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Analysis of structure–activity relationships for the 'B-region' of *N*-(3-acyloxy-2-benzylpropyl)-*N*′-[4-(methylsulfonylamino)-benzyl]thiourea analogues as vanilloid receptor antagonists: discovery of an *N*-hydroxythiourea analogue with potent analgesic activity

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Abstract—The structural modifications on the B-region of the potent and high affinity vanilloid receptor (VR1) lead ligand *N*-(3-acyloxy-2-benzylpropyl)-*N*′-[4-(methylsulfonylamino)benzyl]thiourea were investigated by the replacement of the thiourea with diverse isosteric functional groups. Structure–activity analysis indicated that the A-region in this series was the primary factor in determining the agonistic/antagonistic activities regardless of the B-region. The N_C-hydroxy thiourea analogues (**12**, **13**) showed excellent analgesic activities in the acetic acid writhing assay compared to the parent thiourea analogues.

1. Introduction

The vanilloid receptor (VR1) is a member of the transient receptor potential (TRP) superfamily. Members of this family are nonvoltage activated cation channel proteins that play critical roles in processes ranging from sensory physiology to vasorelaxation and male fertility, and share structural similarities, such as six transmembrane segments.[1,2] The vanilloid or capsaicin receptor (VR1[3] or TRPV1) has been cloned from dorsal root ganglia (DRG) of rat,[4] the human,[5] the chicken,[6] and the guinea pig.[7] Other vanilloid receptor homologues were also cloned recently from different organisms but are not believed to be sensitive to vanilloids.[1] VR1, which is expressed predominantly on unmyelinated pain-sensing nerve fibers (C-fibers) and small Aδ fibers in the dorsal root, trigeminal, and nodose ganglia, is a molecular integrator of nociceptive stimuli. VR1 is activated by protons,[8] heat,[9] natural ligands such as capsaicin (CAP),[10] resiniferatoxin (RTX),[11] and endogenous substances such as anandamide[12] and the lipoxygenase product 12-HPETE.[13] Since VR1 functions as a nonselective cation channel with high Ca^{2+} permeability, its activation by these agents leads to an increase in intracellular Ca^{2+} that results in excitation of primary sensory neurons and ultimately the central perception of pain.

The involvement of this receptor in both pathological and physiological conditions suggests that the blocking

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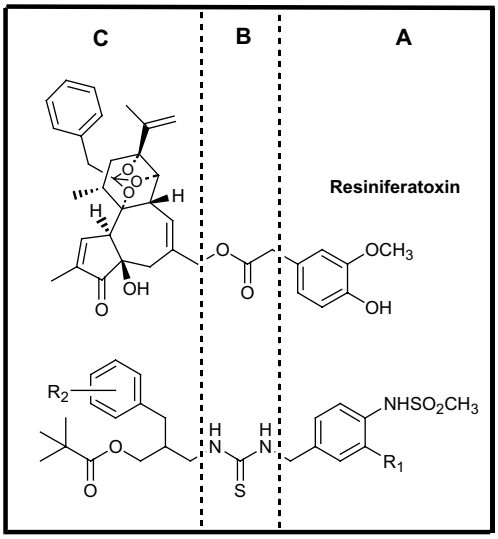
of this receptor activation, by desensitization or antagonism, would have considerable therapeutic utility. Among its therapeutic targets, pain is of particular interest. The validation of VR1 as a molecular target for the treatment of chronic pain was confirmed using transgenic mice lacking functional VR1 receptors. These mice exhibited an impairment in the perception of thermal and inflammatory pain.[14]

VR1 antagonists have attracted much attention as promising drug candidates to inhibit the transmission of painful signals from the periphery to the CNS and to block other pathological states associated with this receptor. The therapeutical advantage of VR1 antagonism over agonism is that it lacks the initial excitatory effect preceding the desensitization. The initial acute pain associated with capsaicin treatment has proven to be the limiting toxicity. After the discovery of capsazepine as the first VR1 antagonist,[15] a number of antagonists have been reported such as ruthenium red,[16] capsazocaine,[17] 5-iodo-RTX,[18] trialkyglycines,[19] hexapeptides,[20] SB-366791,[21] amine derivatives,[22] BCTC,[23] halogenated capsaicin analogues,[24] thioureas,[25] and thiocarbamate analogues.[26] 5-Iodo-RTX appears to be the most potent among them in vitro and blocked capsaicin-induced current in oocytes expressing VR1 with an $IC_{50} = 3.9$ nM.[18] BCTC was characterized in vivo as a potent, orally-effective, VR1 selective antagonist.[23]

We have previously reported that isosteric replacement of the phenolic hydroxyl group in potent vanilloid receptor agonists with the alkylsulfonamido group provided a series of compounds, which are effective antagonists to the action of capsaicin on rat VR1 heterologously expressed in Chinese hamster ovary (CHO) cells. Among these compounds, methylsulfonamides **1**, **2**, and **4** showed high binding affinities with K_i values of 29.3, 54, and 49 nM, respectively, for the inhibition of [^{3}H]RTX binding (Fig. 1). For comparison, the $K_i = 1300$ nM for capsazepine in the same system.[27] Whereas the 4-methylsulfonamido analogue **1** antagonized $^{45}Ca^{2+}$ uptake in response to capsaicin with an $IC_{50} = 67$ nM and displayed partial agonism,[28] its 3-fluoro analogue, **2**, was a full and potent antagonist, with a $K_i = 7.8$ nM (compared to 520 nM for capsazepine) and, conversely, its 3-methoxy analogue, **4**, was instead a full agonist with an $EC_{50} = 22$ nM.[27] From our structure–activity analysis, we demonstrated that the modifications in both the A- and C-regions of 4-methylsulfonamide ligands combined to influence the pattern of VR1 agonism/antagonism.[27]

As a continuation of our effort to optimize in vitro and in vivo activities of 4-methylsulfonamide VR1 ligands, we have investigated the structure–activity relationships in the B-region of the high affinity lead compounds **1**, **2**, and **4**. We have modified the thiourea group of the lead compounds with diverse isosteric functional groups, such as *N*-hydroxy thiourea, thiocarbamate, amide, and ester groups to provide novel VR1 ligands. Herein, we describe the syntheses, in vitro characterization using CHO cells transfected with rat VR1, and analgesic activities of the B-region analogues, and we analyze the structure–activity relationships, which these compounds reveal.

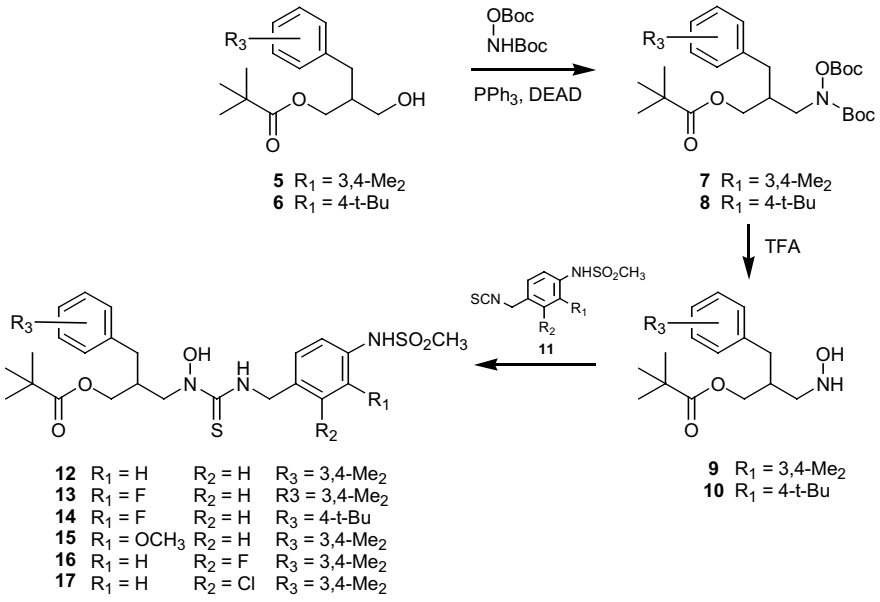


1 R_1 = H R_2 = 3,4-Me_2
2 R_1 = F R_2 = 3,4-Me_2
3 R_1 = F R_2 = 4-t-Bu
4 R_1 = OCH_3 R_2 = 3,4-Me_2

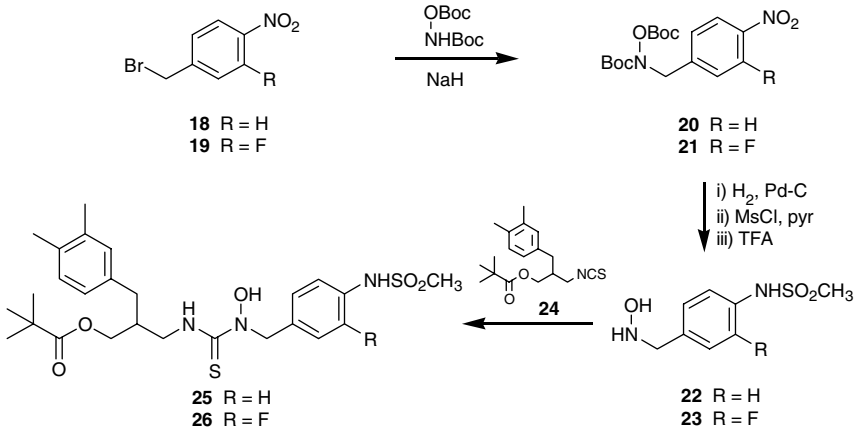
Figure 1.

2. Chemistry

The reference thiourea **3** was prepared according to a previous report.[27] The syntheses of the two types of *N*-hydroxy thiourea analogues (N_C and N_A refer to the nitrogens next to the C- and A-regions, respectively) were outlined in Schemes 1 and 2. 3-Pivaloyloxy-2-benzylpropyl alcohols (**5**, **6**), corresponding to the C-region, were synthesized according to a previous report.[29] The alcohols of **5**, **6** were converted into the corresponding *N*,*O*-diBoc hydroxylamine by the Mitsunobu reaction to produce **7** and **8**, respectively. After the hydrolysis of diBoc groups under acidic conditions, the resulting hydroxylamines (**9**, **10**) were condensed with the various isothiocyanates[27] to afford the N_C-hydroxy thioureas (**12**–**17**), respectively. The alkylation of *N*,*O*-diBoc hydroxylamine with **18** and **19**[30] produced **20** and **21**, whose nitro groups were converted into the corresponding methylsulfonamides (**22**, **23**), respectively. The coupling of hydroxylamines (**22** and **23**) with the isothiocyanate (**24**)[31] furnished the N_A-hydroxy thioureas (**25**, **26**), respectively. The syntheses of thiocarbamate analogues are shown in Scheme 3. The O-alkylation of 4-nitrobenzyl alcohol with the isothiocyanate (**24**) followed by reduction and mesylation provided the thiocarbamate **28**. The syntheses of its 3-fluoro and 3-methoxy analogues, **33** and **34**, were accomplished by a similar method from alcohols **30** and **32**, which were prepared starting from **29**[32] and **31**.[33] The syntheses of amide and ester analogues were shown in Scheme 4. 4-(Methylsulfonylamino)phenylacetic acid analogues (**37**–**39**) were synthesized from **29**, **35**, and



Scheme 1. Synthesis of N_C-hydroxy thiourea analogues.



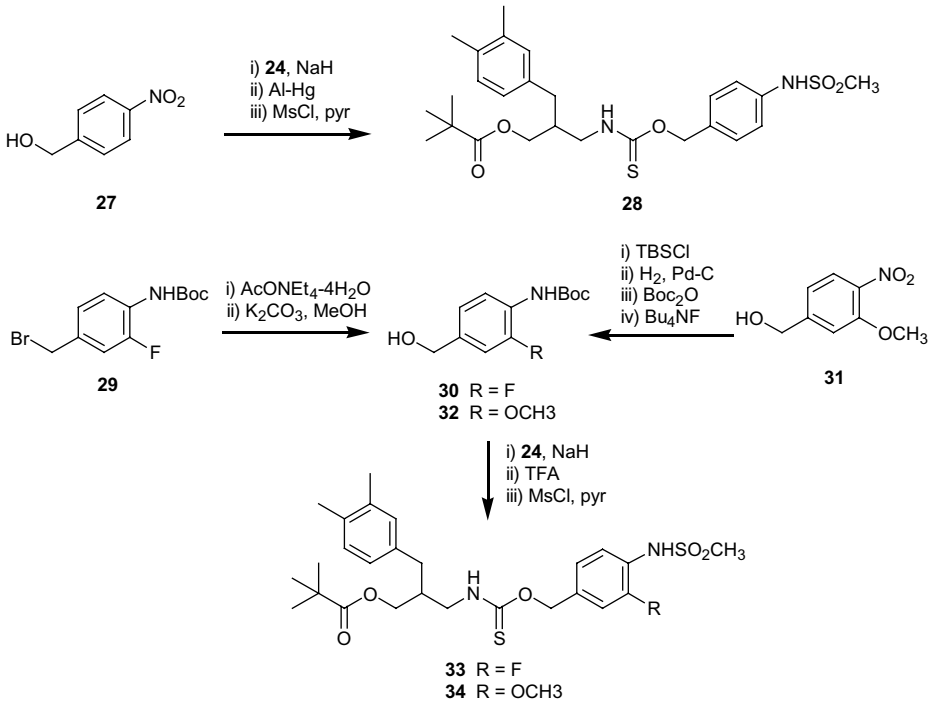
Scheme 2. Synthesis of N_A-hydroxy thiourea analogues.

36,[33] respectively. The acids (**37**–**39**) were condensed with **5** and **40**[29] under EDC coupling to provide the corresponding amides (**41**–**44**) and the ester (**45**) analogue, respectively.

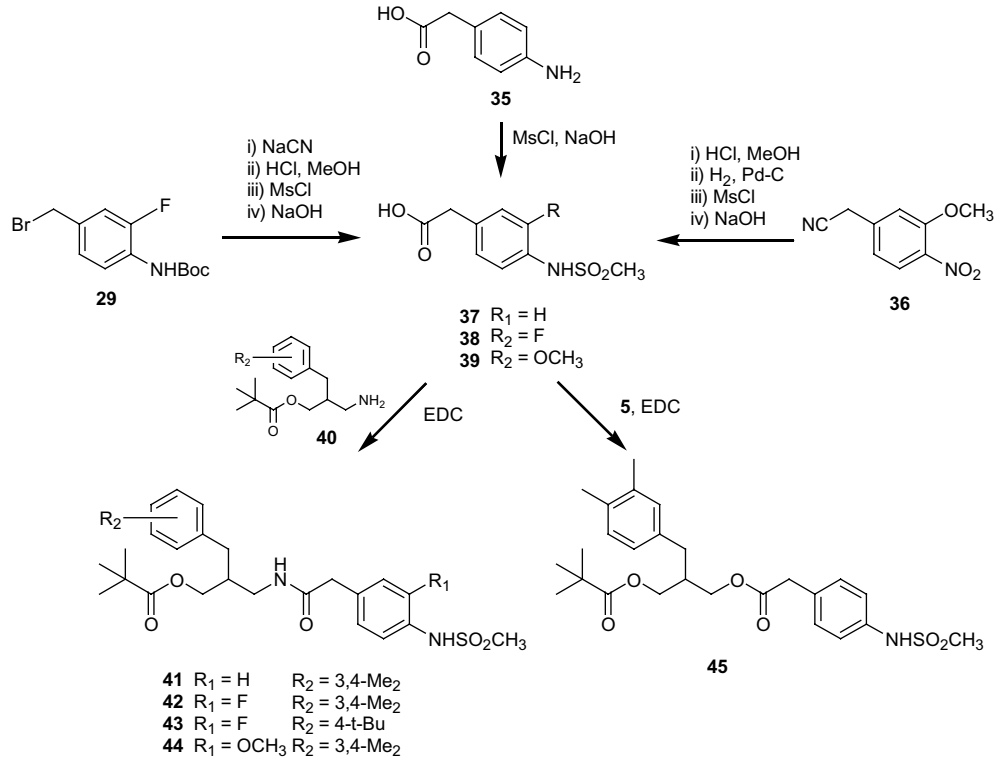
3. Results and discussion

The binding affinities and agonistic/antagonistic potencies of the synthesized VR1 ligands were assessed in vitro by a competition assay with [^{3}H]RTX and a $^{45}Ca^{2+}$ uptake assay using rat VR1 heterologously expressed in Chinese hamster ovary (CHO) cells,[34] as previously described.[28,35] The results are summarized in Table 1. The biochemical behavior and potencies of reference compounds (**1**, **2**, **4**, and capsazepine) were described in detail in previous reports.[27,28]

The thiourea **3**, a 4-*tert*-butylbenzyl surrogate of **2**, displayed 2-fold greater potency in binding affinity ($K_i = 22.6$ nM) but 7-fold lower potency for antagonism ($K_i = 52.4$ nM), as well as a shift to partial agonism, compared to **2**. The N_C-hydroxy thiourea analogues (**12**–**15**) were compared with the corresponding lead thioureas (**1**–**4**), respectively. Their binding affinities were attenuated by 4–35-fold. Nevertheless, their functional characteristics were similar to those found in the thioureas and depended on the effect of the 3-substituent; namely, **1/12** (3-hydrogen) were antagonists with limited partial agonism, **2/13** (3-fluoro) were full antagonists, and **4/15** (3-methoxy) were full agonists. Among the 2-substituent analogues, whereas the 2-fluoro N_C-hydroxy thiourea analogue (**16**) was a partial agonist with reduced binding affinity, the 2-chloro N_C-hydroxy thiourea analogue (**17**) was a full antagonist with equal binding affinity and reduced antagonistic potency compared to **13**. The N_A-hydroxy thiourea analogues (**25**–**26**) were also examined. However, they



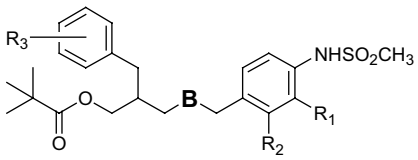
Scheme 3. Synthesis of thiocarbamate analogues.

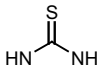
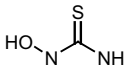
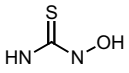


Scheme 4. Syntheses of amide and ester analogues.

exhibited reduced potencies in binding affinities and antagonisms compared to N_C-hydroxy thiourea analogues. The thiocarbamate analogues (**28**, **33**, **34**), N_A-oxa analogues of thiourea, showed moderate reduction (ca. 4–10-fold) in binding affinities and potencies in the functional assays compared to the thioureas (**1**–**2**, **4**). Like the N_C-hydroxy thioureas, their functional characteristics followed the patterns of the A-region as determined for the thioureas. The amide analogues (**41**–**44**) were likewise weaker congeners of the corresponding thioureas. The ester analogue (**45**) was much less potent than the amide surrogate (**41**).

Table 1. Potencies of vanilloid ligands for binding to rat VR1 and for inducing calcium influx in CHO/VR1 cells



B	R_1	R_2	R_3	Binding affinity K_i (nM)	Agonism EC_{50} (nM)	Antagonism IC_{50} (nM)
Capsazepine				1300 (±150)	NE	520 (±12)
						
1	H	H	3,4-Me_2	29.3 (±7.6)	WE[a]	67 (±25)
2	F	H	3,4-Me_2	54 (±28)	NE	7.8 (±3.0)
3	F	H	4-*t*-Bu	22.6 (±2.7)	WE[a]	52 (±17)
4	OCH_3	H	3,4-Me_2	49 (±15)	22 (±10)	NE
						
12	H	H	3,4-Me_2	1042 (±73)	WE[a]	212 (±85)
13	F	H	3,4-Me_2	212 (±40)	NE	94 (±14)
14	F	H	4-*t*-Bu	405 (±15)	WE[a]	10,100 (±4500)
15	OCH_3	H	3,4-Me_2	396 (±62)	809 (±126)	NE
16	H	F	3,4-Me_2	620 (±150)	WE[a]	NE
17	H	Cl	3,4-Me_2	220 (±54)	NE	757 (±65)
						
25	H	H	3,4-Me_2	481 (±67)	WE[a]	331 (±78)
26	F	H	3,4-Me_2	546 (±53)	WE[a]	NE
						
28	H	H	3,4-Me_2	336 (±38)	WE[a]	440 (±140)[b]
33	F	H	3,4-Me_2	210 (±120)	WE[a]	170 (±37)
34	OCH_3	H	3,4-Me_2	199 (±97)	373 (±155)[a]	NE
						
41	H	H	3,4-Me_2	500 (±100)	WE[a]	460 (±250)
42	F	H	3,4-Me_2	157 (±38)	WE[a]	150 (±36)[b]
43	F	H	4-*t*-Bu	74 (±13)	WE[a]	440 (±190)[b]
44	OCH_3	H	3,4-Me_2	430 (±120)	244 (±45)[a]	NE
						
45	H	H	3,4-Me_2	1980 (±240)	NE	3602 (31)

NE: not effective, WE: weakly effective at 30 μM.

[a] Only fractional calcium uptake compared to 300 nM capsaicin (**1**, 15%; **3**, 5%; **12**, 12%; **14**, 13%; **16**, 20%; **25**, 12%; **26**, 24%; **28**, 11%; **33**, 7.5%; **34**, 50%; **41**, 5.6%; **42**, 10%; **43**, 7%; **44**, 40%).

[b] Only fractional antagonism (**28**, 79%; **42**, 65%; **43**, 56%).

Previously, we have demonstrated that the A-region makes a major contribution to the extent of agonism/antagonism in a series of thiourea analogues;[27] namely, substitution of the 4-phenolic hydroxyl of the A-region

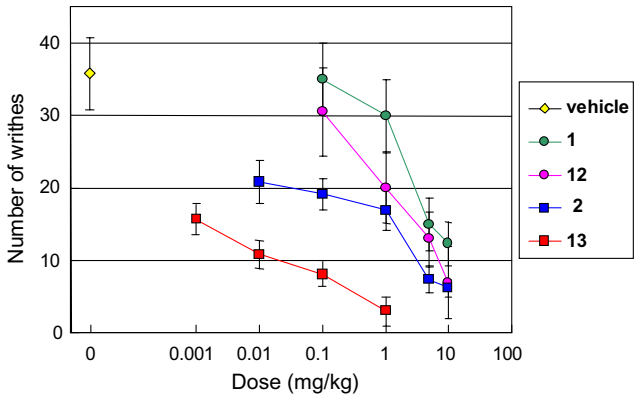


Figure 2. Analgesic activities of compounds **1**, **2**, **12**, and **13** by acetic acid-induced writhing test.

in agonists with a 4-methylsulfonamido group shifted the ligands from agonism toward antagonism, and a 3-fluoro substitution further favored antagonism, whereas a 3-methoxy group favored agonism. This observation was also examined in this structural series with different B-regions. Although their potencies in the binding and functional assays were less than those of thiourea analogues, their extent of agonism/antagonism depended primarily on the A-region as was the case for the thiourea analogues described previously, regardless of B-region. In this structural series, 4-methylsulfonamido compounds showed antagonism with a low level of agonism. The incorporation of the 3-fluoro further enhanced the antagonism; the 3-methoxy group shifted the ligands to full agonists.

The synthesized compounds were screened for their analgesic activities in the acetic acid-induced writhing model, according to the previous protocol.[27,36] Among them, N_C-hydroxy thiourea analogues (**12**, **13**) showed promising activity compared to those of the thiourea analogues (**1**, **2**). Whereas compound **12** displayed comparable potency to thiourea **1**, compound **13** exhibited dramatically enhanced analgesic potency that was approximately two orders of magnitude more potent than that of thiourea **2** in the writhing assay (Fig. 2). Since the in vitro potencies of N_C-hydroxy thiourea analogues were less than those of thioureas, we initially surmised that the N_C-hydroxy thiourea was converted to the corresponding thiourea in the body. However, a preliminary study of their metabolism indicated that the N_C-hydroxy thiourea **12** was not a prodrug of thiourea **1** because **1** was not detected among the metabolites of **12**.[37] The detailed mechanistic and pharmacokinetic analysis of the N_C-hydroxy thiourea analogues are ongoing in an effort to explain their high analgesic potencies.

In summary, we have modified the B-region thiourea group of potent and high affinity VR ligands (**1**, **2**, and **4**) by substitutions with *N*-hydroxythiourea, thiocarbamate, amide, and ester groups to investigate their structure–activity relationships. Although the modifications generally conferred modest decreases in binding affinities and agonistic/antagonistic potencies, their SAR analysis indicated that the functional characteristics of the 4-methylsulfonamide series depended primarily on the A-region in which 3-fluoro favored antagonism and 3-methoxy favored agonism. Despite its relatively weak in vitro potency, compound **13**, an N_C-hydroxy thiourea analogue, exhibited high analgesic potency in the writhing assay. Compound **13** has been chosen for further investigation as an analgesic candidate and for further mechanistic study.

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34. Receptor binding affinities were assessed in terms of the ability of the compounds to compete with specific [^{3}H]RTX binding in the CHO/VR1 system and were expressed as $K_i \pm$ SEM. Potencies as agonists were expressed as $EC_{50} \pm$ SEM, and absolute levels of $^{45}Ca^{2+}$ uptake were compared with that induced by a maximally effective concentration of capsaicin in this system (300 nM). The in vitro antagonistic potencies of the compounds were evaluated by measuring antagonism of the $^{45}Ca^{2+}$ uptake induced by 50 nM capsaicin and expressed as the $IC_{50} \pm$ SEM, respectively, correcting for competition by capsaicin. All K_i and EC_{50} values represent the mean of at least three experiments. Levels of partial agonism are from one to four experiments.
35. Wang, Y.; Szabo, T.; Welter, J. D.; Toth, A.; Tran, R.; Lee, J.; Kang, S. U.; Lee, Y.-S.; Min, K. H.; Suh, Y.-G.; Park, M.-K.; Park, H.-G.; Park, Y.-H.; Kim, H.-D.; Oh, U.; Blumberg, P. M.; Lee, J. (erratum published in *Mol. Pharmacol.* **2003**, *63*, 589) *Mol. Pharmacol.* **2002**, *62*, 947.
36. Experimental protocols involving animals in this study were reviewed by the Animal Care and Use Committee of Digital Biotech Co., according to the NIH guidelines (NIH publication number 85-23, revised 1985) of 'Principles of Laboratory Animal Care'.
37. Since **1** and **12** are structurally related, we have studied whether **1** may be generated after the systemic administration of **12**. Thus, 5 mg/kg of **12** was administered intravenously to male Sprague–Dawley rats (250–280 g) and 0.25 mL of blood was collected at various times up to 120 min. Plasma was collected and an aliquot (0.15 mL) was deproteinated by the addition of 0.3 mL acetonitrile. An aliquot of the supernatant (0.35 mL) was evaporated to dryness under a stream of nitrogen and the residue was reconstituted by the addition of 0.1 mL of mobile phase (water/methanol/acetonitrile = 20:40:40). An aliquot of the mixture was injected directly onto the LC–MS system. Although both **1** and **12** could be readily quantified by the LC–MS system, the concentration of **12**, but not **1**, was detected in the sample. Since we could not have detected any conversion of **12** into **1**, we conclude that the action of **12** cannot be explained by its conversion to **1** in vivo.