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Bioorganic & Medicinal Chemistry 12 (2004) 3411–3420
Bioorganic & Medicinal Chemistry

Analysis of structure–activity relationships with the *N*-(3-acyloxy-2-benzylpropyl)-*N*′-[4-(methylsulfonylamino)benzyl]thiourea template for vanilloid receptor 1 antagonism

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Received 23 March 2004; revised 30 April 2004; accepted 30 April 2004

Abstract—In a continuing effort to elucidate the structure–activity relationships of the lead antagonist *N*-[2-(3,4-dimethylbenzyl)-3-pivaloyloxypropyl]-*N*′-[4-(methylsulfonylamino)benzyl]thiourea (**1**), the distances between the proposed four pharmacophores in **1** have been varied by insertion or deletion of one carbon to optimize their fit to the receptor. In addition, the acyloxy group of the C region was replaced with amide and *N*-hydroxy amide to identify the pharmacophoric importance of the ester group in the C2 region. The results indicated that the pharmacophoric arrangement of **1** was optimal for receptor binding affinity and antagonism, and the ester of the C2 region was significant for receptor binding. Among the derivatives, compound **19** showed distinct behavior with a 2-fold improvement in antagonism but a 13-fold reduction in binding affinity compared to **1**. The partial separation of pharmacophoric requirements of these two assays has been noted before and compound **19** is thus selective for the calcium entry-linked receptor population. The conformational analysis of **1** generated three distinct conformers having different types of hydrophobic interactions, which will be utilized for exploring the active conformation of the VR1 ligand.

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. They share structural similarities such as six transmembrane segments and an oligmeric structure.[1,2] The vanilloid or capsaicin receptor (VR1[3] or TRPV1) has been cloned from dorsal root ganglia (DRG) of the rat,[4] the human,[5] the chicken,[6] the guinea pig,[7] and the rabbit.[8] Vanilloid receptor homologues have also been cloned but are not believed to be sensitive to vanilloids.[1] VR1, which is expressed predominantly on thin, unmyelinated sensory 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,[9] heat,[10] natural exogenous ligands such as capsaicin (CAP)[11] or resiniferatoxin (RTX),[12] and endogenous substances such as anandamide[13] and the lipoxygenase product 12-HPETE.[14] 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. Chronic stimulation of VR1 leads to desensitization/defunctionalization of the neurons, probably reflecting multiple mechanisms.

The involvement of VR1 in both pathological and physiological conditions suggests that the blocking of

Keywords: Vanilloid receptor 1; TRPV1; Antagonist; Capsaicin; Resiniferatoxin.
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doi:10.1016/j.bmc.2004.04.040

this receptor, by desensitization or by 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 impairment in the perception of thermal and inflammatory pain.[15]

The therapeutical advantage of VR1 antagonism over desensitization subsequent to agonism is that it avoids 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,[16] a number of antagonists have been reported both with structures related and unrelated to agonists.[17] Among them, 5-Iodo-RTX,[18] SC0030,[19] halogenated capsaicin analogues,[20] BCTC,[21] SB-366791,[22] 7-hydroxynaphthalen-1-yl urea,[23] and IBTU[24] were characterized in detail as potent VR1 competitive antagonists.

We have previously reported that isosteric replacement of the phenolic hydroxyl group in potent vanilloid receptor agonists[25] 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 (Fig. 1).[26] As a prototype, *N*-[2-(3,4-dimethylbenzyl)-3-pivaloyloxypropyl]-*N*′-[4-(methylsulfonylamino)-benzyl]thiourea (**1**) showed a high binding affinity with a K_i value of 29.3 nM for the inhibition of [^{3}H]RTX binding and potent antagonism with an IC_{50} value of 67 nM for the inhibition of $^{45}Ca^{2+}$ uptake in response to capsaicin, displaying partial agonism.[27] The SAR of the prototype has been examined extensively based on the pharmacophoric regions represented in Figure 1.[26,28]

In the SAR of the A region, the 3-fluoro analogue of **1** was a full and potent antagonist with an $IC_{50} = 7.8$ nM and, conversely, the 3-methoxy analogue of **1** was instead a full agonist with an $EC_{50} = 22$ nM.[26] The results generalized that the A region makes a major contribution to the extent of agonism/antagonism in a series of thiourea analogues; namely, substitution of the 4-phenolic hydroxyl of the A region 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.

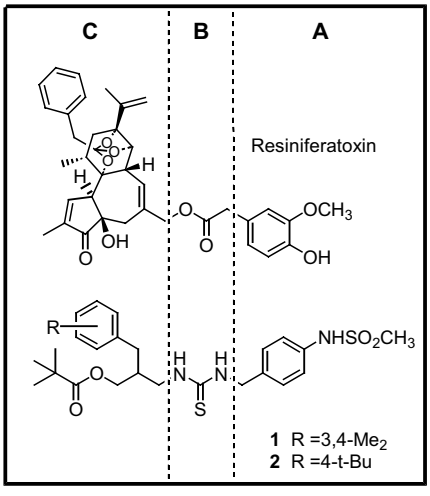


Figure 1. The pharmacophoric comparison between RTX and *N*-(2-benzyl-3-pivaloyloxypropyl)-*N*′-[4-(methylsulfonylamino)benzyl]thiourea.

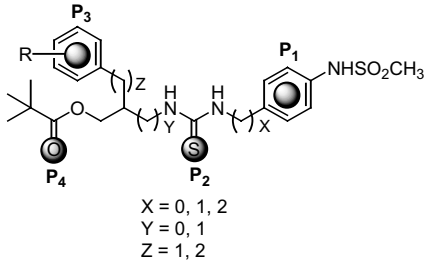


Figure 2. The four principal pharmacophores of antagonist.

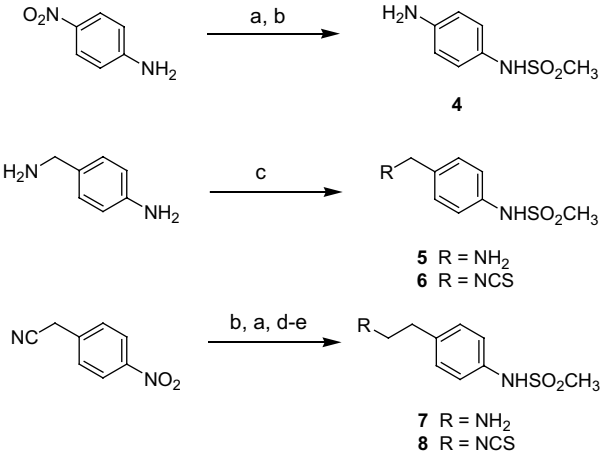
For the SAR of the B region, we modified the thiourea group of **1** with diverse isosteres, including *N*-hydroxy thiourea, thiocarbamate, amide, and ester groups.[28] Structure–activity analysis indicated that the thiourea group provided the best in vitro profile and the A region was a primary factor in determining the agonistic/antagonistic activities regardless of the B region. Interestingly, the N_C-hydroxy thiourea analogues showed excellent analgesic activities in the acetic acid writhing assay compared to the parent thiourea analogues despite relatively less potency in agonism and in binding affinity.[28]

In a continuation of these studies, we have now examined further aspects of the SAR. The four pharmacophores in the lead antagonist **1**, denoted as P_1–P_4 in Figure 2, were derived from our RTX-derived pharmacophore model, which incorporates the 4-hydroxy-3-methoxyphenyl (A region), C_{20}-ester (B region), orthophenyl (C_1 region) and C_3-keto (C_2 region) groups. For the purpose of optimizing the spatial arrangement of these pharmacophores, we have varied the distances between the four pharmacophores by lengthening or shortening them by one carbon, respectively. A similar approach was examined previously in a series of agonists and provided a plausible active conformation of RTX.[29] In addition, structural modification of the acyloxy group of the C region, the so-called C2 region, was examined by replacing it with amide and *N*-hydroxy amide to define its pharmacophoric importance. In this paper, we describe the synthesis and biological activity profiles of these VR1 ligands, and analyze their SAR.

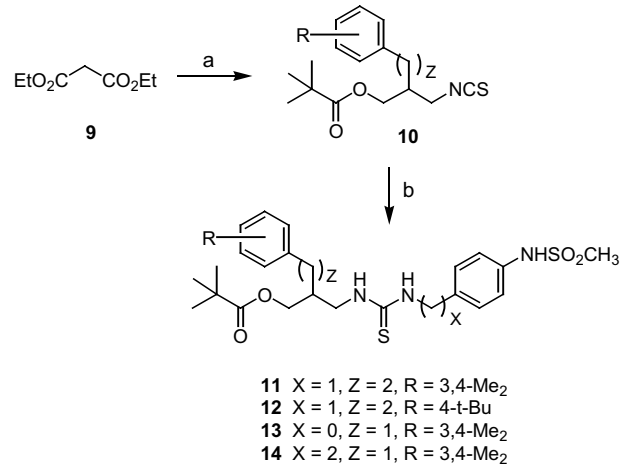
2. Chemistry

General syntheses of the final target thioureas were achieved by the coupling between the corresponding isothiocyanates and amines. The amines (**4**, **5**, **7**) and

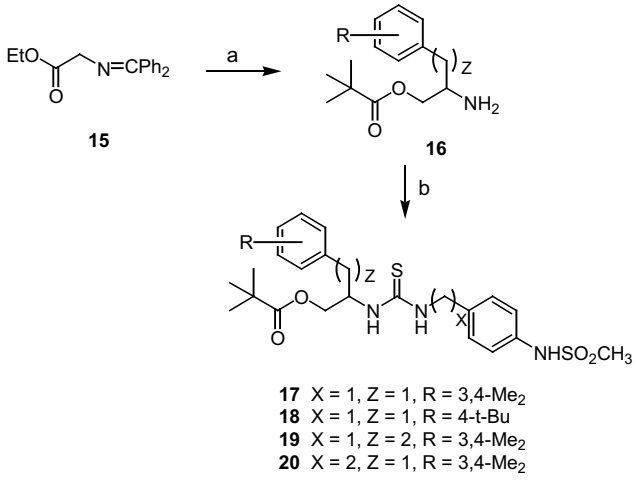
isothiocyanates (**6**, **8**) corresponding to the A region were prepared starting, respectively, from 4-nitroaniline (X = 0), 4-aminobenzyl amine (X = 1), and 4-nitrophenylacetonitrile (X = 2) by conventional methods[26] (Scheme 1). The isothiocyanates of the C region having Y = 1 (**10**) were synthesized from ethyl malonate as reported previously[25,29] and then coupled with amines (**4**, **5**, **7**) to provide thioureas **11**–**14** (Scheme 2). The amines of the C region having Y = 0 (**16**) were prepared from *N*-(diphenylmethylene)glycine ethyl ester by the procedure reported previously[29] and then reacted with isothiocyanates **6** and **8** to afford thioureas **17**–**20**, respectively (Scheme 3). For the synthesis of the amide congener of the pivaloyloxy group in **1**, the hydroxyl group of 2-(3,4-dimethylbenzyl)-3-hydroxypropyl azide (**21**)[25] was converted to the corresponding amine by the Gabriel method, and was then acylated with pivaloyl chloride to give **23**. Azido reduction of **23** followed by coupling with isothiocyanate **6** afforded the target amide analogue **24** (Scheme 4). For the synthesis of the *N*-hydroxyamide congener, the hydroxyl group of **21** was transformed into the corresponding hydroxylamine **26** via the Mitsunobu reaction with di-(*t*-butoxycarbonyl)hydroxylamine followed by acid hydrolysis. The pivaloylation and coupling with **6** as described in



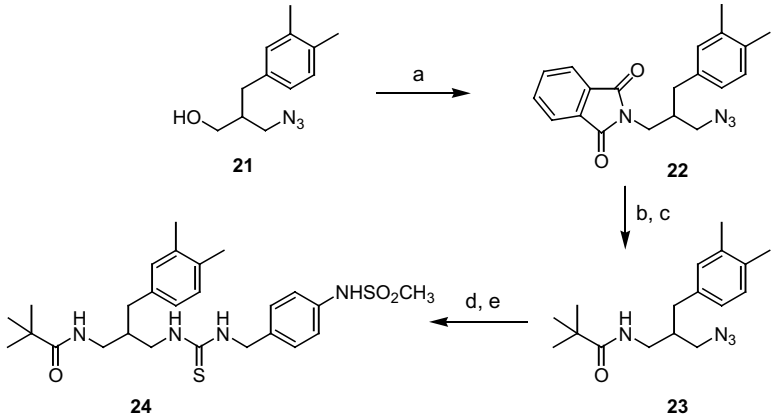
Scheme 1. Synthesis of A region. (a) MsCl, pyridine; (b) H_2, Pd–C, MeOH; (c) Ref. 26; (d) H_2, c-HCl, MeOH; (e) 1,1′-thiocarbonyldiimidazole, CH_2Cl_2.



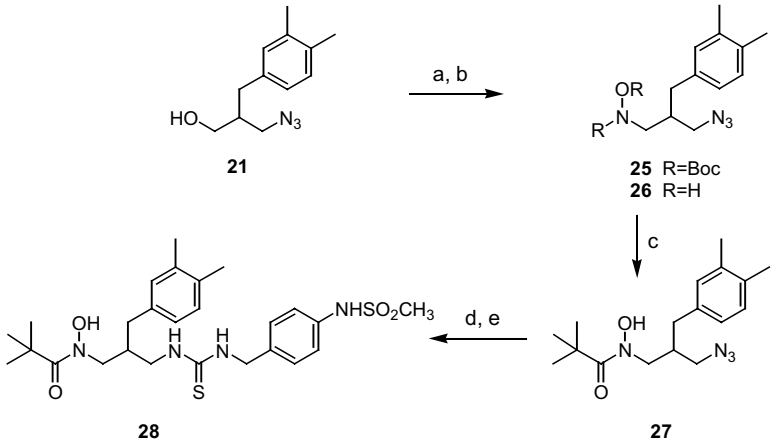
Scheme 2. Synthesis of target compounds with Y = 1. (a) Ref. 25; (b) **4** (or **5**, **7**), CH_2Cl_2.



Scheme 3. Synthesis of target compounds with Y = 0. (a) Ref. 29; (b) **6** (or **8**), CH_2Cl_2.



Scheme 4. Synthesis of amide congener. (a) Phthalimide, PPh_3, DEAD, THF; (b) NH_2NH_2, EtOH; (c) Me_3CCOCl, Et_3N, CH_2Cl_2; (d) H_2, Lindler cat, EtOH; (e) **6**, CH_2Cl_2.



Scheme 5. Synthesis of *N*-hydroxyamide congener. (a) NH(Boc)OBoc, PPh_3, DEAD, THF; (b) CF_3CO_2H; (c) Me_3CCOCl, Et_3N, CH_2Cl_2; (d) H_2, Lindler cat, EtOH; (e) **6**, CH_2Cl_2.

Scheme 3 provided the target *N*-hydroxyamide analogue **28** (Scheme 5).

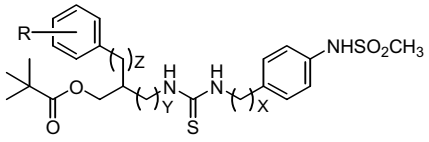
3. Results and discussion

3.1. Biological activity

The potencies and activities as agonists and antagonists of the synthesized VR1 ligands were assessed in vitro by assay of $^{45}Ca^{2+}$ uptake, which was carried out using rat VR1 heterologously expressed in Chinese hamster ovary cells (CHO/VR1 cells) as previously described.[19,26] 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 $K_i \pm$ SEM, correcting for competition by capsaicin. All compounds were also evaluated as agonists. 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, namely 300 nM. Receptor binding affinities were assessed in terms of the ability of the compounds to compete for specific binding of [^{3}H]RTX in the CHO/VR1 system and were expressed as the $K_i \pm$ SEM. All values±SEM represent the mean of at least three experiments. The results are summarized in Tables 1 and 2. In the CHO/VR1 system, the parent antagonists, **1** and **2** (X = Y = Z = 1), showed high binding affinities with K_i values of 29.3 and 64 nM, respectively, and potent antagonism with K_i values of 67 and 86 nM, displaying partial agonism,[26] respectively. Their potencies represented 44- and 20-fold enhancements in binding affinity and 8- and 6-fold enhancements in antagonism compared to capsazepine ($K_i = 1300$ nM in binding affinity, $K_i = 520$ nM in antagonism).

In the variation of the Z part of the template, one-carbon lengthening of the benzyl group in the parent

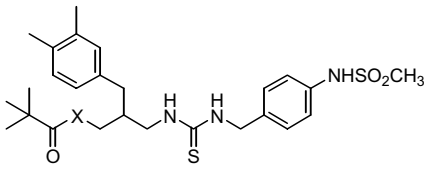
Table 1. Potencies of vanilloid ligands for binding to rat VR1 and for inducing calcium influx in CHO/VR1 cells



	X	Y	Z	R	K_i (nM) binding affinity	EC_{50} (nM) agonism	K_i (nM) antagonism
Capsazepine					1300 (±150)	NE	520 (±12)
1	1	1	1	3,4-Me_2	29.3 (±7.6)	WE[a]	67 (±25)
2	1	1	1	4-t-Bu	64 (±21)	WE[a]	86 (±17)
11	1	1	2	3,4-Me_2	1430 (±390)	WE[a]	121 (±39)
12	1	1	2	4-t-Bu	690 (±250)	1675 (±106)	NE
13	0	1	1	3,4-Me_2	4670 (±800)	WE[a]	WE[b]
14	2	1	1	3,4-Me_2	1850 (±360)	WE[a]	790 (±260)
17	1	0	1	3,4-Me_2	580 (±130)	WE[a]	WE[b]
18	1	0	1	4-t-Bu	416 (±44)	WE[a]	WE[b]
19	1	0	2	3,4-Me_2	390 (±100)	WE[a]	33.7 (±2)
20	2	0	1	3,4-Me_2	3170 (±590)	NE	1750 (±580)

NE: not effective, WE: weakly effective at 30 μM.
[a] Only fractional calcium uptake compared to 30 μM capsaicin **1** (at 300 nM), 15%; **2**, 40%; **11**, 23%; **13**, 21%; **14**, 6%; **17**, 19%; **18**, 8%; **19**, 5%.
[b] Only fractional inhibition **13**, 72%; **17**, 48%; **18**, 31%.

Table 2. Potencies of vanilloid ligands for binding to rat VR1 and for inducing calcium influx in CHO/VR1 cells



	X	K_i (nM) binding affinity	EC_{50} (nM) agonism	K_i (nM) antagonism
1	O	29.3 (±7.6)	WE	67 (±25)
24	NH	7700 (±2900)	NE	NE
28	NOH	2270 (±120)	NE	1870 (±180)

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

compounds **1** and **2** (Z = 1), providing *N*-(3-pivaloyl-2-phenethylpropyl) analogues **11** and **12** (Z = 2), led to reduced potencies in binding affinity with values of $K_i = 1430$ and 690 nM (ca. 50-fold in **11**, ca. 10-fold in **12**) as compared to **1** and **2**, respectively. Interestingly, this modification showed a complicated result. Whereas the 3,4-dimethyl analogue **11** showed antagonism with weak partial agonism like the parent compound, the 4-*tert*-butyl analogue **12** exhibited full agonism although the potency was low. This finding is yet another example that functional receptor activity is sensitive to even small structural modification, as we reported previously.[30]

In the variation of the X part of the template, one-carbon shortening of **1** (X = 0), producing the *N*-[4-(methylsulfonylamino)phenyl] derivative **13**, resulted insubstantial loss of potencies in binding affinity and antagonism. Likewise, one-carbon lengthening of **1** (X = 2), producing the *N*-[4-(methylsulfonylamino)phenethyl] derivative **14**, also led to reduced potencies in binding affinity (63-fold, $K_i = 1850$ nM) and antagonism (12-fold, $K_i = 790$ nM), but their activities were a little better than those of the shortened derivative **13**.

In the variation of the Y part of the template, one-carbon shortening of **1** and **2** (Y = 0) provided the *N*-(2-pivaloyl-1-benzylethyl) analogues **17** and **18**, for which the binding affinities were reduced 20-fold and 7-fold ($K_i = 580$ nM in **17**, $K_i = 416$ nM in **18**), respectively, and the potencies as agonists and antagonists were substantially lost.

The simultaneous variation of Y and Z of **1** to provide **19** (Y = 0 and Z = 2) showed a complicated but very interesting result. Whereas the binding affinity of **19** was reduced by 13-fold ($K_i = 390$ nM) compared to that of **1**, its potency as an antagonist increased by 2-fold ($K_i = 33.7$ nM). This finding is yet another example that receptor binding affinity is not fully correlated with antagonist activity. Such divergence between binding affinity and antagonism was recently described in detail with biochemical characterization of an antagonist, *N*-(4-chlorobenzyl)-*N*′-(4-hydroxy-3-iodo-5-methoxybenzyl)thiourea (IBTU).[24] Whereas IBTU showed potent antagonism of vanilloid-induced calcium uptake ($K_i = 99$ and 93 nM for capsaicin and RTX, respectively), at 30 μM it inhibited [^{3}H]resiniferatoxin binding to VR1 by less than 10%. These distinct potencies reflect different fractions of VR1 in the two assay systems: namely, a plasma membrane fraction controlling $^{45}Ca^{2+}$ uptake, and an intracellular fraction that dominates the [^{3}H]RTX binding measurements. Therefore, as with IBTU, the divergence of **19** in binding affinity and antagonism may be explained by marked selectivity between subpopulations of VR1, and a prediction is that while it blocks the increase in intracellular calcium from the medium by occupying the VR1 at the plasma membrane level, it will be less effective in blocking that released from internal stores. Unlike compound **19**, compound **20**, derived from the variation of X and Y of **1**, exhibited much reduced potencies in binding affinity and antagonism by 108-fold and 26-fold, respectively.

The acyloxy group of the C region, defined as the C2 region in the lead **1**, has been proposed as a pharmacophore derived from the C_3-keto group in RTX. In order to identify the pharmacophoric importance of its ester group, it was replaced with amide and *N*-hydroxyamide to provide **24** and **28**, respectively. The amide analogue **24** showed almost complete loss of activity in binding affinity and in the functional assay, and the *N*-hydroxyamide analogue **28**, whose hydroxyl was designed to mimic the C_4-hydroxyl in RTX, also exhibited weak activities. These results indicated that a hydrogen bonding acceptor may be required to interact with the receptor.

3.2. Conformational analysis

Since compound **1** showed the highest binding affinity among the synthesized derivatives with diverse dispositions of the pharmacophoric groups, conformational analysis was performed to identify its active conformation for binding to VR1. The structure of **1** was minimized in the sybyl program and 48 unique conformations of **1** were found after eliminating the other conformations based on energy cutoff (3 kcal/mol), on chirality, or steric interference (bump check). The conformation with the lowest energy (conformer A) has an energy value of −14.61 kcal/mol. All 48 conformers were matched with the lowest energy conformer A, and the RMS overlap values were calculated. The RMS overlap versus energy values for all conformers were plotted in Figure 3. The energy values ranged from

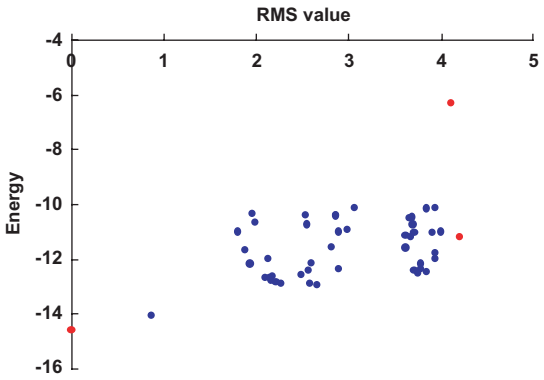


Figure 3. RMS versus energy values for the conformers of **1**.

−6.27 to −14.61 kcal/mol. The maximum and minimum RMS values were 0.00 and 4.21 Å, respectively. The lowest energy (conformer A) and highest energy (conformer C) conformers and the conformer with the most dissimilar topology (conformer B), that is with the highest RMS value, were selected as representative conformations of **1**, and are indicated with the red symbols in Figure 3.

The pharmacophore model of **1** consists of two hydrophobic ring systems and two hydrogen bond acceptors as indicated in Table 3. The interatomic distances of the center of the (4-methylsulfonylamino)phenyl ring (A region), the sulfur atom of the thiourea (B region), the center of the 3,4-dimethylphenyl ring (C1 region), and the carbonyl of the ester (C2 region) in the three conformers were measured for the structural analysis (Table 3). Their three-dimensional conformations were displayed in Figure 4. The structures of the three conformers feature different types of hydrophobic interactions between the three hydrophobic groups: the A ring, the C1 ring, and the pivaloyl group of the C2 region. While the two phenyls of the A region and the C1 region are close (distance of 5.95 Å) in conformer A and the phenyl of the A region and the pivaloyl group of the C2 region are close in conformer B, the hydrophobic groups of the C1 and C2 regions are as far away as possible from the phenyl of the A region in conformer C. This finding indicates that hydrophobic interactions in **1** play a crucial role in energy stabilization of the conformations. The conformational analysis of **1** provided three representative conformations with unique structural features characteristic of different hydrophobic interactions between the three hydrophobic groups; these will be utilized for providing a plausible model of the active conformation.

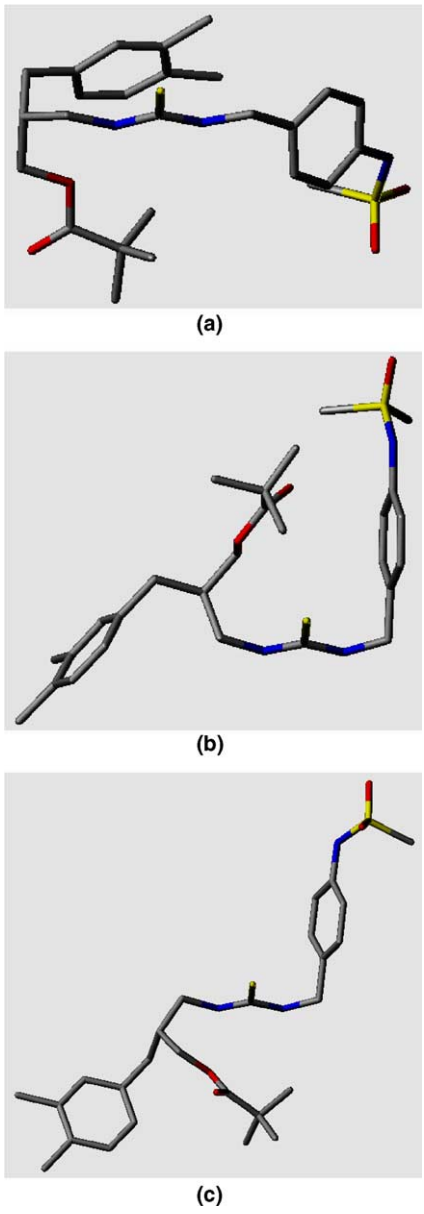
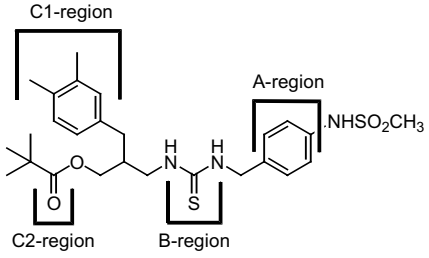


Figure 4. Proposed representative conformations of **1**. (a) Conformer A; (b) conformer B; (c) conformer C.

Table 3. Interatomic distances of four pharmacophoric features



	Conformer A	Conformer B	Conformer C
Energy (kcal/mol)	−14.61	−11.18	−6.27
RMS (Å)	0.00	4.21	4.11
Distance (A–B)	5.91	3.79	3.90
Distance (A–C1)	5.95	9.52	10.95
Distance (A–C2)	8.47	3.38	8.14
Distance (B–C1)	5.27	6.90	7.47
Distance (B–C2)	7.20	4.13	4.49
Distance (C1–C2)	5.43	7.69	5.31

In summary, we have modified the prototype antagonist (**1**) to expand the analysis of SAR in two ways. (1) The distances between the four pharmacophores were varied

by lengthening or shortening of one carbon between them for the purpose of optimizing the spatial arrangement. (2) The acyloxy group of the C region was replaced with amide and with *N*-hydroxy amide for identifying the pharmacophoric importance of the ester group of the C2 region. We conclude that the pharmacophoric distances of **1** are optimal for binding affinity and antagonism and that the ester of the C2 region is significant for receptor binding. Compound **19** showed partial separation of potencies, with enhanced potency as an antagonist but with weaker potency in the binding assay compared to **1**. Based on our current understanding, this suggests that **19** should be more selective for the calcium entry-linked VR1 receptor subpopulation. The conformational analysis of **1** generated three distinct conformers with different hydrophobic interactions, which should be of use for modeling of the active conformation.

4. Experimental section

4.1. General method

All chemical reagents were commercially available. melting points were determined on a melting point Büchi B-540 apparatus and are uncorrected. Silica gel column chromatography was performed on silica gel 60, 230–400 mesh, Merck. Proton NMR spectra were recorded on a JEOL JNM-LA 300 at 300 MHz. Chemical shifts are reported in ppm units with Me_4Si as a reference standard. Mass spectra were recorded on a VG Trio-2 GC–MS. Combustion analyses were performed on an EA 1110 Automatic Elemental Analyzer, CE Instruments.

4.1.1. 4-(Methylsulfonylamino)aniline (4, KMJ-308). White solid, ^{1}H NMR ($CDCl_3$) δ 7.06 (bd, 2H), 6.66 (bd, 2H), 6.06 (br s, 1H, $NHSO_2$), 3.73 (br s, 2H, NH_2), 2.94 (s, 3H, SO_2CH_3).

4.1.2. 4-(Methylsulfonylamino)benzyl amine (5) and 4-(methylsulfonylamino)benzyl isothiocyanate (6). The compounds were prepared according to the previous procedure.[26]

4.1.3. 2-[4-(Methylsulfonylamino)phenyl]ethyl amine hydrochloride (7, KMJ-276). White solid, ^{1}H NMR (DMSO-d_6) δ 7.20 (dd, 4H), 3.15 (m, 2H, $CH_2NH_3^+$), 2.7–3.0 (m, 5H, SO_2CH_3 and CH_2Ar).

4.1.4. 2-[4-(Methylsulfonylamino)phenyl]ethyl isothiocyanate (8, KMJ-338). White solid, ^{1}H NMR ($CDCl_3$) δ 7.22 (s, 4H), 6.51 (br s, 1H, $NHSO_2$), 3.73 (t, 2H, $J = 6.7$ Hz, CH_2NCS), 3.02 (s, 3H, SO_2CH_3), 2.98 (t, 2H, $J = 6.7$ Hz, CH_2Ar).

4.2. General procedure for the synthesis of thiourea

A solution of amine salt (0.5 mmol) in dimethylformamide (1 mL) was treated with triethylamine (0.5 mmol) and stirred for 30 min at room temperature. To the mixture was added isothiocyanate (0.5 mmol). After being stirred for 24 h at room temperature, the mixture was diluted with water and extracted with ethyl acetate several times. The combined organic layers were washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo and the residue was purified by flash column chromatography over silica gel with EtOAc/hexanes (1:1) as eluant to afford the thiourea.

4.2.1. *N*-[4-(3,4-Dimethylphenyl)-2-(pivaloyloxymethyl)-butyl]-*N*′-[4-(methylsulfonylamino)benzyl]thiourea (11, KJM-481). 73% yield, white solid, mp = 50–51 °C; ^{1}H NMR ($CDCl_3$) δ 7.33 (d, 2H, $J = 8.5$ Hz), 7.18 (d, 2H, $J = 8.5$ Hz), 6.9–7.1 (m, 3H), 6.42 (s, 1H, $NHSO_2$), 6.36 (t, 1H, NH), 6.06 (br s, 1H, NH), 4.59 (d, 2H, $J = 5.3$ Hz, $CSNHCH_2Ar$), 4.18 (dd, 1H, $J = 3.7$, 11.5 Hz, CH_2OCO), 3.95 (dd, 1H, $J = 5.1$, 11.5 Hz, CH_2OCO), 3.75 (m, 1H, $CHCH_2NHCS$), 3.22 (m, 1H, $CHCH_2NHCS$), 3.00 (s, 3H, SO_2CH_3), 2.65 (m, 2H, CH_2CH_2Ar), 2.2–2.3 (m, 7H, $2\times CH_3$ and CH), 1.5–1.65 (m, 2H, CH_2CH_2Ar), 1.20 (s, 9H, $C(CH_3)_3$); MS (FAB) 534 (MH^+); Anal. Calcd for $C_{27}H_{39}N_3O_4S_2$: C, 60.76; H, 7.36; N, 7.87; S, 12.02. Found: C, 60.96; H, 7.38; N, 7.83; S, 11.98.

4.2.2. *N*-[4-(4-*tert*-Butylphenyl)-2-(pivaloyloxymethyl)-butyl]-*N*′-[4-(methylsulfonylamino)benzyl]thiourea (12, KJM-459). 77% yield, white solid, mp = 34 °C; ^{1}H NMR ($CDCl_3$) δ 7.33 (d, 2H, $J = 8.3$ Hz), 7.31 (d, 2H, $J = 8.5$ Hz), 7.18 (d, 2H, $J = 8.5$ Hz), 7.13 (d, 2H, $J = 8.3$ Hz), 6.54 (br s, 1H, $NHSO_2$), 6.41 (t, 1H, NH), 6.14 (br s, 1H, NH), 4.60 (d, 2H, $J = 4.9$ Hz, $CSNHCH_2Ar$), 4.19 (dd, 1H, $J = 3.7$, 11.5 Hz, CH_2OCO), 3.96 (dd, 1H, $J = 4.9$, 11.5 Hz, CH_2OCO), 3.76 (m, 1H, $CHCH_2NHCS$), 3.24 (m, 1H, $CHCH_2NHCS$), 2.99 (s, 3H, SO_2CH_3), 2.70 (m, 2H, CH_2CH_2Ar), 2.04 (m, 1H, CH), 1.5–1.7 (m, 2H, CH_2CH_2Ar), 1.30 (s, 9H, $C(CH_3)_3$), 1.20 (s, 9H, $C(CH_3)_3$); MS (FAB) 562 (MH^+); Anal. Calcd for $C_{29}H_{43}N_3O_4S_2$: C, 62.00; H, 7.71; N, 7.48; S, 11.42. Found: C, 62.20; H, 7.74; N, 7.44; S, 11.36.

4.2.3. *N*-[2-(3,4-Dimethylbenzyl)-3-(pivaloyloxy)propyl]-*N*′-[4-(methylsulfonylamino)phenyl]thiourea (13, KMJ-246). 64% yield, white solid, mp = 43 °C; ^{1}H NMR ($CDCl_3$) δ 7.86 (br s, 1H), 7.27 (d, 2H, $J = 8.5$ Hz), 7.16 (d, 2H, $J = 8.5$ Hz), 6.85–7.05 (m, 3H), 6.49 (br s, 1H), 4.10 (m, 1H, CH_2OCO), 3.80 (m, 2H, CH_2OCO and CH_2NHCS), 3.34 (m, 1H, CH_2NHCS), 3.01 (s, 3H, SO_2CH_3), 2.5–2.7 (m, 2H, CH_2Ar), 2.34 (m, 1H, CH), 2.15–2.25 (m, 6H, $2\times CH_3$), 1.17 (d, 9H, $C(CH_3)_3$); MS (FAB) 506 (MH^+); Anal. Calcd for $C_{25}H_{35}N_3O_4S_2$: C, 59.38; H, 6.98; N, 8.31; S, 12.68. Found: C, 59.62; H, 7.01; N, 8.27; S, 12.61.

4.2.4. *N*-[2-(3,4-Dimethylbenzyl)-3-(pivaloyloxy)propyl]-*N′*-{2-[4-(methylsulfonylamino)phenyl]ethyl}thiourea (14, KMJ-282). 72% yield, white solid, mp = 46 °C; ^{1}H NMR ($CDCl_3$) δ 7.18 (s, 4H, Ar), 7.15 (br s, 1H, $NHSO_2$), 6.9–7.1 (m, 3H, Ar), 6.45 (br s, 1H, NHCS), 6.00 (br s, 1H, NHCS), 4.24 (m, 1H, CH_2OCO), 3.82 (m, 2H, CH_2OCO, and CH_2NHCS), 3.53 (m, 2H, CSNHCH_2), 3.20 (m, 1H, CH_2NHCS), 2.98 (s, 3H, SO_2CH_3), 2.85 (m, 2H, CH_2CH_2Ar), 2.5–2.7 (m, 2H, CHCH_2Ar), 2.2–2.35 (m, 7H, 2×CH_3, and CH), 1.25 (d, 9H, $C(CH_3)_3$); MS (FAB) 534 (MH^+); Anal. Calcd for $C_{27}H_{39}N_3O_4S_2$: C, 60.76; H, 7.36; N, 7.87; S, 12.02. Found: C, 61.00; H, 7.39; N, 7.83; S, 11.97.

4.2.5. *N*-[3-(3,4-Dimethylphenyl)-1-pivaloyloxy-2-propyl]-*N′*-[4-(methylsulfonylamino)benzyl]thiourea (17, MSK-483). 66% yield, white solid, mp = 63–65 °C; ^{1}H NMR ($CDCl_3$) δ 7.27 (d, 2H, J = 8.5 Hz), 7.18 (d, 2H, J = 8.5 Hz), 6.9–7.1 (m, 3H), 6.25 (br s, 1H, NH), 6.00 (d, 1H, J = 7.8 Hz, NH), 4.56 (br s, 3H, NHCH_2Ar, and C*H*NHCS), 4.18 (dd, 1H, J = 4.9, 11.5 Hz, CH_2OCO), 3.98 (dd, 1H, J = 5.1, 11.5 Hz, CH_2OCO), 3.00 (m, 4H, SO_2CH_3, and CH_2Ar), 2.75 (m, 1H, CH_2Ar), 2.27 (d, 3H, J = 5.4 Hz, CH_3), 2.23 (s, 3H, CH_3), 1.19 (s, 9H, $C(CH_3)_3$); MS (FAB) 506 (MH^+); Anal. Calcd for $C_{25}H_{35}N_3O_4S_2$: C, 59.38; H, 6.98; N, 8.31; S, 12.68. Found: C, 59.60; H, 7.02; N, 8.26; S, 12.62.

4.2.6. *N*-[3-(4-*tert*-Butylphenyl)-1-pivaloyloxy-2-propyl]-*N′*-[4-(methylsulfonylamino)benzyl]thiourea (18, MSK-423). 68% yield, white solid, mp = 90–92 °C; ^{1}H NMR ($CDCl_3$) δ 7.25–7.35 (m, 4H), 7.12–7.2 (m, 4H), 6.60 (s, 1H, $NHSO_2$), 6.50 (t, 1H, NH), 6.08 (d, 1H, J = 7.8 Hz, NH), 4.59 (br s, 3H, NHCH_2Ar, and C*H*NHCS), 4.20 (dd, 1H, J = 4.9, 11.5 Hz, CH_2OCO), 3.97 (dd, 1H, J = 5.1, 11.5 Hz, CH_2OCO), 3.00 (m, 4H, SO_2CH_3, and CH_2Ar), 2.75 (dd, 1H, J = 8.3, 13.6 Hz, CH_2Ar), 1.30 (s, 9H, $C(CH_3)_3$), 1.18 (s, 9H, $C(CH_3)_3$); MS (FAB) 534 (MH^+); Anal. Calcd for $C_{27}H_{39}N_3O_4S_2$: C, 60.76; H, 7.36; N, 7.87; S, 12.02. Found: C, 60.98; H, 7.39; N, 7.84; S, 11.98.

4.2.7. *N*-[4-(3,4-Dimethylphenyl)-1-pivaloyloxy-2-butyl]-*N′*-[4-(methylsulfonylamino)benzyl]thiourea (19, JYL-1413). 73% yield, white solid, mp = 52 °C; ^{1}H NMR ($CDCl_3$) δ 7.50 (br s, 1H, $NHSO_2$), 7.32 (d, 2H, J = 8.5 Hz), 7.22 (d, 2H, J = 8.5 Hz), 6.9–7.1 (m, 3H), 6.78 (br s, 1H, NH), 6.19 (br s, 1H, NHCS), 4.68 (br s, 2H, $CSNHCH_2$Ar), 4.3–4.4 (m, 2H, CH_2OCO, and CH), 3.97 (br s, 1H, CH_2OCO), 3.02 (s, 3H, SO_2CH_3), 2.6–2.8 (m, 2H, CH_2Ar), 2.25 (m, 6H, 2×CH_3), 1.8–2.0 (m, 2H, CH_2CH_2Ar), 1.18 (s, 9H, $C(CH_3)_3$); MS (FAB) 520 (MH^+); Anal. Calcd for $C_{26}H_{37}N_3O_4S_2$: C, 60.09; H, 7.18; N, 8.09; S, 12.34. Found: C, 60.28; H, 7.23; N, 8.03; S, 12.27.

4.2.8. *N*-[3-(3,4-Dimethylphenyl)-1-pivaloyloxy-2-propyl]-*N′*-{2-[4-(methylsulfonylamino)phenyl]ethyl}thiourea (20, KMJ-342). 76% yield, white solid, mp = 54 °C; ^{1}H NMR ($CDCl_3$) δ 7.16 (m, 4H, Ar), 6.9–7.1 (m, 3H, Ar), 6.82 (br s, 1H, $NHSO_2$), 6.20 (br s, 1H, NHCS), 6.03 (br t, 1H, NHCS), 4.50 (br s, 1H, C*H*NH), 4.16 (m, 1H, CH_2OCO), 3.94 (m, 1H, CH_2OCO), 3.61 (m, 2H, CSNHCH_2), 2.98 (s, 3H, SO_2CH_3), 2.86 (t, 2H, CH_2CH_2Ar), 2.7–2.8 (m, 2H, CHCH_2Ar), 2.2–2.3 (m, 6H, 2×CH_3), 1.20 (d, 9H, $C(CH_3)_3$); MS (FAB) 520 (MH^+); Anal. Calcd for $C_{26}H_{37}N_3O_4S_2$: C, 60.09; H, 7.18; N, 8.09; S, 12.34. Found: C, 60.29; H, 7.21; N, 8.04; S, 12.29.

4.2.9. *N*-[3-Azido-2-(3,4-dimethylbenzyl)propyl]phthalimide (22, JYL-1575). A cooled mixture of **21** (0.55 g, 2.3 mmol), phthalimide (0.508 g, 3.45 mmol), triphenylphospine (0.905 g, 3.45 mmol) in THF (10 mL) at 0 °C was treated with diethyl azodicarboxylate (1.5 mmol, 0.543 mL) and stirred at room temperature for 18 h. The solvent was removed in vacuo and the residue was purified by flash column chromatography over silica gel with EtOAc/hexanes (1:10) as eluant to afford **22** as a colorless oil (0.753 g, 94%); ^{1}H NMR ($CDCl_3$) δ 7.65–7.9 (m, 4H), 6.85–7.0 (m, 3H, Ar), 3.65–3.8 (m, 2H, CH_2N), 3.2–3.4 (m, 2H, CH_2N_3), 2.72 (d, 1H, J = 7.8 Hz, CH_2Ph), 2.64 (d, 1H, J = 7.1 Hz, CH_2Ph), 2.45 (m, 1H, CH), 2.1–2.3 (m, 6H, 2×CH_3).

4.2.10. *N*-[3-Azido-2-(3,4-dimethylbenzyl)propyl]-2,2-dimethylpropanamide (23, JYL-1581). A mixture of **22** (0.753 g, 2.16 mmol) and hydrazine monohydrate (0.126 mL, 2.59 mmol) in ethanol (8 mL) was stirred at room temperature for 16 h and concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (10 mL) and treated with pivaloyl chloride (0.317 mL, 2.59 mmol). After 3 h of stirring at room temperature, the mixture was diluted with H_2O and extracted with EtOAc several times. The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography over silica gel with EtOAc/hexanes (1:3) as eluant to afford **23** as a yellow oil (0.392 g, 60%); ^{1}H NMR ($CDCl_3$) δ 6.85–7.1 (m, 3H, Ar), 5.75 (br s, 1H, NH), 3.2–3.4 (m, 4H, CH_2N, and CH_2N_3), 2.5–2.7 (m, 2H, CH_2Ph), 2.2–2.3 (m, 6H, 2×CH_3), 2.09 (m, 1H, CH), 1.13 (s, 9H, $COC(CH_3)_3$).

4.2.11. *N*-[2-(3,4-Dimethylbenzyl)-3-(*N*-pivaloylamino)-propyl]-*N′*-[4-(methylsulfonylamino)phenyl]thiourea (24, JYL-1585). A suspension of **23** (0.302 g, 1 mmol) and Lindlar's catalyst (0.5 g) in ethanol (50 mL) was hydrogenated under a balloon of hydrogen for 1 h. The reaction mixture was filtered and the filtrate was concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (50 mL) and treated with 4-(methylsulfonylamino)benzyl isothiocyanate (0.242 g, 1 mmol). After being stirred for 3 h at room temperature, the reaction mixture was concentrated in vacuo and the residue was purified by flash column chromatography over silica gel with EtOAc/hexanes (1:1) as eluant to afford **24** as a white solid (0.394 g, 76%); mp = 86 °C; ^{1}H NMR ($CDCl_3$) δ 7.29 (d, 2H, J = 8.3 Hz), 7.15 (d, 2H, J = 8.3 Hz), 6.8–

7.1 (m, 3H), 6.58 (m, 1H, NH), 6.08 (m, 1H, NH), 4.59 (br s, 2H, $NHCH_2Ar$), 3.65–3.85 (m, 2H, $CHCH_2NH$), 3.1–3.5 (m, 2H, $CONHCH_2$), 2.96 (s, 3H, SO_2CH_3), 2.70 (ddd, 1H, CH_2Ar), 2.1–2.5 (m, 8H, $2\times CH_3$, CH_2Ar, and CH), 1.12 (d, 9H, $J = 4.9$ Hz, $C(CH_3)_3$); MS (FAB) 519 (MH^+); Anal. Calcd for $C_{26}H_{38}N_4O_3S_2$: C, 60.20; H, 7.38; N, 10.80; S, 12.36. Found: C, 60.39; H, 7.42; N, 10.74; S, 12.29.

4.2.12. *tert*-Butyl *N*-(3-azido-2-(3,4-dimethylbenzyl)propyl)-*N*-[(*tert*-butoxycarbonyl)oxy] carbamate (25, JYL-1459). A cooled mixture of **21** (0.5 g, 2.3 mmol), *tert*-butyl-*N*-(*tert*-butoxycarbonyloxy)carbamate (0.536 g, 2.3 mmol) and triphenylphosphine (0.905 g, 3.45 mmol) in THF (10 mL) at 0 °C was treated with diethyl azodicarboxylate (0.543 mL, 1.5 mmol) and stirred at room temperature for 18 h. The reaction mixture was concentrated in vacuo, and the residue was purified by flash column chromatography over silica gel with EtOAc/hexanes (1:10) as eluant to afford **25** as a colorless oil (0.97 g, 97%); ^{1}H NMR ($CDCl_3$) δ 6.85–7.1 (m, 3H, Ar), 3.5–3.75 (m, 2H, CH_2NO), 3.3–3.5 (m, 2H, CH_2N_3), 2.5–2.8 (m, 2H, CH_2Ph), 2.0–2.3 (m, 7H, CH, and $2\times CH_3$), 1.52 (s, 9H, $C(CH_3)_3$), 1.48 (s, 9H, $C(CH_3)_3$).

4.2.13. *N*-[3-Azido-2-(3,4-dimethylbenzyl)propyl]hydroxylamine (26, JYL-1527). To cooled trifluoroacetic acid (3 mL) at 0 °C was added **25** (0.97 g, 2.23 mmol). The reaction mixture was stirred at room temperature for 20 min. The solvent was removed in vacuo to afford **26** as a brown oil (0.7 g, 90%), which was used in next step without any further purification; ^{1}H NMR ($CDCl_3$) δ 6.85–7.25 (m, 3H, Ar), 3.5–3.7 (m, 2H, CH_2 NOH), 3.3–3.5 (m, 2H, CH_2N_3), 2.45–2.8 (m, 3H, CH_2Ph, and CH), 2.1–2.3 (m, 6H, $2\times CH_3$).

4.2.14. *N*-[3-Azido-2-(3,4-dimethylbenzyl)propyl]-*N*-hydroxy-2,2-dimethylpropanamide (27, JYL-1533). A solution of **26** (0.7 g, 2 mmol) in H_2O (2 mL) was treated with sodium bicarbonate (0.2 g, 2.4 mmol) and stirred at room temperature for 30 min. After addition of CH_2Cl_2 (5 mL), the reaction mixture was treated with pivaloyl chloride (0.2 mL, 0.7 mmol) dropwise and stirred at room temperature for 2 h. After 2 h of stirring, the mixture was extracted with EtOAc several times. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography over silica gel with EtOAc/hexanes (1:4) as eluant to afford **27** as a yellow oil (0.333 g, 52%); ^{1}H NMR ($CDCl_3$) δ 7.40 (br s, 1H, NOH), 6.85–7.1 (m, 3H, Ar), 3.6–3.8 (m, 2H, CH_2NOH), 3.25–3.5 (m, 2H, CH_2N_3), 2.5–2.8 (m, 2H, CH_2Ph), 2.38 (m, 1H, CH), 2.2–2.3 (m, 6H, $2\times CH_3$), 1.25 (s, 9H, $COC(CH_3)_3$).

4.2.15. *N*-[2-(3,4-Dimethylbenzyl)-3-(*N*-pivaloyl-*N*-hydroxyamino)propyl]-*N*′-[4-(methylsulfonylamino)phenyl]-thiourea (28, JYL-1535). The same procedure as described for the synthesis of **24** was followed starting from **27** to provide **28** as a white solid in 54% yield; mp = 84 °C; ^{1}H NMR ($CDCl_3$) δ 7.32 (d, 2H, $J = 8.3$ Hz), 7.19 (d, 2H, $J = 8.3$ Hz), 6.9–7.1 (m, 3H), 5.58 (t, 1H, NH), 5.51 (t, 1H, NH), 4.7–4.9 (m, 2H, $CSNHCH_2Ar$), 3.5–3.8 (m, 2H, $HONCH_2$), 2.75–3.0 (m, 2H, $CHCH_2NHCS$), 2.99 (s, 3H, SO_2CH_3), 2.3–2.5 (m, 3H, CH_2Ar, and CH), 2.24 (dd, 6H, $2\times CH_3$), 0.93 (d, 9H, $J = 6.6$ Hz, $C(CH_3)_3$); MS (FAB) 535 (MH^+); Anal. Calcd for $C_{26}H_{38}N_4O_4S_2$: C, 57.78; H, 6.79; N, 10.78; S, 12.34. Found: C, 58.00; H, 6.82; N, 10.74; S, 12.29.

4.3. Molecular modeling

The conformational analysis of the lead antagonist **1** was performed to identify its active conformation on binding to VR1. The structure of **1** was built and minimized using the Tripos force field (method: conjugate gradient, termination: gradient 0.01 kcal/mol Å, and max iterations: 10,000) implemented in the sybyl 6.5 program. The partial atomic charges were calculated by the Gasteiger–Hückel method. Then, the three-dimensional structure was subjected to conformational analysis using the Random Search method with the following non-default options (Maximum Cycles: 3,000 and Chirality: *R* configuration). In the analysis, 48 unique conformations of **1** were found, and the other conformations were eliminated based on energy cutoff (3 kcal/mol), chirality, or bump check.

Acknowledgements

This work was supported by a Fund 2002 grant (ES0024) from the Korea Research Foundation.

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