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Synthesis and In Vitro Evaluation of a Novel Iodinated Resiniferatoxin Derivative that is an Agonist at the Human Vanilloid VR1 Receptor

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Abstract—Using a 'directed' iodination procedure, novel iodo-resiniferatoxin congeners were synthesized from 4-acetoxy-3-methoxyphenylacetic acid and resiniferinol- 9,13,14-*ortho*-phenylacetate (ROPA). The 2-iodo-4-hydroxy-5-methoxyphenylacetic acid ester of resiniferinol **5** displayed high affinity binding ($K_i = 0.71$ nM) for the human vanilloid VR1 receptor and functioned as a partial agonist. © 2002 Elsevier Science Ltd. All rights reserved.

The pungency and antinociceptive properties of *Capsicum* species have been recognized and used therapeutically for centuries. The active ingredient, capsaicin, (*trans*-8-methyl-*N*-vanillyl-6-nonenamide)[1] acts upon a population of mammalian sensory C- and Aδ-fibers causing excitation of primary afferents followed by neuronal desensitization. The cloning of the human and rat VR1 receptors[2–4] as the putative pharmacological target of capsaicin has reinvigorated research in vanilloids with the promise of developing novel analgesic agents that are not irritants.[5,6] To date, the most potent natural agonist at VR1 is resiniferatoxin (RTX) **1** (Fig. 1), a phorbol terpenoid isolated from the cactus *Euphoria resinifera* that binds to VR1 with subnanomolar affinity.

Tritiated-RTX (^{3}H-RTX) is a suitable radioligand in receptor binding assays; however, its usefulness is limited by its low specific activity. We sought to synthesize novel RTX derivatives that might similarly exhibit high affinity for VR1 but could incorporate an iodine radiolabel (i.e., ^{125}I). Here we report the synthesis and in vitro evaluation of novel iodinated resiniferatoxin congeners. A salient feature of our work is the use of a 'directed' iodination protocol that installed the iodine substituent at the 2-position of the homovanillic terminus, adjacent to the benzylic carbon center.

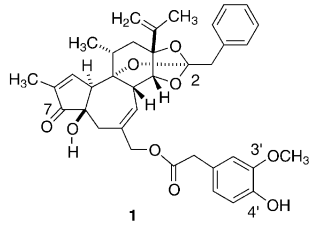

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Figure 1. The structure of resiniferatoxin (RTX).

Homovanillic acid was protected as the *O*-acetate upon reaction with acetic anhydride in the presence of catalytic sulfuric acid. Iodination was accomplished by treatment with a slight excess of elemental iodine and silver trifluoroacetate in methylene chloride.[7] Analysis (HPLC and MS) of the crude reaction revealed that iodination occurred exclusively at a single position and NMR analysis revealed this site was *meta* to the aryl hydroxy substituent and *para* to the methoxy group of the homovanillic moiety. The position of iodination is apparently due to the *o*,*p*-directing effects of the methoxy group in conjunction with *meta* direction from the electron-withdrawing *O*-acetate protecting group. Interestingly, iodination of homovanillic acid itself as well as the corresponding ethyl carboxy ester gave complex mixtures of products using the same conditions. Thus the acetate group not only masks the phenolic OH and directs iodination specifically to a lone carbon center, but also ameliorates reactivity of the aryl ring affording only mono-iodinated product **3**.

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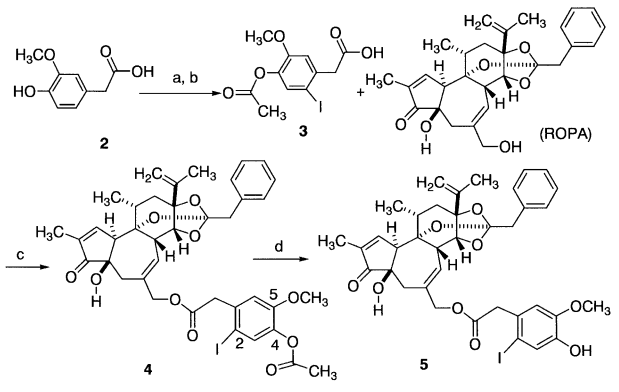
4-Acetoxy-2-iodo-5-methoxyphenylacetic acid **3** was then coupled to resiniferinol (ROPA) using carbodiimide, base and catalytic DMAP to afford iodoresiniferatoxin *O*-acetate **4**.[8] Cleavage of the acetate group by treatment with pyrrolidine gave 9,13,14-orthophenylacetylresiniferonyl-20-(4-hydroxy-2-iodo-5-methoxyphenylacetate) **5**[9] (Scheme 1).

The VR1 binding assay[10] used was a modified version of that previously reported by Szallasi and Blumberg.[11] Membranes harvested from a HEK293 cell line stably expressing human VR1 cDNA (hVR1/HEK293)[12] were incubated with ^{3}H-RTX and the test compounds **1** and **4**–**6**. After incubation and centrifugation, bound ^{3}H-RTX was quantified.

The permeability of VR1 to Ca^{2+} enabled the functional activity of the iodinated RTX analogues to be determined using a Ca^{2+}-sensitive fluorescent dye and FLIPR™ technology (Molecular Devices, Inc.).[13] The magnitude of agonist-induced increases in intracellular Ca^{2+} was compared to that evoked by a subsequent challenge with capsaicin at a concentration eliciting a maximal response.[14] All compounds (**1**, RBI; **6**,[15] Tocris) were tested for their ability to block a subsequent capsaicin response eliciting ~80% of the maximal response. The whole cell configuration of the patch clamp technique was used to compare the magnitude of currents induced by **5** ($10\times EC_{50}$) with that of capsaicin (3 μM). Cells were maintained in 0 Ca^{2+} buffer to allow multiple challenges to the same cell.[12]

Utilization of our 'directed' iodination protocol enabled the synthesis of new isomeric iodo-RTX analogues that retained high affinity for VR1 but displayed unexpected vanilloid receptor pharmacology. Resiniferatoxin (RTX) **1** bound to human VR (Table 1) with high affinity and functioned as a full agonist of the receptor. The iodinated-RTX congener **5** exhibited sub-nanomolar affinity for VR1 whereas the acetate precursor **4** was slightly less potent (Table 1). In functional assays, both iodinated resiniferatoxin derivatives (**4** and **5**) induced increases in intracellular Ca^{2+} in VR1-expressing cell lines. These effects were mediated through the vanilloid channel since the VR1 antagonists capsazepine and ruthenium red abolished calcium flux and since control (non-transfected) HEK293 cells were not affected by the RTX congeners. The EC_{50} values for **4** and **5** were 935 and 160 nM, respectively (Table 1), ~640- and 285-fold less potent than their ability to inhibit ^{3}H-RTX binding.



Scheme 1. (a) Ac_2O, cat. H_2SO_4 (97%); (b) I_2 (1.1 equiv), AgO_2CCF_3 (1.1 equiv)/CH_2Cl_2 (89%); (c) DCC, cat. DMAP, pyrrolidine (30 equiv)/CH_2Cl_2 (58%); (d) pyrrolidine/CH_2Cl_2 (68%).

We determined whether the compounds reached equilibrium in the 5 min functional assay by determining the concentration needed to half-block a subsequent capsaicin response after 60 min incubation. The potency of **5** did increase 10-fold, however, the potency of **4** was unaltered. Degradation of **4** is unlikely to account for its weak potency at human VR1 since cleavage of the acetate group leads to the more potent **5**. Similar discrepancies in binding and functional activity have been noted for other ion channels (e.g., nicotinic acetylcholine receptors) and have been attributed to increased affinity of the radioligand for the desensitized receptor, a state not detected in functional assays. Interestingly, the iodinated RTX congener **5** functioned as a partial agonist at human VR1, eliciting only 50% of the maximal response to CAP (Table 1), whereas **5** was a full agonist at rat VR1 ($92\pm6\%$, $n=7$). The partial agonism of **5** was confirmed using whole cell patch clamp technology (personal communication).

Wahl recently reported that reaction of RTX with sodium iodide and chloramine-T afforded the 2-iodo-4-hydroxy-3-methoxy phenylacetic acid ester of resiniferinol **6** (Fig. 2) and demonstrated that this iodinated RTX analogue is a antagonist of rat VR1.[15] We confirmed this finding and further demonstrated that **6** is an antagonist at human VR1 (Table 1).

As reported here, a 'directed-iodination' protocol was used to synthesize novel RTX analogues that retained high affinity for the human VR1 receptor. Iodo-RTX **5** bound to human VR1 with subnanomolar affinity and functioned as a partial agonist, compared to the full agonism observed with RTX. It is striking that the site

Table 1. Human VR1 binding affinities and functional activity of RTX and iodinated RTX congeners[a]

Compd	Binding affinity K_i (nM)	Functional activity (nM)	Efficacy (% capsaicin response)
1 (RTX)	0.48 ± 0.07 ($n=5$)	$EC_{50}=1.9\pm1.0$ ($n=9$)	116 (±6)
4	1.46 ± 0.55 ($n=5$)	$EC_{50}=935\pm280$ ($n=7$)	75 (±15)
5	0.71 ± 0.27 ($n=5$)	$EC_{50}=130\pm105$ ($n=9$)	50 (±13)
6	$1.4\pm$ ($n=1$)	$IC_{50}=27\pm11$ ($n=3$)	n.a.[b]

[a]Values are means ± standard deviation of no. experiments (n).
[b]n.a., not applicable (compound is not an agonist).

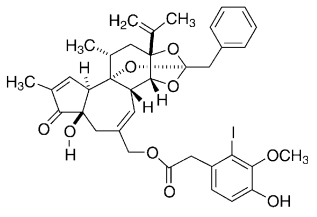


Figure 2. Iodinated resiniferatoxin reported by Wahl.

of iodination plays such an important role in functionality in light of an isomeric iodo-RTX reported to be a antagonist of rat VR1.[15] The use of radioactive ligands with partial or opposing functional activity will aid in understanding the RTX/capsaicin binding pocket of VR1.

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7. **4-Acetoxy-2-iodo-5-methoxyphenylacetic acid** (**3**): 4-Acetoxy-3-methoxyphenylacetic acid (200 mg, 0.89 mmol), silver-trifluoroacetate (217 mg, 0.98 mmol) and iodine (237 mg, 0.93 mmol) were combined and stirred in dry dichloromethane (20 mL) for 2 h under argon. The reaction was filtered. The filtrate was washed (satd NaCl solution), dried ($MgSO_4$), and solvents removed to afford the title compound **3** as a white powder (yield = 279 mg; 89%). Analytical reverse-phase HPLC (gradient 10–90% MeCN, 0.1% TFA) $t_R = 3.56$ min, ~99%; ^{1}H NMR (CD_3OD, 300 MHz) δ 7.46 (s, 1H), 7.10 (s, 1H), 3.79 (s, 3H), 3.78 (s, 2H), 2.24 (s, 3H); ^{13}C NMR (CD_3OD, 300 MHz) δ 174.4, 170.7, 153.3, 140.9, 138.8, 134.1, 116.5, 88.8, 56.9, 46.9, 20.7. Anal. calcd for $C_{11}H_{11}O_5I_1$: C, 37.74; H, 3.17. Found: C, 37.93; H, 3.31.
8. **9,13,14-Orthophenylacetylresiniferonyl-20-(4-acetoxy-2-iodo-5-methoxyphenylacetate)** (**4**): To a round-bottom flask (10 mL) the following reagents were added: dichloromethane (4 mL), resiniferonol-9,13,14-orthophenylacetate (10 mg, 0.02 mmol), 4-dimethylaminopyridine (0.28 mg, 0.0022 mmol), dicyclohexylcarbodiimide (4.8 mg, 0.023 mmol) and 2-iodo-4-acetoxy-5-methoxyphenylacetic acid (8.2 mg, 0.023 mmol). The reaction was stirred at room temperature, under argon, and in the dark for 24 h. The solvent was removed in vacuo and the residue chromatographed on silica gel (chloroform). Solvent was removed from appropriate fractions to yield adduct **4** as a white solid (yield = 10 mg; 59%). Analytical reverse-phase HPLC (gradient 60–90% MeCN, 0.1% TFA) $t_R = 4.27$ min, ~95%; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.44 (s, 1H), 7.35 (s, 1H), 7.29 (d, 2H, $J = 6.8$ Hz), 7.22–7.15 (m, 2H), 6.84 (s, 1H), 5.80 (s, 1H), 4.63 (s, 2H), 4.51 (s, 2H), 4.15 (d, 2H, $J = 2.1$ Hz), 3.79–3.66 (m, 6H), 3.13 (s, 2H), 2.99 (d, 2H, $J = 14.3$ Hz), 2.51 (t, 1H, $J = 7.6$ Hz), 2.39 (s, 1H), 2.26 (s, 1H), 2.22 (s, 3H), 2.09–1.96 (m, 3H), 1.74 (s, 1H), 1.43 (s, 1H), 0.88 (d, 4H, $J = 7$ Hz); ^{13}C NMR ($CDCl_3$, 500 MHz) δ 208.2, 169.7, 168.9, 157.8, 151.4, 146.3, 139.0, 136.5, 136.3, 134.9, 134.0, 133.0, 130.8, 128.6, 127.6, 126.5, 117.7, 114.2, 110.7, 87.9, 84.4, 81.0, 80.5, 73.1, 71.3, 56.0, 55.2, 46.3, 40.9, 39.9, 39.0, 35.6, 33.9, 32.9, 24.9, 20.6, 19.9, 18.8, 10.3. Accurate mass (FAB MH^+): calcd for $C_{39}H_{41}I_1O_{10}$, 797.1951; found, 797.1822.
9. **9,13,14-Orthophenylacetylresiniferonyl-20-(4-hydroxy-2-iodo-5-methoxyphenylacetate)** (**5**). 9,13,14-Orthophenylacetylresiniferonyl-20-(3-acetoxy-4-methoxy-6-iodophenylacetate) (17 mg, 0.02 mmol) was dissolved in dichloromethane (1 mL) and stirred at room temperature under argon. Pyrrolidine (59.7 uL, 0.71 mmol) was then added and the reaction stirred for 2 h after which time no starting material was visible by TLC. The solvent was removed in vacuo and the residue was purified on a TLC preparative plate (silica gel, $CHCl_3$). The product was extracted (chloroform) from the silica and the solvent evaporated after filtration to give the desired iodo-RTX congener **5** as a white solid (yield = 11 mg; 68%). Analytical reverse-phase HPLC (gradient 40–90% MeCN, 0.1% TFA) $t_R = 4.83$ min, ~90%; IR (KBr) 2850, 1732, 1705, 1504, 1270, 1027 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.37 (t, 1H, $J = 1.9$ Hz), 7.31 (d, 2H, $J = 1.5$ Hz), 7.23–7.15 (m, 1H), 6.76 (d, 1H, $J = 8.0$ Hz), 6.72–6.67 (m, 2H), 5.80 (s, 1H), 5.48 (s, 1H), 4.63 (s, 2H), 4.59 (q, 2H, $J = 12.1$ Hz), 4.12 (d, 1H, $J = 2.7$ Hz), 3.8 (s, 3H), 3.49 (s, 2H), 3.14 (s, 2H), 3.0 (d, 2H, $J = 13.4$), 2.47 (t, 1H, $J = 7.5$ Hz), 2.35 (d, 1H, 18.9), 2.09–1.99 (m, 3H), 7.5 (s, 3H), 1.61–1.59 (s, 1H broad), 1.44 (s, 3H), 0.88 (d, 4H, $J = 7.1$ Hz); ^{13}C NMR ($CDCl_3$, 500 MHz): δ 208.4, 171.4, 158.2, 146.5, 146.2, 136.5, 134.9, 134.0, 130.8, 128.5, 127.7, 126.5, 125.6, 122.2, 117.7, 114.3, 111.7, 110.7, 84.4, 81.0, 80.5, 73.3, 70.5, 65.8, 55.9, 55.3, 41.0, 40.9, 39.9, 39.0, 35.6, 33.0, 19.8, 18.8, 10.3. Accurate mass (FAB MH^+): calcd for $C_{37}H_{39}I_1O_9$, 755.1741; found, 755.1717.
10. **[^{3}H]RTX binding assay using hVR1/HEK293 cell membranes. Preparation of membranes.** hVR1/HEK293 were homogenized with a Polytron twice and centrifuged at 3000 rpm for 10 min in HEPES buffer containing 20 mM HEPES, pH 7.4, NaCl 5.8 mM, sucrose 320 mM, $MgCl_2$ 2 mM, $CaCl_2$ 0.75 mM and KCl 5 mM. The supernatant was centrifuged at 18,000 rpm for 20 min. The pellet was saved in a tube and 10 mL assay buffer was added into the tube. The pellet and buffer were mixed with a Polytron. **Incubation procedure.** Incubations for 60 min at 37 °C were performed in a total volume of 0.5 mL that contained 120 μg/mL membrane protein and 0.3–0.6 nM ^{3}H-RTX (NEN, Boston MA, USA) in the HEPES buffer. After incubation, the samples were cooled on ice and 100 μg of α-acid glycoprotein were added followed by centrifugation at 13,000 rpm for 15 min. The supernatant was aspirated and the tips of tubes were cut off into 6 mL vials. Nonspecific binding was measured in the presence of 200 nM unlabeled RTX in 4 mL scintillation liquid using a Packard scintillation counter. **Data analysis.** Percent (%) inhibition = (total binding − total binding in presence of compound)*100/(total binding − nonspecific binding). K_i values were calculated using Prism (GraphPad, San Diego, CA, USA).

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14. hVR1/HEK cells[4] or cells stably expressing rat VR1[2] were seeded on poly-D-lysine coated 96-well, black-walled plates (BD 354640) and 2 days later loaded with Fluo-3/AM for 1 h and subsequently tested for agonist-induced increases in intracellular Ca^{2+} levels using FLIPR™ technology.[13] Rat VR1 was cloned from a rat thalamus cDNA library and is identical to Genbank accession #AF029310. Rat and hVR1/HEK cells were

occasionally seeded on different halves of the same plate for more direct comparisons of compound effects on VR1 from these species. Cells were challenged with single concentrations of compound (**1**, **4**, **5**, and **6**) and intracellular Ca^{++} was measured for 5 min prior to the addition of CAP to all wells to achieve a final CAP concentration of 3 μM to fully activate VR1. Cells in columns 1–11 received compounds in increasing concentrations by half-log intervals from 0.1 nM (**4**) or 0.01 nM (**5**); column 12 received buffer. Antagonist potency was determined using a similar protocol except that after incubation with **1**, **4**, **5** and **6**, cells were challenged with CAP at a concentration eliciting ~80% maximal response. Efficacy determinations were made by normalizing the maximum iodo-RTX-induced fluorescence change to the change elicited by a supramaximal concentration (3 μM) of capsaicin. Data were analyzed using Prism software.

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