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Research Article

Synthesis of Analogues of BCTC Incorporating a Pyrrolidinyl Linker and Biological Evaluation as Transient Receptor Potential Vanilloid 1 Antagonists

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A series of novel pyrrolidinyl linker TRPV1 antagonists were prepared in an effort to lower the hyperthermic side-effects of first-generation antagonist BCTC. These compounds were investigated for antagonism of *h*TRPV1 activation by capsaicin and acid *in vitro*. Preliminary results suggested the compounds 10a, 10b, 10c and 10j had favorable TRPV1 antagonism activity. In further studies in vivo, 10b, comparable to BCTC, showed potent analgesic activity in capsaicin-induced and heat-induced pain models. In addition, 10b indicated a reduced risk of body temperature elevation. All of these demonstrated that 10b can be considered as a safe candidate for the further development of analgesic drugs.

Key words: analgesic, BCTC, hyperthermia, transient receptor potential vanilloid type 1

Abbreviations: %MPE, percent maximal possible effect; BCTC, N-(4-(tert-butyl) phenyl)-4-(3-chloropyridin-2-yl) piperazine-1-carboxamide; TLC, thin layer-chromatography; TRPV1, transient receptor potential vanilloid type 1.

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TRPV1 is a non-selective cation channel that is polymodal, being activated by multiple unrelated stimuli such as exogenous ligands (e.g. capsaicin from chili peppers, peptide toxins from tarantula venom) (1), heat (> 43 °C), low pH (< 5.9), and certain endogenous substances [e.g. anandamide (2), *N*-arachidonoyl-dopamine (3)] produced by inflamed tissues. TRPV1 channel blocked leads to analgesia in several animal models, which renders TRPV1 as next molecular target for treating pain (4–7). During the past decade, various classes of TRPV1 antagonists with high efficacy and potency have been identified (8,9). However, few candidates have progressed into clinical trials because of serious hyperthermic side-effect (10–12). Therefore, the key challenge in developing TRPV1 antagonists for pain management is to try to avoid hyperthermia.

In 2003, Sun *et al.* (13) reported a potent and orally available TRPV1 antagonist, BCTC(**1**) effectively reversed the behavioral effects of inflammatory and neuropathic pain in rats, but its hyperthermia side-effect had led to poor patient compliance, thus hindering its development (14,15). We therefore initiated our SAR effort with the aim of identifying novel potent TRPV1 antagonists based on BCTC with minimized hyperthermic side-effect.

During the past decade, diverse BCTC analogues have been synthesized to improve the pharmaceutical and pharmacological profile. These structural modifications mainly focused on these positions (Figure 1). (i) Modification of the linker 1: Central piperazine ring in BCTC was replaced with a phenyl ring or tetrahydropyridine ring to yield compounds with significant distribution into the CNS (16). (ii) Modification of the region B: The urea in BCTC act as H-bond interactions can be transformed into amide (17) or quinazoline (18) and 6,6-heterocycle (19). (iii) Modification of the region B and C: Refinements of the urea motif, such as restricting the number of accessible conformations, have provided examples such as the benzimidazole and indazolone analogues (20,21). Analysis of the structure of BCTC and analogues of BCTC indicated that the linker between regions B and C is absent (22). On the other hand, most recently, Rami *et al.* (23) reported a pyrrolidinyl urea derivative (SB-705498, **2**) as a potent antagonist toward multiple modes of activation of TRPV1, which was reported to significantly reduce both (i) capsaicin-evoked flare and heat-evoked pain on non-sensitized skin and (ii) heat-evoked pain after ultraviolet B-evoked inflammation in Phase I trials (24). It is worth pointing out that hyperthermia was not described as one of the adverse effects for this compound, despite suggesting redundancy in Phase II trials (25). Furthermore, pyrrolidinyl skeleton has an extensive history of use in the pharmaceutical industry. With these concerns in mind, we first synthesized novel analogues of BCTC incorporating a pyrrolidinyl linker into regions B and C in an attempt to lower the hyperthermic side-effect.

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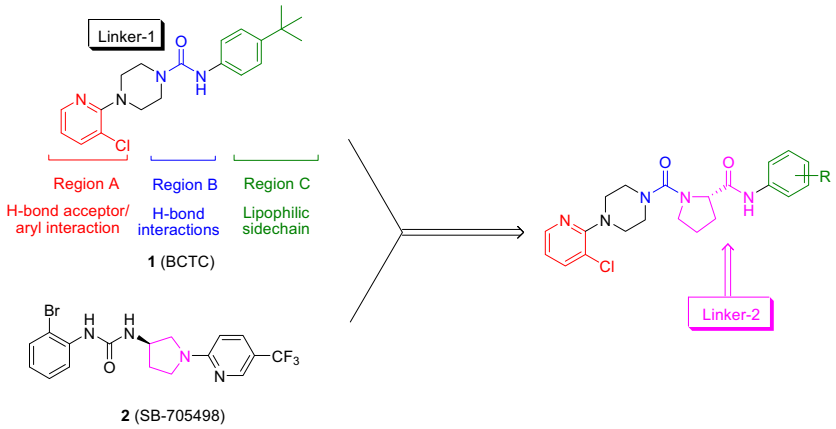


Figure 1: Design of analogues based on BCTC as new TRPV1 antagonists.

Methods and Materials

General

All reagents were purchased from Shanghai Chemical Reagent Company, Shanghai, China. Column chromatography (CC, 100-200 mesh silica gel60) and thin-layer chromatography (TLC) were purchased from Qingdao Ocean Chemical Company, Qingdao, China. The structures of target compounds were ensured by NMR, MS, IR, and elementary analysis. NMR spectra were recorded in $CDCl_3$ on a Bruker ACF-300Q instrument (300 MHz for 1H, 75 MHz for 13C; Bruker Instruments, Inc., Billerica, MA, USA). Chemical shifts are expressed in δ values in ppm with tetramethylsilane (TMS) as internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constant (Hz), and integration. ESI-MS was recorded with Waters ACQUITY UPLC Systems with Mass (Waters, Milford, MA, USA). IR spectra were recorded on Shimadzu FTIR-8400S spectrophotometer (Shimadzu, Tokyo, Japan). Elemental analysis was recorded on the CHN-O-Rapid instrument. Melting points were measured using a Mel-TEMP II melting point apparatus, which was uncorrected. Percentage purity of the target compounds (> 97%) were determined by HPLC analysis (Shimadzu).

Pharmacology

The synthesized compounds were investigated for TRPV1 antagonistic activity *in vitro*, analgesic activity *in vivo,* and the effect on body temperature. The test compounds and the standard drugs were administered in the form of a suspension (using 0.5% sodium carboxymethyl cellulose as a vehicle) by intragastric administration. Separate groups of KM male mice (n = 8), weighing 18–22 g, were pretreated with compounds (30 mg/kg unless otherwise indicated) 30 min before the test. The animals were procured from the Comparative Medicine Centre of Yangzhou University (Jiangsu, China) and were maintained in colony cages at 25 ± 2 °C, relative humidity 45–55%, under a 12-h light/dark cycle; they were fed standard animal feed. All the animals were acclimatized for a week before use. The institutional animal ethics committee has approved the protocol adopted for the experimentation of animals.

Transient receptor potential vanilloid type1 antagonistic activity assays in vitro

Culture plates with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline supplemented with 5 mM ethylenediaminetetra-acetic acid were used for the TRPV1 aequorin cells (Perkin Elmer, Waltham, MA, USA) growth. The cells were pelleted for 2 min at 1000 *g*, resuspended in Dulbecco's minimum essentialmedium-F12 medium with 15 mM HEPES (pH 7.0) and 0.1% BSA (assay buffer) at a density of 3×10^5 cells/mL, and incubated for 4 h in the dark in the presence of 5 mM coelenterazine h (Promega, Madison, WI, USA). After loading, cells were diluted with assay buffer to a concentration of 5×10^6 cells/mL. Twenty microliters of cells was injected over 20 μL of the sample solution plated on 384-well plates, respectively, unless otherwise indicated. The digitonin, ATP (Sigma-aldrich, St Louis, MO, USA), and assay buffer were added in the blank control wells for reference, and final concentration of digitonin and ATP was 100 and 50 μM. The sample solutions at different concentrations and the cells were incubated for 2.5 min before the addition of agonists capsaicin (Tocris, Bristol, UK) and HCl solution at pH 5 and then immediately detected. The light emission was recorded during variable times using EnVision2014 Multilabel Reader (PerkinElmer; 26,27).

Analgesic activity

Capsaicin test

As previously described, we evaluated analgesic activity in the capsaicin-induced pain model (28). Mice were grouped randomly according to the weight, and each group had eight mice. Twenty microliters of solution of capsaicin (16 μg/20 mL) was injected s.c. under the skin of the dorsal surface of the right hindpaw. The mouse was then placed in an individual cage. The amount of time spent

licking the injected paw was measured and expressed as the cumulative licking time for 5 min after the capsaicin injection.

Abdominal constriction test

Abdominal constriction test was performed as described previously to assess analgesia of pain activated by acid (26). Mice were grouped randomly according to the weight, and each group had eight mice. We placed mice in individual glass cylinders for a 30-min acclimatization period, injected with 0.6% acetic acid (0.1 mL/10 g/mouse i.p.), and immediately placed inside transparent glass cylinders. The number of writhes was recorded for 15 min.

Tail-flick test

Tail-flick test was carried out as previously performed (29). Mice were grouped randomly according to the weight, and each group had eight mice. Briefly, in a water bath maintained at 52 °C, the distal one-third of the mouse tail was immersed. Latency times until a tail-flick response were recorded before and after drug treatment. The antinociception response was presented as percent maximal possible effect (%MPE) as defined by %MPE = 100% × (drug response time − basal response time)/(cutoff time − basal response time). A cutoff time of 12 seconds was applied to avoid tissue damage.

Effect on body temperature

Mice were grouped randomly according to the weight, and each group had eight mice. They were intragastric administered with synthesized compounds (30 mg/kg, i.g.), BCTC (30 mg/kg, i.g.), or an equal volume of vehicle. The body temperature of mice was monitored by inserting the electric probe thermometer (MT-1C/F; Ruidien, Shenzhen, China) into the anus at 0, 30, 60, 90, and 120 min after dosing. The effect on body temperature was presented as △ temperature = the temperature at the certain time after dosing − the temperature at 0 min after dosing.

Statistical analysis of the data

Statistical analyses were performed using specific software (GRAPHPAD INSTAT version 5.00; GraphPad Software, San Diego, CA, USA). Comparisons were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, unless otherwise stated. $p < 0.05$ is regarded as statistically significant.

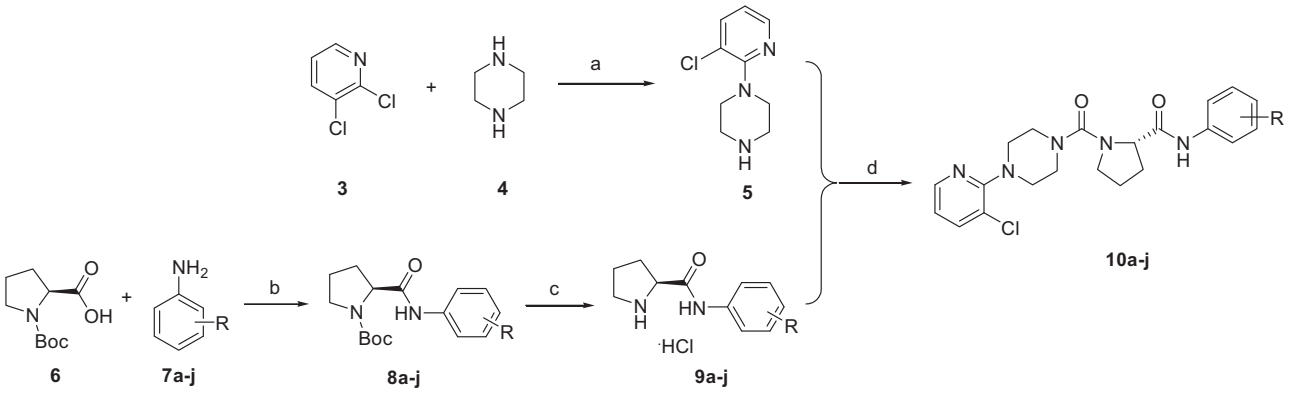
Results and Discussion

Chemistry

The synthetic route employed to prepare the target compounds is shown in Scheme 1. Commercially available 2,3-dichloropyridine(**3**) reacted with piperazine in *n*-butanol to produce the intermediate **5**. Reaction of *N*-Boc-L-proline (**6**) with substituted anilines produced intermediates **8a**–**j**. The Boc protecting group was removed by treatment with 1N HCl in acetic ether, leading to **9a**–**j** as hydrochloride salt in high yield. Subsequently, the target compounds **10a**–**j** were prepared by coupling **9a**–**j** with intermediate **5** in the presence of triphosgene. The chemical synthesis details were presented as supporting information.

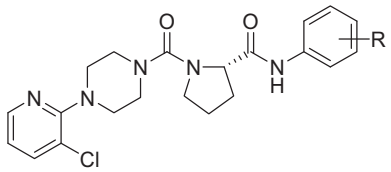
Pharmacology

The compounds were evaluated for their ability to block capsaicin (CAP) or low pH-induced activation of human TRPV1 channels and the results are shown in Table 1. We found that the potency at TRPV1 was sensitive to the substitution on the aryl amides. Compounds possessing electron-withdrawing substituent showed no antagonist activity in either the pH or CAP assays. In contrast, the unsubstituted analogue (**10a**) and electron-donating substituents,



Scheme 1: Synthesis of the target compounds **10**. Reagents and conditions:(a) n-butanol, reflux; (b) EDCI/DMAP; (c) HCl/EtOAc; (d) CH_2Cl_2, triphosgene/Et_3N, one pot.

Table 1: *In vitro* ability of compounds to inhibit the activation of hTRPV1 receptors



Compounds	R	hTRPV1(CAP) IC_{50} (μM)[a]	hTRPV1(pH) IC_{50} (μM)[b]
10a	H	0.115	0.013
10b	4-tBu	0.237	0.356
10c	2-CH_3	0.334	0.264
10d	2-OCH_3	3.3	ND
10e	4-CH_3	5.4	ND
10f	4-F	ND	ND
10g	3-Cl	ND	ND
10h	4-Cl	ND	ND
10i	2-Cl	ND	ND
10j	2-C_2H_5	0.314	0.0058
BCTC		0.017	0.0032
SB-705498		0.048	0.0088

ND, not determined.
[a]Human TRPV1 receptor activated by capsaicin.
[b]Human TRPV1 receptor activated by low pH (5.0). Unless otherwise stated, all values are the mean (SEM of at least three separate experiments).

such as *p*-tert-butyl (**10b**), *o*-Me (**10c**), and *o*-Et (**10j**), showed slight decrease when compared to compound BCTC, while more potent than SB-705498 in CAP assay. In pH assay, these compounds showed dramatic decrease in potency than BCTC. So the compounds **10a**, **10b**, **10c** and **10j** that demonstrated acceptable *in vitro* potency were advanced into our preliminary *in vivo* studies (Table 1).

To attest the preferable compounds, three different models of pain were selected for antinociceptive activity *in vivo* (Figure 2). The total time spent licking the paw was reduced by all test compounds in the capsaicin test, especially **10b** and **10j** were superior to positive control BCTC. It indicated that all test compounds are effective in treatment of capsaicin-induced pain. In the abdominal constriction test, all compounds significantly reduced the number of writhes in proton-induced pain models and compound **10j** exhibited better potency than BCTC, while **10b** had much weaker effects compared to other compounds. In the tail-flick test, compounds **10a** and **10b** showed similar %MPE to BCTC, while **10j** exhibited no effects in treatment of heat-induced pain, although it exhibited best analgesic activity in treatment of proton-induced pain. All the test compounds had antinociceptive activity to a certain extent. Compound **10a** showed effective treatment in proton- and heat-induced pain, and no improvement in the tail-flick test. Compound **10b** had significant effect on capsaicin-induced pain and good effect in the tail-flick test; however, it exhibited very weak effect in the abdominal constriction test, which was consistent with its *in vitro* ability. We assume that the different effects of test compounds in the three models of pain may be attributed to the different analgesic mechanisms with different effects on the body temperature.

In a follow-up experiment, we next conducted body temperature study for compounds **10a**, **10b**, **10c** and **10j**, and compared their effects with positive control BCTC. In our study, we found that compounds **10a**, **10j**, and BCTC significantly increased the temperature relative to vehicle from 30 min through 90 min after dosing, and with a maximum of △temperature occurring at 60 min (Figure 3). In contrast, compound **10b** did not exhibit significant effects. It has previously been reported that blocking proton-induced TRPV1 activation evokes the unwanted side-effect of hyperthermia (30). These findings are particularly striking when considered relative to BCTC, an agent which induces robust increases in body temperature in mice in 30 mg/kg after oral administration and a potent blocker of both capsaicin and acid activation of TRPV1 *in vitro*. In this case, considering the data in the three analgesic tests, **10b** was effective in treatment of capsaicin-induced and heat-induced pain and showed a reduced risk of body temperature elevation when compared to comparator BCTC.

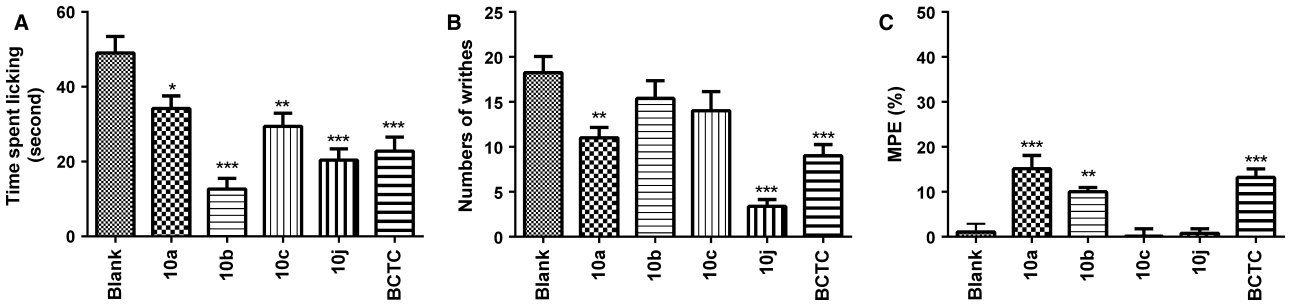


Figure 2: Analgesic activities of compounds in 30 mg/kg after oral administration. (A) The antinociceptive effects in the capsaicin test; (B) suppression of acetic acid-induced writhing response; (C) inhibition of thermal nociception. Each bar represents the mean ± SEM (n = 8). Statistical analysis was evaluated using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. *p < 0.05; **p < 0.01; ***p < 0.001 compared with the vehicle group.

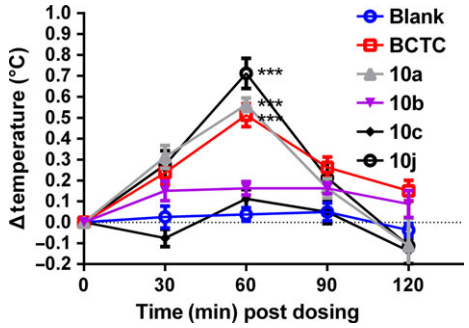


Figure 3: The effects of compounds in 30 mg/kg after oral administration on body temperature in mice. The changes of body temperature after dose. Data are expressed as mean ± SEM (n = 8). *p < 0.05, **p < 0.01; ***p < 0.001 by Dunnett's multiple comparison test compared with the vehicle-treated group.

Conclusions

In conclusion, we have conducted SAR studies with the goal of identifying a novel development analgesic candidate with minimized hyperthermic effects. This was achieved through incorporating a pyrrolidinyl linker into regions B and C of the lead molecule BCTC. Part of synthesized compounds showed TRPV1 antagonistic potency and exhibited different analgesic effects in the three models of pain. In particular, **10b** showed good analgesic potency in capsaicin-induced and heat-induced pain models with no meaningful hyperthermic effects. Therefore, **10b** may be considered as a promising candidate for the development of potent agents for the treatment of pain with minimized side-effects.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Materials.