 $[M + Na]^+$ $[C_{35}H_{51}NO_5]$.

Dihydrophenylacetylrinvanil (1i): To a solution of phenylacetylrinvanil (300 mg, 0.54 mmol) in methanol (2 ml), Pd(C) was added (ca. 5 mg), and the stirred solution was hydrogenated (balloon pressure). After 12 h, the reaction was worked up by filtration over Celite and evaporation to afford 196 mg (quantitative) of 1i as a colorless syrup. IR (KBr) cm^{-1}: 3273, 1731, 1651, 1372, 1273, 1074, 1034, 721. ^{1}H NMR (300 MHz): δ 7.27 (m, 5H), 6.86 (d, J = 7.9 Hz, 1H), 6.84 (d, J = 2.0 Hz, 1H), 6.80 (dd, J = 7.9, 2.0 Hz, 1H), 5.67 (br s, 1H), 4.85 (q, J = 6 Hz), 4.35 (d, J = 5.8 Hz, 2H), 3.86 (s, 3H), 3.57 (s, 2H), 2.18 (t, J = 7.4 Hz), 1.63–1.52 (m, 6H), 1.27 (br m), 0.80 (br t, J = 7.1 Hz, 3H). ESI-MS: 576 $[M + Na]^+$ $[C_{34}H_{51}NO_5]$.

ent-*Phenylacetylrinvanil (1j):* To a solution of rinvanil (300 mg, 0.69 mmol) in tetrahydrofuran (10 ml), phenylacetic acid (90 mg, 0.69 mmol), 180 mg of triphenylphosphine (0.69 mmol, 1 mol Eq), and DIAD (0.136 ml, 140 mg, 0.69 mmol, 1 mol Eq) were sequentially added. After stirring at room temperature for 2 days, the reaction was worked up by evaporation. Toluene (ca. 5 ml) was then added, and the solution was stored overnight at −18°C. After filtration, the solution was evaporated and purified by gravity column chromatography (10 g of silica gel, petroleum ether/ethyl acetate 7:3 as eluant) to afford 124 mg (32%) 1i. $[\alpha]_D^{25}$: −3.0 (methanol, *c* 1.2).

Synthesis of 5′- and 6′-Iodophenylacetylrinvanyl (3a and 3b). The exemplicative synthesis of 3b is as follows:

O-*Mem-6′-iodorinvanil:* To a solution of ricinoleic acid (666 g, 1.46 mmol) in dry dichloromethane (5 ml), 6-iodovanillamine (1.07 g, 2.9



Scheme 1. General synthetic scheme for the synthesis of the acylrinvanils 1c–f and 1g–i (A), 1j (B), and 3a,b (C). Reagents and conditions: i, trichloroethanol, DCC, DMAP, quantitative. ii, RCOOH, DCC, DMAP, quantitative. iii, Zn, acetic acid, methanol, 50–60%. iv, vanillamine, PPAA, triethylamine, 30–40%. v, MCPBA, dichloromethane, 35%. vi, Et_2Zn, CH_2I_2, 40%. vii, H_2, Pd(C), quantitative. viii, DIAD, triphenylphosphine, $PhCH_2COOH$, 32%. ix, PPAA, triethylamine, 30–40%. x, $PhCH_2COCl$, pyridine, 60–70%. xi, $SnCl_4$, tetrahydrofuran, 75–80%.

mmol, 2 mol Eq), triethylamine (0.816 ml, 0.59 g, 5.9 mmol, 4 mol Eq), and PPAA (50% in ethanol, 1.1 ml, 0.56 g, 1.76 mmol, 1.2 mol Eq) were added. The reaction was stirred at room temperature for 3 h and then worked up by evaporation. The residue was purified by gravity column chromatography (15 g of silica gel, petroleum ether/ethyl acetate 7:3 as eluant) to afford 331 mg (35%) of a colorless oil. IR (KBr) cm^{-1}: 3305, 1641, 1543, 1260, 999, 723, 694. ^1H NMR (300 MHz, $CDCl_3$): δ 7.63 (s, 1H), 6.97 (s, 1H), 5.99 (br t, J = 5.6 Hz, 1H), 5.54 (m, 1H), 5.39 (m, 1H), 5.27 (s, 2H), 4.40 (d, J = 5.1 Hz, 2H), 3.83 (s, 3H), 3.77 (m, 2H), 3.61 (quint, J = 6.0 Hz, 1H), 3.57 (m, 2H), 3.39 (s, 3H), 2.33 (m, 2H), 2.19 (t, J = 7.6 Hz), 1.99 (m, 2H), 1.67 (m, 2 H), 1.46 (m, 2H), 1.27 (br m), 1.20 (br m), 0.87 (br t, J = 7.1 Hz, 3H). Chemical ionization-mass spectrometry: 648 $[M + H]^+$ $[C_{30}H_{50}INO_6 + H]^+$.

6′-Iodophenylacetylrinvanil (3b): To a solution of *O*-mem-6′-iodorinvanil (383 mg, 0.5 mmol) in pyridine (5 ml), phenylacetylchloride (0.33 ml, 156 mg, 1 mmol, 2 mol Eq) was added. After stirring overnight at room temperature, the reaction was worked up by the addition of water/methanol 9:1 (5 ml) and extraction with ethyl acetate. The organic phase was sequentially washed with 2N H_2SO_4, sat. $NaHCO_3$ and brine and evaporated. The residue was purified by gravity column chromatography (10 silica gel, petroleum ether/ethyl acetate 7:3 as eluant) to afford 239 mg of mem-protected 3b (70%) as a gum. The latter was dissolved in dry tetrahydrofuran (5 ml) and treated with $SnCl_4$ (5 drops). After stirring at room temperature for 48 h, the reaction was worked up by dilution with ethyl acetate and washed with sat. $NaHCO_3$. After removal of the solvent, the residue was purified by gravity column chromatography (10 g silica gel, petroleum ether/ethyl acetate 7:3 as eluant) to afford 190 mg (80%) of 3b as a colorless oil. IR (KBr) cm^{-1}: 3240, 1728, 1669, 1598, 1497, 1440, 1376, 1256. ^1H NMR (300 MHz, $CDCl_3$): δ 7.34 (s, 1H), 7.28 (m, 5H), 6.94 (s, 1H), 6.05 (br t, J = 5.9 Hz, 1H), 5.40 (m, 1H), 5.30 (m, 1H), 4.87 (quint, J = 6.0 Hz, 1H), 4.39 (d, J = 5.8 Hz, 2H), 3.86 (s, 3H), 3.59 (br s, 2H), 2.28 (m, 2H), 2.19 (t, J = 7.4 H, 2H), 1.975 (m, 2H), 1.60 (m, 2 H), 1.53 (m, 2H), 1.27 (br m), 1.20 (br m), 0.87 (br t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.9 (s), 171.3 (s), 147.0 (s), 145.7 (s), 134.2 (s), 133.08 (d), 132.5 (s), 129.6 (s), 129.5 (d), 129.1 (d), 129.4 (d), 124.9 (d), 124.6 (d), 120.1 (d), 112.6 (d), 87.6 (s), 74.4 (d), 55.9 (d), 47.7 (t), 47.6 (t), 41.6 (t), 36.6 (t), 33.4 (t), 31.8 (t), 31.5 (t), 29.6 (t), 29.1 (t), 29.0 (t), 28.9 (t), 28.9 (t), 27.1 (t), 25.5 (t), 25.1 (t), 22.45 (t), 14.0 (t). Chemical ionization-mass spectrometry: 678 $[M + H]^+$ $[C_{34}H_{48}INO_5 + H]^+$.

TRPV1 Assays

Human embryonic kidney (HEK)-293 cells overexpressing the human TRPV1 were kindly donated by GlaxoSmithKline (Uxbridge, Middlesex, UK). The pharmacological responses of this cell line to vanilloid agonists and antagonists are very similar to those previously described by Witte et al. (2002) for the same cell line overex-

pressing the human TRPV1. 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%:5% O_2/CO_2 at 37°C. The effect of the substances on $[Ca^{2+}]_i$ was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+} (Di Marzo et al., 2002b). One day prior to experiments, cells were transferred into six-well dishes coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and grown in the culture medium mentioned above. On the day of the experiment, the cells (50–60,000 per well) were loaded for 2 h at 25°C with 4 μM Fluo-3 methylester (Molecular Probes, Eugene, OR) in dimethyl sulfoxide containing 0.04% pluoronic. After the loading, cells were washed with Tyrode's solution pH = 7.4, trypsinized, resuspended in Tyrode, and transferred to the cuvette of the fluorescence detector (PerkinElmer LS50B; PerkinElmer Life and Analytical Sciences, Boston, MA) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25°C (λ_{EX} = 488 nm, λ_{EM} = 540 nm) before and after the addition of the test compounds at various concentrations. In antagonism experiments, varying doses of PhAR were added 10 min prior to capsaicin (100 nM). Data were expressed for the agonists as the concentration exerting a half-maximal effect (EC_{50}) and for the antagonists as the concentration exerting a half-maximal inhibition (IC_{50}), both calculated by GraphPad. The efficacy of the agonists was determined by comparing it to the analogous effect observed with 4 μM ionomycin.

Assays for the Interactions with Proteins of the Endocannabinoid System

Binding Assays. For CB_1 and CB_2 receptor binding, [^{3}H]-(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol (CP-55,940) (K_d = 690 pM) was incubated with P_2 membranes from COS cells transfected with either the human CB_1 or CB_2 receptor as described by the manufacturer (PerkinElmer Life and Analytical Sciences). Displacement curves were generated by incubating drugs with 0.5 nM [^{3}H]CP-55,940. In all cases, K_i values were calculated by applying the Cheng-Prusoff equation to the IC_{50} values (obtained by GraphPad Software Inc., San Diego, CA) for the displacement of the bound radioligand by increasing concentrations of the test compounds.

Fatty Acid Amide Hydrolase Assays. The effect of compounds on the enzymatic hydrolysis of [^{14}C]anandamide (6 μM) was studied by using membranes prepared from rat brain incubated with increasing concentrations of compounds in 50 mM Tris-HCl, pH 9, for 30 min at 37°C (Di Marzo et al., 2002b). [^{14}C]Ethanolamine produced from [^{14}C]anandamide hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ (2:1 by volume).

Anandamide Membrane Transporter (AMT) Assays. The effect of compounds on the uptake of anandamide by RBL-2H3 cells was studied as described previously (Di Marzo et al., 2002b). Cells were incubated with [^{14}C]anandamide (4 μM) for 5 min at 37°C in the presence or absence of varying concentrations of the inhibitors. Residual [^{14}C]anandamide in the incubation media after extraction with $CHCl_3/CH_3OH$ (2:1 by volume) was used as a measure of the anandamide that was taken up by cells. Data are expressed as the concentration exerting 50% inhibition of anandamide uptake (IC_{50}) calculated with GraphPad Software Inc.

Assays on Dorsal Root Ganglion Neurons

Newborn rats (2–3 days old) were terminally anesthetized and decapitated. The dorsal root ganglia (DRG) were removed and rapidly placed in cold phosphate-buffered solution (PBS) before being transferred to collagenase/dispase (1 mg ml^{-1} dissolved in Ca^{2+}-Mg^{2+}-free PBS) for 35 min at 37°C. Enrichment of the fraction of nociceptive neurons was obtained following the methods reported previously (Gilabert and McNaughton, 1997). After the enzymatic treatment, ganglia were rinsed three times with Ca^{2+}-Mg^{2+}-free PBS and then placed in 2 ml of cold Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, heat inactivated), 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. The ganglia were then dissociated into single cells by several passages through a series of syringe needles (23G down to 25G). Finally, the complex of medium and ganglia cells were sieved through a 40-μm filter to remove debris and topped off with 8 ml of DMEM and centrifuged (200*g* for 5 min). The final cell pellet was resuspended in DMEM [supplemented with 100 ng ml^{-1} mouse nerve growth factor (mouse-NGF-7S) and cytosine-b-D-arabinofuranoside free base (ARA-C) 2.5 μM]. Cells were plated on poly-L-lysine (8.3 μM)- and laminin (5 μM)-coated 25-mm glass coverslips and kept for 2 to 5 days at 37°C in a humidified incubator gassed with 5% CO_2 and air. Cells were fed on the second day (and subsequent alternate days) with DMEM (with 1% instead of 10% FBS). Experiments were performed as previously reported (Tognetto et al., 2001). Briefly, plated neurons (2 to 5 days) were loaded with Fura-2/acetoxymethyl ester (3 μM) in Ca^{2+} buffer solution with the following composition: 1.4 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM $MgSO_4$, 135 mM NaCl, 5 mM D-glucose, and 10 mM HEPES with 0.1% bovine serum albumin at pH 7.4 for 40 min at 37°C, washed twice with the Ca^{2+} buffer solution, and transferred to a chamber on the stage of Nikon eclipse TE300 microscope. The dye was excited at 340 and 380 nm to indicate relative $[Ca^{2+}]_i$ changes by the F_{340}/F_{380} ratio recorded with a dynamic image analysis system (Laboratory Automation 2.0; RCS, Florence, Italy). The effect of PhAR (1 pM–100 μM) was studied in the presence or absence of the TRPV1 antagonist, 5′-iodoresiniferatoxin (10 nM). In antagonism studies, capsaicin (0.1 μM) was used as the agonist, added 10 min after increasing concentrations of the antagonists.

Rat Urinary Bladder Assay

Rats were sacrificed by cervical dislocation, and the urinary bladder was removed. Strips of urinary bladders (approximately 1 cm in length) were suspended in an organ bath with a resting tension of 1 g, bathed (37°C), and aerated (95% O_2 and 5% CO_2) with Krebs' solution with the following composition: 119 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.5 mM $MgSO_4$, 2.5 mM $CaCl_2$, 4.7 mM KCl, 11 mM D-glucose, 0.001 mM phosphoramidon, and 0.001 mM captopril. Tissues were allowed to equilibrate for 60 min prior to the beginning of each set of experiments (washed every 5 min). In all experiments, tissues were first contracted with carbachol (1 μM), then after another 60 min, cumulative concentration-response curves with PhAR (0.1 pM–1 μM) were performed. The effects of PhAR were also tested in the presence of 5′-iodoresiniferatoxin (10 nM) or its respective vehicles.

Rat Urinary Incontinence Assay

Female Sprague-Dawley rats (200–225 g) were used in this investigation. Procedures involving animals and their care were conducted in compliance with local institutional guidelines from the University of Milan. Animals were anesthetized with ketamine (40 mg/kg) and xylazine (20 mg/kg). The urethra was exposed by a low midline abdominal incision. According to Steers and De Groat (1988), a double-silk (4.0) ligature was placed around the urethra and tied in the presence of an extraluminally placed (1-mm o.d.) polyethylene tubing which was then removed. Sham-operated rats underwent urethral manipulation with no urethral ligature. Six to eight weeks from partial urethral ligature, rats were anesthetized with urethane (1.1 g/kg i.p.), and they underwent anesthetized cystometry. The bladder was exposed by a low midline abdominal incision, the intravesical urine was removed, and its volume was measured. Then, a double-lumen catheter was inserted into the bladder. The outside catheter (0.8-mm o.d.) was connected to a pressure transducer (Gould P23 ID), and the intravesical pressure was recorded continuously on an ink-write two-channel Gemini7070 (Basile, Comerio, Italy). The inner catheter (0.5-mm o.d.) was connected to a peristaltic

pump Peri-Star (World Precision Instruments, New Haven, CT), and saline at 37°C was infused at an infusion rate set to 0.11 ml/min for control and operated animals. Distal urethra was catheterized with a polyethylene tube to collect and measure the volume of fluid during each voiding phase. Bladder outflow obstruction was successfully produced 6 to 8 weeks from the surgical operation in about 70% of the animals, and about 40% of these animals also showed detrusor overactivity. Acute retention was probably the cause of death in 10% of animals during the first week after operation. The residual 20% of animals showed abnormal cystometry and were not included in the experiments. The cystometric parameters evaluated in anesthetized rats were: resting pressure, pressure threshold, maximal amplitude of contraction, micturition volume, residual volume, bladder capacity, and frequency of micturition. The effect of PhAR (10–100 nM) and that of the reference compound resiniferatoxin (10–100 nM) on cystometric parameters was studied after bladder instillation for 30 min. Both drugs were solubilized in 100% ethanol, and dilutions (10, 50, and 100 nM) were obtained in 1% ethanol. At this concentration, ethanol did not alter cystometric parameters.

Results

Functional Activity of the Novel Compounds at Human TRPV1 Receptors in HEK-293 Cells. We first assessed the activity at the human TRPV1 of the vanyllamide of ricinoleic acid by using a typical functional assay of TRPV1 activity, the enhancement of intracellular Ca^{2+} in HEK-293 cells overexpressing the human receptor. Rinvanil, as we named this new vanyllamide (Fig. 1), exhibited less potency, albeit higher efficacy, than olvanil (Table 1). The activity of rinvanil could be modulated by acylation of the hydroxyl group on the fatty acid moiety. Thus, acetylation afforded a compound with vanilloid potency comparable with that of olvanil (Table 1). Acylation of rinvanil with benzoic-, phenylacetic-, and β-phenylpropionic acid provided compounds that exhibited significantly higher potency and efficacy than olvanil and rinvanil (Figs. 1–2, Table 1). In particular, PhAR is the first compound structurally unrelated to resiniferatoxin that shows efficacy comparable with the natural product in assays of vanilloid activity with potency, although 8-fold lower, in the same picomolar EC_{50} range (Table 1). Epoxydation, cyclopropanation, and hydrogenation of the *cis*-9,10 double bond did not ameliorate the potency and efficacy of PhAR, and neither did the inversion of its configuration at the only chiral carbon atom to *ent*-PhAR (Table 1). None of the compounds exerted any notable activity in wild-type, nontransfected HEK-293 cells (data not shown).

Functional Activity of PhAR at Native Rat TRPV1 Channels. PhAR was also extremely potent at the native rat TRPV1 by elevating Ca^{2+} in neurons from rat dorsal root ganglia in a way sensitive to the highly selective TRPV1 antagonist 5′-iodoresiniferatoxin and with an EC_{50} = 11.3 ± 0.15 nM (Fig. 3a) similar to that of resiniferatoxin (6.20 ± 0.05 nM) when using a Ca^{2+} imaging assay in intact cultured neurons. The potency of PhAR and resiniferatoxin in this assay was lower than that using recombinant human TRPV1 overexpressed in HEK-293 cells, but nevertheless both compounds were extremely powerful in contracting the rat urinary bladder with EC_{50} = 0.55 ± 0.19 and 0.17 ± 0.02 nM, respectively (Fig. 3b and data not shown).

Activity of PhAR and Its Analogs on Proteins of the Endocannabinoid System. *N*-Acyl-vanyllamides were reported to interact with proteins of the endocannabinoid system with low to moderate affinity, for cannabinoid CB_1 receptors, or as inhibitors of the activity of the proteins mediating endocannabinoid degradation, the fatty acid amide hydrolase (FAAH), and the putative AMT (Di Marzo et al., 2002b). Therefore, we decided to test the activity of PhAR on recombinant human CB_1 and CB_2 receptors as well as on [^{14}C]anandamide cellular uptake in RBL-2H3 cells and hydrolysis by rat brain membranes. As shown in Table 1, PhAR was inactive on FAAH up to 50 μM, although it significantly interacted with human CB_2 receptors in a typical binding assay (K_i = 300 nM) and was weakly active at human CB_1 receptors (K_i = 2.2 μM) and on the AMT (K_i = 12.2 μM). The other compounds did not exert any strong activity in any of the assays.

Epoxydation, cyclopropanation, and hydrogenation of the *cis*-9,10 double bond of PhAR significantly decreased its af-

TABLE 1

Summary of the pharmacological effects of the novel compounds developed in this study

Data on the maximal effect at TRPV1 are expressed as percentage of the maximal observable effect induced by 4 μM ionomycin. For a comparison, the EC_{50} of resiniferatoxin tested side-by-side and under the same conditions was 0.011 ± 0.002 nM, whereas the IC_{50} (nM) of other TRPV1 antagonists tested side-by-side and under the same conditions are: 5′-I-resiniferatoxin, 0.6 ± 0.2 nM; SB-366791, 32.0 ± 5.1 nM; capsazepine, 50.0 ± 0.6 nM.

	K_i				TRPV1	
	Rat		Human			
	AMT	FAAH	CB_1	CB_2	EC_{50} (IC_{50} against capsaicin, 100 nM)	Percentage of Maximum Effect
	μM				*nM*	
Capsaicin (1a)	>25	>50	>10	>10	40 ± 0.3	66.2 ± 4.1
Olvanil (1b)	7.3 ± 1.6	>50	>10	>10	0.7 ± 0.05	67.2 ± 6.2
Rinvanil (1)	>25	>50	>10	>10	6.0 ± 0.3	85.0 ± 4.9
Acetylrinvanil (1c)	>25	>50	>10	>10	0.50 ± 0.07	93.5 ± 5.9
PhAR (1e)	12.2 ± 2.3	>50	2.2 ± 0.3	0.3 ± 0.02	0.09 ± 0.02	98.2 ± 2.1
ent-PhAR (1j)	7.4 ± 0.4	>50	2.4 ± 0.4	0.4 ± 0.01	0.10 ± 0.02	95.2 ± 2.3
Benzoylrinvanil (1d)	10.2 ± 1.4	24.3 ± 2.2	1.3 ± 0.2	1.4 ± 0.3	0.13 ± 0.02	86.1 ± 2.2
Phenylpropionylrinvanil (1f)	NT	>50	NT	NT	0.20 ± 0.02	98.4 ± 2.7
Epoxy-PhAR (1g)	>25	>50	9.5 ± 1.1	6.0 ± 0.8	5.0 ± 0.2	71.3 ± 3.2
Cycloproyl-PhAR (1 h)	15.9 ± 3.6	>50	5.6 ± 0.8	1.4 ± 0.2	0.13 ± 0.02	80.1 ± 2.3
Dihydro-PhAR (1i)	9.4 ± 1.3	>50	>5	1.1 ± 0.1	0.59 ± 0.03	72.2 ± 3.5
6-I-PhAR (3b)	17.6 ± 2.6	>50	2.5 ± 0.4	2.2 ± 0.2	>10 μM (6.0 ± 0.5)	ND
5-I-PhAR (3a)	9.1 ± 1.3	>50	>5	1.5 ± 0.2	>10 μM (900 ± 97)	ND

NT, not tested; ND, not determinable; SB-366791, *N*-(3-methoxyphenyl)-4-chlorocinnamide.

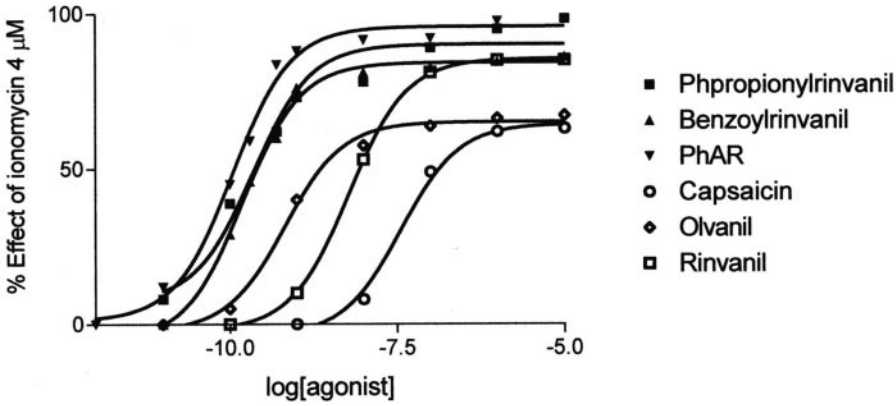


Fig. 2. Activity of some of the compounds synthesized in this study on human recombinant TRPV1 channels. Activity was measured as the capability of increasing the intracellular Ca^{2+} concentration in HEK-293 cells overexpressing the human TRPV1 and is expressed as percentage of the maximal observable effect induced by 4 μM ionomycin. Data are the means of $n = 3$ separate experiments, and the standard error bars (never higher than 10% of the means) are not shown for the sake of clarity. No effect was observed in wild-type HEK-293 cells. Phpropionylrinvanil, phenylpropionylrinvanil.

finity for both cannabinoid receptor subtypes (Table 1). Inversion of configuration to *ent*-PhAR, however, improved only the inhibitory activity on AMT and did not modify the affinity for either cannabinoid receptor subtype (Table 1).

Activity of PhAR in a Rat Model of Urinary Incontinence. We used anesthetized rats with ligature-intact partial urethral obstruction as a well established animal model of this disorder. Figure 4a shows a typical cystometrogram obtained in an anesthetized control rat; the infusion rate of 0.11 ml · min^{-1} induced a slowly developing increase in bladder pressure. Cystometrograms obtained in anesthetized rats with partial urethral obstruction (6–8 weeks) showed a more pronounced spontaneous activity than those obtained in control rats during the filling phase (Fig. 4b). Bladder capacity increased from 0.91 ± 0.05 in controls to 4.45 ± 0.39 ml in obstructed rats. Residual volume increased from 0.23 ± 0.04 to 3.95 ± 0.48 ml in obstructed rats. Micturition volume slightly decreased from 0.67 ± 0.05 ml in controls to 0.49 ± 0.07 ml in obstructed rats, and frequency of micturition increased from 15.3 ± 0.68 to 30.7 ± 3.23 h^{-1} in obstructed rats. Bladder instillation with either PhAR (10–50 and 100 nM) or resiniferatoxin (10–50 nM) inhibited detrusor overactivity, restored the resting pressure, slightly increased the micturition volume compared with obstructed rats, and significantly decreased the frequency of micturition (Fig. 4, c–e). Resiniferatoxin (100 nM) instilled in the bladder for 30 min blocked micturition reflexes in three of six obstructed rats.

Iodination of PhAR Leads to TRPV1 Antagonists. Iodination of the 5′ or 6′ carbon atoms of the vanillyl moiety of PhAR totally abolished its agonist activity at the human TRPV1 in HEK-293 cells and, in the case of 6′-iodo-PhAR, led to a potent TRPV1 antagonist with a IC_{50} = 6 nM, which is 10-fold higher than that of the most potent TRPV1 antagonist developed to date, 5′-iodoresiniferatoxin (Table 1, Fig. 5a). The compound behaved as a noncompetitive antagonist (K_d = 0.54 nM, Schild plot slope = 0.62 ± 0.06) (Fig. 5b). The 6′-iodo-PhAR was also tested on the effect of capsaicin (100 nM) on cytoplasmatic Ca^{2+} concentration in cultured rat DRG neurons (Fig. 5c). Similar to the shift observed for the agonist (i.e., PhAR), the IC_{50} for 6′-iodo-PhAR against capsaicin (747 nM) was 125-fold higher in rat DRG neurons compared with transfected HEK-293 cells. 5′-Iodoresiniferatoxin retained its potency between the two assays (IC_{50}, 0.87 ± 0.01 nM in DRG neurons), whereas capsazepine was 3-fold less potent than 6′-iodo-PhAR (Appendino et al., 2003).

Discussion

We have used a medicinal chemistry approach to generate novel and ultra-potent TRPV1 agonists suitable for use in vitro and in vivo and with a high potential for therapeutic use

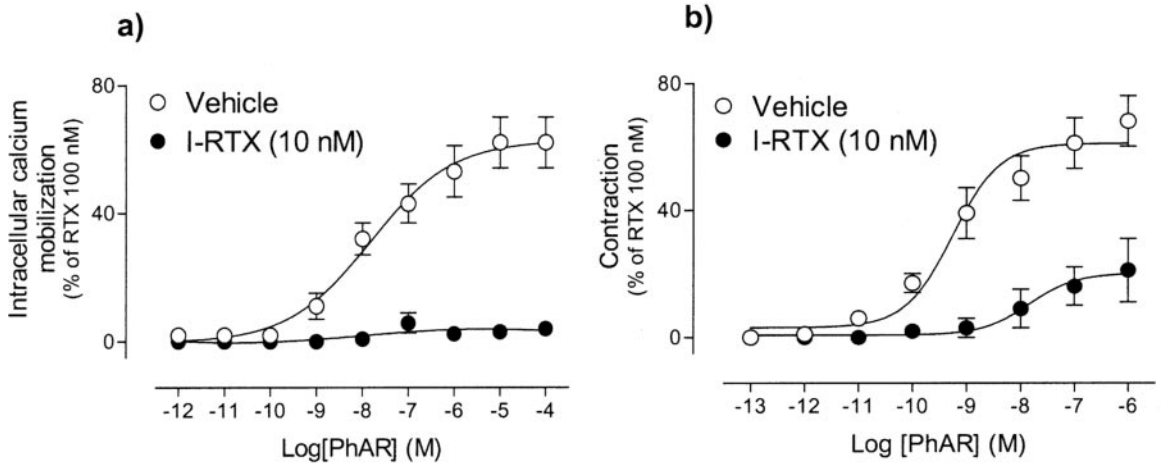


Fig. 3. Effects of PhAR (IDN5890, ○) on cytoplasmatic Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured rat DRG neurons (a) and isolated strips of rat urinary bladder (b). In both models, the TRPV1 antagonist, 5′-iodoresiniferatoxin (I-RTX) (10 nM, ●), significantly reduced the effect of PhAR demonstrating its selective agonistic activity at the capsaicin receptor. PhAR-induced contractions and $[Ca^{2+}]_i$ mobilization were expressed as percentage of the maximal effect elicited by 100 nM resiniferatoxin. Data are means ± S.E.M. of at least six experiments.

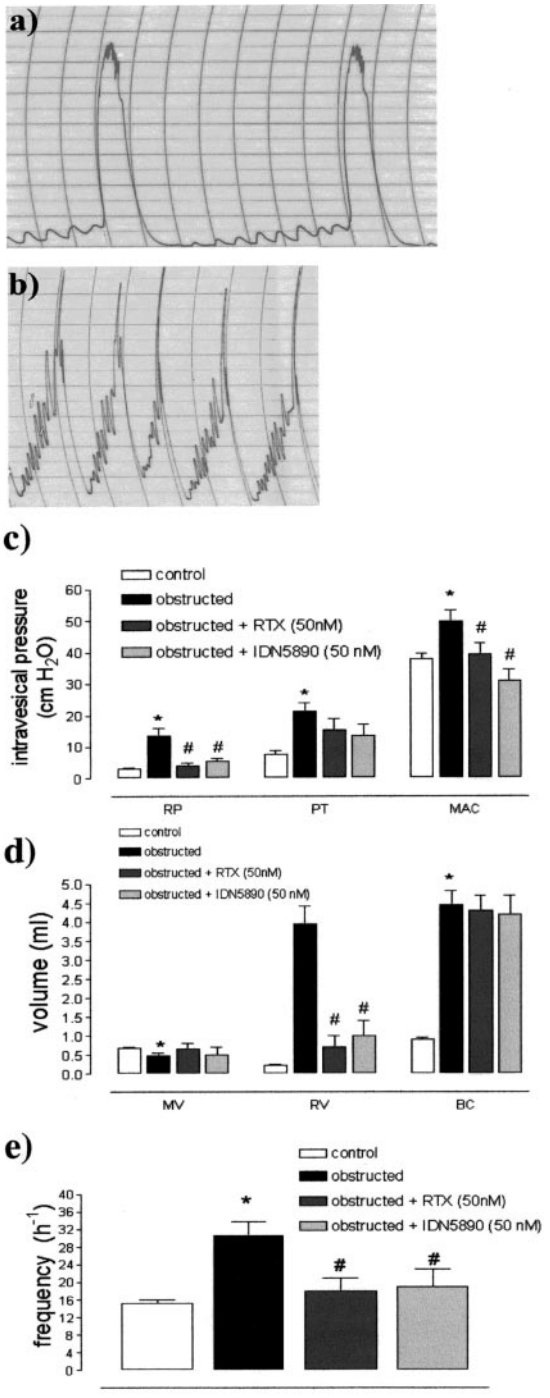


Fig. 4. Original tracing showing a cystometry from control rat (a), and from rat with partial urethral obstruction (8 weeks) (b). Six to eight weeks after surgical operation, rat and bladder mean weights were 295 ± 13 versus 293 ± 8 g and 102 ± 9 versus 462 ± 93 mg in sham-operated (control) and in partial obstructed rats, respectively. In rats with outflow obstruction, spontaneous bladder contractions (detrusor overactivity) were often observed during the filling phase (infusion rate of 0.11 ml min^{-1}). c, d, and e, collected data obtained in anesthetized controls (n = 10), obstructed (n = 10), obstructed after bladder instillation of PhAR (IDN5890, 50 nM) (n = 8), or resiniferatoxin (RTX) (50 nM) (n = 8). The two drugs were also tested at 10 and 100 nM and found not to be significantly different from "obstructed" and as active as the 50 nM concentration, respectively (not shown). Statistical significance (one-way ANOVA with Tukey post hoc test, $p < 0.05$): ∗, versus control; #, versus obstructed. RP, resting pressure; PT, pressure threshold; MAC, maximal amplitude of contraction; MV, micturition volume; RV, residual volume; BC, bladder capacity.

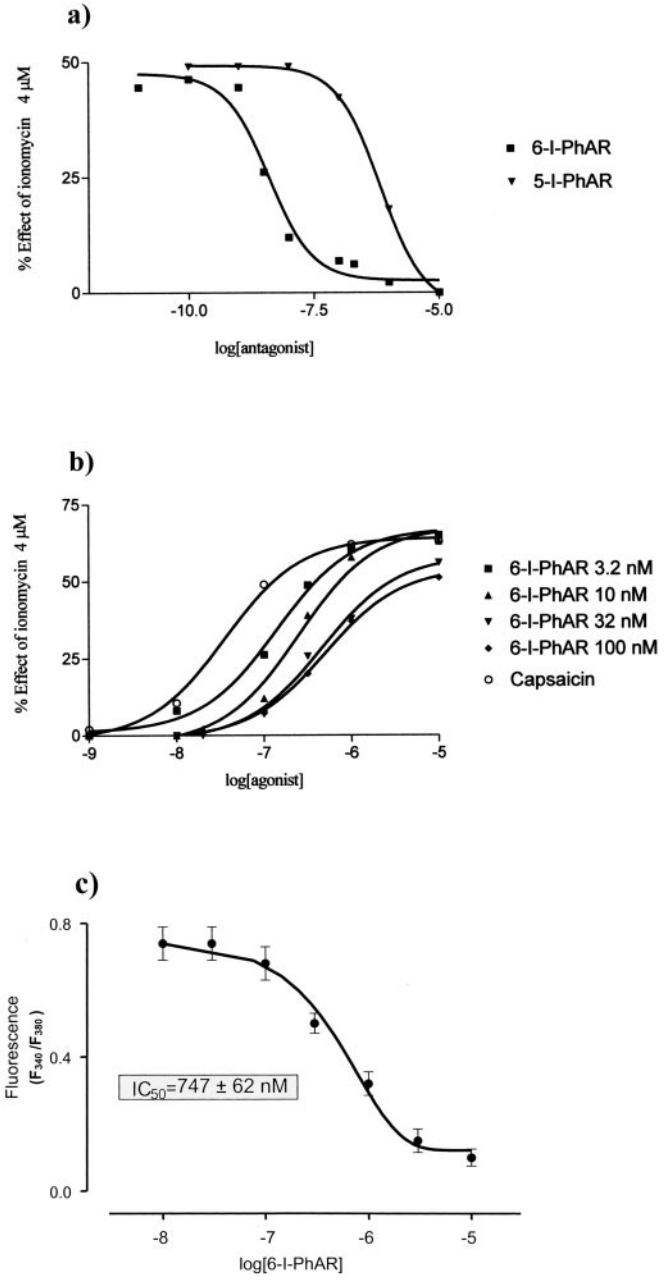


Fig. 5. Antagonistic activity of iodo-phenylacetylrinvanil (I-PhAR) isomers on human recombinant and rat native TRPV1 channels. a, effect of increasing concentrations of 5′- and 6′-I-PhAR on the action of capsaicin (100 nM) on the intracellular Ca^{2+} concentration in HEK-293 cells overexpressing the human TRPV1. b, effect of different doses of 6′- I-PhAR on the dose-response curve of capsaicin. Data are expressed as percentage of the maximal observable effect induced by 4 μM ionomycin and are means of n = 3 separate experiments. Standard error bars (never higher than 10% of the means) are not shown for the sake of clarity. c, effects of increasing doses 6′-iodo-phenylacetylrinvanil on the effect of capsaicin (100 nM) on cytoplasmatic Ca^{2+} concentration ($[Ca^{2+}]_i$) in cultured rat DRG neurons. Capsaicin-induced $[Ca^{2+}]_i$ mobilization was measured as detailed under *Materials and Methods*. Data are expressed as the net increase in the F_{340}/F_{380} ratio and are means of n = 5 separate experiments The IC_{50} of 6′-I-PhAR is shown.

against urinary incontinence. Furthermore, we have used one of these agonists to generate a very potent antagonist by simply iodinating its vanillyl moiety. Olvanil (*N*-oleoylvanillamine), a validated TRPV1 agonist devoid of pungency, served as the starting structural scaffold, and an intracellu-

lar Ca^{2+} assay widely employed to determine TRPV1 activity was used as the initial endpoint.

By introducing in the acyl chain of olvanil a hydroxy group highly suitable to further derivatization and starting from observation that resiniferatoxin shows an aromatic moiety on its lipophilic diterpenoid core (Fig. 1), we first synthesized rinvanil and then derivatized it with acids containing aryl moieties. Using this strategy, we developed PhAR, the most potent capsaicinoid TRPV1 agonist ever reported, and the first compound structurally unrelated to resiniferatoxin that shows efficacy comparable with the natural product in assays of vanilloid activity with potency, although 8-fold lower, in the same two-digit picomolar EC_{50} range. The ultra-potency of PhAR was only decreased by further chemical modifications of its fatty acid chain but was conserved in assays of TRPV1 activity carried out with the native rat receptor. In fact, the very low EC_{50} observed for PhAR in the TRPV1-mediated contraction of the rat urinary bladder suggested its testing in an animal model of urinary incontinence.

Detrusor overactivity, which is characterized by involuntary bladder contractions and loss of urine, is a major cause of urinary incontinence, a common health problem for both genders. Anticholinergic drugs are still considered as the first-line therapy for treating overactive bladder, although their clinical use is limited by their well known side effects. Inhibiting afferent C-fiber pathway from the bladder by intravesical administration of vanilloid compounds such as resiniferatoxin or capsaicin is an attractive option and might represent a possible alternative to the anticholinergic drugs. The effects of these two compounds has already been investigated on patients with spinal cord injures (Giannantoni et al., 2002) and also on patients with idiopathic detrusor overactivity (Silva et al., 2002). In these patients, detrusor overactivity was abolished or reduced for 2 to 3 months from bladder instillation, revealing that C-fibers seem to have an important role in the generation of this bladder dysfunction. Here, we used anesthetized rats with ligature-intact partial urethral obstruction as a well established animal model of this disorder. Our results show that this procedure, in agreement with published results (Steers and De Groat, 1988), leads to a significant increase in bladder weight, capacity, frequency of micturition, and to the development of a detrusor overactivity in the majority of the animals. These effects were similar to those observed in humans (Groutz et al., 2000). In our experimental conditions, a bladder instillation of PhAR was able to abolish spontaneous bladder contractions in anesthetized rats, and similarly, to resiniferatoxin, it was able to reduce frequency of micturition. Both compounds exhibited a very long duration of action, more than 4 to 6 h (not shown). This is actually an advantage compared with the short duration of action (less than 1 h) of other compounds such as the ATP-sensitive potassium channel openers (Fabiyi et al., 2003), which are claimed to be an alternative to the anticholinergic drugs.

Since olvanil and other *N*-acyl-vanyllamides have been reported to interact with proteins of the endocannabinoid system, including the cannabinoid CB_1 receptor, FAAH and the putative AMT (Di Marzo et al., 2002b), we decided to test the activity of PhAR and its analogs on these proteins. However, in most cases, the compounds were weakly active or inactive in these assays with the notable exception of PhAR which, unlike other *N*-acyl-vanyllamides, exhibited a significant affinity for the human CB_2 receptor. Given the analgesic and anti-inflammatory activity of both CB_2 (Malan et al., 2003) and TRPV1 agonists (see Introduction), this finding provides a strong rationale to conduct future studies that assess the functional activity of PhAR at CB_2 receptors and its effects on inflammatory pain in vivo.

Since iodination of the vanillyl moiety of capsaicin and resiniferatoxin causes a dramatic switch in vanilloid functional activity (Wahl et al., 2001; Appendino et al., 2003), we also investigated the effect of iodination on PhAR. As expected, this modification totally abolished PhAR agonist activity and, in the case of the 6′-iodo-PhAR, led to a TRPV1 antagonist exhibiting a potency in vitro comparable with that of some other recently developed TRPV1 antagonists (Appendino et al., 2003; Rami et al., 2004; Szallasi and Appendino, 2004, for review). The activity in vivo of this compound will need to be assessed before suggesting its possible therapeutic value.

In conclusion, by using a stepwise medicinal chemistry approach, we have developed a novel ultra-potent TRPV1 agonist that, by reducing the C-fiber input, appears to significantly reduce idiopathic detrusor overactivity associated with bladder incontinence. This compound and its analogs, by lacking the potential tumorigenicity of resiniferatoxin, are likely to represent a safer alternative to "resiniferoids" to treat this disorder. Finally, we have described two more potential applications of the further chemical modification of PhAR, consisting of the possible future development of either novel cannabinoid CB_2 receptor ligands or potent TRPV1 antagonists. Further studies will be required to assess the efficacy in the clinic of PhAR and then the potential to develop novel analgesics and anti-inflammatory agents from PhAR-derived TRPV1 antagonists and/or CB_2 agonists.

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