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Analysis of structure–activity relationships for the 'B-region' of *N*-(4-*t*-butylbenzyl)-*N*′-[4-(methylsulfonylamino)benzyl]-thiourea analogues as TRPV1 antagonists

Jeewoo Lee,[a,*] Mi-Kyoung Jin,[a] Sang-Uk Kang,[a] Su Yeon Kim,[a] Jiyoun Lee,[a] Myoungyoup Shin,[a] Jaemin Hwang,[a] Sookhyun Cho,[a] Yeon-Sil Choi,[a] Hyun-Kyung Choi,[a] Sung-Eun Kim,[a] Young-Ger Suh,[a] Yong-Sil Lee,[a] Young-Ho Kim,[b] Hee-Jin Ha,[b] Attila Toth,[c] Larry V. Pearce,[c] Richard Tran,[c] Tamas Szabo,[c] Jacqueline D. Welter,[c] Daniel J. Lundberg,[c] Yun Wang,[c] Jozsef Lazar,[c] Vladimir A. Pavlyukovets,[c] Matthew A. Morgan[c] and Peter M. Blumberg[c]

[a]*Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea*
[b]*Digitalbiotech, Ansan, Kyounggi-Do 425-839, Republic of Korea*
[c]*Laboratory of Cellular Carcinogenesis and Tumor Promotion, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892, USA*

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Abstract—The structure–activity relationships for the 'B-region' of *N*-(4-*t*-butylbenzyl)-*N*′-[4-(methylsulfonylamino)benzyl]thiourea analogues have been investigated as TRPV1 receptor antagonists. A docking model of potent antagonist **2** with the sensor region of TRPV1 is proposed.

1. Introduction

TRPV1, which is regulated by endogenous substances and signaling pathways in concert with low pH and elevated temperature, is a key nociceptor for the central perception of pain and is expressed with enhanced density during inflammation. TRPV1 antagonists, to block endovanilloid signaling, have thus emerged as novel and promising analgesic and antiinflammatory agents, particularly for chronic pain and inflammatory hyperalgesia. Following the identification of capsazepine as the first competitive TRPV1 antagonist[1] and of ruthenium red[2] as a noncompetitive antagonist, a growing number of antagonists have been reported such as capsazocaine,[3] trialkyglycines,[4] hexapeptides,[5] 5-iodo-RTX,[6,7] halogenated capsaicinoids,[8] SB-366791/452533,[9,10] naphthylureas,[11–13] BCTC,[14] and thioureas.[15–23] The discovery and development of these antagonists have been reviewed in detail.[24,25]

We previously demonstrated that a series of *N*-4-(methylsulfonylamino)benzyl thiourea analogues showed potent TRPV1 antagonism with high affinity in the rTRPV1/CHO system and, in the preceding paper, we investigated the structure–activity relationships (SAR) in the A-region of *N*-(4-*t*-butylbenzyl)-*N*′-[4-(methylsulfonylamino)benzyl]thiourea analogues. We describe here the SAR analysis for the B-region of the high-affinity prototype antagonists (**1**–**3**) (see Fig. 1).

2. Chemistry

The syntheses of the two types of *N*-hydroxy thiourea analogues (N_A and N_C refer to the nitrogens next to the A- and C-regions, respectively) are outlined in Schemes 1 and 2. 4-*tert*-Butylbenzyl bromide was converted to the corresponding hydroxylamine by N-alkylation of *N*,*O*-diBoc hydroxylamine followed by acid

Keywords: TRPV1 antagonist; Vanilloid Receptor 1 antagonist; Molecular modeling; Capsaicin; Resiniferatoxin.
* Corresponding author. Tel.: +82 2 880 7846; fax: +82 2 888 0649; e-mail: jeewoo@snu.ac.kr

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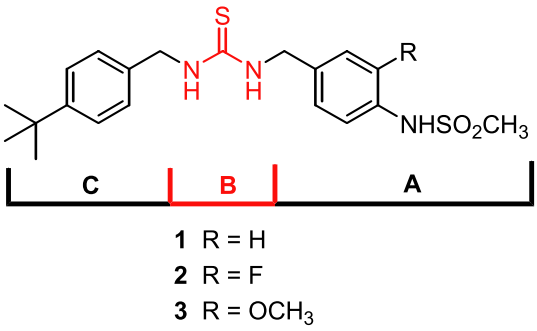
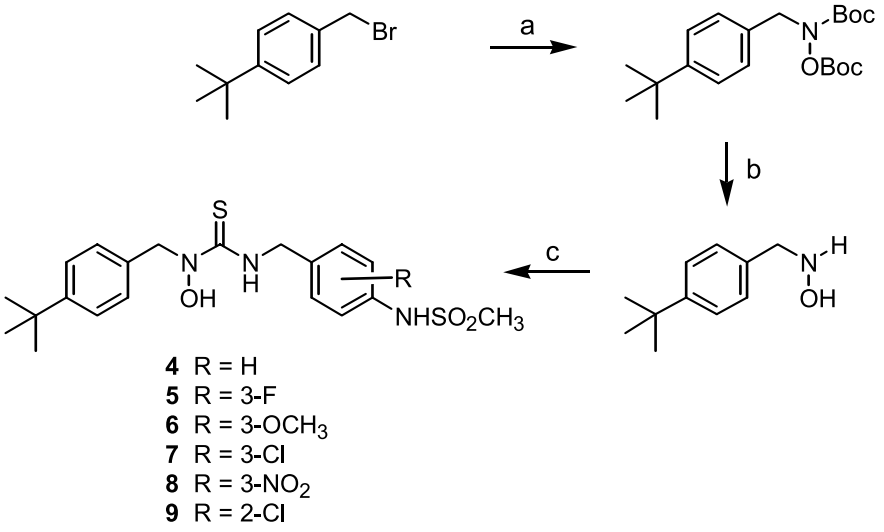


Figure 1.

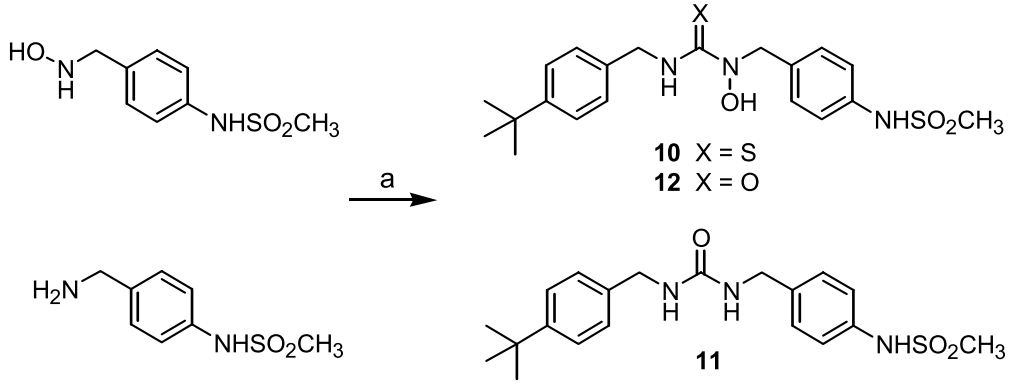
hydrolysis; the hydroxylamine was then condensed with isothiocyanates to afford the N_C-hydroxy thioureas **4**–**9**. The N_A-hydroxy thiourea analogue (**10**) was obtained by the coupling between *N*-[4-(methylsulfonylamino)benzyl]hydroxylamine[26] and 4-*t*-butylbenzyl isothiocyanate. The urea (**11**) and N_A-hydroxy urea (**12**) congeners were prepared by the same protocol employed for the syntheses of the thioureas except that the corresponding isocyanates were used.

The syntheses of the two types of thiocarbamate analogues (O_A and O_C refer to the oxygens next to the A- and C-regions, respectively) are shown in Schemes 3 and 4. The O_A-thiocarbamate analogues **13**–**15** were synthesized by the O-alkylation of 4-(butoxycarbonylamino)benzyl alcohols[26] with the 4-*t*-butylbenzyl isothiocyanate followed by acid hydrolysis and mesylation, respectively. The syntheses of O_C-thiocarbamate analogues (**16**, **17**) were accomplished employing the above method, starting from the 4-(butoxycarbonylamino)benzyl isothiocyanates, which were prepared from 4-aminobenzyl amine and 3-methoxy-4-nitrobenzyl alcohol, respectively, by straightforward functional group interconversion.

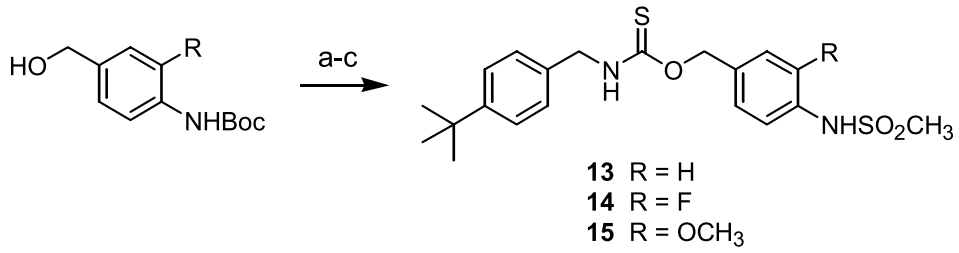
The amide, *N*-hydroxyamide, and its reverse analogues (**18**–**21**) were obtained by the coupling of (4-*t*-butylphenyl)acetic acid or 4-(methylsulfonylamino)phenylacetic acid with the corresponding amines/hydroxylamines as outlined in Scheme 5. The hydrazinecarbothioamide analogues, N_A-type (**22**) and N_C-type (**23**), were readily obtained by the coupling between the corresponding isothiocyanates and hydrazines, respectively, as shown



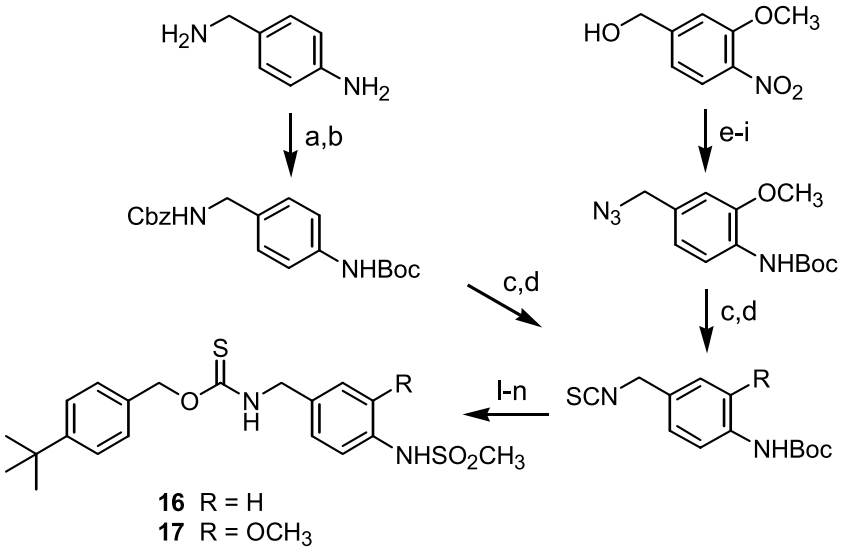
Scheme 1. Synthesis of N_C-OH thiourea analogues. Reagents: (a) BocONHBoc, NaH, DMF, 95%; (b) CF_3CO_2H, CH_2Cl_2; then $NaHCO_3$; (c) $ArCH_2NCS$, DMF, 60–80% for two steps.



Scheme 2. Synthesis of N_A-OH thiourea, N_A-OH urea and urea analogues. Reagents: (a) (4-*t*-Bu)$PhCH_2NCO$ (or NCS), DMF, 70–95%.



Scheme 3. Synthesis of O_A-thiocarbamate analogues. Reagents: (a) NaH, (4-*t*-Bu)PhCH$_2$NCS, DMF, 60–75%; (b) CF_3CO_2H, CH_2Cl_2; (c) MsCl, pyridine, 85–94% for two steps.



Scheme 4. Synthesis of O_C-thiocarbamate analogues. Reagents: (a) CbzCl, NEt_3, 92%; (b) Boc_2O, THF, 95%; (c) H_2, Pd–C, MeOH, 95–98%; (d) TDI, CH_2Cl_2, 80–90%; (e) TBSCl, imidazole, DMF, 94%; (f) H_2, Pd–C, MeOH, 98%; (g) Boc_2O, THF, 92%; (h) Bu_4NF, THF, 92%; (i) DPPA, PPh_3, DEAD, 86%; (l) 4-*t*−BuPhOH, NaH, DMF, 80–90%; (m) CF_3CO_2H, CH_2Cl_2; (n) MsCl, pyridine, 85–92% for two steps.

in Scheme 6. The reverse amide analogues (**24**–**27**) were prepared by the same method employed for the synthesis of **20**.

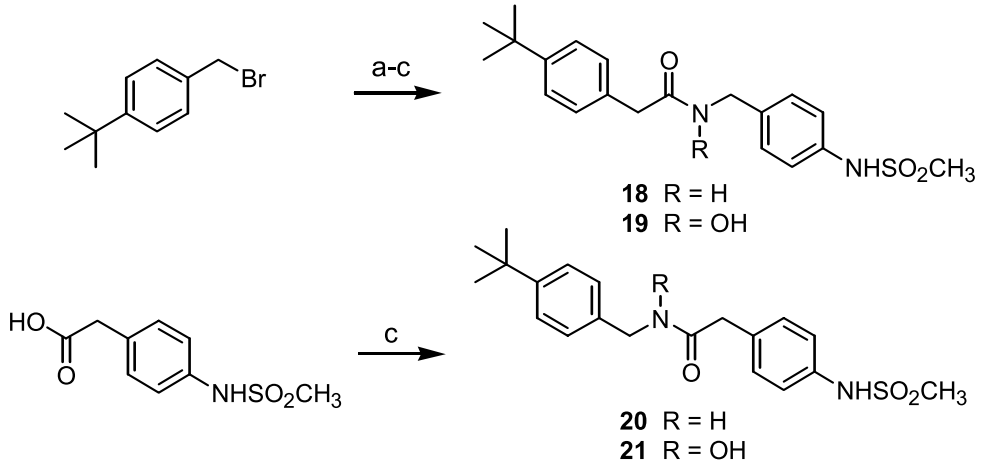
3. Results and discussion

The binding affinities and agonistic/antagonistic potencies of the synthesized VR1 ligands were assessed in vitro by a competition binding assay with [^{3}H]RTX and a functional $^{45}Ca^{2+}$ uptake assay using rat TRPV1 heterologously expressed in Chinese hamster ovary (CHO) cells (rTRPV1/CHO), as previously described.[21–23] The results are summarized in Tables 1 and 2.

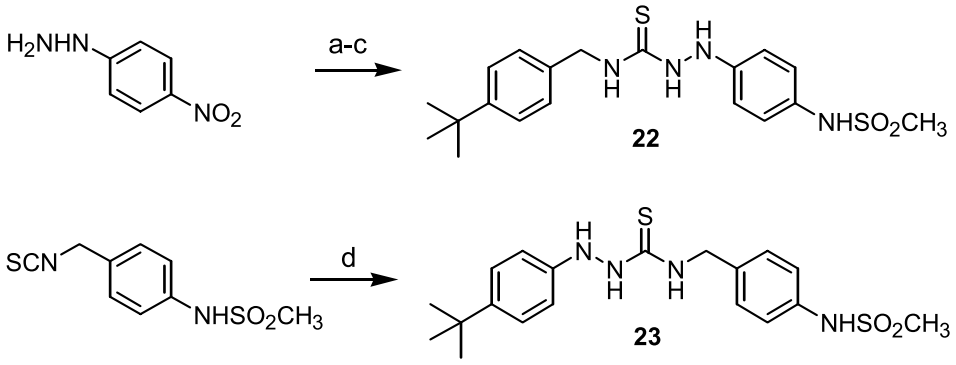
The N_C-hydroxy thiourea analogues (3-hydrogen: **4**, 3-fluoro:**5**, and 3-methoxy:**6**) were compared with the corresponding lead thioureas (**1**–**3**), respectively. Their binding affinities were attenuated by 15- to 20-fold. Furthermore, **5** and **6** were shifted in their pattern of activity to be weak agonists although **4** remained an antagonist with weak agonism. Other substituents (3-chloro: **7**, 3-nitro: **8**, and 2-chloro: **9**) did not change the weak potencies of the N_C-hydroxy thiourea analogues. The N_A-hydroxy thiourea analogue **10** was also examined. It likewise exhibited reduced potency in binding affinity and antagonism comparable to the N_C-hydroxy thiourea.

A series of compounds with urea as the B-region have been reported as potent TRPV1 antagonists.[11–14] However, the urea analogue **11** was a weak congener of the corresponding thiourea (**1**) with 40- and 4-fold less potency in binding affinity and antagonism. The N_A-hydroxy urea **12** showed a greater reduction in binding affinity compared to the urea, but it was a weakly potent, full agonist.

The thiocarbamate analogues were also examined as thiourea congeners and compared with the corresponding thioureas. Whereas the O_A-thiocarbamate analogues **13**–**15** were moderately weaker surrogates of the corresponding thioureas with 4- to 30-fold reduced binding affinity and antagonism, the O_C-thiocarbamate analogues **16** and **17** showed a dramatic decrease in binding affinity and antagonism. The result indicated that the N_C-hydrogen of thiourea would be more significant than the N_A-hydrogen for the interaction with the receptor.



Scheme 5. Synthesis of amide and *N*-hydroxyamide analogues. Reagents: (a) NaCN, DMF, 94%; (b) NaOH, THF, 92%; (c) EDC, R-NH_2 or R-NHOH, 85–95%.



Scheme 6. Synthesis of hydrazinecarbothioamide analogues. Reagents and conditions: (a) H_2, Pd–C, MeOH, 98%; (b) MsCl, pyridine, 0 °C, 80%; (c) 4-*t*-BuPhCH_2NCS, DMF, 52%; (d) 4-*t*-BuPhNHNH_2, CH_2Cl_2, 82%.

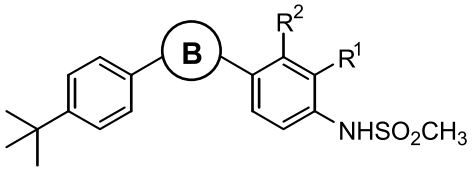
Substitutions of the thiourea in **1** with the amide (**18**), *N*-hydroxyamide (**19**), reverse amide (**20**), and reverse *N*-hydroxyamide (**21**) as its isosteres also led to much reduced potencies in binding affinity and antagonism except for the *N*-hydroxy amide (**19**). This compound was a weakly potent full agonist as was the N_A-hydroxy urea (**12**), which contains an *N*-hydroxy amide moiety. We examined hydrazinecarbothioamide isosteres containing a nitrogen next to the thiourea. Both N_A-type (**22**) and N_C-type (**23**) analogues exhibited a large decrease in binding affinity and antagonism.

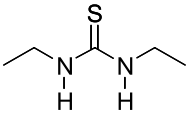
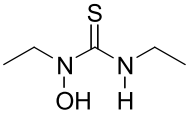
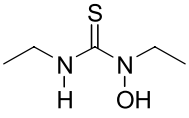
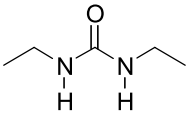
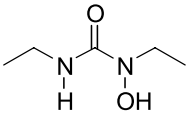
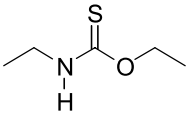
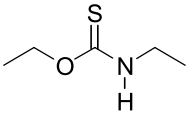
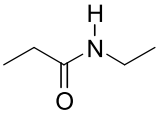
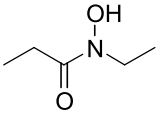
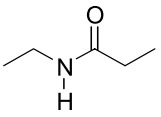
Since a 3-fluoro substituent on the A-region enhanced receptor antagonism, we explored the effect of a 3-fluoro substituent in the reverse amide surrogates as shown in Table 2. Although 4-methylsulfonamide analogues **20** and **25** were weak antagonists with low affinities, incorporation of the 3-fluoro group (**24**, **26**) improved both binding affinity and antagonism as they did in the thiourea series. The increased potency was consistently 3- to 4-fold. The oleyl amide analogue (**27**), like the corresponding thiourea analogue,[27] was a poor ligand for the receptor.

4. Molecular modeling

The structural identification of the binding motif of the TRPV1 receptor is essential to determine in detail the mechanism of action of the TRPV1 ligands. In recent publications, molecular determinants and models for the vanilloid binding site were proposed based on species-specific differences in TRPV1 sequence, site-directed mutagenesis, homology modeling of the transmembrane domain of TRPV1, and docking studies of capsaicin or RTX bound to the putative binding site.[28–30] The experimental results indicated that Tyr511,[28] Ser512,[28] Met547 (only present in rTRPV1),[29,30] and Thr550[29] within the transmembrane domain (S1–S4) were critical for vanilloid sensitivity. Based on these experimental findings, several hypothetical models of RTX or capsaicin binding to TRPV1 were suggested. In the model of Jordt and Julius, an aromatic residue, Tyr511, on the intracellular S2/S3 loop interacts with the vanillyl-moiety of capsaicin. Additional polar residues, such as S512 or R491, could interact with capsaicin via hydrogen bonds, whereas lipophilic residues in TM3 might contribute to hydrophobic interactions with the aliphat-

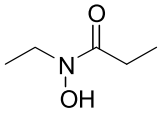
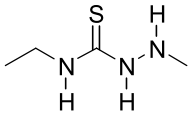
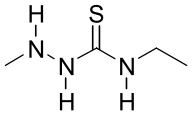
Table 1. Potencies of TRPV1 ligands for binding to rat VR1 and for inducing calcium influx in CHO/VR1 cells



B		R^1	R^2	K_i (nM)[c] binding affinity	EC_{50} (nM)[c] agonism	K_i (nM)[c] antagonism
Capsazepine				1300 (±150)	NE	520 (±12)
	**1**	H	H	63 (±10)	NE	54 (±8.7)
	2	F	H	53.5 (±6.5)	NE	9.16 (±1.6)
	3	OCH_3	H	50.4 (±16.5)	WE[a]	3.4 (±0.5)[b]
	**4**	H	H	1090 (±150)	WE[a]	470 (±200)[b]
	5	F	H	800 (±190)	>7060	NE
	6	OCH_3	H	926 (±74)	2000 (±200)[a]	NE
	7	Cl	H	1310 (±210)	NE	579 (±43)
	8	NO_2	H	1330 (±310)	NE	635 (±52)
	9	H	Cl	2270 (±730)	NE	NE
	**10**	H	H	4260 (±370)	NE	470 (±100)[b]
	**11**	H	H	2560 (±370)	NE	225 (±65)
	**12**	H	H	3500 (±620)	1055 (±36)	NE
	**13**	H	H	772 (±21)	NE	222 (±71)
	14	F	H	450 (±170)	WE[a]	120 (±21)
	15	OCH_3	H	461 (±41)	NE	110 (±9.6)
	**16**	H	H	4600 (±1400)	WE[a]	6250 (±2300)
	17	OCH_3	H	2170 (±150)	NE	8400 (±2700)
	**18**	H	H	5220 (±1500)	WE[a]	6760 (±760)
	**19**	H	H	5300 (±730)	1960 (±400)	NE
	**20**	H	H	2100 (±440)	NE	398 (±78)

(*continued on next page*)

Table 1 (*continued*)

B		R^1	R^2	K_i (nM)[c] binding affinity	EC_{50} (nM)[c] agonism	K_i (nM)[c] antagonism
	**21**	H	H	6400 (±3100)	WE[a]	6600 (±2000)[b]
	**22**	H	H	12100 (±4100)	NE	1460 (±420)
	**23**	H	H	6400 (±3200)	NE	2010 (±580)

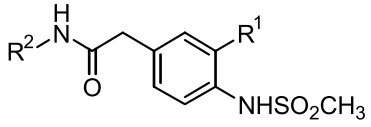
NE: not effective, WE: weakly effective.

[a] Only fractional calcium uptake compared with that induced by 300 nM capsaicin (**4**, 16%; **6**, 72%; **14**, 7%; **16**, 13%; **18**, 20%; **21**, 32%).

[b] Fractional antagonism at 10 μM capsaicin (**4**, 61%; **10**, 78%; **21**, 87%).

[c] Values represent the means ± SEM of three or more experiments. Values for extent of partial agonism or partial antagonism are from one to three experiments. The extent of agonism was compared to the effect of a maximal concentration of capsaicin (300 nM) determined in the same assay. Antagonist values were determined from inhibition of the response evoked by 50 nM capsaicin. WE and NE values were determined at ligand concentrations of 10–30 μM.

Table 2. Potencies of TRPV1 ligands for binding to rat VR1 and for inducing calcium influx in CHO/VR1 cells



	R^1	R^2	K_i (nM) binding affinity	EC_{50} (nM) agonism	K_i (nM) antagonism
20	H	4-*t*-BuPhCH_2	2100 (±440)	NE	398 (±78)
24	F	4-*t*-BuPhCH_2	472 (±134)	NE	118 (±35)
25	H	3,4-Me_2Ph$(CH_2)_3$	7180 (±770)	NE	13,555 (±6905)
26	F	3,4-Me_2Ph$(CH_2)_3$	2710 (±700)	NE	4656 (±1638)
27	H	Oleyl	>25,000	NE	NE

ic moiety of capsaicin within the plane of the membrane.[28] In the model of Gavva and co-workers, Tyr511, Met547, and Thr550 are suggested to be present in the binding pocket, since all three residues are important molecular determinants for vanilloid sensitivity. This model has the interactions of the vanillyl-moiety with Thr550 and the 'tail end' hydrophobic group of capsaicin or RTX interacting with Tyr511.[29] In the model of Middleton and co-workers, Met547 and Tyr555 interact with the vanillyl-moiety and Tyr511 contacts the orthophenyl group of RTX. The model also shows the additional interactions of the C_3-carbonyl and the C_2-methyl of RTX with Asn551 and Leu515, respectively.[30] The most distinctive difference among the three models is that whereas Tyr 511 interacts with the vanillyl group (A-region) in the model of Jordt and Julius, it contacts the hydrophobic ends of RTX or capsaicin (C-region) in the models of Gavva or Middleton and co-workers.

To investigate the binding mode of the thiourea TRPV1 antagonists, the potent antagonist **2** was docked into the transmembrane helices TM3/4 of TRPV1, which was constructed through homology modeling by Gavva and colleagues.[31] Two alternative binding modes are possible in the molecular docking simulations. However, the molecular surface mapping of ligand **2** and of the receptor is able to distinguish their regions of hydrophobic and hydrophilic surface. The calculations indicated that, in ligand **2**, the 4-*tert*-butylbenzyl group represents the area of highest lipophilicity (brown), and the sulfonylaminobenzyl segment represents the area of highest hydrophilicity (blue), as shown in Fig. 2a. In the receptor, the hydrophobic binding pocket is surrounded by Trp549, Met552, and Leu553, and the hydrophilic pocket is formed by Ser510, Try511, and Ser512. On the basis of this mapping, the docking study was conducted such that the nonpolar part of the ligand was fitted to the hydrophobic region of the pocket, and the polar subunit was bound to the hydrophilic region. The resulting binding model is shown in Fig. 2b. In this model, the NH of the sulfonamide acts as a hydrogen bond donor for the phenolic hydroxyl group of Tyr511 (2.01 Å) and the 3-fluoro atom engages in a hydrogen bond to the amide proton of Gly563 (1.77 Å). The sulfur atom of the thiourea group makes a hydrogen bond to the side chain of

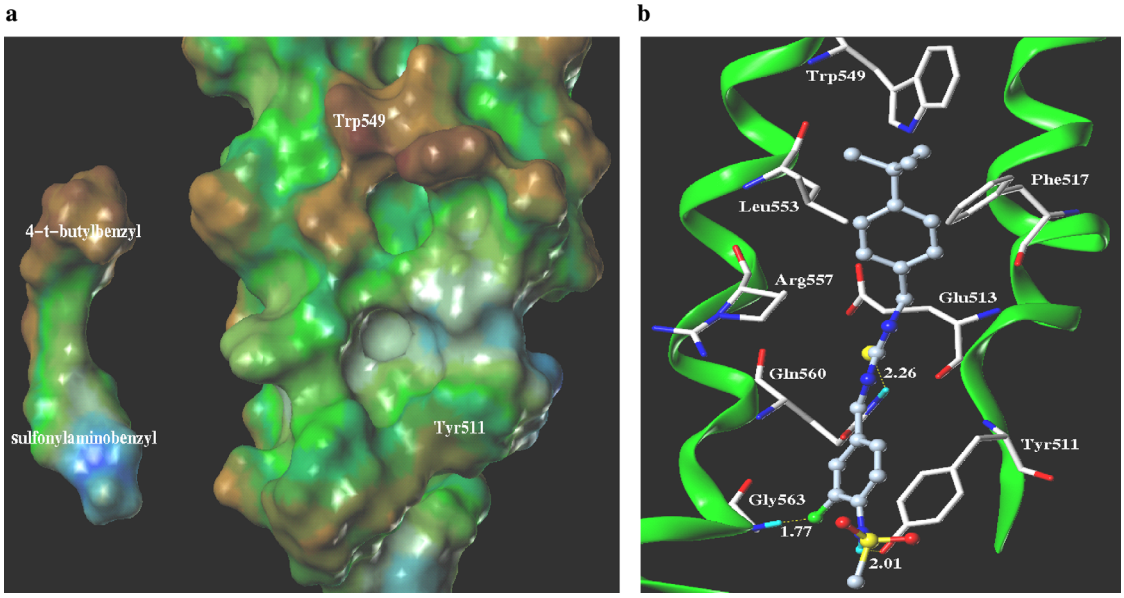


Figure 2. (a) Surface maps color-coded by lipophilic potential (LP) of antagonist **2** and TRPV1. The color for LP ranges from brown (highest lipophilic area of the molecule) to blue (highest hydrophilic area); (b) proposed model of **2** bound to TRPV1 binding site.

Gln560 (2.26 Å). The hydrophobic 4-*tert*-butylbenzyl group contacts closely the hydrophobic parts of Phe517, Trp549, Leu553, and Arg557. The 4-(methylsulfonylamino)phenyl ring (A-region) forms a parallel stack with the 4-hydroxyphenyl ring of Tyr511 with an interplanar stacking distance of 3.2 Å.

In summary, we have modified the B-region thiourea group of potent and high-affinity TRPV1 ligands (**1**–**3**) by substitutions with *N*-hydroxythiourea, urea, *N*-hydroxyurea, thiocarbamate, amide, *N*-hydroxy amide, and hydrazine carbothioamide groups to investigate their SARs. Although the modifications generally conferred modest to dramatic decreases in binding affinities and agonistic/antagonistic potencies, their SAR analysis indicated that both hydrogens, which act as H-bonding donors, and the sulfur atom in the thiourea, which functions as a H-bonding acceptor, are crucial for high-binding affinity and potent antagonism in this series of *N*-(4-*t*-butylbenzyl)-*N*′-[4-(methylsulfonylamino)benzyl]thioureas. In addition, we found that a 3-fluoro substituent enhanced antagonism in the various B-region analogues. Finally, the docking study of the potent antagonist **2** with the homology model of TRPV1 suggested a binding model for thiourea antagonists with the receptor, which may aid in optimizing the antagonistic activity of drug candidate and in understanding the gating mechanism of TRPV1 channels.

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31. The structure of compound **2** was built using the Sybyl molecular modeling program (Tripos, Inc.), and then the geometry was fully optimized using the Tripos force field with the following nondefault options: (method) conjugate gradient; (termination) gradient 0.01 kcal mol^{-1} $Å^{-1}$; (max iterations) 10,000. The partial atomic charges were calculated by the Gasteiger–Hückel method in the Sybyl program. The molecular surface maps color-coded by lipophilic potential of the binding site of TRPV1 and compound **2** were calculated using the MOLCAD program implemented in Sybyl 6.9, prior to the docking study. The docking study was conducted using the program DOCK implemented in Sybyl 6.9. Distance constraint docking was applied to keep the closest atom–atom pairs from hydrogen-bonding interactions with 2.3 Å constraint and the force field constant set to 200, which is used in calculating penalty for deviation from the equilibrium distance, for the NH of the sulfonamide to the sp^3 oxygen of Tyr511 and the S atom of the thiourea group to the NH of Gln560. All computational work was done on a Silicon Graphics O_2 R10000 workstation.