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BCTP, a classic polymodal inhibitor of TRPV1 with a reduced liability for hyperthermia, is analgesic and ameliorates visceral hypersensitivity

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Running Title: BCTP, an inhibitor of TRPV1 with a reduced liability for hyperthermia

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ABBREVIATIONS: TRPV1, Transient Receptor Potential vanilloid-type 1; CRD, colorectal distension; VMR, visceromotor response; $[Ca^{2+}]_i$, intracellular calcium concentration; IBS, irritable bowel syndrome

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ABSTRACT

The therapeutic potential of TRPV1 antagonists for chronic pain has been recognised for over a decade. However, pre-clinical and clinical data revealed that acute pharmacological blockade of TRPV1 perturbs thermoregulation resulting in hyperthermia, which is a major hurdle for clinical development of these drugs. Here we describe the properties of BCTP, a TRPV1 antagonist with excellent analgesic properties that does not induce significant hyperthermia in rodents at doses providing maximal analgesia. BCTP is a classical polymodal inhibitor of TRPV1, blocking activation of the human channel by capsaicin and low pH with IC_{50} values of 65.4 nM and 26.4 nM, respectively. Similar activity was observed at rat TRPV1 and the inhibition by BCTP was competitive and reversible. BCTP also blocked heat-induced activation of TRPV1. In rats, inhibition of capsaicin-induced mechanical hyperalgesia was observed with a D_{50} value of 2 mg/kg, *p.o.*. BCTP also reversed visceral hypersensitivity and somatic inflammatory pain, and using a model of neuropathic pain in TRPV1 null mice we confirmed that its analgesic properties were solely through inhibition of TRPV1. Surprisingly, BCTP administered orally induced only a maximal 0.6 °C increase in core body temperature at the highest tested doses (30 and 100mg/kg), contrasting markedly with AMG517, a clinically tested TRPV1 antagonist, which induced marked hyperthermia (>1 °C) at doses eliciting sub-maximal reversal of capsaicin-induced hyperalgesia. The combined data indicate that TRPV1 antagonists with a classical polymodal inhibition profile can be identified where the analgesic action is separated from effects on body temperature.

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Introduction

Transient receptor potential vanilloid type-1 or TRPV1 is a non-selective cation channel highly expressed by nociceptive sensory neurons that is sensitive to noxious heat, protons and endogenous lipid mediators such as anandamide, in addition to capsaicin (Caterina et al, 2000; Hwang et al., 2000; McIntyre et al., 2001). The role of TRPV1 in somatic inflammatory and neuropathic pain has been extensively characterized, with multiple TRPV1 inhibitors shown to be effective analgesics both clinically and pre-clinically in conditions with marked hyperalgesia (reviewed Szallasi et al., 2007; Gunthorpe and Chizh, 2008; Broad et al., 2008; Pal et al., 2009).

There is also a growing body of evidence for an involvement of TRPV1 in altered visceral sensation. The majority of primary sensory afferents innervating the colon express TRPV1 (Robinson et al., 2004) and TRPV1 expressing neurons are more prevalent within colonic afferents than muscle or skin (Christianson et al., 2006) suggesting that TRPV1 will have at least as important a role in visceral pain conditions as in somatic pain. TRPV1 channels are markedly up-regulated and/or sensitized in inflammatory conditions (Yiangou et al., 2001; Chan et al., 2003; Geppetti and Trevisani, 2004) and mucosal biopsy samples from irritable bowel syndrome (IBS) patients show elevated TRPV1 expression that correlates with the degree of reported pain (Akbar et al., 2008). Activation of TRPV1 in the viscera also elicits pain in humans (Hammer et al., 1998; Drewes et al., 2003; Schmidt et al., 2004) and mechanosensitivity of mouse colon is impaired in TRPV1 knockout mice (Jones et al., 2005). Furthermore, recent work has shown that activation of TRPV1 plays a key role in the development of visceral pain and hypersensitivity in animal

models (Winston et al., 2007; Miranda et al., 2007; Hong et al., 2009; Ravnefjord et al., 2009).

Despite the early promise shown by TRPV1 inhibitors in clinical trials of inflammatory pain (Chizh et al., 2007), there is concern that the associated hyperthermia induced by TRPV1 blockade may ultimately limit their therapeutic potential (Gavva, 2008). Thus, administration of AMG517 resulted in a body temperature of 40.2 °C in one patient during a clinical trial, effectively terminating further development of this compound (Gavva et al., 2008). Moreover, although tolerance to hyperthermia is evident upon repeat dosing in pre-clinical species (Gavva et al., 2007a), body temperatures remained elevated with administration of multiple doses of AMG517 to humans (Gavva et al., 2008). At present the mechanisms underlying the effect of TRPV1 inhibitors on body temperature are unclear but they do not appear to involve a direct effect on the thermoregulatory centres of the hypothalamus, rather they appear to arise from inhibition of TRPV1 on afferents innervating the viscera (Steiner et al., 2007; Romanovsky et al., 2009). Although it was originally suggested that all TRPV1 inhibitors share the same hyperthermic liability (Gavva et al., 2007b), more recent work has shown that compounds with different profiles of block to the classical polymodal inhibitors fail to induce hyperthermia (Gavva, 2008; Lehto et al., 2008). For example, Lehto et al. (2008) described a compound, AMG8562, with novel pharmacology; inhibition of capsaicin-induced activation of TRPV1, no effect on noxious heat-induced activation and potentiation of proton-induced activation that had no effect on core body temperature. However, compounds of this type provide only modest analgesia in preclinical models of inflammatory pain (Lehto et al., 2008). Moreover, the potential detrimental effect of potentiating proton-induced activation of TRPV1 in inflammatory conditions

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where local acidification can occur may be of concern for their future clinical development.

Here we demonstrate the key role played by TRPV1 in inflammatory visceral pain by studying TRPV1 null mice and describe a novel TRPV1 inhibitor 7-tert-butyl-6-(4-chloro-phenyl)-2-thioxo-2,3-dihydro-1H-pyrido[2,3-d]pyrimidin-4-one (BCTP). BCTP is a polymodal inhibitor of TRPV1 that is an effective analgesic and reverses inflammation- and stress-induced visceral hypersensitivity in animal models. In contrast to other TRPV1 inhibitors, BCTP does not induce hyperthermia at doses providing significant analgesia. This important finding suggests that it may be possible to avoid a major hurdle to the clinical development of TRPV1 inhibitors.

Materials and Methods

Materials. For *in vitro* experiments all compounds were dissolved in DMSO and diluted in assay buffer to give final DMSO concentrations of 0.5 – 3 %. For *in vivo* studies capsaicin (Tocris Cookson, UK) was dissolved to a stock solution of 10 mM with dilutions in saline and administered intraplantar in a volume of 10 µl. BCTP, AMG517 (Doherty et al., 2007), alosetron and diclofenac (Sigma) were dissolved (v/v) in 0.5% methylcellulose, 20% cremophor EL/80% saline (0.9%) or saline for oral, intravenous or subcutaneous administration, respectively. WIN 55212-2 (Tocris Cookson, UK) was administered subcutaneously (2.5 ml/kg) in a mixture of 20 % DMSO, 1 % Tween 80, 1 % EtOH and 78 % saline.

Animals. Colorectal distension studies were carried out using adult male Sprague Dawley rats (250-280 g; Charles River, UK), water avoidance stress and mechanical hyperalgesia studies were carried out using adult male Wistar rats (280-350 g; Charles River. UK). Abdominal allodynia experiments used male C57BL/6

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mice (25-30 g; Charles River, UK) or TRPV1 knockout mice (Caterina et al., 2000; Jackson Laboratories, USA). Animals were housed in plastic cages with up to six animals per cage with a 12-h-light / 12-h-dark cycle (lighting from 6 a.m. to 6 p.m.) and had free access to food and water. The housing facilities were kept at constant temperature and humidity. Animals were used in groups of 6-8 and were assigned randomly to treatment groups with the experimenter blind to treatments. All experiments were performed according to Home Office (United Kingdom) guidelines and with approval of the local Novartis Animal Welfare and Ethics Committee.

***In vitro* pharmacology.** Experiments were carried out using CHO cells expressing either human or rat TRPV1. Changes in intracellular calcium concentration $[Ca^{2+}]_i$ evoked by capsaicin and acidic solutions were measured in cells loaded with the fluorescent Ca^{2+} indicator Fura-2 as previously described (McIntyre et al., 2001) using a Flexstation (Molecular Devices). Fluorescence emission (>510nm) was measured at 5 seconds intervals with alternate excitation at 340 and 380 nm before and after injection of either capsaicin or pH5.5 solution as the agonist. The mean of the peak fluorescence ratio after agonist injection minus the basal ratio was used for plotting concentration response curves. Experiments were performed in quadruplicate and data represent the mean ± S.E.M. of 3-4 independent experiments.

The effects of BCTP on heat evoked increases in $[Ca^{2+}]_i$ were determined using a microscope based imaging system as described by Andersson et al (2007). Coverslips of Fura-2-loaded CHO cells were mounted in a chamber (volume 200µl) and locally superfused (3ml/min) with extracellular solution. Temperature of the superfusate was regulated by a peltier device attached to a temperature control unit (Marlow Industries SE5010) and monitored by a thermistor attached at the tip of the inflow tube. The baseline temperature was maintained at 30°C and heat responses

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evoked by two linear temperature ramps to 48°C (60 second duration; 1st ramp - control, 2nd ramp - test solution) with a 5 minute interval at 30°C between heat challenges. Responses to capsaicin (1μM) were tested at the end of each experiment after washout of BCTP to ensure expression of TRPV1. Increases in Fura-2 ratio evoked by the heat ramps were determined for 30-90 cells per coverslip and the mean increase in Fura-2 ratio for the 2nd heat challenge expressed as a percentage of the 1st (control) response for each coverslip. Each concentration of BCTP was tested on 3-5 coverslips and the results expressed as mean ± S.E.M.

For determinations of antagonist IC_{50} values (concentrations of antagonist that inhibits responses to either pH 5.5 or capsaicin by 50%) the non-linear regression analysis in Origin v5.0 (Microcal) was used. To determine the pA_2 for BCTP, concentration response curves to capsaicin were carried out in the presence of various concentrations of BCTP. The dose ratio for each concentration of antagonist was calculated and the Log (Dose Ratio-1) plotted against the Log [antagonist] to give a Schild plot. The pA_2 value was then determined as the intercept of the x-axis.

Pharmacokinetics. Male Wistar rats (approx. 200 g) were used for these studies. For oral administration studies animals were fasted for at least 18 h (no longer than 24 h) prior to dosing. BCTP was either ground in 0.5 % methylcellulose for oral administration (1 ml) as a suspension or dissolved in cremophor before diluting to 20% cremophor with PBS for *i.v.* administration (0.3 ml injection volume). Blood was collected from animals at a single time point post administration via cardiac puncture, centrifuged and the plasma stored at -20°C prior to analysis. Brains were removed and then washed with distilled water before freezing at –20°C prior to analysis.

Plasma samples were extracted from 200 μl plasma of animals dosed with BCTP using a 50 mg C8 SPE column and analyzed using LC/MSD (Hewlett Packard

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HP1100 MSD). The results obtained were then compared to a standard curve which was obtained using similarly processed plasma samples from naïve rats spiked with compound, and plasma from animals that had received vehicle only (1 h following oral administration and 2 min following *i.v.* administration). Brain samples were extracted from 100 µl of a 20% homogenate of the brain (in water). The homogenate was extracted for 1 h in 1.25 ml diethyl ether, the sample was then spun down and the supernatant dried. The dried sample was made up in 100 % methanol and analyzed by LC/MSD and compared to a standard curve of known concentrations of BCTP extracted from brain homogenates at the same time.

Capsaicin-induced hyperalgesia model. Mechanical hyperalgesia was assessed by measuring hindpaw withdrawal thresholds to an increasing pressure stimulus using an analgesymeter (Ugo-Basile) with a cut-off threshold of 250 g. The end point was taken as the first sign of pain response (struggling, vocalization or paw withdrawal). Withdrawal thresholds of both hind paws were measured immediately prior to oral administration of vehicle or BCTP. Capsaicin (10 nmol) was injected intraplantar into one hind paw 1 h later, and paw withdrawal thresholds determined after a further period of 30 minutes. Reversal of established hyperalgesia was calculated according to the formula:

$$\%\ Reversal = \frac{Postdose\ threshold - Predose\ threshold}{Naive\ threshold - Predose\ threshold}\ X\ 100 \qquad \textbf{(1)}$$

Inflammatory somatic pain. A similar method to that outlined above was used to assess BCTP in an inflammatory pain model. Paw withdrawal thresholds to a mechanical stimulus were measured with an analgesymeter before and 24 h following intraplantar injection of 25µl Complete Freund's Adjuvant (CFA) into one hind paw, and then up to 24 h following oral administration of vehicle or BCTP. The COX-2 inhibitor celebrex was included as a positive control.

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Inflammation-induced allodynia model. Mice were anesthetized with isoflurane and mustard oil (0.25 % in 70% ethanol; 50 µl per mouse) administered by inserting a fine cannula with a rounded tip (3 cm long) into the colon via the anus. Following administration of mustard oil the animals were immediately placed in a clear Perspex box and after allowing 10 minutes for the mice to recover from the anesthetic, spontaneous pain-related behaviors (e.g. licking of the abdomen, abdominal writhing and retractions) were observed and counted for 20 minutes.

Withdrawal thresholds to the application of von Frey filaments (Semmes-Weinsten Monofilaments, North Coast Medical Inc., CA) to the abdomen were measured as a test for referred allodynia prior to (baseline) and 24-48 h following administration of mustard oil. Animals were placed in a raised perspex ventilated box with a wire mesh floor and allowed to acclimatize. Allodynia was tested by touching the lower to mid abdomen with von Frey filaments in ascending order of force (0.02 to 15 g) for up to 6 seconds. A sharp abdominal retraction, jumping or immediate licking or scratching of site of application of the filament was taken as a response. The lowest amount of force required to elicit a response was recorded as withdrawal threshold (in grams).

Inflammation-induced visceral hypersensitivity model. Trinitrobenzene sulfonic acid (TNBS; 40 mg/kg in 1:1 ETOH:saline; 1 ml/kg) was administered intracolonically through a polyethylene catheter introduced 7 cm through the anus under light anaesthesia with isoflurane. Control rats were treated with an identical volume of vehicle (saline). Visceral hypersensitivity to colorectal distension was determined after resolution of overt inflammation (10 days).

Animals were habituated to the experimental environment for 1 h daily for 5 to 6 days to minimize stress during the experiment. At the beginning of each

experiment, a balloon (5 cm long, 0.5 cm diameter) attached to a catheter was inserted through the anus into the colon of the animals for the application of colorectal distension stimuli. A pair of disposable cutaneous EMG-electrodes (8 x 12 mm) was temporarily attached on to the abdominal skin (bilaterally 10 mm distal to the rib bones; 10 mm apart from each other) for the measurement of striated muscle contractions reflecting visceromotor pain responses to colorectal distension (CRD). The balloon catheter and the wires of the electrodes were taped on to the tail of the animals. Thereafter, the animals were placed in a Bollman cage and CRD was performed by graded increases in intensity of phasic isobaric distensions (baseline = 0, 10, 20, 30, 40, 50 and 60 mm Hg) using a computer-controlled barostat system. Each isobaric distension lasted for 3 min separated by a 1 min break. The EMG activity of the abdominal musculature in response to CRD was recorded, amplified and stored for later analysis (PowerLab).

Acute stress-induced visceral hypersensitivity model. Rats were submitted to a 1 h session of water-avoidance stress or sham stress (control). The procedure involved placing the rat on a Perspex platform (6 x 6 cm) in the middle of a plastic tank filled with water (room temperature) up to 1 cm to the top of the platform. Rats tried to avoid the aversive stimulus (water) by remaining on the platform for 1 h. Control rats were placed on the same platform in a waterless container for 1 h. Visceral hypersensitivity to colorectal distension was assessed as described for TNBS above.

Neuropathic pain model. Mechanical hyperalgesia was assessed 11-15 days after partial sciatic nerve ligation as described previously (Fox et al., 2003). Withdrawal thresholds were measured on the ipsilateral (ligated) and contralateral (non-ligated) hind paws using the paw pressure technique described above, prior to

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and up to 24 h following oral administration of vehicle or BCTP. Data are combined from 3 separate experiments for each compound. Lamotrigine (10 mg/kg) administered subcutaneously in saline was included in these behavioral experiments as a positive control. Reversal of hyperalgesia at each time point was calculated according to the following formula:

$$\%\ Reversal = \frac{Postdose\ ipsilateral\ threshold - Predose\ ipsilateral\ threshold}{Predose\ contralateral\ threshold - Predose\ ipsilateral\ threshold}\ X\ 100 \quad \textbf{(2)}$$

Body temperature measurement. Rectal temperatures were measured using a rectal probe thermister before and then 1, 3 and 6 hours after oral administration of BCTP.

Statistics. For behavioral studies statistical analysis was carried out on raw data (paw withdrawal threshold, latency and temperature) using a two-way ANOVA followed by Dunnetts' or post hoc Tukey's tests comparing time-matched drug treated groups to vehicle. Plots for percentage reversal of hyperalgesia versus dose were fitted by linear or nonlinear regression for calculation of D_{50} values defined as a 50% reversal of hyperalgesia.

Results

***In vitro* characterization of BCTP.** BCTP was identified from a medicinal chemistry lead optimization programme following a high-throughput screen (Culshaw et al., 2006; Fig. 1). BCTP antagonized both capsaicin- and low pH-evoked increases in intracellular calcium in CHO cells expressing human (Fig. 1A) or rat (Fig. 1B) TRPV1. The compound was slightly more potent at inhibiting rat (IC_{50} 11.4 ± 3.3 nM) than human TRPV1 (IC_{50} 65.4 ± 6.7 nM) when capsaicin was the agonist, but showed no clear species selectivity for acid-induced activation (human TRPV1; IC_{50} 26.4 ± 6.3 nM and rat TRPV1; 28.2 ± 1.5 nM). BCTP (1 μM) also inhibited the

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response of human and rat TRPV1 to a noxious heat stimulus (48^{o}C, Fig. 1C) with IC_{50} values of 10.4 ± 1.6nM (human) and 12.2 ±1.8nM (rat). The inhibition of TRPV1 by BCTP was reversible following 1 h washing (data not shown) and it behaved as a competitive antagonist for capsaicin at both human (Fig. 2A) and rat (Fig. 2B) TRPV1. Incubation with increasing concentrations of BCTP caused a surmountable rightward shift in the log (concentration)-response curves for capsaicin indicating competitive antagonism. Schild analysis gave pA_2 values of 7.24 ± 0.13 (slope 1.28 ± 0.13) for human (Fig. 2C) and 7.93 ± 0.29 (slope 1.02 ± 0.14) for rat (Fig. 2D) TRPV1.

The selectivity of BCTP was tested in binding assays against a panel of 61 common ion channels and G protein-coupled receptors at the Novartis Central Receptor Screening Laboratory. No significant binding to the majority of targets was noted (IC_{50} > 10 μM), although high concentrations of BCTP inhibited binding of specific ligands to the dopamine transporter (IC_{50} = 1.6 μM) and phosphodiesterase 4D (IC_{50} = 22 μM).

Pharmacokinetic properties of BCTP. To determine the pharmacokinetic profile of BCTP, plasma concentrations were measured at different time points following *i.v.* (0.3 mg/kg; 20% Cremophor EL-saline solution) or oral administration (3 mg/kg; suspension in 0.5% methyl cellulose) after terminal bleeding and sampling (Fig. 3). Two minutes after *i.v.* administration, BCTP plasma levels were 3478 ± 337 pmol ml^{-1}, which fell to 75 ± 12 pmol ml^{-1} by 24 h post administration. Following oral administration, BCTP was detectable in the plasma at 10 min with levels of 1559 ± 194 pmol ml^{-1} and reached a Cmax of 8573 ± 449 pmol ml^{-1} by 1 h post administration falling to 698 ± 94 pmol ml^{-1} by 24 h. The terminal half-life of the compound after oral administration was ~7.5 h with an estimated bioavailability of

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100 %. Brain exposure for BCTP reached levels of 2873 ± 213 pmol g^{-1} 1 h and 2947 ± 237 pmol g^{-1} 3 h post oral administration giving mean brain/plasma ratios of 3 and 2.6, respectively. In a separate series of experiments exposure of BCTP (10 mg/kg *p.o.*) in the hypothalamus was found to closely match levels detected in the brain (data not shown). A summary of the key pharmacokinetic parameters for BCTP following oral dosing are shown in Table 1.

BCTP inhibits TRPV1 activation *in vivo*. The ability of BCTP to inhibit capsaicin-induced hyperalgesia in the rat was examined to provide *in vivo* proof-of-mechanism (Fig. 4). Intraplantar injection of capsaicin produced a pronounced mechanical hyperalgesia with a reduction in ipsilateral paw withdrawal threshold from a naïve level of approximately 105 g to 40 – 45 g following capsaicin. BCTP (3-30 mg/kg) administered orally 30 minutes before capsaicin, inhibited the development of hyperalgesia in a dose-dependent manner with a D_{50} value of approximately 2 mg/kg (Fig. 4A). Near complete inhibition (97%) was observed with the highest dose tested (30 mg/kg). BCTP was also effective at reversing CFA-induced mechanical hyperalgesia (Fig. 4B, D_{50} value 5.6 mg/kg), confirming that BCTP has similarly analgesic properties to other TRPV1 antagonists for somatic inflammatory pain in rodents (Pomonis et al., 2003; Gavva et al., 2004; Honore et al., 2005).

Effects of BCTP are absent in TRPV1$^{-/-}$ mice. To confirm that the anti-hyperalgesic effects of BCTP were due to a specific action at TRPV1 we compared its effects in normal and TRPV1 null mice. The development of hyperalgesia following inflammation is impaired in mice lacking TRPV1 (Caterina et al., 2000; Davis et al., 2000) and so this model could not be used. We noted, however, that the development and magnitude of mechanical hyperalgesia following partial nerve ligation is not different in wild-type compared to TRPV1 knock-out mice and so used this model to

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investigate the specificity of the compound. In wild-type mice BCTP displayed a dose-dependent anti-hyperalgesic effect, with a maximal reversal of 57 % observed 1 hour following oral administration (Fig. 5A). In contrast, no significant anti-hyperalgesic effect was noted in the TRPV1 knockout mice (Fig. 5B). The reversal of hyperalgesia by the sodium channel blocker lamotrigine was similar in wild-type and TRPV1 knockout mice (Fig. 5A & B).

TRPV1 mediates visceral pain. To determine the role of TRPV1 in acute and established visceral pain we first studied inflammation-evoked effects in TRPV1 knockout mice. As shown in Fig. 6A, instillation of mustard oil into the colon of wild-type mice evoked a marked acute increase in spontaneous pain behaviours compared to age-matched TRPV1 knockout mice. Moreover, there was a profound abdominal allodynia 24 h following mustard oil administration with withdrawal thresholds reduced from >6 g before treatment to less than 2 g following mustard oil treatment (Fig. 6B, grey bar). This response was absent in mice lacking the TRPV1 channel where withdrawal thresholds were comparable to vehicle treated controls (Fig. 6B, diagonal lines bar). These data strongly support an involvement of TRPV1 in visceral pain, in addition to its more widely established role in somatic pain.

BCTP prevents mustard oil-induced visceral pain. The role of TRPV1 in mustard oil-induced visceral pain was further confirmed using the novel antagonist BCTP. Intracolonic mustard oil administration induced spontaneous pain behaviours for 30 min following administration (24.0 ± 3.0 behaviours; Fig. 7A). BCTP (3-30 mg/kg; *p.o.*; dosed 1h pre mustard oil instillation) reduced the number of pain behaviours in a dose dependent manner. The maximum dose tested (30 mg/kg) produced an equivalent degree of inhibition (8.1 ± 3.7 behaviours) to the 5-HT_3

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receptor antagonist alosetron (1 mg/kg; 7.9 ± 1.6 behaviours) and diclofenac (30 mg/kg; 13.4 ± 0.8 behaviours), a non-steroidal anti-inflammatory drug.

Abdominal allodynia was assessed using calibrated Von Frey filaments 24 h before and 48 h following mustard oil treatment (Fig. 7B). Mustard oil produced a powerful allodynia (72.9 ± 4.5 % reduction in pain threshold) which was dose-dependently inhibited in mice dosed with BCTP (3-30 mg/kg; *p.o.*; dosed 1h pre allodynia assessment). Here, the maximum dose tested (30 mg/kg) produced an equivalent degree of inhibition as alosetron (1 mg/kg; -3.3 ± 11 and 1.7 ± 12.9 % reduction in pain threshold, respectively). Diclofenac did not have any effect on the mustard oil induced allodynia.

Visceral hypersensitivity. Rats treated with TNBS intracolonically and tested 7 days post treatment were hypersensitive to colorectal balloon distension (as indicated by a leftward shift of the visceromotor response to increasing pressures with respect to vehicle control). Significant reversal of the TNBS-induced hypersensitivity was observed with the 5-HT_3 receptor antagonist alosetron (1 mg/kg *p.o.*) whilst the NSAID diclofenac (30 mg/kg *p.o.*) was ineffective (data not shown). Oral administration of BCTP (1-10 mg/kg) produced dose-dependent reversal of the TNBS-induced visceral hypersensitivity (Fig. 8A). At both 3mg/kg and 10mg/kg BCTP fully reversed hypersensitivity (95.4 ± 10.2 and 110.4 ± 6.2 % reversal, respectively). Brief exposure of rats to water avoidance stress (WAS) also produced a hypersensitivity to colorectal balloon distension. Oral administration of BCTP (10-30 mg/kg) produced a dose-dependent reversal of this hypersensitivity. At 10 mg/kg BCTP fully reversed the stress-induced visceral hypersensitivity (99.6 ± 39.1 %). Alosetron (3 mg/kg) also fully reversed (104.3 ± 40.1 %) the stress-induced visceral hypersensitivity (Fig. 8B).

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Body temperature. Oral administration of BCTP (3-100 mg/kg) produced a modest increase in core body temperature at higher doses (30 and 100 mg/kg only) that reached a maximum of 0.6 ^{o}C (100 mg/kg) 1 h following administration (Fig. 9A). The cannabinoid receptor-1 agonist WIN 55,212-2 (6 mg/kg), included as a positive control produced a significant hypothermic response that reached a maximum at 3h post dose (-1.23 ^{o}C change in core temperature; Fig. 9A). Plotting the effect of different doses of BCTP against inflammation-induced visceral hypersensitivity and core-body temperature revealed a 10-fold window between the two parameters (Fig. 9B), with 3 mg/kg providing maximal reversal of the hypersensitivity and 30 mg/kg the threshold for induction of hyperthermia. The analgesic and hyperthermic effects effects of BCTP were compared with another TRPV1 antagonist AMG517, which induced significant hyperthermia in clinical trials (Gavva et al., 2008). Comparison of the reversal of capsaicin-induced mechanical hyperalgesia and change in body temperature showed that AMG517 evoked hyperthermia at low doses that were not analgesic. In contrast marked analgesia was seen with BCTP at doses that did not raise body temperature (Fig. 9C).

Discussion

Visceral pain is a key symptom associated with multiple gastrointestinal conditions including inflammatory bowel disease, gastroesophageal reflux disease, functional dyspepsia and irritable bowel syndrome (Sengupta, 2009; Knowles and Aziz, 2009). For IBS in particular, clinical studies have revealed that 50-70 % of patients exhibit a heightened sensitivity to colorectal distension, termed visceral hypersensitivity that appears to underlie the pathophysiology of the persistent abdominal pain/discomfort (Delvaux 2002). The current hypotheses around visceral

hypersensitivity suggest that tissue irritation, inflammation and/or stress-induced changes in gut function result in sensitization, in particular to mechanical stimuli, of extrinsic sensory nerves as well as second order spinal neurones (Mayer and Gebhart 1994; Azpiroz et al., 2007; Akbar et al., 2009). This sensitization is reminiscent of the hyperalgesia observed following somatic inflammation (Sandkühler, 2008). Our studies with TRPV1 knockout mice have revealed that TRPV1 plays an important role in the development of visceral pain after inflammation and we therefore characterized the effects of BCTP, a novel TRPV1 inhibitor, in a range of visceral pain paradigms.

In vitro BCTP acts as a polymodal inhibitor and inhibited TRPV1 activation by capsaicin, low pH and noxious heat with similar potency and was nearly equipotent at human and rat channels. The compound acts as a competitive antagonist, presumably at the capsaicin binding site, as shown by Schild analyses of capsaicin concentration-response curves, which yielded slopes close to unity for both human and rat TRPV1. *In vivo*, BCTP showed excellent oral bioavailability and inhibited the development of mechanical hyperalgesia evoked by a local injection of capsaicin into the paw, a mechanistic model for TRPV1 activation *in vivo*, with a D_{50} value of 2-3 mg/kg. Visceral pain differs from somatic pain in that it is poorly localized, diffuse and is often referred to the body wall through sensitization of common second order spinal neurons following activation of visceral sensory afferent nerves. Intracolonic instillation of mustard oil models this referred allodynia to the somatic tissues and oral administration of BCTP prevented both the acute spontaneous pain as well as attenuated the delayed referred allodynia to the flank, following intracolonic administration of mustard oil in mice. BCTP also reversed inflammation (TNBS)- and stress-induced visceral hypersensitivity to colorectal distension. Importantly, in separate studies we found that neuropathic pain due to sciatic nerve injury, in contrast

to inflammatory visceral pain, developed normally in TRPV1 knockout mice and capitalized on this finding to confirm that the analgesic properties of BCTP occur solely through an action on TRPV1. The efficacy of BCTP in a model where genetic knockout is ineffective represents something of a paradox, however, this is likely due to compensatory mechanisms during development that allow nociceptive responses to be retained in the knockout mice, which is a recognized phenomenon in pain research (Mogil et al., 2000).

The site of action of BCTP for inhibition of visceral pain in these animal models following systemic exposure is currently uncertain, but is likely to include a combination of peripheral and central activity. It was originally thought that TRPV1 was expressed exclusively in peripheral sensory neurons, but more recent evidence shows that TRPV1 is also located at sites within the CNS. TRPV1 is expressed both pre- and post-synaptically in the superficial laminae of the spinal cord (Valtschanoff et al., 2001) and at supraspinal sites involved in the transmission or modulation of pain (Szabo et al., 2002; Roberts et al., 2004), including the periaqueductal gray where microinjection of capsaicin evokes hyperalgesia (McGaraughty et al., 2003). In common with other TRPV1 antagonists, BCTP readily crosses the blood-brain barrier, and produced a moderate reversal of hyperalgesia following direct injection into the spinal cord (data not shown) so a peripheral site of action cannot be assumed. Although it is likely that inhibition of TRPV1 on nociceptive sensory neurons is a major mechanism for the anti-hyperalgesic effects of BCTP, additional actions at central sites could contribute to the behavioral effects of the compound.

It is well established that central administration of capsaicin in experimental animals produces hypothermia via regulation of warm sensitive neurones in the pre-optic/ anterior hypothalamic nucleus via TRPV1 (Jancsó-Gábor et al., 1970; reviewed

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Gavva, 2008; Romanovsky et al., 2009). In addition to direct injection of capsaicin into the hypothalamus, administration of brain penetrant TRPV1 agonists can directly activate warm-sensitive neurons in the hypothalamus (Caterina, 2007) to elicit robust hypothermic responses. Although such data do not *per se* indicate that TRPV1 antagonists will affect body temperature, recent reports provide evidence that hyperthermia is a pharmacodynamic effect of TRPV1 inhibition (Gavva et al., 2007ab; Gavva, 2008). This hyperthermic response appears to be triggered by blockade of the action of an as yet unidentified endogenous agonist of TRPV1 expressed on peripheral afferents innervating the viscera (Steiner et al., 2007) that poly-synaptically activate neurons in the preoptic/anterior hypothalamus.

Our data are in agreement with other reports that TRPV1 antagonists can elicit a transient and acute hyperthermia of about 1ºC (Swanson et al., 2005; Gavva et al., 2007ab; Steiner et al., 2007, Mills et al., 2008), an effect that appears to be mediated via TRPV1 as for one compound, at least, the hyperthermic effect is absent in TRPV1 knockout mice (Steiner et al., 2007). In the present study, however, there was a clear separation between the analgesic and hyperthermic activity of BCTP such that significant hyperthermia was observed with only the highest doses of BCTP tested, whilst lower doses that gave good reversal of hyperalgesia in a number of models did not evoke significant increases in body temperature. This contrasts markedly with data on the clinical candidate, AMG517, which elicited significant hyperthermia at doses lower than those that reversed mechanical hyperalgesia. This lack of therapeutic window was previously noted in clinical trials. The difference in profiles of BCTP and AMG517 suggests that not all polymodal TRPV1 inhibitors have the same hyperthermic liabilities (Gavva et al., 2008).

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Recently, non-hyperthermic TRPV1 antagonists have been described (Lehto et al., 2008; Watabiki et al., 2011). Such compounds differ from BCTP in that they exhibit a distinct pharmacology compared to the classical polymodal inhibitors of TRPV1. Although they inhibit the activity of vanilloid agonists like capsaicin, they show either a weak ability to block proton activation (Watabiki et al., 2011) or even potentiate proton activation of TRPV1 (Lehto et al., 2008). On the basis of such findings Garami et al. (2010) proposed that inhibition of proton activation of TRPV1 is the critical determinant for hyperthermia induction. Our data demonstrate that it is possible to separate the analgesic and hyperthermic properties of polymodal TRPV1 inhibitors with a compound such as BCTP. *In vitro* BCTP was almost equipotent at inhibiting capsaicin and proton evoked activation of TRPV1 (~2-fold difference in IC_{50} values) but there was a 20-fold difference between the analgesic and hyperthermic doses *in vivo*. The lack of hyperthermia at almost maximally effective analgesic doses is therefore difficult to reconcile with the proton inhibition hypothesis. The mechanism underlying the reduced hyperthermic liability with BCTP remains uncertain. It is possible that differences in tissue exposure, temporal onset of TRPV1 inhibition or a failure to block the effect of the endogenous ligand mediating body temperature changes might explain the observed differences on body temperature between BCTP and AMG516, however further work will be required to clarify this.

In conclusion, BCTP is a novel, potent and polymodal TRPV1 inhibitor that shows good anti-hyperalgesic activity in animal models of somatic and inflammatory- and stress-induced visceral pain. Experiments with TRPV1-null mice demonstrate unequivocally that the analgesic effects of BCTP are mediated by inhibition of TRPV1. In contrast to the more marked increases in body temperature observed with

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other TRPV1 inhibitors (e.g. AMG517; Gavva et al., 2007b; 2008), BCTP induced only a modest hyperthermia at doses above those providing maximal therapeutic analgesia. The combined data support the potential clinical utility of TRPV1 inhibitors in the treatment of visceral pain conditions such as IBS and suggest that it is possible to limit the potential for hyperthermia, which currently represents a major hurdle to the future clinical development of TRPV1 inhibitors. Although there is a window between analgesic and hyperthermic doses of BCTP in pre-clinical studies it will be essential to carefully monitor temperature in any clinical trial to avoid possible hyperthermia.

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Authorship Contributions

Participated in research design: Nash, McIntyre, Groarke, Lilley, Fox, Bevan.

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Contributed new reagents or analytic tools: Culshaw, Hallett.

Performed data analysis: Nash, Groarke, Lilley, Panesar, Bevan.

Wrote or contributed to the writing of the manuscript: Nash, McIntyre, Fox, Bevan

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Figure Legends

Fig. 1. BCTP inhibited responses to capsaicin (A), pH 5.5 (B) and noxious heat (C) in CHO cells recombinantly expressing either human or rat TRPV1. The results represent the mean increase in fluorescence ratio evoked by (A) capsaicin, 0.1 μM (human) or 0.03 μM (rat), (B) pH 5.5 or (C) an increase in temperature to 48°C in the presence of different concentrations of BCTP, as a percentage of the control response. Data points in (C) also show the percentage responses of two heat challenges in the absence of BCTP and the inhibition seen in the presence of a higher (1μM) concentration of BCTP. Data in are the mean ± S.E.M. from 3-5 independent experiments.

Fig. 2. Competition experiments for BCTP at human (A) and rat (B) TRPV1. The data represent the increase in the fluorescence ratio evoked by capsaicin in the presence of the concentrations shown of BCTP and are mean ± S.E.M. from 4 (human) or 3 (rat) independent experiments performed in quadruplicate. Schild plots for BCTP against capsaicin for the human (C) and rat (D) TRPV1. Data are the log (dose ratio minus 1) plotted against log concentration of BCTP.

Fig. 3. Mean plasma concentrations of BCTP following oral (3 mg/kg, 0.5% w/v aqueous methylcellulose) and intravenous (0.3 mg/kg, 20% Cremophor in saline) administration. Pharmacokinetic parameters are summarized in Table 1.

Fig. 4. BCTP inhibits capsaicin- (A) and CFA- (B) induced mechanical hyperalgesia. BCTP administered orally in 0.5% methylcellulose 30 min prior to capsaicin (10 nmol) injection into one hindpaw. (B) BCTP or the COX-2 inhibitor Celecoxib were administered subcutaneously 24 h following CFA injection into the hindpaw and paw

withdrawal thresholds monitored. The data show mean ± S.E.M. ipsilateral paw withdrawal thresholds from 6 animals / group. *, $P<0.05$ compared to vehicle by ANOVA followed by Tukey's HSD test.

Fig. 5. Effect of BCTP on mechanical hyperalgesia induced by partial ligation of the sciatic nerve. The left sciatic nerves of wild-type (A) and TRPV1 -/- (B) mice were partially ligated and significant hyperalgesia to mechanical pressure applied to the paw observed after 11-15 days. Lamotrigine reversed the hyperalgesia in both strains 1 h post-dose, whilst BCTP was only effective in wild-type mice. Data are mean ± S.E.M. from 6 animals/ group. *, $P<0.05$ compared to vehicle by ANOVA followed by Tukey's post-hoc test.

Fig. 6. Effect of genetic knockout of TRPV1 on visceral pain. Spontaneous pain (A) and delayed referred allodynia to the flank (B) following intracolonic treatment with mustard oil were reduced in TRPV1 knockout mice relative to wild-type controls. Data are mean ± S.E.M. from 8 mice per treatment group. **, $P<0.01$ and ***, $P<0.001$ compared to wild-type vehicle control using ANOVA followed by Dunnett's test.

Fig. 7. BCTP inhibited mustard oil-induced acute spontaneous pain behaviours (A) and referred allodynia (B). BCTP, alosetron or diclofenac were administered orally in 0.5 % methylcellulose or subcutaneously in saline, respectively, 1 h prior to mustard oil and again 48 h later, 1 h prior to allodynia testing. Each column represents mean ± S.E.M. from 8 animals per group. * $P< 0.05$, ** $P<0.01$, *** $P<0.001$ compared to wild-type vehicle control using ANOVA followed by Dunnett's test.

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Fig. 8. Intracolonic administration of TNBS induced a marked visceral hypersensitivity monitored as an increase in abdominal contractions (the visceromotor response) to increasing pressures of colorectal distension and oral administration of BCTP in 0.5 % methylcellulose dose-dependently reversed this hypersensitivity (A). Acute water avoidance stress induced a similar visceral hypersensitivity to colorectal distension that was reversed by BCTP and alosetron (B). Data are mean ± S.E.M. from 8 animals per group. * $P< 0.05$, ** $P<0.01$, *** $P<0.001$ compared to TNBS- or WAS-vehicle control using ANOVA followed by Dunnett's test.

Fig. 9. Effect of BCTP on core body temperature. Dose response curve for the effect of BCTP on core body temperature following oral administration (A). Data are mean ± S.E.M. from 6 rats per group. * $P<0.05$, *** $P<0.001$ compared to time matched vehicle control using ANOVA followed by Dunnett's test. (B) Plot comparing the effects of BCTP on visceral hypersensitivity to colorectal distension following intracolonic instillation of TNBS and core body temperature. Data are from Fig. 8A and Fig. 9A, respectively. (C) Plot comparing the effects of BCTP with AMG517 on capsaicin-induced mechanical hyperalgesia and core body temperature. Data are mean ± S.E.M. from 6-8 animals per group.

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Table 1. Pharmacokinetic properties of BCTP (3 mg/kg *p.o.*)

Parameter	**Value**
Bioavailability (BAV)	~100%
Maximum plasma concentration (C_{max})	8573 pmol ml^{-1}
Time to reach Cmax	1 h
Terminal half-life (t½)	7.5 h
Mean Residence Time	7.8 h
Area under curve (AUCiv(0-inf))	480433 pmol min^{-1} ml^{-1}
Plasma clearance (CL)	0.36 ml min^{-1}
Steady state volume of distribution (V_{ss})	169 ml

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Figure 1

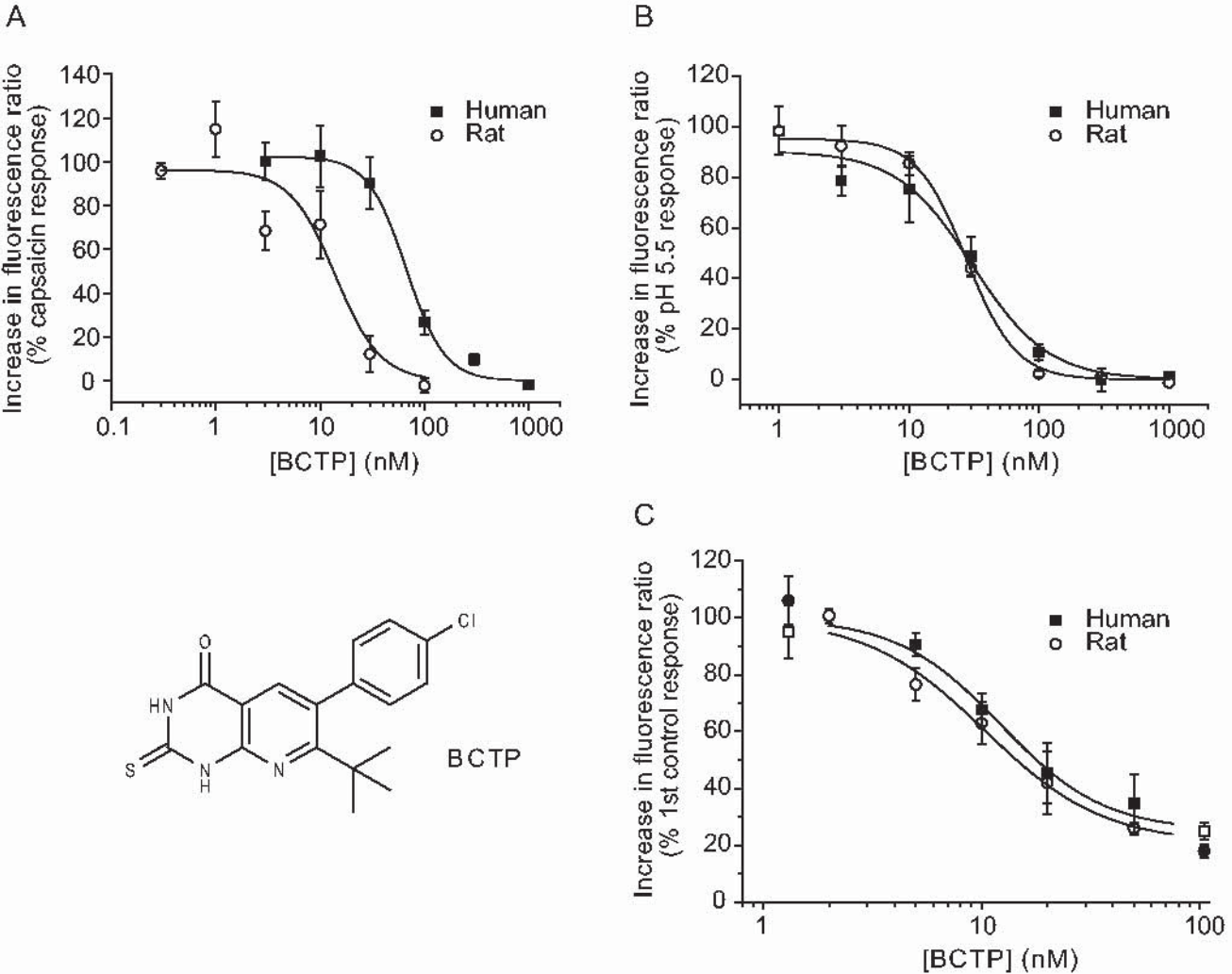

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Figure 2

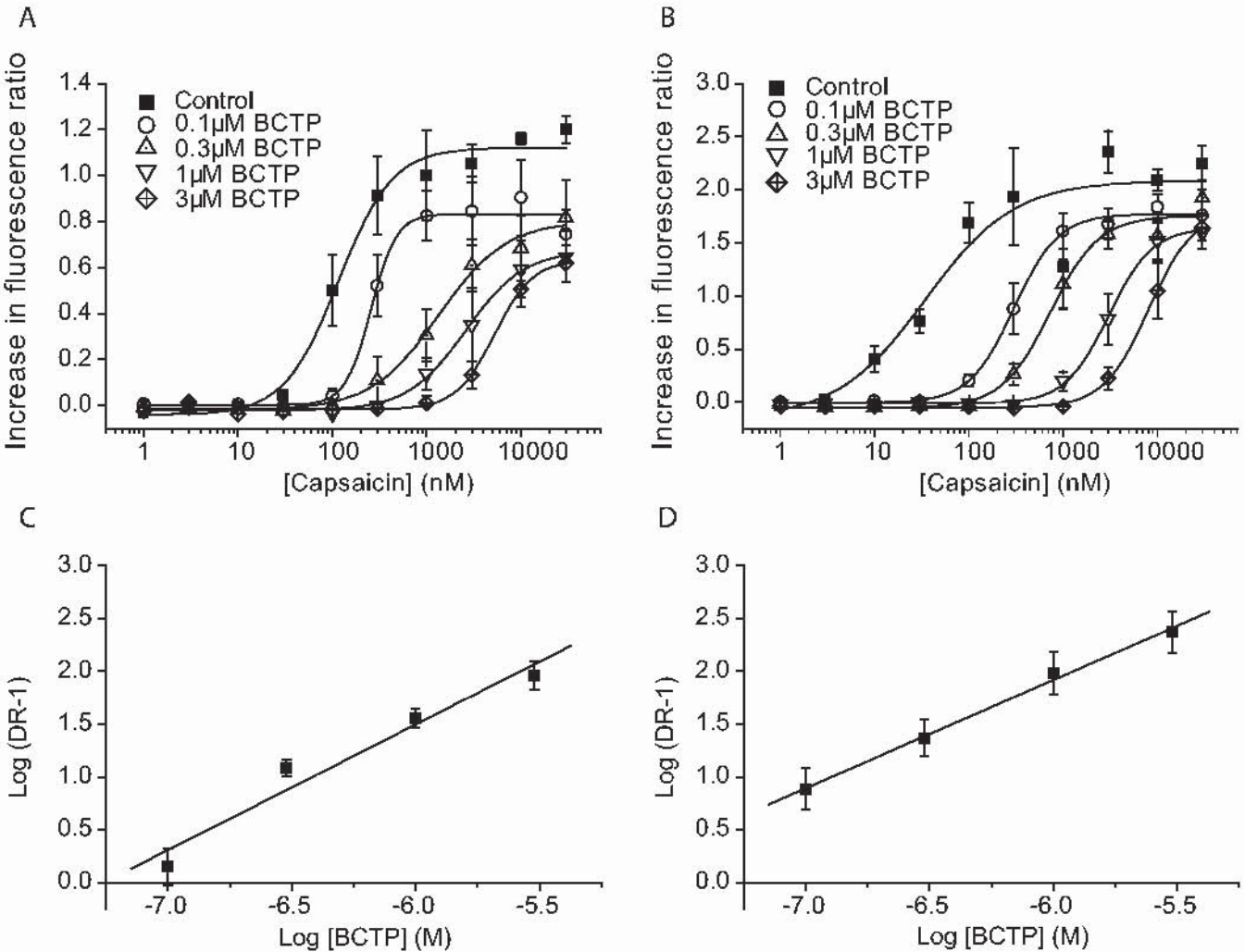

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Figure 3

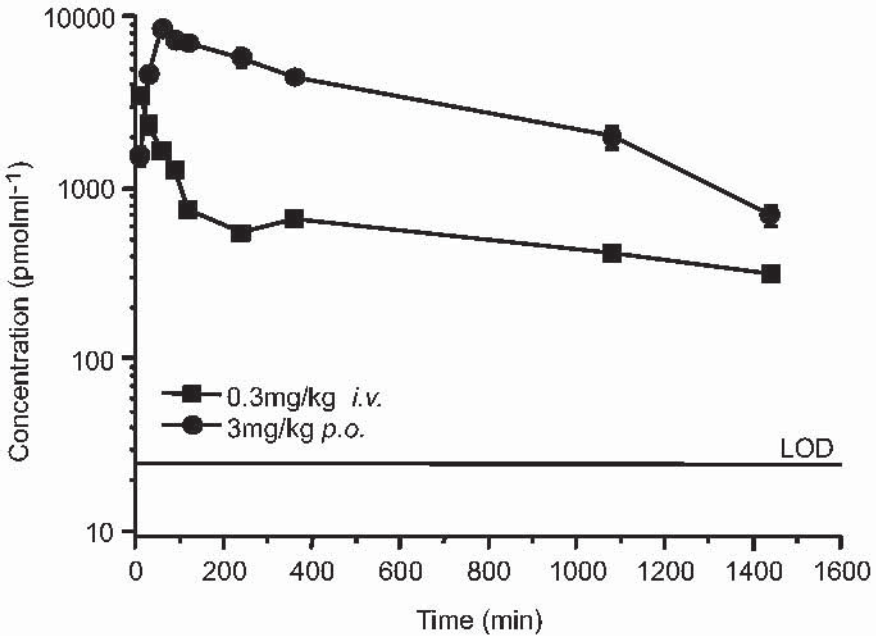

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Figure 4

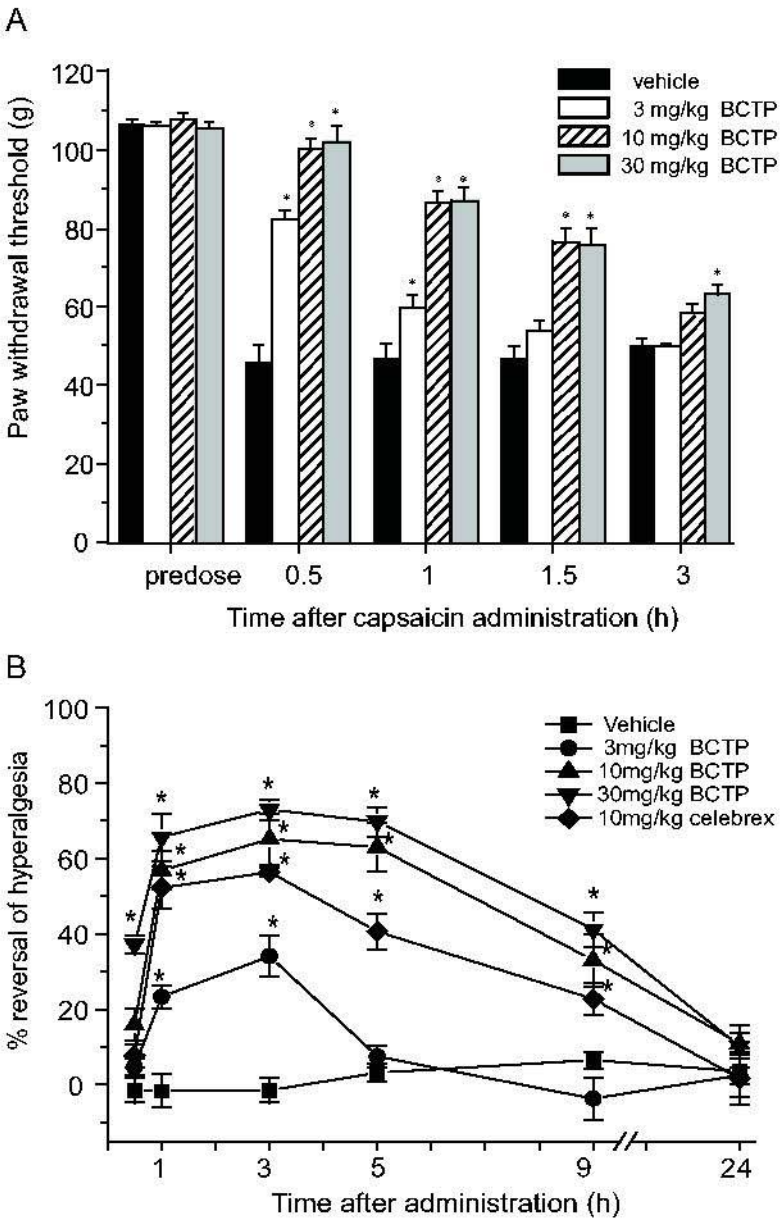

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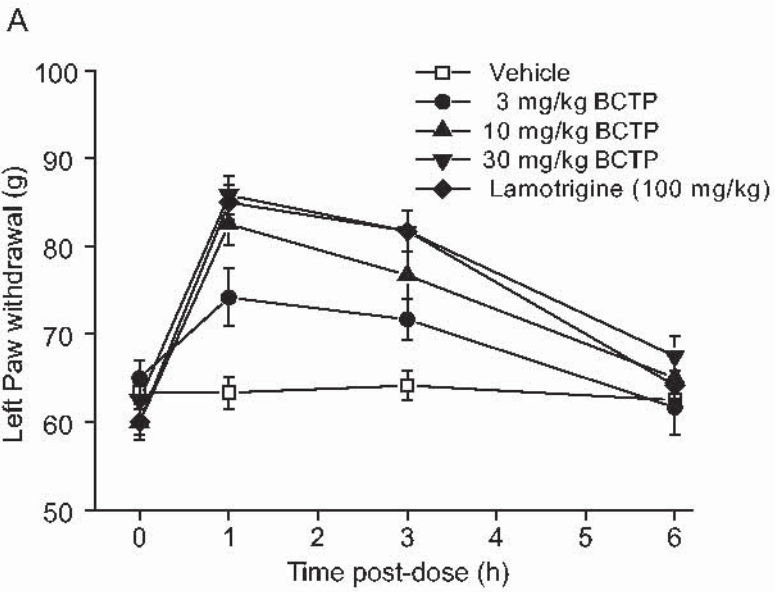
Figure 5



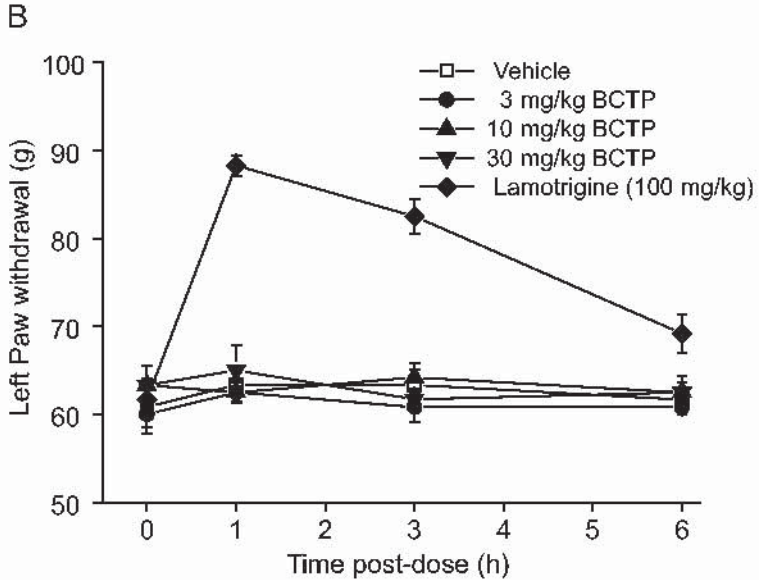


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Figure 6

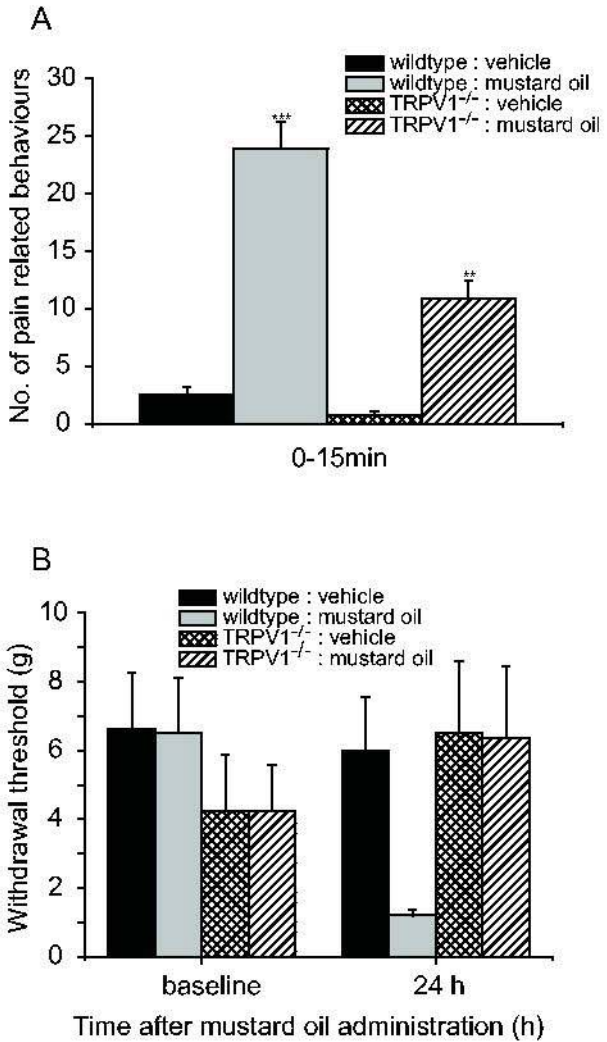

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Figure 7

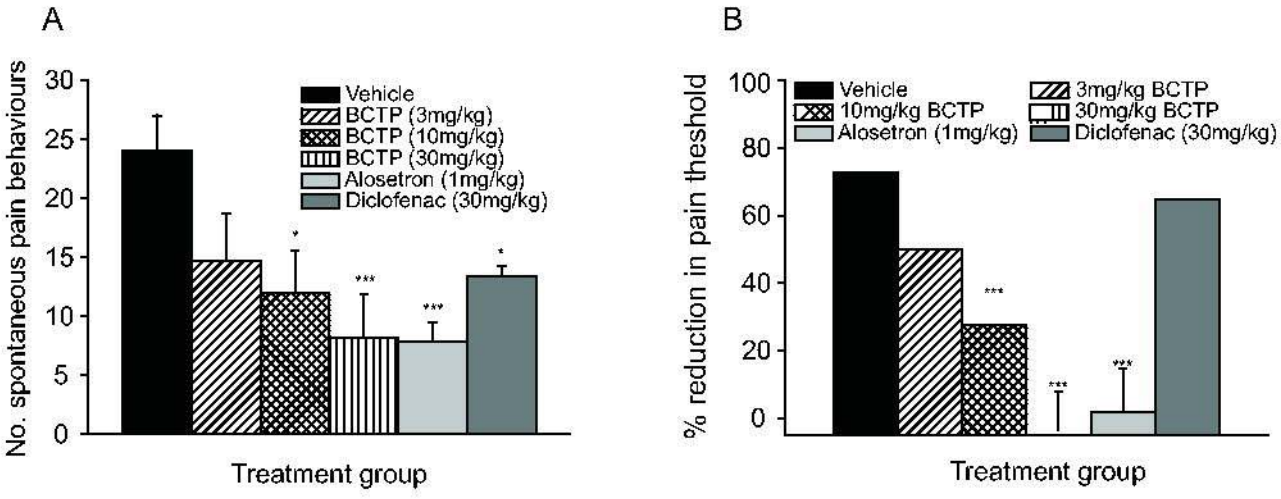

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Figure 8

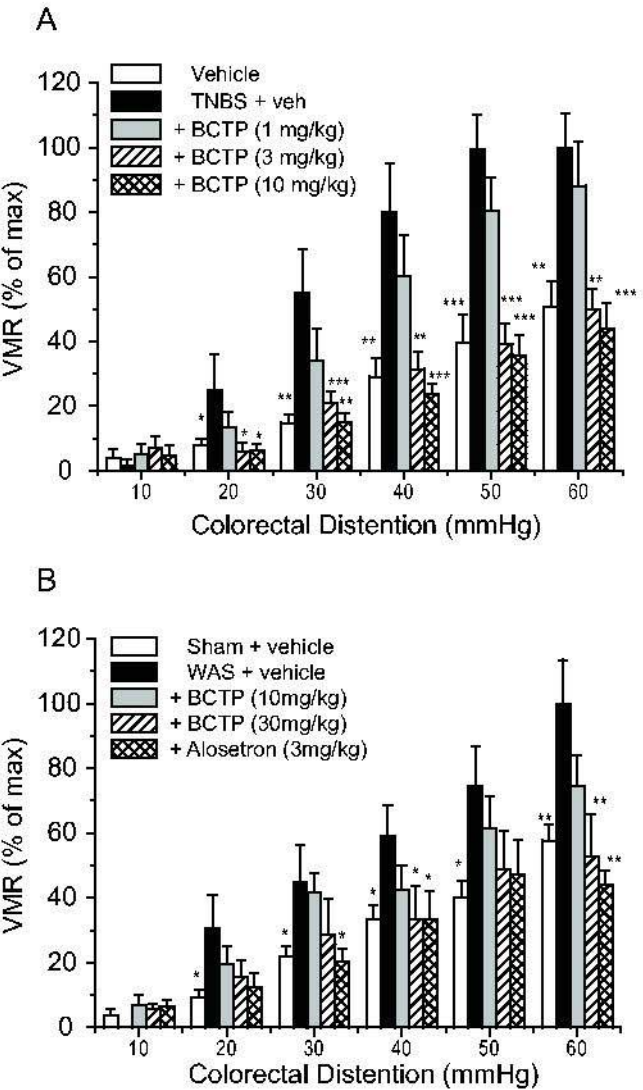

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Figure 9