BCTC (N-(4-tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tetrahydropryazine-1(2H)-carbox-amide), a novel, orally-effective vanilloid receptor 1 antagonist with analgesic properties: II. In vivo characterization in rat models of inflammatory and neuropathic pain.

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Running Title: Selective blockade of vanilloid receptor 1 in rat models of pain

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Abbreviations: BCTC, N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide; DRG: dorsal root ganglion, FCA: Freund's complete adjuvant, FW50: Median fiber weight, NSAID: nonsteroidal anti-inflammatory drug, PWL: paw withdrawal latency, PWT: paw withdrawal threshold, VR1: vanilloid receptor 1

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ABSTRACT

The vanilloid receptor 1 (VR1) is a cation channel expressed predominantly by nociceptive sensory neurons and is activated by a wide array of pain-producing stimuli including capsaicin, noxious heat and low pH. Although the behavioral effects of injected capsaicin and the VR1 antagonist capsazepine have indicated a potential role for VR1 in the generation and maintenance of persistent pain states, species differences in the molecular pharmacology of VR1 and a limited number of selective ligands have made VR1 difficult to study in vivo. BCTC is a recently described inhibitor of capsaicin- and acid-mediated currents at rat VR1 (Valenzano et al., accompanying manuscript). Here we report the effects of BCTC on acute, inflammatory, and neuropathic pain in rats. Administration of BCTC (30 mg/kg, p.o.) significantly reduced both mechanical and thermal hyperalgesia induced by intraplantar injection of 30 µg capsaicin. In rats with Freund’s complete adjuvant-induced inflammation, BCTC significantly reduced the accompanying thermal and mechanical hyperalgesia (3 mg/kg and 10 mg/kg, p.o., respectively). BCTC also reduced mechanical hyperalgesia and tactile allodynia 2 weeks following partial sciatic nerve injury (10 and 30 mg/kg, p.o.). BCTC did not affect motor performance on the rotarod following administration of doses up to 50 mg/kg p.o. These data suggest a role for VR1 in persistent and chronic pain arising from inflammation or nerve injury.
The vanilloid receptor type 1 (VR1) is a pivotal molecular integrator of noxious stimuli that is expressed on somatic and autonomic primary afferent neurons. VR1 has been confirmed as a ligand-gated ion channel following its cloning from rat and human tissues, and has been shown to be highly expressed in small diameter primary afferent neurons (Caterina et al., 1997; Hayes et al., 2000; McIntyre et al., 2001). *In vitro* studies have shown that, like the native vanilloid receptor, recombinant VR1 can be activated by a variety of chemical as well as physical stimuli. *In vitro*, VR1 responds to plant-derived compounds including capsaicin, a pungent component of chilli peppers, lipid mediators such as anandamide (Smart et al., 2000), the lipoxygenase product 12-(S) HPETE (Hwang et al., 2000), as well as noxious heat (Caterina et al., 1997), and low pH (Tominaga et al., 1998).

A potential role for VR1 in nociception has been evident for some time as injection of the VR1 agonist capsaicin induces nocifensive and hyperalgesic behaviors in rodents and pain in humans (Szolcsányi, 1977; Carpenter and Lynn, 1981; Simone et al., 1987; Simone et al., 1989; Gilchrist et al., 1996). Further support for VR1 as a therapeutic target arose from experiments involving capsazepine. Capsazepine is a VR1 antagonist that has been shown to competitively inhibit capsaicin-mediated responses in isolated dorsal root ganglion (DRG) neurons (Bevan et al., 1992a) and tissues from rat (Bevan et al., 1992b; Cholewinski et al., 1993; Maggi et al., 1993; Santicioli et al., 1993; Jerman et al., 2000), mouse (Urban and Dray, 1991) and guinea-pig (Ellis and Undem, 1994; Fox et al., 1995; Auberson et al., 2000). *In vivo*, capsazepine has been shown to inhibit nocifensive and hyperalgesic responses to capsaicin in mice, rats and guinea pigs (Santos and Calixto, 1997; Walker et al., 2002). However, both *in vitro* and *in vivo* studies have indicated that capsazepine also has species-dependent activity. *In vitro*, capsazepine has been shown to block low pH-mediated activation of human or guinea
pig, but not rat VR1 (Lou and Lundberg, 1992; Satoh et al., 1993; Fox et al., 1995; McIntyre et al., 2001; Savidge et al., 2002). These in vitro results correlate with the finding that capsazepine reverses inflammatory and neuropathic hyperalgesia in the guinea pig, but not in the rat (Walker et al., 2002).

Together, these data suggest that blockade of low pH-induced VR1 activation may be predictive of antihyperalgesic efficacy in vivo. If this hypothesis is valid, then molecules that inhibit pH-induced activation of rat VR1, in vitro, would also produce a reduction in hyperalgesia in rat models of chronic pain. In the accompanying report, Valenzano and colleagues describe N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide (BCTC) as a potent, selective and orally bioavailable antagonist of rat VR1 (Valenzano et al., accompanying manuscript). In contrast to capsazepine, BCTC not only blocks the activation of rat VR1 by capsaicin, but also by low pH at the native rat VR1 in a skin-nerve preparation. Thus, BCTC has provided us with an opportunity to test our hypothesis that the inhibition of low pH-induced activation of VR1 confers in vivo efficacy in models of chronic pain. This report describes the effects of BCTC in models of, inflammatory, neuropathic, and capsaicin-induced pain in the rat. The efficacy and side effect profile of BCTC in these models were compared to those of non-steroidal anti-inflammatory drugs (NSAIDs) and anti-epileptic drugs currently used for the clinical therapy of inflammatory and neuropathic pain, respectively.
MATERIALS AND METHODS

Compounds and administration procedures. BCTC was synthesized according to known methods and was used in all experiments as its free base (molecular weight = 372.89). Indomethacin (Sigma, St. Louis, MO) and BCTC were administered orally in 2% beta-cyclodextrin (Sigma, St. Louis, MO) by gastric gavage in a dose volume of 10 ml/kg body weight. Gabapentin (Kemprotec, Middlesborough, UK) was dissolved in saline, and administered via i.p. injection in a dose volume of 2 ml/kg. Capsaicin (Sigma, St. Louis, MO) was used in all experiments as its free base. The procedure of Gilchrist et al (1996) was used to dissolve the capsaicin. Briefly, 6 mg of capsaicin was first dissolved in 0.14 mL (20) sorbitan monooleate (Tween 80) by gently heating the solution to approximately 70°C. The solution was then diluted with 1.86 mL 0.9% sodium chloride using an ultrasonic bath, and passed through a 0.20 µm filter. The final concentration of the capsaicin solution was 3 µg/µL. Intraplantar injections of the capsaicin solution were given in a 10 µL volume using a 100 µL Hamilton syringe fitted with a 27-gauge needle. The Tween 80/saline vehicle was used for control injections.

Animals. The Purdue Institutional Animal Care and Use Committee (IACUC) approved all animal procedures according to the guidelines of the Office of Laboratory Animal Welfare. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY), weighing 180-200g at the start of acute and inflammatory experiments, or 90-110g at the start of nerve ligation experiments were used. Animals were group-housed and had free access to food and water at all times, except prior to oral administration of drugs when food was removed for 16 h before dosing. For comparison to compound-treated groups, animals treated with the appropriate drug vehicle were included in each experiment. The volume
of administration was identical for vehicle- and compound-treated rats and rats were identical with respect to all other experimental procedures.

_Capsaicin-induced hyperalgesia._ Intraplantar injection of capsaicin (30 µg) was used to induce mechanical and thermal hyperalgesia, as previously described (Gilchrist et al., 1996). The paw pressure assay was used to assess capsaicin-induced mechanical hyperalgesia. For this assay, hind paw withdrawal thresholds (PWT) to a noxious mechanical stimulus were determined using an analgesymeter (7200, Ugo Basile, Varese, Italy) (Walker et al., 2001). Cut-off was set at 250 g and the end point was taken as complete paw withdrawal. PWT were determined once for each rat at each time point. All rats were tested for baseline PWT and one hour later, animals received a single dose of 1, 3, 10, or 30 mg/kg BCTC or vehicle (p.o., volume = 10 mL/kg, n = 10/group). Thirty minutes later, under isofluorane/oxygen anesthesia, rats received a single intraplantar injection of 30 µg capsaicin or vehicle in a 10 µL volume. Ninety minutes after the intraplantar injection of capsaicin, PWT were determined again.

The plantar test was used to assess capsaicin-induced thermal hyperalgesia (n = 8/group). For this test, hind paw withdrawal latencies (PWL) to a noxious thermal stimulus were determined using the technique described by Hargreaves et al (1988) using a plantar test apparatus (Model # 7370-371, Ugo Basile, Varese, Italy). Cut-off was set at 32 seconds, and any directed paw withdrawal from the heat source was taken as the end point. To assess the effects of BCTC on the development of capsaicin-induced thermal hyperalgesia rats were treated as described above for capsaicin-induced mechanical hyperalgesia, but PWL were measured 30 min after capsaicin injection.
Inflammatory hyperalgesia. The efficacy of BCTC against hyperalgesia associated with inflammation was investigated using the Freund’s Complete Adjuvant (FCA) model (Walker et al., 2001). Mechanical and thermal hyperalgesia were measured in separate groups of rats (n = 8-20/group) according to the procedure described above. Baseline PWT or PWL were determined, and rats were then anaesthetized with isofluorane/oxygen and received an intraplantar injection of 100% FCA (50 µL). Twenty-four hours following FCA treatment, pre-drug PWT or PWL measurements were taken and then rats received a single dose of 1, 3, 10, or 30 mg/kg BCTC, 30 mg/kg indomethacin, or vehicle (p.o., volume = 10 mL/kg). PWT or PWL were determined again 2, 4, 6, and 24 hours post-drug administration.

Hyperalgesia and allodynia following nerve-injury. The partial sciatic nerve ligation model was used as a model of nerve-injury-related pain in rats, as previously described by Seltzer and colleagues (Seltzer et al., 1990). Partial ligation of the left sciatic nerve was performed under isoflurane/O₂ inhalation anesthesia. Following induction of anesthesia, the left thigh was shaved and cleaned. The sciatic nerve was exposed at high thigh level through a small incision and was carefully cleared of surrounding connective tissues at a site near the trocanther just distal to the point at which the posterior biceps semitendinosus nerve branches off the common sciatic nerve. A 7-0 silk suture was inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle, and tightly ligated so that the dorsal 1/3 to 1/2 of the nerve thickness was held within the ligature and the wound was closed in two layers. Sham-operated control rats underwent an identical dissection on the left hind limb, but the sciatic nerve was not dissected away from the surrounding muscle nor ligated. Following surgery animals were weighed and placed on a warm pad until they recovered from anesthesia.
The effects of BCTC on mechanical hyperalgesia and tactile allodynia were measured 14-17 days post-surgery (n = 8-10/group). Mechanical hyperalgesia was assessed using the paw pressure technique prior to, 2, 4, 6 and 24 hours after drug administration. To assess tactile allodynia, rats (N = 10/group) were placed in clear, plexiglass compartments with a wire mesh floor and were allowed to habituate for a period of at least 15 minutes. After habituation, a series of von Frey monofilaments was presented to the plantar surface of the left (operated) foot of each rat. The series consisted of six monofilaments of increasing diameter, with the smallest diameter fiber presented first. Five trials were conducted with each filament, with each trial separated by approximately 2 minutes. Each presentation lasted for a period of 4-8 seconds, or until a nocifensive withdrawal behavior was observed. Flinching, paw withdrawal, or licking of the paw were all considered nocifensive behavioral responses. Tactile allodynia was assessed prior to and 2 hours post drug administration.

Ataxia/motor coordination. To examine the potential effects of BCTC on motor performance, rats were tested using the rotarod assay. The rotarod speed was maintained at 30 rpm, with the maximum time spent on the rotarod set at 300 sec. Rats (N = 8-10/group) received two training trials on the first day, were fasted overnight, and then received a single dose of either 10, 30, or 50 mg/kg BCTC, or 100 mg/kg gabapentin (i.p.) as a positive control. Rats were tested on the rotarod 2 and 4 hours after drug administration.

Statistical analysis. For studies involving mechanical or thermal hyperalgesia, tail flick, or rotarod latencies, untransformed data were analyzed using a one-way analysis of variance. In instances where a significant main effect was detected, planned
comparisons were made using Fisher’s PLSD test. The level of significance was set at p <0.05. For studies involving tactile allodynia, analyses were performed using an analysis of covariance model for each time, with log value of the median fiber weight (FW$_{50}$) required to evoke a nocifensive response at Hour 0 (baseline) as the covariate. A t-test for each pair wise comparison based on this model was performed, using PROC GLM of SAS (Version 6.12).
RESULTS

Capsaicin-induced hyperalgesia. Injection of 30 µg of capsaicin into the plantar surface of the left hindpaw induced a profound, but short-lasting decrease in PWL to thermal stimulation. Initial experiments revealed that this thermal hyperalgesia was present from 15-45 min after injection, and was most robust 30 min post-injection (data not shown). We therefore tested whether pretreatment of rats with BCTC would prevent the development of capsaicin-mediated thermal hyperalgesia. Injection of 30 µg capsaicin resulted in a significant decrease in PWL (27.1 ± 1.0 sec versus 9.5 ± 2.25 sec for vehicle- or capsaicin-treated rats, respectively). Pre-treatment with BCTC (30 mg/kg, p.o.) prior to capsaicin injection significantly inhibited the development of capsaicin-induced thermal hyperalgesia (18.3 ± 2.34 sec, p < 0.01), as compared to rats treated with capsaicin alone.

As reported previously (Gilchrist et al., 1996) intraplantar injection of capsaicin (30 µg) also induced mechanical hyperalgesia that lasted longer than the coincident thermal hyperalgesia. Our preliminary experiments indicated that maximal decreases in PWT occurred 90 min post-capsaicin injection (data not shown). There was a significant main effect of treatment on PWT, indicating a dose-dependent reversal of capsaicin-induced mechanical hyperalgesia by BCTC (F(5,41) = 4.008, p < 0.01). Pre-treatment with BCTC (30 mg/kg) 30 min prior to capsaicin injection significantly inhibited capsaicin-induced mechanical hyperalgesia relative to rats treated with capsaicin alone (p < 0.05, Fig. 1).

BCTC reduces thermal and mechanical hyperalgesia associated with inflammation. Intraplantar injection of 50 µL FCA resulted in the development of thermal hyperalgesia as indicated by decreased PWL to a noxious thermal stimulus (Fig. 2). Oral
administration of BCTC produced a dose-dependent reduction in thermal hyperalgesia (F(5,42) = 8.892, p < 0.001) and 4 hours post-administration (F(5,42) = 6.306, p < 0.001). BCTC (3 and 10 mg/kg, p.o.) produced significant antihyperalgesia 2 hours following administration. (p < 0.05). These effects were comparable both in terms of efficacy and duration of action to the NSAID, indomethacin (30 mg/kg, p.o; p < 0.05, Fig. 2). The effects of BCTC were restricted to the hyperalgesia associated with inflammation or injury and do not reflect an acute analgesic activity as PWT from the non-injured hind paw were not affected by systemic BCTC administration (data not shown).

Intraplantar injection of 50 µL FCA also resulted in the development of mechanical hyperalgesia, determined using the paw pressure assay (Fig. 3). BCTC (1-30 mg/kg, p.o.) produced a dose-dependent reversal of FCA-induced mechanical hyperalgesia at 2, 4 and 6 hours post-administration (p < 0.001 at each time point). BCTC significantly reduced FCA-induced mechanical hyperalgesia following oral administration at doses as low as 3 mg/kg (4 h) and at higher doses (10 and 30 mg/kg, p.o.) were effective for up to 6 h (Fig. 3). The effects of BCTC were similar to indomethacin (30 mg/kg, p.o.) in terms of efficacy, but indomethacin was only effective at 2 and 4 h following administration.

**BCTC reduces mechanical hyperalgesia associated with nerve injury.** Partial ligation of the sciatic nerve resulted in the development of mechanical hyperalgesia within two weeks of surgery. Administration of BCTC to rats 14-18 days after partial ligation of the sciatic nerve resulted in a significant and dose-dependent reversal of this mechanical hyperalgesia (F(6,89) = 10.675, p < 0.001, Fig. 4). Oral administration of BCTC (1-30 mg/kg) produced a significant reversal of mechanical hyperalgesia in the partially denervated rat hind paw for at least 6 h following administration (p < 0.01). The effects of administration of BCTC (10 mg/kg, p.o.) were similar to those seen following
administration of gabapentin (100 mg/kg, i.p.), but with a longer duration of action (6 vs. 4 h, Fig. 4).

**BCTC reduces tactile allodynia associated with nerve injury.** Partial ligation of the sciatic nerve also resulted in the development of tactile allodynia that was evident within 14 days of surgery (Fig. 5A). Administration of 10 mg/kg BCTC to rats 14-18 days after partial ligation of the sciatic nerve resulted in the development of tactile allodynia as determined by a significant decrease in the median fiber weight, or the fiber weight required to evoke a hindpaw withdrawal with a 50% frequency (FW₅₀) relative to vehicle-treated controls. Administration of 10 mg/kg BCTC significantly increased the log FW₅₀ at 2 hours post-administration (p <0.01, Fig. 5B). Administration of 100 mg/kg gabapentin (i.p.) also significantly increased the log FW₅₀ in nerve-injured rats 2 hours post-administration (p < 0.01 Fig. 5B).

**Side effect profiling: ataxia.** A common side effect of compounds used to treat neuropathic pain is ataxia, which can confound the interpretation of behavioral assays. A role for VR1 in motor function has not been described, however we tested rats for motor function using the rotarod assay. BCTC did not affect rotarod performance at 2 or 4 h following administration at doses up to 50 mg/kg, whereas gabapentin (100 mg/kg, i.p.) produced in a significant decrease in rotarod performance 2 hours post-administration (p < 0.05, Fig. 6).
DISCUSSION

VR1 has been extensively investigated as an intriguing target for the pharmacotherapy of pain. Its expression is predominantly restricted to nociceptive neurons, and it is activated by pathological rather than physiological stimuli, including well-known nociceptive stimuli such as acid, temperatures above 48°C, and the pungent spice capsaicin. Here we report that systemic administration of the novel VR1 antagonist, BCTC, significantly reversed mechanical and thermal hyperalgesia associated with injection of capsaicin, with FCA-induced inflammation, or following nerve injury. These effects were seen in the absence of ataxia that is commonly associated with anti-epileptic drugs used to treat neuropathic pain.

The VR1 antagonist capsazepine has also been used in many studies to investigate the role of VR1 in acute and chronic nociception. However, recent studies have revealed species-specific pharmacological actions of capsazepine that may limit its applicability in some animal models (McIntyre et al., 2001; Savidge et al., 2002; Walker et al., 2002). Although capsazepine can inhibit activation of rat VR1 by capsaicin, Valenzano et al., (accompanying manuscript) found it to be ineffective at blocking acid-mediated VR1 currents, and is similarly ineffective at reversing pain behaviors associated with inflammation in rats (Walker et al., 2002). However, the pharmacological profile of capsazepine is markedly different in guinea pigs compared with rats. Capsazepine is effective at inhibiting both the acid- and capsaicin-induced VR1 currents at guinea pig VR1 (Savidge et al., 2002), and has been shown to reduce inflammatory and nerve-injury related pain in guinea pigs (Walker et al., 2002). Thus, the species-dependent activity of capsazepine to inhibit low pH-induced currents at VR1 correlates with its ability to reduce hyperalgesia in models of chronic pain.
In contrast to capsazepine, BCTC inhibits the activation of rat VR1 by either capsaicin or low pH (Valenzano et al., accompanying manuscript). The unique pharmacological profile for BCTC at the rat VR1 provided us with an opportunity to test the hypothesis that the effects of VR1 blockers in chronic pain models may be predicted by their ability to inhibit low pH-induced activation of VR1. We found that pretreatment with BCTC effectively inhibits the VR1 agonist effects of capsaicin in the rat in vivo (Fig. 1). These results were consistent with the findings of Valenzano, et al., (accompanying manuscript) who demonstrated using an ex-vivo rat skin-nerve preparation that BCTC completely inhibited the activation of nociceptors by capsaicin.

However the most striking findings of this study were those indicating that BCTC is effective in reversing established thermal and mechanical hyperalgesia associated with inflammation (Figs. 2 and 3) or nerve injury (Figs. 4 and 5). The effects of BCTC were restricted to the hyperalgesia associated with inflammation or injury and do not reflect an acute analgesic activity as PWT from the non-injured hind paw were not affected by systemic BCTC administration (data not shown). Likewise, BCTC did not produce any impairment of locomotor activity over and above the dose range that produced anti-hyperalgesic effects (Fig. 6). These findings are interesting in light of the recent study by Walker et al. (2002) indicating that systemic administration of the VR1 antagonist capsazepine effectively reverses mechanical hyperalgesia associated with inflammation or nerve injury in the guinea pig but was ineffective in rat models of chronic pain. These differences are reflected by the different molecular pharmacological profiles of capsazepine vs. BCTC at rat VR1, in vitro. Valenzano et al., (accompanying manuscript) have demonstrated that unlike capsazepine, BCTC potently antagonizes low pH-induced activation of recombinant or native rat VR1. This suggests that the in vivo efficacy of VR1 antagonists in models of chronic pain may be predicted by their ability to inhibit pH-
induced activation of VR1. Since both capsazepine and BCTC antagonize capsaicin-induced activation of VR1, in vitro and in vivo, this action appears to be less predictive of a VR1 antagonist’s efficacy in chronic pain states.

We have found that the VR1 antagonist, BCTC (3-30 mg/kg, p.o.), effectively reverses both thermal and mechanical hyperalgesia associated with inflammation in the rat (Figs. 2 and 3). However, whereas Walker et al., (2002) reported that capsazepine was weakly active against thermal hyperalgesia associated with inflammation in the guinea pig, we found that VR1 blockade via BCTC provided effective reversal of inflammatory hyperalgesia in the rat. Although this may indicate further differences in the molecular pharmacology of BCTC vs. capsazepine at VR1, we cannot rule out the possibility that different behavioral phenotypes make the measurement of thermal hyperalgesia more robust in the rat as opposed to the guinea pig. Regardless the explanation, we know that the hyperalgesic effects of heat cannot be explained by the action of VR1 alone, since mice lacking VR1 retain the sensation of noxious heat (Davis et al., 2000; Caterina et al., 2000) and two VR1 homologues, VRL-1 (TRPV2) and TRPV3 have been reported to be insensitive to capsaicin or protons but respond to either high or low threshold heat stimulation (Caterina et al., 1999; Peier et al., 2002).

We have found that BCTC effectively reversed hypersensitivity to mechanical stimuli following inflammation or nerve injury. Stimuli that activate VR1 have been shown to induce mechanical hypersensitivity. To this end, intraplantar injection of either capsaicin (Simone et al., 1987; Simone et al., 1989; Gilchrist et al., 1996) or hyaluronic acid (Hamamoto et al., 1998) results in the development of mechanical hyperalgesia. In fact, the mechanical hyperalgesia observed after capsaicin injection is more robust than the thermal hyperalgesia both in terms of duration of action and the size of the area of
secondary hyperalgesia (Gilchrist et al., 1996). There is also evidence that the mechanisms underlying the thermal and mechanical hyperalgesia following capsaicin injection differ. Thermal hyperalgesia is thought to be due to sensitization of nociceptors because it is restricted to the immediate area of injection, whereas central mechanisms may underlie the mechanical hyperalgesia as the sensitivity is seen in a larger area surrounding the site of injection (Simone et al., 1987; Simone et al., 1989; LaMotte et al., 1991). It has been shown that intradermal capsaicin injection facilitates the responses of dorsal horn neurons due to input of low threshold mechanoreceptors and nociceptors (LaMotte et al., 1991; Simone et al., 1991). In inflamed tissue, local pH decreases may ultimately activate VR1, sensitizing nociceptors and low threshold mechanoreceptors, decreasing the threshold at which mechanical stimuli result in the detection of noxious stimuli.

The ability of BCTC to reduce tactile allodynia and mechanical hyperalgesia following partial ligation of the sciatic nerve may be attributed, in part, to altered expression of VR1 in this nerve injury model. Hudson and colleagues (2001) recently demonstrated that partial ligation of the sciatic nerve results in decreased VR1 immunoreactivity in damaged (ligated) neurons, but increased VR1 immunoreactivity in undamaged neurons. In another rat model of neuropathy, tight ligation of the L5 spinal nerve resulted in a significant decrease of VR1-immunoreactivity in the injured L5 dorsal root ganglion (DRG) neurons, with a concomitant increase in VR1-immunoreactivity in the uninjured L4 DRG neurons. Interestingly, the increased VR1-immunoreactivity was present not only in C fibers, but also in myelinated A fibers, as well, which could also explain the ability of BCTC to block mechanical sensitivity following nerve injury (Hudson et al., 2002). The increased levels of VR1 may then also prime the sensory neurons to respond to other physiological consequences of nerve damage that occur post injury,
such as the release of inflammatory mediators from macrophages during Wallerian degeneration (Tracey and Walker, 1995).

The findings reported here provide strong support for a role for VR1 in the pathology of chronic pain, both of inflammatory and neuropathic origin. Moreover, the *in vitro* characterization of BCTC (Valenzano et al., accompanying manuscript) indicates that the ability of VR1 antagonists to block low pH-induced channel opening may be a key component in predicting the *in vivo* efficacy of VR1 antagonists in models of chronic pain.
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REFERENCES


LEGENDS FOR FIGURES

Figure 1. Pretreatment with BCTC dose-dependently inhibits capsaicin-induced mechanical hyperalgesia. Rats received oral administration of beta-cyclodextrin vehicle (V) or BCTC 30 minutes prior to an intraplantar injection of 30 µg capsaicin or saline/Tween vehicle (V). Administration of 30 mg/kg BCTC significantly reduced capsaicin-induced mechanical hyperalgesia 90 min post-capsaicin injection. Asterisks denote significance (P < 0.05) from capsaicin (CAP)/V group according to Fisher’s PLSD test, error bars represent s.e.m (n = 9-10 rats/group).

Figure 2. BCTC dose-dependently inhibits inflammatory thermal hyperalgesia. Rats received an intraplantar injection of 50 µL saline (open squares) or 100% FCA (all other groups), followed 24 hours later by oral administration of BCTC, indomethacin or vehicle. Asterisks denote significance (P < 0.05) from FCA/vehicle group according to Fisher’s PLSD test, error bars represent s.e.m (n = 8 rats/group).

Figure 3. BCTC dose-dependently reverses inflammatory mechanical hyperalgesia. Rats received an intraplantar injection of 50 µL saline (open squares) or 100% FCA (all other groups), followed 24 hours later by oral administration of BCTC, indomethacin, or vehicle. Asterisks denote significance (P < 0.05) from FCA/vehicle group according to Fisher’s PLSD test, error bars represent s.e.m (n = 10-20 rats/group).

Figure 4. BCTC dose-dependently reverses mechanical hyperalgesia associated with nerve injury. Rats underwent surgery involving partial ligation of the sciatic nerve.
days post-surgery, rats received oral administration of vehicle or BCTC, or intraperitoneal administration of gabapentin (100 mg/kg, open circles). Asterisks denote significance (P < 0.05) from vehicle-treated control group according to Fisher’s PLSD test, error bars represent s.e.m (n = 10-20 rats/group.

**Figure 5.** BCTC significantly reduces tactile allodynia associated with nerve injury. Rats underwent surgery involving partial ligation of the sciatic nerve. Rats were tested for tactile allodynia 14-17 days post-surgery by application of von Frey monofilaments prior to (A), and 2 hours (B) post-administration of vehicle, BCTC, or intraperitoneal administration of gabapentin (100 mg/kg, open circles). N = 10 rats/group.

**Figure 6.** BCTC has no effect on motor performance as measured using the rotarod assay. Rats were placed on a rotarod set at a fixed speed of 30 rpm and the latency to fall off the rotarod was recorded 2 and 4 hours post-administration of vehicle, BCTC (p.o.), or 100 mg/kg gabapentin (i.p.). Asterisks denote significance (P < 0.05) from vehicle-treated group, error bars represent s.e.m. N = 8 rats/group.
Figure 1

Paw Withdrawal Threshold (g)

BCTC (mg/kg)

0 100 200

V/V CAP/V

1 3 10 30

*
Figure 2

Paw Withdrawal Latency (seconds)

- Sham
- Vehicle
- 1 mg/kg BCTC
- 3 mg/kg BCTC
- 10 mg/kg BCTC
- 30 mg/kg Indomethacin

Time Post-Administration (hours)
Figure 3

![Graph showing paw withdrawal threshold (grams) over time post-administration (hours) for different treatments:
- Sham
- Vehicle
- 1 mg/kg BCTC
- 3 mg/kg BCTC
- 10 mg/kg BCTC
- 30 mg/kg BCTC
- 30 mg/kg Indomethacin

*Significant differences from baseline at each time point.](image-url)
Figure 4

Paw Withdrawal Threshold (grams)

Time Post-Administration (hours)

- Sham
- Vehicle
- 1 mg/kg BCTC
- 3 mg/kg BCTC
- 10 mg/kg BCTC
- 30 mg/kg BCTC
- 100 mg/kg Gabapentin

* Indicates statistical significance compared to Sham group.
Figure 5

A

Pre-dose

Response Frequency (%)

Fiber Weight (grams)

B

2 hrs post-dose

Response Frequency (%)

Fiber Weight (grams)

- Vehicle
- 3 mg/kg BCTC
- 10 mg/kg BCTC
- 30 mg/kg BCTC
- 100 mg/kg Gabapentin
- Sham
Figure 6