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Synthesis and evaluation of pyridazinylpiperazines as vanilloid receptor 1 antagonists

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Abstract—A structurally biased chemical library of pyridazinylpiperazine analogs was prepared in an effort to improve the pharmaceutical and pharmacological profile of the lead compound *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carboxamide (BCTC). The library was evaluated for VR1 antagonist activity in capsaicin-induced (CAP) and pH 5.5-induced (pH) FLIPR assays in a human VR1-expressing HEK293 cell line. The most potent VR1 antagonists were found to have IC_{50} values in the range of 9–200 nM with improved pharmaceutical and pharmacological profiles versus the lead BCTC. These compounds represent possible second-generation BCTC analogs.

The vanilloid receptor 1 (VR1, recently renamed TRPV1) is a member of the superfamily of transient receptor potential (TRP) cation channels, that is, expressed in peripheral sensory neurons.[1,2] VR1 is a non-selective cation channel (although it has a preference for Ca^{++} ions) that has been cloned from rat dorsal root ganglia[3] and human.[4] It is activated by various chemical stimulants, such as capsaicin (CAP) and resiniferatoxin (RTX)[2,5] (Fig. 1), as well as noxious heat and low pH.[3,6] Activation of VR1 induces excitation of primary sensory neurons and the sensation of burning pain in animals and humans. However, prolonged nociceptor excitation via chronic agonist administration is followed by a long-term desensitization, ultimately showing an analgesic effect.[5,7] Several potent VR1 agonists have been identified, but their initial excitatory effects on primary sensory neurons have led to poor patient compliance thus hindering their development.[8] For this

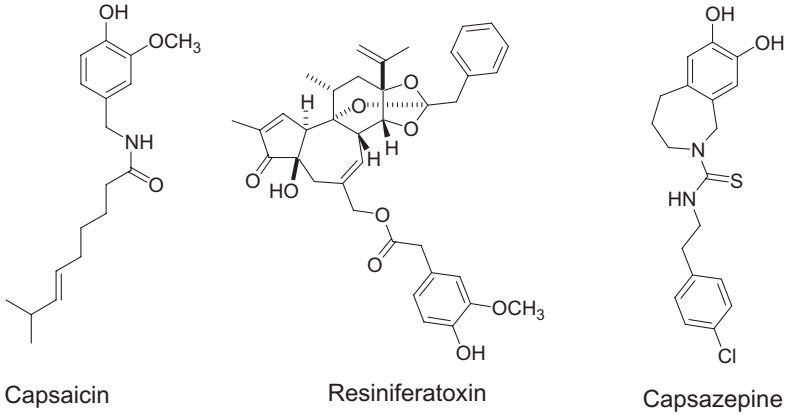

Figure 1. Structures of capsaicin, resiniferatoxin, and capsazepine.

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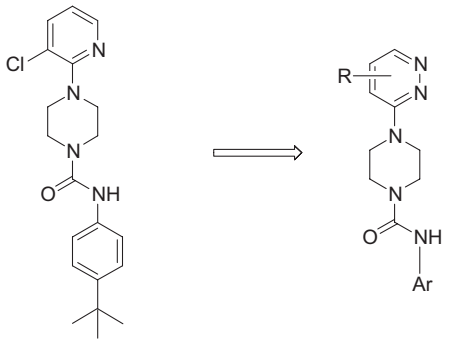
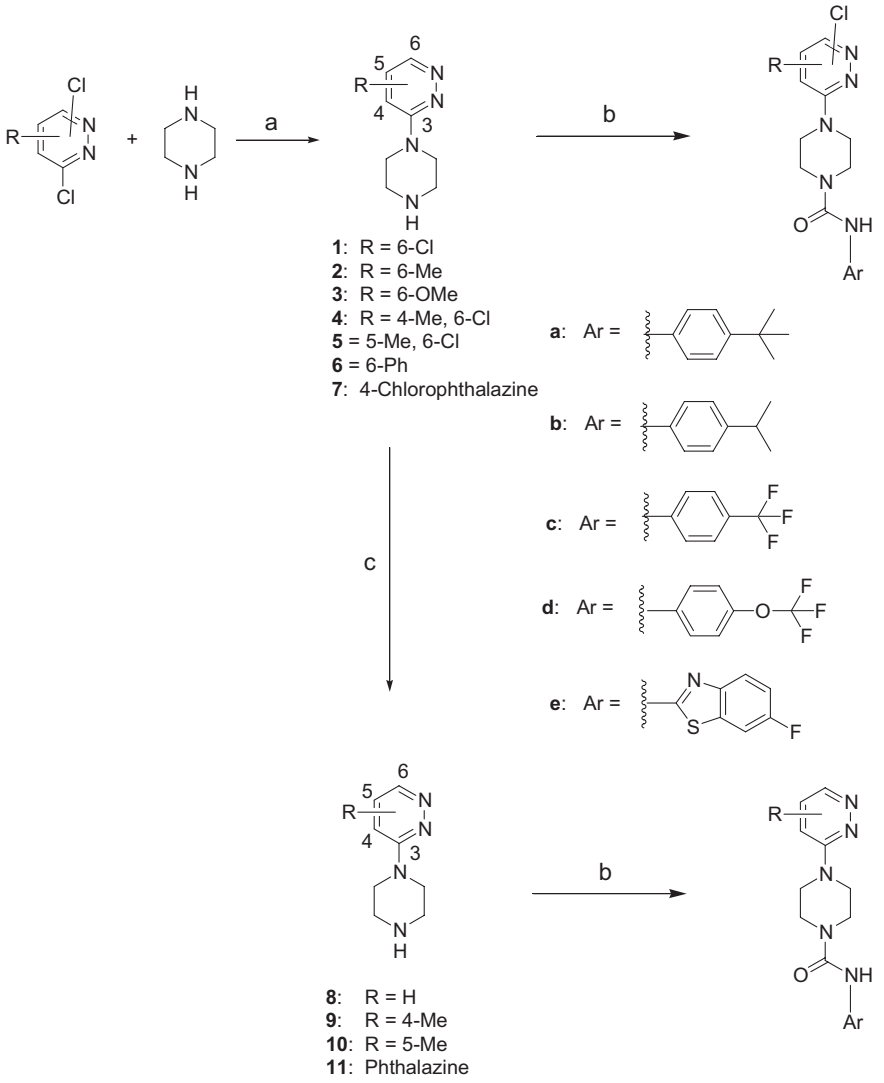


Scheme 1. Proposed pyridazinylpiperazine analogs based on BCTC.

reason, the use of VR1 antagonists as potent analgesics has attracted much attention. Capsazepine (Fig. 1) was the first well-characterized synthetic antagonist.[9–11]

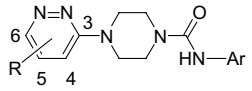
Recently, several different structural classes of VR1 antagonists have been reported.[12–15] Previously, we described the design and synthesis of a series of 4-(2-pyridyl)piperazine-1-carboxamide analogs as potent VR1 antagonists.[15] Our group reported that *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carboxamide (BCTC), a member of that new chemical series, was a highly potent VR1 antagonist that effectively reverses the behavioral effects of inflammatory and neuropathic pain in rats.[15–17] From a pharmaceutical development perspective, BCTC has several possible shortcomings including poor metabolic stability, short half-life, poor aqueous solubility, and moderate oral bioavailability. In addition, BCTC did not produce dose-proportionate pharmacokinetics when dosed orally to rats. In our continuing efforts to improve upon the pharmaceutical and pharmacological properties of BCTC, here we report the synthesis and evaluation of a series of pyridazinylpiperazine compounds as VR1 antagonists (Scheme 1).

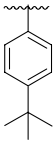
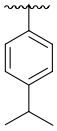
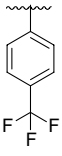
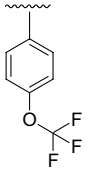
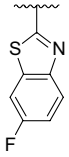
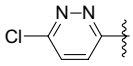
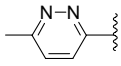
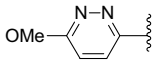
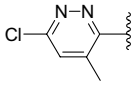
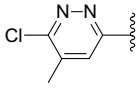
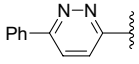
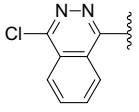
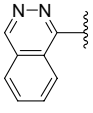
A biased chemical library of pyridazinylpiperazines was prepared in a straightforward manner as shown in Scheme 2. Pyridazine systems were explored as



Scheme 2. Reagents and conditions: (a) DMSO, 100 °C, Et_3N, 6 h; (b) Ar–NCO, THF, rt, 2 h; (c) Pd/C, H_2, MeOH, rt, 3 h.

Table 1. High throughput values for pyridazinylpiperazines in the crude form; IC_{50} values (nM) CAP/pH

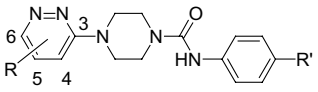


	Pyridazine	Ar				
		a	**b**	**c**	**d**	**e**
						
1		ND	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000
2		>10,000/>10,000	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000
3		ND	ND	ND	ND	>10,000/>10,000
4		ND	ND	**2796**/>25,000	ND	**2178/3262**
5		>10,000/>10,000	>10,000/>10,000	ND	>10,000/>10,000	>10,000/>10,000
6		ND	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000
7		ND	ND	ND	ND	ND
8		**340/>25,000**	**5745/13,286**	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000
9		**28/50**	ND	**1465/5861**	**1386/2727**	**6169**/>25,000
10		**893/2642**	**5021/20,336**	**4867**/13,602	>10,000/>10,000	>10,000/>10,000
11		>10,000/>10,000	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000	>10,000/>10,000

Bold-face represents discovered hits.

replacements for the 3-chloropyridine portion of BCTC. In order to compare the pharmacology of the proposed library with BCTC, various 4-substituted arylisocyanates **a**–**d** were selected as building blocks based on the structure of BCTC. The building block **e** was included in order to increase the diversity of the proposed library. The intermediates 3-piperazin-1yl-pyridazines **1**–**7** were synthesized by reacting various substituted 3-chloro-pyridazines with piperazine in DMSO at 100 °C using triethylamine as a base. The

Table 2. Results of the purified pyridazine analogs



No.	R	R′	IC_{50} (nM) pH	IC_{50} (nM) CAP
12	H	*t*-Bu	>10,000	679.4 ± 141.4
13	6-Cl	*t*-Bu	>10,000	3825.8 ± 2727.9
14	4-Me	*t*-Bu	220.7 ± 50.5	47.2 ± 9.9
15	4-Me, 6-Cl	*t*-Bu	300.7 ± 50.5	72.6 ± 58.5
16	5-Me	*t*-Bu	>10,000	>10,000
17	5-Me	CF_3	>10,000	>10,000
18	4-Me	*i*-Pr	2783.7 ± 1072.2	176.6 ± 66.7
19	4-Me	Ph	5727.7 ± 1321.8	279.0 ± 120.5
20	4-Me	CF_3	2263.0 ± 409.6	2154.0 ± 351.3
21	4-Me	OCF_3	4332.0 ± 499.4	4222.7 ± 501.0

IC_{50} values are the mean of at least three determinations.

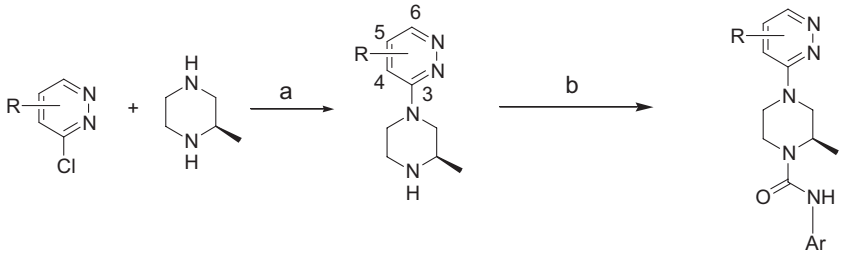
piperazin-1yl-pyridazines **1**, **4**, **5**, **7** were further reduced with Pd/C in methanol using H_2 to give additional head groups **8**–**11** thereby increasing diversity of the proposed library. The intermediates **1**–**11** were then reacted with isocyanates **a**–**e** for 3 h in THF at room temperature to give the desired products.

The compounds were evaluated for human VR1 antagonist activity in a HEK293 cell line against CAP and pH 5.5 activation. The assays were conducted essentially as described previously.[16] The tests were carried out measuring 100 nM CAP- or pH 5.5-mediated calcium influx with a Fluorometric Imaging Plate Reader (FLIPR).[18]

Our library purity cutoff for the initial pharmacological assay was set to be greater than 70% (determined by LC/MS[19]). A total of 39 compounds, out of 55 attempted syntheses, passed the library purity criteria and were assayed at 10 μM concentration. The results are as shown in Table 1. The wells showing >50% inhibition (10 hits total) of the maximal CAP or pH response were considered positive and were followed up by additional experiments to determine their IC_{50} values. The pyridazine analogs made using head groups **1**, **2**, **3**, **5**, and **6** with a substitution at the 6-position showed no antagonist activity in either the pH or CAP assays. However, moderate in vitro potency was observed in the CAP assay when a substituent is added at the 4-position, as in analogs made using head group **4**. This result showed that having substitution at the 4-position is crucial for the in vitro potency, consistent with BCTC series. The use of unsubstituted pyridazine **8** resulted in analogs with moderate potency in the CAP assay and little or no activity when evaluated in the pH assay. When the substitution at the 6-position is removed as in analogs made using the head groups **9** and **10**, the in vitro potencies are significantly improved in both the pH and CAP assays. Comparison of analogs prepared using head groups **9** and **10** gives a clear example where substitution at the 4-position is preferred over the 5-position. The methyl group at the 4-position gave an analog, that is, 31-fold more potent in the CAP assay and 52-fold more potent in the pH assay compared to the 5-methyl analog. The phthalazine analogs prepared using **11** did not show VR1 antagonist activity in either assays.

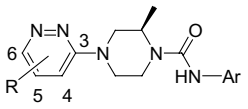
Guided by the initial screening results of the library compounds, some compounds were selected and purified to greater than 98% purity via flash chromatography on silica gel as demonstrated by two differing RP-HPLC methods.[19] The correct molecular weights were confirmed by mass spectroscopy[19] and structures were further confirmed by ^1H NMR spectroscopy.[20] Upon purification and reassay of the library hits, no significant change to the IC_{50} values was observed as compared to the crude molecules. These results are shown in Table 2. The use of an unsubstituted pyridazine head group, as in **12**, gave a product that has moderate potency in the CAP assay and no activity in the pH assay. Introduction of 6-chloro, as in **13**, resulted in a 5-fold decrease in potency in the CAP assay compared to **12**. The introduction of a 4-methyl group on the pyridazine ring, **14**, resulted in a significant increase in potency in both assays, >50-fold in the pH assay and >14-fold in the CAP assay compared to **12**. The introduction of a 6-chloro substitution, as in **15**, did not change the potency in the pH assay but reduced the potency by 2-fold in the CAP assay compared to **14**. The use of 5-methyl substitutions on pyridazine head groups, as in **16** and **17**, resulted in analogs with no VR1 antagonist activity. The use of *i*-propyl or phenyl substitution in the tail group instead of the *t*-butyl in **14**, as in **18** and **19**, reduced the potency by 12–26-fold in the pH assay and by 4–6-fold in the CAP assay. Replacing the *t*-Bu group in **14** with CF_3 or OCF_3, resulted in analogs **20** and **21** that have much reduced in vitro potency, 10–20-fold in the pH assay and 45–90-fold in the CAP assay.

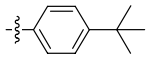
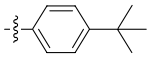
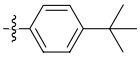
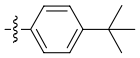
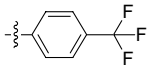
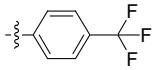
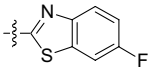
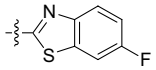
From the SAR study (unpublished) that was developed for BCTC series, we had observed that introduction of a methyl group in the *R* stereoisomer configuration on the



Scheme 3. Reagents and conditions: (a) DMSO, 100 °C, Et_3N, 6 h; (b) Ar–NCO, THF, rt, 2 h.

Table 3. Results of the *R*-methyl pyridazine analogs



No.	Ar	R	IC_{50} (nM) pH	IC_{50} (nM) CAP
BCTC			29.8 ± 9.3	34.0 ± 11.0
22		4-Me	15.2 ± 3.5	8.5 ± 3.6
23		5-Me	>25,000	8590.7 ± 477.2
24		4-Me, 6-Cl	67.5 ± 26.9	10.0 ± 1.0
25		5-Me, 6-Cl	>25,000	8118.8 ± 1449.0
26		4-Me	250.7 ± 14.3	57.7 ± 23.4
27		5-Me	>25,000	>10,000
28		4-Me, 6-Cl	226.3 ± 61.7	103.3 ± 30
29		5-Me, 6-Cl	13,469.7 ± 4385.2	1623.0 ± 477.0

IC_{50} values are the mean of at least three determinations.

piperazine ring generally increases the potency of analogs by at least 10-fold. Therefore, pyridazine analogs that showed promising in vitro potency from the screening library were selected and resynthesized with an *R*-methyl group on the piperazine ring. The syntheses of the *R*-methyl analogs were accomplished in a similar manner to the library synthesis by using *R*-2-methylpiperazine (AstaTech) instead of simple piperazine as shown in Scheme 3.[15] The results are as shown in Table 3. The introduction of the *R*-methyl substitution on the piperazine ring dramatically improved the in vitro potency of the resulting analog **22** compared to **14**, 14-fold in the pH assay and 5-fold in the CAP assay. Compound **22** is more potent than BCTC, 2-fold in the pH assay and 4-fold in the CAP assay. In agreement with the previous observation, all of the analogs with 5-methyl substitutions on the pyridazine ring **23**, **25**, **27**, and **29** were inactive whereas the analogs with 4-methyl substitutions **22**, **24**, **26**, and **28** were significantly more potent. The introduction of the *R*-methyl substitution on the piperazine ring did not improve the in vitro potency of **23** and **27** when compared to the unsubstituted analogs **16** and **17**. The *R*-methyl substitution did improve the potency of **24** by 4-fold in the pH assay and 7-fold in the CAP assay when compared to the unsubstituted piperazine analog **15**. Introduction of the 6-chloro on the pyridazine ring, as in compound **24**, did not affect the potency in the CAP assay when compared to **22**. However, the pH assay indicated a 4-fold decrease in potency. The in vitro potency of **26** also showed improvement over the unsubstituted piperazine analog **20**, 9-fold in the pH assay and 37-fold in the CAP assay. Comparison of benzothiazole analog **28** with a similar analog in the screening library revealed once again that the introduction of the *R*-methyl group on the piperazine ring improves the potency significantly, 21-fold in the CAP assay and 14-fold in the pH assay. Moving the methyl substitution on the pyridazine ring from the 4- to the 5-position, as in **28** and **29**, was again shown to significantly decrease the potency in the benzothiazole analogs. Even though the benzothiazole analog **28** is not the most potent pyridazine analog, it is structurally different from the usual aniline tail groups used and it was presumed that it would result in a different pharmaceutical profile.

Compounds that inhibit the human ether-a-go-go related gene (hERG) channel have a potential liability of prolonging the cardiac QT interval causing ventricular arrhythmias and fibrillation.[21] BCTC and compounds **22**, **24**, and **28** were screened for their abilities to block hERG channel expressed in HEK-293 cells at 1 μM concentration using a standard whole cell voltage-clamp protocol.[22] The results are as shown in Table 4. It was discovered that BCTC has a significantly high inhibition (87%) whereas the pyridazine analogs **22** and **24** show

only 7% and 6% inhibitions, respectively. Compound **28** has 36% inhibition at 1 μM concentration. The use of the pyridazine head groups has significantly decreased the ability of the piperazine-1-carboxamide analogs to inhibit the hERG channel.

The analogs with IC_{50} values <500 nM were selected and their rat liver microsomal (RLM) stability was evaluated. This was done by incubating the analogs with rat liver microsomes in the presence of NADPH and oxygen. After 30 min, the amount of the parent compound was measured by LC/MS. The results are as shown in Table 4. The use of pyridazine groups instead of the pyridine in BCTC seems to have improved RLM stability of the resulting analogs. The RLM stabilities of **22** and **24** were 35% and 26%, respectively. These are significant improvements over BCTC that only had 0.3% of parent compound remaining after a 30 min incubation. Changing the *t*-butyl group of **22** to a CF_3, as in **26**, has improved the RLM significantly to 74%. The use of benzothiazole, as in **28**, instead of the usual aniline also had a favorable effect in the RLM assay (63%).

In order to facilitate the compound selection process, initial pharmacokinetic (PK) screening studies were conducted. The PK profiles were determined following intravenous (iv) administration (3 mg/kg) of compounds in 25% aqueous HPBCD. The mean plasma concentrations ($N = 3$ rats) were used to calculate the PK parameters utilizing noncompartmental analysis (NCA) algorithm of WinNonlin software. Analysis of the in vivo PK parameters revealed that compounds **22**, **24**, and **26** have very short half-lives and showed no improvement over BCTC, as shown in Table 5. Surprisingly, compound **28** showed a significantly longer half-life of 6 h compared to the usual aniline analogs.

Additional studies were done in order to investigate the oral bioavailability and dose proportionality of compound **28**. Following a single iv and escalating oral doses under fasted conditions, the corresponding bioavailability was calculated as the ratio of the oral dose-adjusted AUC to the dose-adjusted AUC for iv administration (Fig. 2). The bioavailability of the 10 mg/kg oral dose was calculated to be 62% (with a maximum plasma concentration of 3957 ± 755 ng/mL at 4 h) whereas the bioavailability of 3 mg/kg oral dose was calculated to be 70% (with a maximum plasma concentration of 2689 ± 143 ng/mL at 3 h). This is a significant improvement over BCTC that showed marginal oral bioavailability only at high oral dose (40 mg/kg). Unlike BCTC, compound **28** showed good aqueous solubility and high RLM stability that most likely led to its marked increases in bioavailability and dose proportional PK.

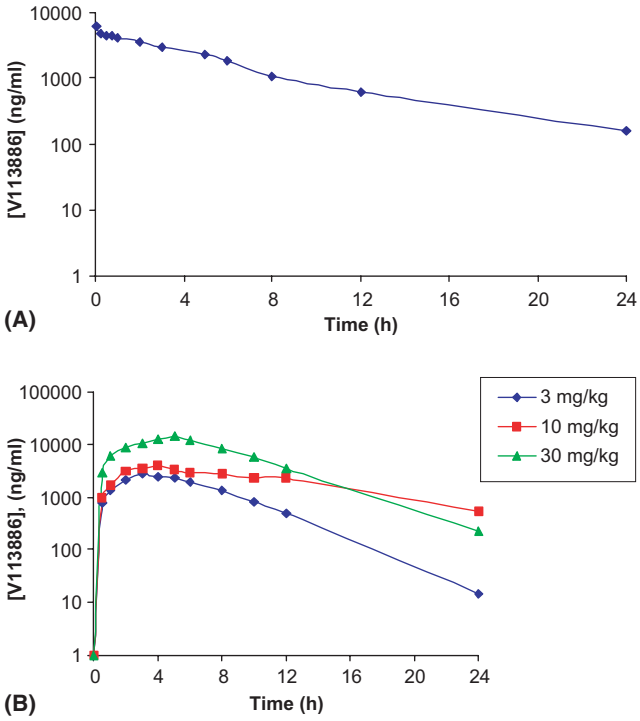


Figure 2. In vivo pharmacokinetic profile of compound **28**. Compound **28** was dosed to rats intravenously (A) or orally (B) and plasma samples withdrawn at time points indicated.

Table 4. Rat liver metabolic stability and hERG channel inhibitions for BCTC and potent pyridazine VR1 antagonists

No.	Metabolic stability (% remaining)	HERG (% inhibition)
BCTC	0.3	87 ± 4
22	35	8 ± 2
24	26	7 ± 5
26	74	ND
28	63	36 ± 3

Table 5. In vivo pharmacokinetic profile of BCTC, **22**, **24**, **26**, and **28**

No.	Half-life (h)	Clearance (L/h/kg)	Volume of distribution (L/kg)
BCTC	0.9	4.6	5.8
22	0.5	7.0	5.1
24	0.2	6.5	1.44
26	0.9	3.35	4.26
28	6.0	0.11	0.93

In summary, we have synthesized and evaluated a structurally biased chemical series of pyridazinylpiperazines analogs as VR1 antagonists. The most potent VR1 antagonists were found to have IC_{50} values in the range of 9–200 nM. Moreover, we have identified a potent analog **28** that has demonstrated an improved pharmaceutical and pharmacological profile versus the lead compound BCTC. This compound represents a second-generation BCTC analog.

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18. PCR amplification of human VR1 from spinal cord cDNA, subcloning into the pIND/V5-His-TOPO vector and generation of an inducible, stable cell line were performed as previously described for rat VR1.[16] Primers flanking the coding region of human VR1 were designed as follows: forward primer, GAAGATCTTCGCTGGTTGCACACTGGGCCACA, and reverse primer, GAAGATCTTCGGGGACAGTGACGGTTGGATGT. In addition, the capsaicin- and acid (pH 5.5)-based assays were also as previously described for the rat VR1.[16]
19. Liquid chromatography/mass spectrometry (LC/MS) was performed on Agilent Series 1100 MSD instrument with an electrospray sample inlet system. ZORBAX Eclipse XDB column (4.6 × 50 mm) was used with a gradient of 15–90% B over 4.3 min (A = 0.1% TFA in H_2O, B = 0.1% TFA/CH_3CN). Two unique HPLC methods were used to access purity of the purified compounds. The first method uses Discovery HS C-18 (3 μ, 4.6 × 150 mm) column with a gradient of 15–95% B over 12.1 min (A = 0.1% TFA in H_2O, B = 0.1% TFA/CH_3CN). The second HPLC method uses LUNA C-18 (3 μ, 4.6 × 150 mm) column with a gradient of 10–95% B over 43 min (A = 0.05% TFA in H_2O, B = 0.05% TFA/CH_3CN).
20. NMR spectra were obtained on a Bruker Avance 400 (^{1}H at 400 MHz). The NMR data for compounds **22**, **24**, **26**, **28** is as follows; **22**: ^{1}H NMR ($CDCl_3$): δ 8.77 (d, 1H, J = 4.60 Hz), 7.34–7.32 (m, 4H), 7.20 (d, 1H, J = 4.60 Hz), 6.46 (br s, 1H), 4.50–4.39 (m, 1H), 3.98 (br d, 1H, J = 12.50 Hz), 3.69–3.61 (m, 1H), 3.53–3.44 (m, 2H), 3.30 (dd, 1H, J = 12.50 Hz, J = 3.73 Hz), 3.16 (dt, 1H, J = 12.50 Hz, J = 3.50 Hz), 2.38 (br s, 3H), 1.45 (d, 3H, J = 6.58 Hz), 1.32 (s, 9H). Compound **24**: ^{1}H NMR ($CDCl_3$): δ 7.379–7.29 (m, 4H), 7.25–7.23 (m, 1H), 6.42 (br s, 1H), 4.48–4.39 (m, 1H), 3.97 (br d, 1H, J = 13.15 Hz), 3.66–3.58 (m, 1H), 3.66–3.58 (m, 1H), 3.53–3.41 (m, 2H), 3.26 (dd, 1H, J = 3.72 Hz, J = 12.28 Hz), 3.13 (dt, 1H, J = 3.72 Hz, 12.72 Hz), 2.37 (br d, 3H, J = 1.09 Hz), 1.43 (d, 3H, J = 6.57 Hz), 1.31 (s, 9H). Compound **26**: ^{1}H NMR ($CDCl_3$): δ 8.77 (d, 1H, J = 4.60 Hz), 7.60–7.53 (m, 4H), 7.22 (d, 1H, J = 4.60 Hz), 6.89 (br s, 1H), 4.55–4.45 (m, 1H), 4.03 (br d, 1H, J = 12.93 Hz), 3.70–3.64 (m, 1H), 3.57–3.45 (m, 2H), 3.31 (dd, 1H, J = 3.73 Hz, 12.71 Hz), 3.18 (dt, 1H, J = 3.44 Hz, J = 12.28 Hz), 1.47 (d, 1H, J = 6.79 Hz). Compound **28**: ^{1}H NMR ($CDCl_3$): δ 8.84 (br s, 1H), 7.61–7.53 (m, 1H), 7.49–7.42 (m, 1H), 7.25–7.21 (m, 1H), 7.16–7.07 (m, 1H), 4.54–4.43 (m, 1H), 4.08 (br d, 1H, J = 12.93 Hz), 3.64–3.56 (m, 1H), 3.54–3.37 (m, 2H), 3.23 (dd, 1H, J = 3.73 Hz, J = 12.49 Hz), 3.11 (dt, 1H, J = 3.51 Hz, J = 12.71 Hz), 2.34 (br d, 3H, J = 0.44 Hz), 1.44 (d, 3H, J = 6.79 Hz).
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22. The assays were conducted on hERG channel expressed in HEK-293 cells (obtained from Wisconsin Alumni Research Foundation) at 1 μM concentration using a standard whole cell voltage-clamp protocol, that is, pre-pulsing from −80 mV to +20 mV for 2 s and then pulsing to −40 mV for 1 s every 15 s. The peak amplitude of the tail current elicited by the second, test, pulse was used to assess the degree of inhibition. Percent inhibition was measured at least in three cells for each compound, with astemizole, 100 nM as a positive control; data is presented as mean ± SEM.