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Brief Article

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Novel Radiolabeled Vanilloid with Enhanced Specificity for Human Transient Receptor Potential Vanilloid 1 (TRPV1)

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ABSTRACT: Transient receptor potential vanilloid 1 (TRPV1) has emerged as a promising therapeutic target. While radiolabeled resiniferatoxin (RTX) has provided a powerful tool for characterization of vanilloid binding to TRPV1, TRPV1 shows 20-fold weaker binding to the human TRPV1 than to the rodent TRPV1. We now describe a tritium radiolabeled synthetic vanilloid antagonist, 1-((2-(4-(methyl-[^{3}H])piperidin-1-yl-4-[^{3}H])-6-(trifluoromethyl)pyridin-3-yl)methyl)-3-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)urea ([^{3}H]MPOU), that embodies improved absolute affinity for human TRPV1 and improved synthetic accessibility.

■ INTRODUCTION

TRPV1, the transient receptor potential vanilloid 1, represents the site of action of capsaicin, the pungent ingredient in red pepper, and is a central nociceptor on sensory afferent neurons.[1] TRPV1 integrates noxious signals including those from exogenous ligands like capsaicin or resiniferatoxin, from endogenous vanilloids like 5-HPETE or arachidonoyl dopamine, and from physical stimuli like elevated temperature and low pH. TRPV1 is further subject to modulation by signaling pathways in the cell, permitting it to respond in a holistic fashion to perturbations in the cellular environment. Protein kinase C, protein kinase A, protein kinase D, and Ca^{2+}/calmodulin-dependent protein kinase II all phosphorylate TRPV1 and influence its sensitivity for ligands, its desensitization, and the functional consequences of ligand interaction.[2,3] Current intense interest is directed at the relative responses of TRPV1 to various stimulatory modulators, because it is thought that a side effect impacting the therapeutic development of vanilloids, *viz*. hyperthermia, is related to whether vanilloid antagonists block responses both to capsaicin and low pH or whether they selectively antagonize capsaicin only.[4,5]

Our identification of resiniferatoxin (RTX, **1**) as an ultrapotent capsaicin analog impacted the understanding of capsaicin pharmacology at three levels.[6] It revealed that it was possible with appropriate structures to achieve biological potencies up to four orders of magnitude greater than those that had been found at the time for capsaicin analogs.[7-9] It proved that different biological consequences of capsaicin action could be dissected, with desensitization / defunctionalization – a potential therapeutic strategy with vanilloid agonists – being partially separated from induction of acute pain – a side effect limiting therapeutic application of vanilloid agonists. Finally, the great potency of **1** made it possible to develop a radioligand binding assay to directly demonstrate the existence of a capsaicin receptor,[10] the existence of which had been hypothesized by Szolcsanyi.[11] This ligand binding assay permitted characterization of the biochemical pharmacology of the capsaicin receptor.[12] While initial characterization was directed at the endogenous receptor in dorsal root ganglia, the subsequent cloning of the capsaicin receptor, designated TRPV1,[13] and its exogenous expression represented a further breakthrough facilitating the current intense efforts in the understanding of vanilloid structure activity relations.[14-16]

Earlier biological work had suggested important differences in the species response to vanilloids. The guinea pig showed distinct behavior compared to the rodent, and rabbits and birds were much less sensitive.[17,18] Comparison of binding of **1** to rat and human TRPV1 showed that there was a marked difference in sensitivity, with the human TRPV1 approximately 20-fold less sensitive than the rat TRPV1 (K_d = 1.47 nM, human TRPV1; 0.064 nM, rat TRPV1). Sequence comparison has implicated a specific residue in this difference. Rodent TRPV1 has Met in position 547, whereas the human TRPV1 has Leu. Our current understanding of ligand – TRPV1 interaction based on homology modeling of the TRPV1 suggests that this functional difference results from the greater steric constraint imposed on the ligand binding site by the Leu at this position.[19,20]

For optimizing structure activity relations, we have argued elsewhere that ligand binding provides a valuable complement to functional assays.[21] The functional assays are subject to issues of spare receptors and to modulation of the TRPV1 over the time course of the assay, reflecting the resultant feedback as calcium enters the cell through the activated receptor channel. Furthermore, we have shown using fluorescent vanilloids that some compounds will penetrate slowly relative to the short time of the typical functional assay.[22] Such assays of course are very different from the long-term effects of the vanilloid interaction in a whole animal or therapeutic setting. Direct ligand binding can thus provide a complementary picture, assisting in understanding of the structure activity relations and in interpretation of functional assays.

While radiolabeled RTX ([^{3}H]RTX, **2**) has proven acceptable for measuring vanilloid interactions with human TRPV1, the assay has been less robust than has been the case with rodent TRPV1. Since the primary therapeutic target is the human, the structure activity relations for the human TRPV1 are of particular interest, although, because preclinical testing is carried out in animals, it is also important to know the degree to which species difference will distort the findings in the animal models. As we have optimized the structure of vanilloids targeting the human TRPV1, we have achieved potencies approaching or exceeding those of **1** for binding to human TRPV1.[24,25] Non-specific binding will in part reflect the partitioning of the ligand into the membranes in which TRPV1 resides. We therefore sought a ligand combining high absolute potency for human TRPV1, a low octanol-water partition coefficient (logP), and ease of radiolabeling. Among the potent TRPV1 antagonists emerging from our extensive optimization of pharmacophoric regions based on our antagonistic template, 1-((2-(4-methylpiperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)methyl)-3-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)urea (MPOU, **12**), was predicted to embody these characteristics (**Figure 1**).

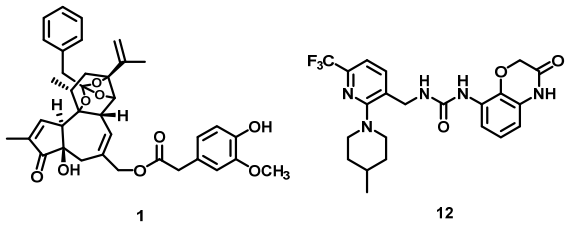


Figure 1. Structures of **1** and **12**

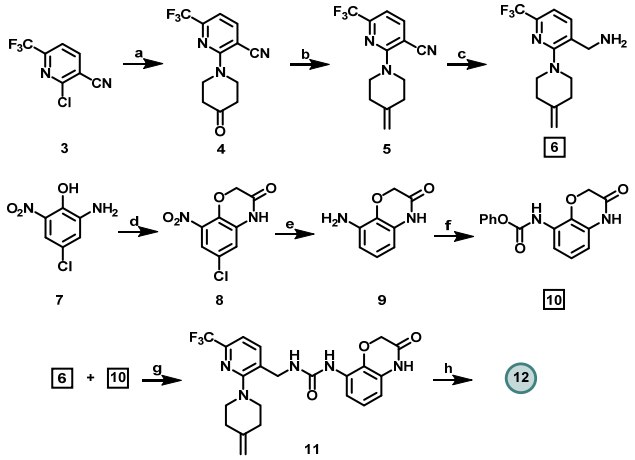
Here, we report its synthesis and characterize its binding to human TRPV1. While still not optimal, 1-((2-(4-(methyl-[^{3}H])piperidin-1-yl-4-[3H])-6-(trifluoromethyl)pyridin-3-yl)methyl)-3-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl) urea ([^{3}H]MPOU, **13**) represents a modest improvement over **2** ([^{3}H]RTX) for characterization of the structure activity relations for vanilloid binding to human TRPV1.

RESULTS AND DISCUSSION

Chemistry. The synthesis of **12** was accomplished by urea coupling between the two corresponding amines (**6**, **9**) as described in **Scheme 1**. The left-hand amine of **12** (**6**) was prepared from commercially available 2-chloro-6-(trifluoromethyl)nicotinonitrile (**3**) in 3 steps through nucleophilic substitution by 4-piperidinone, Wittig reaction, followed by reduction. The synthesis of the right-hand amine (**9**) was accomplished starting from commercially available 2-amino-4-chloro-6-nitrophenol (**7**). The cyclization of **7** with 2-chloroacetyl chloride provided the dihydrobenzo[1,4]oxazin-3-one ring (**8**) efficiently. Dehalogenation followed by reduction of **8** produced the amine (**9**), which was converted to the corresponding phenylcarbamate (**10**) for coupling. The coupling between **6** and **10** provided a precursor of **12** (**11**) that was used for the radiolabeling synthesis of **13** ([^{3}H]MPOU). The catalytic hydrogenation of **11** gave the compound **12**.

The synthesis of **13** was conducted by the radioactive labeling of the precursor **11** employing the reduction with tritium gas over palladium on charcoal and was performed by Quotient Bioresearch (Cardiff, United Kingdom) as was its radiochemical characterization (**Figure 2**).

Scheme 1. Synthesis of the 12[a]



[a] *Reagents & conditions*: (a) 4-piperidinone, DBU, CH_3CN, reflux, 2 h; (b) PPh_3MeBr, n-BuLi, anhyd. THF, -78 ˚C, 1 h; (c) $LiAlH_4$, diethyl ether, 0 ˚C, 3 h; (d) 2-chloroacetyl chloride, K_2CO_3, 18-crown-6, DMF, 50 ˚C, 2 h; (e) 10% Pd/C, H_2, MeOH, 40 ˚C, 15 h; (f) Phenyl chloroformate, pyridine, THF, r.t, 1 h; (g) DMAP, CH_3CN, r.t., 15 h; (h) 10% Pd/C, H_2, MeOH, 40 ˚C, 3 h.

Purity, measured by HPLC, was 99.7%. HPLC conditions were a Luna C18 column (5 µm, 150 x 4.6 mm column) eluted with a gradient of acetonitrile in water. The radiolabeled material co-chromatographed with authentic **12** and its mass spectroscopic spectrum was consistent with the proposed structure and that of authentic **13**. Specific activity, determined by mass spectrometry, was 78 Ci/mmol, a value approximately 2-fold greater than that (30 Ci/mmol) which we had for **2**.

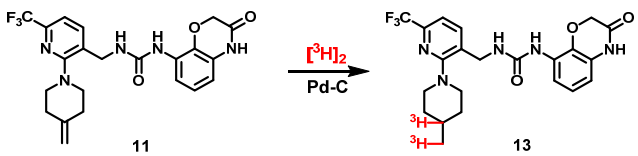


Figure 2. Synthesis of **13**, the radiolabeled **12**.

Biological Activity. Among potent ligands for human TRPV1 that we have investigated, **12** displayed a combination of attractive features as a potential ligand for human TRPV1 binding assays. Assayed for competition of **2** binding to human TRPV1, it yielded a K_i = 0.63 ± 0.19 nM (n = 3) (**Figure 3A**). For comparison, the corresponding K_i of **12** at the rat TRPV1 measured by competition of **2** binding was 10-fold weaker (6.5 ± 2.0 nM, n = 3) (**Figure 3B**). The K_d for **1** for human TRPV1 was previously found to be 1.23 ± 0.22 nM[26]; on rat TRPV1 the K_d of **1** was 0.064 ± 7.4 nM, reflecting the selectivity of **1** for the rat TRPV1 versus human TRPV1.

Vanilloids need to penetrate through the membrane to gain access to their binding site on TRPV1,[27] which extends into the transmembrane region from the inner face of the membrane.[28] As a consequence, potent vanilloids possess substantial lipophilicity. Unfortunately, this lipophilicity necessarily means that the compounds will show corresponding substantial receptor-independent partitioning into the membranes into which the TRPV1 is inserted.

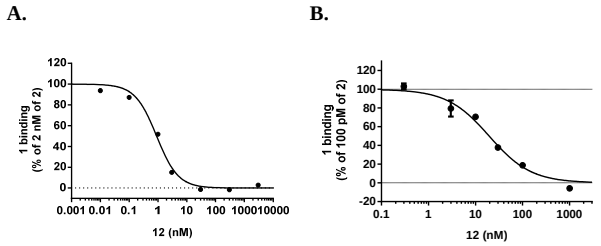


Figure 3. Binding of **12** to A) human TRPV1 and B) rat TRPV1. Binding was measured by competition of **2** binding. Results are of single representative experiments. The concentration of **2** was approximately 2 nM for assay of human TRPV1, 100 pM for assay of rat TRPV1. 3 independent experiments were performed with both human and rat TRPV1. Points represent the mean of triplicate measurements in a single experiment. Bars, SE (when not visible, the errors were smaller than the symbols). Mean K_i values were: human TRPV1, K_i = 0.63 ± 0.19 nM; rat TRPV1, K_i = 6.5 ± 2.0 nM.

As we sought the most attractive candidate for an improved ligand for binding to human TRPV1, we therefore looked for a candidate with the optimal ratio of specific binding to non-specific binding, where non-specific binding was estimated as being proportional to the LogP (the calculated octanol-water partition coefficient) and to the ligand concentration at the K_i of the ligand. By this measure, **12** appeared to be an attractive choice among the potent human TRPV1 ligands that we have been characterizing, with a prediction of significantly improved specific to non-specific binding (**Table 1**). For comparison, a similar analysis is provided for 1-((*R*)-5-*tert*-butyl-indan-1-yl)3-isoquinolin-5-yl-urea (**A-778317**), another ligand that has been used for radioligand binding assays with human TRPV1.[29] Of course, one concern is that predicted LogP values have significant uncertainty. For example, CLogP uses a different algorithm for calculation of the octanol-water partition coefficient than does LogP and the CLogP values for **1** and for **12** predict an 8.3-fold improvement with **12** compared to the 45.6-fold improvement predicted from the LogP values. Moreover, we could not predict the contribution to non-specific binding from binding to other elements in the assay mixture nor the relative efficiency of α1-acid glycoprotein from bovine plasma to reduce non-specific binding by **12** versus **1**.

Table 1.

Compound	**1**	**A-778317**[a]	**12**
K_d	1.23 nM	3.4 nM	0.63 nM
Activity	agonist	antagonist	antagonist
LogP[b]	4.38	4.32	3.01
1[c]	1	0.42	45.6

[a] K_d from ref 29
[b] LogP values calculated by ChemDraw.
[c] Improvement over **1** from the formula $((K_{d\ RTX} \times {}^{10LogP\ RTX})/(K_{i\ compound} \times 10^{LogP\ compound}))$

A further attractive feature of **12** relatives to **1** is that it is synthetic, whereas **1** is a hard-to-obtain natural product, and that there is a straightforward route for its synthesis and for its labeling by reduction with tritium gas over palladium from the precursor **11**.

Finally, **12** is a TRPV1 antagonist, whereas **1** is an agonist. It is not clear whether this difference should be important, but we have described elsewhere that the potency of antagonists for TRPV1 showed less dependence on the cellular environment than was the case for agonists.[30] A theoretical rationale for this difference is that an agonist will necessarily cause a conformation change leading to channel opening. This change in conformation will be subject to whatever other regulatory influences impact the conformational change. An antagonist, in contrast, need not necessarily cause any conformational change with a channel that is already present in the closed conformation.

13 proved to be a satisfactory ligand for measurement of binding to human TRPV1 (**Figure 4A**). Its K_d, 0.59 ± 0.05 nM (n = 8 independent experiments), was in close agreement with the K_i (0.63 nM, see above) measured by competition of **2** binding with non-radioactive **12**. Assay conditions were similar to those used for measuring binding in the presence of **2**.[28] Briefly, membranes (approximately 20 µg total protein) from CHO cells exogenously expressing human TRPV1 were incubated for 45 min at 37°C with **13** in the presence of Dulbecco's phosphate buffered saline and 0.25 mg/ml bovine serum albumin, final volume 350 µl. Samples were then chilled and 100 µl of α1-acid glycoprotein (2 mg/ml) was added and incubation continued for a further 10 min at 0 °C with the goal of complexing unbound ligand. The membranes were then pelleted by centrifugation, the supernatant removed, and bound and free radioactivity was measured by scintillation counting. Non-specific binding was measured in parallel samples treated with 50 nM non-radioactive **1**.

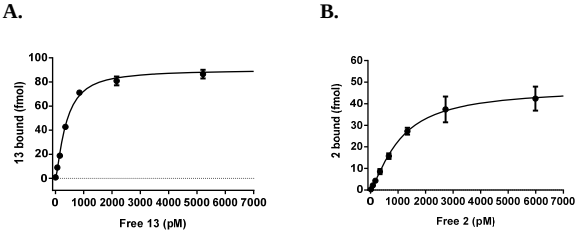


Figure 4. A) Binding of **13** to human TRPV1. B) Binding of **2** to the same membranes. Results shown are for single representative experiments of quadruplicate parallel experiments performed. Points represent the mean of triplicate measurements in a single experiment. Bars, SE (when not visible, the errors were smaller than the symbols). The mean K_d for **12** from these together with additional, independent experiments was 0.589 ± 0.049 nM. For **1** in this series of experiments the mean K_d was 1.47 ± 0.31 nM.

Although the assay procedure is not suitable for detailed analysis of binding kinetics, we confirmed that the specific binding of **13** was largely complete by 5 min, increasing slightly over the next hour (**Figure 5A**). Likewise, at 0 °C the specific binding was stable over the time for assay work-up (**Figure 5B**). Importantly, addition of non-radioactive **1** (50 nM) caused almost full release of specifically bound **13** within 2 min at 37 °C (**Figure 5C**), assuring that competition assays should reach equilibrium.

The reduction in non-specific binding with **13** compared to **2** did not meet the expectations predicted from the comparison of LogP or CLogP. In parallel measurements under assay conditions, the non-specific partitioning of **13** into the membranes was 0.115 ± 0.011 compared to 0.069 ± 0.012 for **2**. Of course, the measured non-specific binding is more complex than that of simple membrane partitioning, since it also reflects the efficiency with which unbound ligand is extracted by the α_1-acid glycoprotein or binds with low affinity to other membrane proteins.

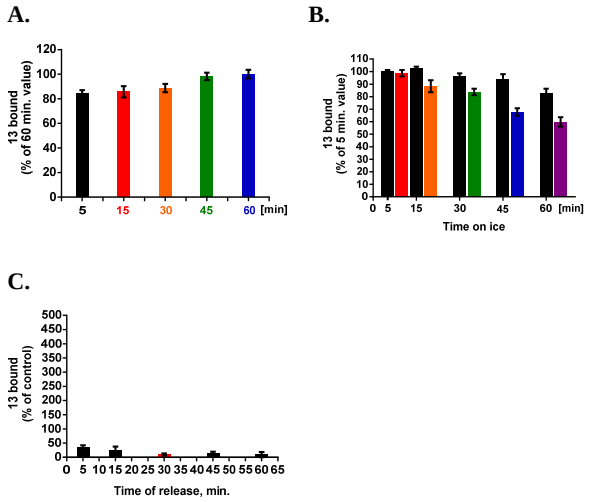


Figure 5. Time course of binding of **13** to human TRPV1 and its release. A) **13** was incubated with human TRPV1 at 37°C for the indicated times and then binding was measured. Results are expressed relative to incubation for 60 min. Values represent the mean of triplicate independent experiments. Bars, SE. B) **13** was bound to human TRPV1 for 60 min at 37°C. The samples were then chilled and non-radioactive **1** (50 nM) was then added and incubation continued at 0°C for the indicated times. Remaining binding was then measured. Values, expressed relative to the 5 min control to which **1** was not added, represent the mean of triplicate experiments. Bars, SE. C) **13** was incubated with human TRPV1 for 60 min at 37 °C. Non-radioactive **1** (50 nM) was then added for the indicated times, after which the samples were cooled to 0°C and binding measured. Values, expressed relative to the control to which **1** was not added, represent the mean of triplicate experiments. Bars, SE.

Nonetheless, the higher affinity of **12** for human TRPV1 made a significant contribution to the ratio of specific to non-specific binding. The non-specific binding is directly proportional to the concentration of ligand, and the higher affinity of the **12** permitted a lower concentration of the ligand to be used in the assays. Thus, in the series of parallel assays illustrated in **Figure 4A**, the % specific binding for **12** and for **1** were 70 ± 4% and 45 ± 6% at their respective K_d values. In the parallel assays, we also noted that the B_{max} values differed somewhat, with the B_{max} for **12** being 1.8 ± 0.2 relative to that for **1**.

For analysis of human TRPV1 binding, specific binding of **13** is defined as that blocked by non-radioactive **1**. The specific binding should therefore reflect similar TRPV1 dependency as does **2** binding, which was also defined as that blocked by non-radioactive compound **1**. Likewise, specific binding was fully inhibited by other vanilloid agonists and antagonists (**Figure 6**). Finally, under our conditions, specific binding as detected with **13** in CHO cells not expressing exogenous human TRPV1 was < 12% of that of the CHO cells expressing the exogenous human TRPV1. Given vanilloid structure activity relations, high affinity binding to other TRP family members would not be expected but was not examined directly.

A primary objective of the human TRPV1 radioligand binding assay is to evaluate the structure-activity relations of non-radioactive ligands by competition. Competition curves using **13** are illustrated (**Figure 6**). We compared the K_i values obtained using **13** and **2**. Good agreement was found, although the K_i values tend to be slightly higher with **13** (**Table 2**, select examples; ratio = 1.57 ± 0.56, mean ± SD, n = 9 compounds).

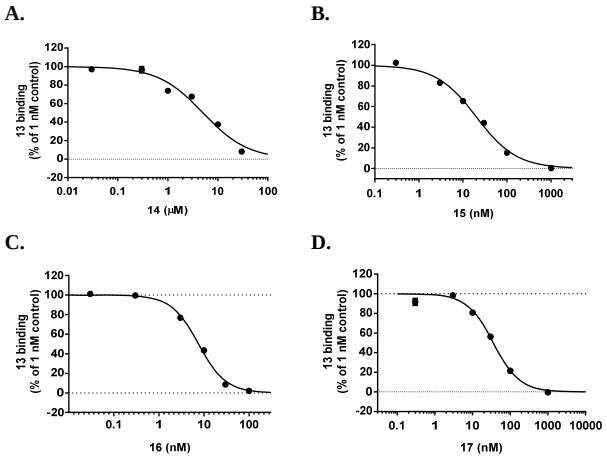
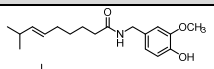
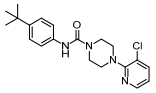
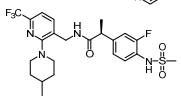
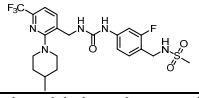


Figure 6. Competition of **13** binding to human TRPV1 by capsaicin (**14**),4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazine carboxamide (BCTC, **15**)[23], (*S*)-2-(3-fluoro-4-(methylsulfonamido)-phenyl)-*N*-((2-(4-methylpiperid-in-1-yl)-6-(trifluoromethyl)pyridin-3-yl)-methyl)propanamide (GRT-12360, **16**)[24], *N*-(2-fluoro-4-(3-((2-(4-methyl-piperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)methyl)-ureido)-benzyl) methanesulfonamide (KMS-1589, **17**)[25]. Binding of **13** to human TRPV1 was measured in the presence of the indicated concentrations of the vanilloids. Points represent the mean of triplicate determinations in a single representative experiment. Bars, SE. (not visible when bars were less than size of symbol). All experiments were performed at least 3 times.

TRPV1 is subject to multiple phosphorylations which can to influence its ability to be activated. In limited experiments, we sought to compare the degree to which changes in factors influencing its state of phosphorylation influenced the measured affinities for the agonist **2** and the antagonist **13**. Cells expressing *h*TRPV1 were treated for 30 min with 1 μM PMA, with 250 nM cyclosporin A added for the final 5 min. The cells were then harvested, membranes prepared, and binding measured. Control samples were prepared in parallel except that PMA and cyclosporin A were not added. Treatment of the cells with this combination of a protein kinase C activator and a protein phosphatase 2B inhibitor caused a 3-fold enhanced affinity for **2** (356 ± 23 pM versus 1140 ± 140 pM, n = 3, mean ± SE) whereas no appreciable change was observed for **13** (518 ± 62 pM versus 534 ± 97 pM, n = 3, mean ± SE). These findings are consistent with our previous results that agonist potencies showed greater sensitivity to modulators of phosphorylation than did the potencies of antagonists, in assays evaluating uptake of $^{45}Ca^{2+}$ mediated by rat TRPV1.[30]

Table 2. Comparison of K_i values for competition binding assays with **2** and **13**.

Compds	Structure	**2** (K_i, nM[a])	**13** (K_i, nM[a])
14		1140 (+/- 120)	2530 (+/- 650)
15		4.32 (+/- 0.47)	10.5 (+/- 1.1)
16[b]		2.95 (+/- 0.73)	2.94 (+/- 0.71)
17[c]		6.77 (+/- 0.48)	11.5 (+/- 2.2)

[a] Mean ± SE of at least 3 independent experiments [b] 49S in ref 24, [c] 27 in ref 25

■ CONCLUSION

We conclude that **13** is a suitable ligand for measurement of ligand binding to human TRPV1. While we failed to capture the full predicted advantage of this ligand over **2**, due to the difference in LogP or CLogP not translating into a corresponding difference in non-specific binding after inclusion of the α_1-acid glycoprotein in the assay, **13** yielded higher specific activity and greater potency, which, by allowing a lower concentration of free ligand, itself reduced interference from non-specific binding. The good although not perfect agreement with values obtained with **2** confirms that **13** is a suitable replacement for **2**, which is no longer commercially available, for human TRPV1. **2** remains the ligand of choice for rodent TRPV1. The modest difference in B_{max} values remains unexplained but could possibly be linked to the twin issues of **12** being an antagonist versus **1** as an agonist and the different influence of the TRPV1 modulatory environment on agonists versus antagonists. Consistent with this explanation, we showed that **13** binding affinity was less subject to modulation by treatments that would affect TRPV1 phosphorylation than was the binding affinity for **2**.

■ EXPERIMENTAL SECTION

General Methods. All chemical reagents were commercially available. Nuclear magnetic resonance spectra were recorded on JEOL JNM-LA 300 spectrometers. Mass spectra were recorded on a VG Trio-2 GC-MS and 6460 Triple Quad LC/MS. All final compounds were purified to >95% purity, as determined by high-performance liquid chromatography (HPLC).

2-(4-Oxopiperidin-1-yl)-6-(trifluoromethyl)nicotinonitrile (4) To a stirred solution of 4-piperidone (0.96 g, 9.68 mmol) in DCM was added DBU (2.17 mL, 14.52 mmol) and 2-chloro-6-(trifluoromethyl)nicotinonitrile **3** (1.00 g, 4.84 mmol) at room temperature and refluxed. After 2 h, the resulting mixture was allowed to warm to ambient temperature. The mixture was extracted with ethyl acetate. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Purification by column chromatography (EtOAc/Hex=20:1) gave **4** (1.13 g), 90% yield. ^{1}H NMR ($CDCl_3$) δ 8.00 (d, 1H, *J* = 7.86 Hz, pyridine), 7.14 (d, 1H, *J* = 7.71 Hz, pyridine), 4.12(t, 4H, *J* = 6.03 Hz, piperidine-N-(CH_2)$_2$-), 2.65 (t, 4H, *J* = 6.21 Hz, piperidine-(CH_2)$_2$-CH_2).

2-(4-Methylenepiperidin-1-yl)-6-(trifluoromethyl)nicotinonitrile (5) To a solution of PPh_3MeBr (2.24 g, 6.28 mmol) in dry THF (20 mL) at -78 ºC was added n-BuLi solution 2.0 M in hexane (2.09 mL) under a nitrogen atmosphere. After 30 min, ketone **4** (1.13 g, 4.19 mmol) in dry THF (4 mL) was added dropwise. The resulting mixture was allowed to warm to ambient temperature and stirred for 1.5 h. Saturated NH_4Cl was added. The mixture was extracted with ethyl acetate. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. Purification by column chromatography (EtOAc/Hex=1:20) gave **5** (0.512 g), 45% yield. ^{1}H NMR ($CDCl_3$) δ 7.92 (d, 1H, *J* = 7.86 Hz, pyridine), 7.14 (d, 1H, *J* = 7.68 Hz, pyridine), 4.38 (s, 2H, methylene-CH_2) 4.12 (t, 4H, *J* = 6.03 Hz, piperidine-N-(CH_2)$_2$-), 2.65 (t, 4H, *J* = 6.21 Hz, piperidine-(CH_2)$_2$$CCH_2$).

(2-(4-Methylenepiperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)methanamine (6) To a stirred solution of $LiAlH_4$ (0.085 g, 2.24 mmol) in diethyl ether (5 mL) at 0˚C, compound **5** (0.3 g, 1.12 mmol) in diethyl ether (3 mL) was added dropwise under nitrogen. After 2 h, the mixture allowed to warm to ambient temperature and stirred for 1 h and then quenched by slow addition of MeOH at 0˚C. When the suspension appeared white, it was filtered over Celite. The combined filtrate was concentrated *in vacuo*. Purification by column chromatography (MeOH/MC=1:20) gave **6** (0.197 g), 65% yield. ^{1}H NMR ($CDCl_3$) δ 8.61 (*b*s, 2H, -NH_2), 7.92 (d, 1H, *J* = 7.86 Hz, pyridine), 7.14 (d, 1H, *J* = 7.68 Hz, pyridine), 4.38 (s, 2H, methylene-CH_2), 4.31 (s, 2H, pyridine-CH_2-), 4.12 (t, 4H, *J* = 6.03 Hz, piperidine-N-(CH_2)$_2$-), 2.65 (t, 4H, *J* = 6.21 Hz, piperidine-(CH_2)$_2$CCH$_2$).

6-Chloro-8-nitro-2H-benzo[b][1,4]oxazin-3(4H)-one (8) A solution of 2-chloroacetyl chloride (1.2 mL, 15.12 mmol) in DMF (3 mL) was added to a suspension of 2-amino-4-chloro-6-nitrophenol **7** (2 g, 12.6 mmol), potassium carbonate (3.5 g, 25.2 mmol), and 18-crown-6 (0.666 g, 2.52 mmol) in DMF (10 mL) at 0° C. The reaction mixture was maintained for an additional 60 min at 0° C. and was then heated at 55° C for 15 h. The insoluble solids were removed by filtration and the filtrate was concentrated *in vacuo*. The residue was diluted with water (500 mL) and the precipitated solids were collected by filtration. The final product was purified by recrystallization from ethanol to provide compound **8** in 72 % yield as a brown solid. ^{1}H NMR ($CDCl_3$) δ 11.22 (s, 1H, benzoxazinone-N*H*-), 7.65 (s, 1H, Ar), 7.13 (s, 1H, Ar), 4.78 (s, 2H, benzoxazinone-CH_2-).

8-Amino-2H-benzo[b][1,4]oxazin-3(4H)-one (9) Compound **8** (1 g, 4.37 mmol) was dissolved in MeOH (10 mL), triethylamine (1.8 mL, 13.11 mmol) and then 10% Pd/C (0.25 g) was added. The mixture was hydrogenated under H_2 at 50 °C for 15 h. The reaction mixture was filtered over Celite and the combined filtrate was concentrated in vacuo. Purification by column chromatography (EtOAc/Hex=1:2) gave **9** (0.552 g), 77% yield. ^{1}H NMR (DMSO-d_6) δ 10.47 (s, 1H, benzoxazinone-N*H*-), 6.62 (t, 1H, *J* = 7.86 Hz, Ar), 6.31 (d, 1H, *J* = 7.89 *Hz,* Ar), 6.13 (d, 1H, *J* = 7.68 Hz, Ar), 4.85 (bs, 2H, Ar-NH_2), 4.49 (s, 2H, benzoxazinone-CH_2-).

Phenyl (3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)-carbamate (10) Phenyl chloroformate (0.44 mL, 3.53 mmol) was added dropwise to a stirred mixture of compound **9** (0.552 g, 3.36 mmol), pyridine (0.32 mL, 1.2 mmol) and THF (20 mL) at 0 °C. The mixture was stirred for 3 h at room temperature, then quenched with water (100 mL) twice, dried over $MgSO_4$, and filtrated. The combined filtrate was concentrated in vacuo. Purification by column chromatography (EtOAc/Hex=1:2) gave **10** (0.792 g), 95% yield. ^{1}H NMR (DMSO-d_6) δ 10.46 (s, 1H, benzoxazinone-N*H*-), 9.33 (s, 1H, Ar-N*H*-CO-), 7.15 (m, 2H, Ar), 6.76-6.86 (m, 3H, Ar), 6.62 (t, 1H, *J* = 7.86 Hz, Ar), 6.31 (dd, 1H, J_{ab} = 1.29 Hz, J_{cd} = 7.95 *Hz,* Ar), 6.13 (dd, 1H, J_{ab} = 1.44 Hz, J_{cd} = 7.71 *Hz,* Ar), 4.85 (bs, 2H, Ar-NH_2), 4.49 (s, 2H, benzoxazinone-CH_2-).

1-((2-(4-Methylenepiperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)methyl)-3-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]-oxazin-8-yl)urea (11) To a stirred solution of carbamate **10** (0.112 g, 0.390 mmol) and amine **6** (0.106 g, 0.390 mmol) in acetonitrile (5 mL) were added 4-dimethylaminopyridine (0.057 g, 0.468 mmol). The reaction mixture was stirred for 15 h at 50 °C. The mixture was dissolved in ethyl acetate (10 mL) and washed with water (50 mL) and brine (10 mL). The organic layer was dried over $MgSO_4$ and filtered. The combined filtrate was concentrated *in vacuo*. The crude material purified by column (EtOAc/Hex=1:2) gave **11** (155 mg) in 86% yield as a

white solid. mp = 234-242 °C . ^{1}H NMR (DMSO-d_6) δ 10.68 (s, 1H, benzoxazinone-N*H*), 8.22 (s, 1H, -N*H*-), 7.77 (d, 1H, *J* = 7.68 Hz, pyridine-*H*), 7.70 (d, 1H, *J* = 7.89 Hz, pyridine-*H*), 7.45 (d, 1H, *J* = 7.68 Hz, pyridine-*H*), 7.37 (t, 1H, *J* = 5.85 Hz, -N*H*-), 6.80 (t, 1H, *J* = 7.89 Hz, Ar), 6.47 (d, 1H, *J* = 7.89 Hz, Ar), 4.62 (s, 2H, benzoxazinone-CH_2-), 4.57 (s, 2H, methylene-CH_2), 4.38 (d, 2H, *J* = 6.36 Hz, pyridine-CH_2-NH-), 3.10 (t, 4H, *J* = 5.31 Hz, piperidine-N-(CH_2)$_2$-), 2.32 (t, 4H, *J* = 5.31 Hz, piperidine-(CH_2)$_2$CCH$_2$). HRMS (FAB) calc. for $C_{22}H_{22}F_3N_5O_3$ $[M + H]^+$ 462.1753, found 462.1748.

1-((2-(4-Methylpiperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)methyl)-3-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)urea (12) To compound **11** (0.030 g, 0.065 mmol) in MeOH (2 mL) was added 10% Pd/C (cat) and the mixture was stirred at 40 ˚C for 3 h. The reaction mixture was then allowed to warm to ambient temperature and filtered over Celite. The combined filtrate was concentrated *in vacuo*. Purification by column chromatography (EtOAc/ Hex 1:2) gave **12** (0.025 g), 83% yield as a white solid. mp = 228-236 °C. ^{1}H NMR (DMSO-d_6) δ 10.67 (s, 1H, benzoxazinone-N*H*), 8.21 (s, 1H, -N*H*-), 7.76 (d, 1H, *J* = 7.68 Hz, pyridine-*H*), 7.70 (d, 1H, *J* = 7. 89 Hz, Ar), 7.44 (d, 1H, *J* = 7.50 Hz, pyridine-*H*), 7.37 (t, 1H, *J* = 5.85 Hz, -N*H*-), 6.80 (t, 1H, *J* = 7.89 Hz, Ar), 6.47 (d, 1H, *J* = 7.89 Hz, Ar), 4.62 (s, 2H, benzoxazinone-CH_2-), 4.30 (s, 2H, pyridine-CH_2-NH-), 3.39-3.43 (m, 2H, , piperidine-N-(CH_2)$_2$-), 2.76 (t, 2H, *J* = 11.73 Hz, piperidine-N-(CH_2)$_2$-), 1.71 (d, 2H, *J* = 11.52 Hz, piperidine), 1.53 (*b*s, 1H piperidine-C*H*CH$_3$), 1.27-1.34 (m, 2H, piperidine-(CH_2)$_2$CCH$_3$), 3.33 (d, 3H, *J* = 6.21, piperidine-CHC*H*$_3$). HRMS (FAB) calc. for $C_{22}H_{24}F_3N_5O_3$ $[M + H]^+$ 464.1909, found 464.1906.

Radiolabeling for [^{3}H]MPOU. Compound **11** was radiolabeled by Quotient Bioresearch (Cardiff, United Kingdom) using reduction with tritium gas over a Pd/charcoal catalyst. The specific activity obtained was 78 Ci/mmol, as determined by mass spectroscopy. Purity was greater than 99.7% as determined by HPLC against an authentic sample of non-radiolabeled MPOU.

■ ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publication website at DOI: Purity of the final compounds, detailed experimental procedures for binding assay and molecular formula strings.

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Notes

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■ ABBREVIATIONS

TRPV1, transient receptor potential vanilloid 1; RTX, resiniferatoxin, MPOU, 1-((2-(4-methylpiperidin-1-yl)-6-(trifluoromethyl)pyridin-3-yl)methyl)-3-(3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-8-yl)urea.

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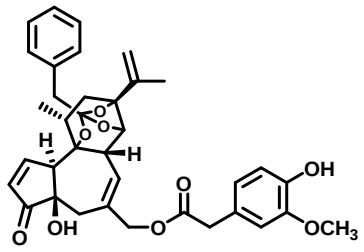

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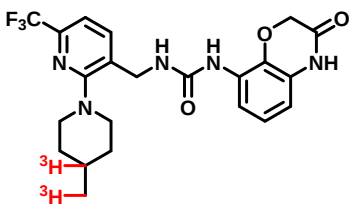

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	1	12
K_d	1.23 nM	0.63 nM
activity	agonist	antagonist
LogP	4.38	3.01
Improvement	1	45.6