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Antinociceptive Pharmacology of *N*-(4-Chlorobenzyl)-*N′*-(4-hydroxy-3-iodo-5-methoxybenzyl) Thiourea, a High-Affinity Competitive Antagonist of the Transient Receptor Potential Vanilloid 1 Receptor

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ABSTRACT

The transient receptor potential vanilloid 1 receptor (TRPV1) is expressed predominantly in a subset of primary afferent nociceptors. Due to its specific anatomical location and its pivotal role as a molecular integrator for noxious thermal and chemical stimuli, there is considerable interest to develop TRPV1 antagonists for the treatment of pain. Recently, *N*-(4-chlorobenzyl)-*N′*-(4-hydroxy-3-iodo-5-methoxybenzyl) thiourea (IBTU) was synthesized, and it was found in vitro to be a high-affinity competitive antagonist of cytoplasmic, but not intracellular, TRPV1. In this study, we examined the in vivo antinociceptive activity of IBTU in several acute and inflammatory pain models in mice. Our emphasis was on nociceptive pathways that are likely mediated by TRPV1, including capsaicin-, noxious heat-, and proton (including inflammation)-induced nociception tests. Capsazepine was used as a positive control in these experiments. IBTU dose-dependently blocked the capsaicin-induced nociception, confirming its antagonism at TRPV1 in vivo. By itself, IBTU produced significant antinociception, because it significantly prolonged the tail-flick latency in a dose-dependent manner. IBTU also blocked both early and late phases of the formalin-induced flinching response as well as acetic acid-induced writhing behavior. Moreover, IBTU inhibited the complete Freund's adjuvant-induced persistent hyperalgesia. Taken together, these data demonstrate that IBTU acts as a TRPV1 antagonist in vivo, and they suggest that it may be of therapeutic use for the treatment of pain.

The transient receptor potential vanilloid 1 receptor (TRPV1, formerly called VR1) is a nonselective cationic ion channel that is predominantly expressed in small-to-medium-diameter sensory neurons (Caterina et al., 1997; Hayes et al., 2000; McIntyre et al., 2001). These pain-sensing primary afferent neurons, also called nociceptors, are thought to release proinflammatory and pronociceptive mediators in the periphery and to transmit nociceptive information to the spinal cord (Szallasi and Blumberg, 1999). TRPV1 is activated by a variety of stimuli, including noxious heat (>43°C), protons (e.g., low pH environment caused by inflammation), and specific ligands such as capsaicin, the pungent ingredient of chili peppers (Caterina et al., 1997, 2000; Tominaga et al., 1998; Szallasi and Blumberg, 1999; Davis et al., 2000). Due to its specific distribution in primary afferent neurons and its pivotal role as a polymodal integrator for noxious chemical and thermal stimuli, there is a considerable interest in designing and developing pharmacological agents that block the actions of TRPV1 for the treatment of pain and other illnesses (Szallasi and Appendino, 2004).

Capsazepine was the first relatively selective TRPV1 antagonist that was shown to competitively inhibit capsaicin-

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ABBREVIATIONS: TRPV1, transient receptor potential vanilloid type 1 receptor; I-RTX, 5-iodoresiniferatoxin; DD161515, *N*-[2-(2-(*N*-methylpyrrolidinyl)ethyl)glycyl]-[*N*-[2,4-dichlorophenethyl]glycyl]-*N*-(2,4-dichlorophenethyl)glycinamide; DD191515, [*N*-[3-(*N*,*N*-diethylamino)propyl]glycyl]-[*N*-[2,4-dichlorophenethyl]glycyl]-*N*-(2,4-dichlorophenethyl)glycinamide; BCTC, *N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2*H*)-carbox-amide; A-425619, 1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea; AMG 9810, (*E*)-3-(4-*t*-butylphenyl)-*N*-(2,3-dihydrobenzo[*b*][1,4] dioxin-6-yl)acrylamide; IBTU, *N*-(4-chlorobenzyl)-*N′*-(4-hydroxy-3-iodo-5-methoxybenzyl) thiourea; CFA, complete Freund's adjuvant; MPO, myeloperoxidase; i.pl., intraplantarly.

mediated responses in vitro and in vivo (Urban and Dray, 1991; Bevan et al., 1992; Santos and Calixto, 1997; McIntyre et al., 2001; Walker et al., 2003). Although the action of capsazepine was not particularly potent (Wang et al., 2002), had limited specificity, and was species-dependent (McIntyre et al., 2001; Walker et al., 2003), it proved in principle the feasibility of developing TRPV1 antagonists. Although desensitization of TRPV1 upon chronic exposure to TRPV1 agonists provided the initial impetus for developing TRPV1-targeted therapeutics, the pain induced upon acute exposure to TRPV1 agonists, the long duration of desensitization/defunctionalization, and the concern over toxicity have caused most current attention to be directed toward antagonists (Appendino et al., 2003). Vigorous efforts by many groups have revealed a diversity of structures with competitive TRPV1 antagonism and good potency. Examples include I-resiniferatoxin (I-RTX) (Wahl et al., 2001), DD161515 and DD191515 (Garcia-Martinez et al., 2002), BCTC (Pomonis et al., 2003), A-425619 (Honore et al., 2005), and AMG 9810 (Gavva et al., 2005).

We recently reported the synthesis of *N*-(4-chlorobenzyl)-*N*′-(4-hydroxy-3-iodo-5-methoxybenzyl) thiourea (IBTU), which was found to be a potent competitive antagonist of TRPV1 in vitro (Toth et al., 2004). The structure of IBTU can be divided into three regions: A, B, and C. The A region corresponds to the same region in I-RTX, which confers antagonistic activity. The B region is thiourea that enhances the affinity of the compound in the calcium uptake assay. The C region is similar to that found in capsazepine (Toth et al., 2004). IBTU was 5-fold more potent than capsazepine in antagonizing increases of intracellular Ca^{2+} levels and $^{45}Ca^{2+}$ uptake induced by capsaicin or resiniferatoxin. However, IBTU was less effective in displacing the binding of [^{3}H]resiniferatoxin to TRPV1 heterologously expressed in Chinese hamster ovary-TRPV1 cells, and it seemed to block resiniferatoxin-induced Ca^{2+} influx from extracellular medium rather than from internal stores. These in vitro data suggest that IBTU is a potent TRPV1 antagonist that blocks cytoplasmic membrane TRPV1 (a minor fraction of total TRPV1) that controls $^{45}Ca^{2+}$ uptake. In contrast, IBTU does not seem to interact effectively with the predominant intracellular fraction of TRPV1 that dominates the [^{3}H]resiniferatoxin binding measurements. How this unique in vitro antagonism profile translates into a functional TRPV1 antagonist in vivo still needs to be studied. In the present study, we examined the potential antinociceptive, antihyperalgesic, and antiallodynic effects of IBTU in several acute and chronic pain models in mice.

Materials and Methods

Materials. Male ICR mice (20–25 g; Harlan, Indianapolis, IN) were maintained on a 12-h light/dark cycle, and they were provided food and water ad libitum before experimental procedures. All experiments were performed in accordance with National Institutes of Health guidelines after approval by the Animal Care and Use Committee of the University of Illinois (Chicago, IL). Capsaicin, capsazepine, acetic acid, formalin, complete Freund's adjuvant (CFA), hexadecyl-trimethylammonium bromide, *O*-diansidine dihydrochloride, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Capsaicin-Induced Nociception. Intraplantar injection of capsaicin (3 μg/20 μl) was used to produce a capsaicin-induced nociception response as described previously (Sakurada et al., 1992; Caterina et al., 2000). Immediately after the injection of capsaicin, mice were placed inside glass cylinders. The number and duration of episodes of licking the injected hindpaw were recorded for 15 min. IBTU or capsazepine was administered either i.p. 30 min before capsaicin or intraplantarly (i.pl.) with capsaicin. Control mice received an equal volume of vehicle.

Tail-Flick Test. Antinociception was determined using a tail-flick test as described previously (Tang et al., 2006). In brief, the distal one third of the tail was immersed in a water bath maintained at 52°C. Latency times until a tail-flick response were recorded before and at different time points after drug treatment. Separate groups of six mice were treated with IBTU, capsazepine, or an equal volume of vehicle. The antinociception response was presented as percent maximal possible effect as defined by percent maximal possible effect = 100% × (drug response time − basal response time)/(cut-off time − basal response time). A cut-off time of 12 s was applied to avoid tissue damage.

Abdominal Constriction Test of Visceral Pain. Testing for inhibition of abdominal constriction was performed as described previously (Yang et al., 2003; Porreca et al., 2006). Mice were placed in individual glass cylinders for a 30-min acclimatization period. Separate groups of nine mice were injected with 0.6% acetic acid (0.1 ml/10g/mouse i.p.). Mice were immediately placed inside transparent glass cylinders, and the number of writhes was recorded for 15 min. To assess the effect of TRPV1 antagonists on writhing behavior, mice were treated with different doses of IBTU (i.p.) or capsazepine (40 mg/kg i.p.) 30 min before the injection of acetic acid.

Formalin Test. Tonic inflammatory pain was induced in separate groups of six mice by a subcutaneous injection of formalin (20 μl of 2% solution/mouse) into the dorsal surface of the left hindpaw, as described previously (Wang et al., 2001). The left hindpaw was observed for 60 min for the number and duration of paw flinching, and the results tabulated for successive 5-min intervals. This test produces a distinct biphasic response. The total number of flinches during the early phase (0–10 min) and late phase (10.01–60 min) were summed, respectively. To test the effect of TRPV1 antagonism, IBTU (30 μg i.pl.) was coadministered with formalin via the same route.

CFA-Induced Hyperalgesia. CFA [*Mycobacterium tuberculosis*, suspended in an oil/saline (1:1) emulsion, 0.5 mg/ml *Mycobacterium*, Sigma-Aldrich] was injected into the dorsal surface of the left hindpaw of mice (20 μl/paw) (Iadarola et al., 1988; Malmberg et al., 2003). Control mice received 20 μl of saline (i.pl.). Tactile allodynia and thermal hyperalgesia were assessed before and on the first, third, and seventh day after CFA injection, by assessing the thermal and mechanical sensitivity (see below). To block TRPV1, IBTU (30 μg/paw i.pl.) was coadministered with CFA. Antihyperalgesia was calculated as follows: percentage of activity = 100 × [(test paw withdrawal latency − post-CFA baseline paw withdrawal latency)/(pre-CFA baseline paw withdrawal latency − post-CFA baseline paw withdrawal latency)]. Antiallodynia was calculated as follows: percentage of activity = 100 × [(test paw withdrawal threshold − post-CFA baseline paw withdrawal threshold)/(pre-CFA baseline paw withdrawal threshold − post-CFA baseline paw withdrawal threshold)].

Assessment of Thermal and Mechanical Sensitivity. Thermal and mechanical sensitivity was assessed as described previously (Wang et al., 2001). Sensitivity to a thermal stimulus was determined by paw withdrawal latency to radiant heat (Hargreaves et al., 1988), using a plantar tester (UGO Basile, Stoelting, Wood Dale, IL). The stimulus intensity was adjusted to produce an ~10-s response in naive control mice (Malmberg et al., 2003). A cut-off time of 20 s was applied. Mechanical sensitivity was determined using a set of calibrated von Frey filaments (Stoelting) using the "up and down paradigm" (Chaplan et al., 1994).

CFA-Induced Edema and Myeloperoxidase Activity. CFA-induced paw edema was determined by measuring the hindpaw

thickness using a caliper (Malmberg et al., 2003). To assess the mechanism of inflammatory action, myeloperoxidase (MPO) activity in tissue supernatants was determined according to a published method (Kowaluk et al., 2000). MPO activity reflects the neutrophil accumulation at the site of inflammation. After the animals were sacrificed, the plantar surface of the CFA-injected hindpaws was quickly dissected, frozen in liquid nitrogen, and stored at −80°C until the assay. For the MPO assay, harvested tissue was homogenized by using a Polytron homogenizer (setting 6 for 30 s; Kinematica, Basel, Switzerland) in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyl-trimethylammonium bromide and then quickly frozen and thawed for three cycles. The homogenate was then centrifuged for 15 min at 40,000*g* at 4°C. Protein content in the supernatant was determined by a modified Bradford method (Bradford, 1976) (Bio-Rad, Hercules, CA). Supernatant (100 μl) was mixed with 50 μl of 50 mM potassium phosphate buffer, pH 6.0, and 25 μl of freshly mixed reaction mixture containing *O*-dianisidine dihydrochloride (0.167 mg/ml final concentration) and H_2O_2 (0.0005% final concentration). The absorbance at 450 nm was determined by a microplate reader (Labsystems Multiskan Plus; Thermo Electron Corporation, Waltham, MA). The relative MPO activity was expressed in optical density$_{450}$ per milligram of protein.

Statistical Analysis. All data are presented as mean ± S.E.M. Differences in responses between the treatment groups were determined using Student's *t* test (two groups) or analysis of variance followed by Dunnett's *t* test (multiple groups). Statistical significance was established at 95%.

Results

Effect of IBTU on Capsaicin-Induced Nocifensive Response. Because capsaicin is an agonist of TRPV1, we first investigated whether IBTU could block the nocifensive responses to capsaicin. Capsaicin produced distinct paw-licking behavior when injected i.pl. into the hindpaw (Sakurada et al., 1992; Caterina et al., 2000) (Fig. 1). Pretreatment with IBTU (i.p., 30 min before capsaicin) dose-dependently reduced both the number of capsaicin-induced hindpaw licks (ED_{50} = 43 ± 10 mg/kg; Fig. 1A) and the total time spent licking the paw (ED_{50} = 58 ± 10 mg/kg; Fig. 1B). IBTU (30–90 μg/20 μl) was also effective when injected intraplantarly, completely blocking the action of capsaicin at the highest dose used (Fig. 2). The ED_{50} value was estimated to be 27 ± 10 and 21 ± 10 μg for suppressing the number of licks and total licking time, respectively. For comparison, capsazepine (30 μg/20 μl or 78 pmol/dose i.pl.), a known TRPV1 antagonist (Urban and Dray, 1991), was included in the study and found to also significantly reduce the paw-licking nocifensive response to capsaicin, although it seemed to be less effective than 30 μg/20 μl (61 pmol/dose) IBTU (i.pl.) ($p <$ 0.01 or better) (Fig. 2).

Effect of IBTU on Thermal Nociception. TRPV1 has been reported to contribute to noxious (>43°C) thermal nociception (Caterina et al., 2000). In this series of experiments, we determined whether IBTU could modify the response to thermal nociception in a tail-flick test. Mice were treated with IBTU (i.p.), and the tail-flick latency was measured at different time points after the injection. The antinociceptive activity of IBTU showed quick onset, because the drug exhibited significant antinociception within 5 to 15 min, and it reached its peak action in 30 min. IBTU-produced antinociception lasted for at least 2 to 3 h (Fig. 3A), and it was dose-dependent (ED_{50} = 37 ± 10 mg/kg; Fig. 3B). As a positive control, mice in another group were treated with capsazepine (40 mg/kg or 0.11 mmol/kg i.p.). Capsazepine also produced antinociception by significantly prolonging withdrawal latency (Fig. 3B); the effect lasted for less than 60 min, shorter than that of IBTU (Fig. 3A). It is concluded that IBTU produced antinociception in a dose-dependent manner.

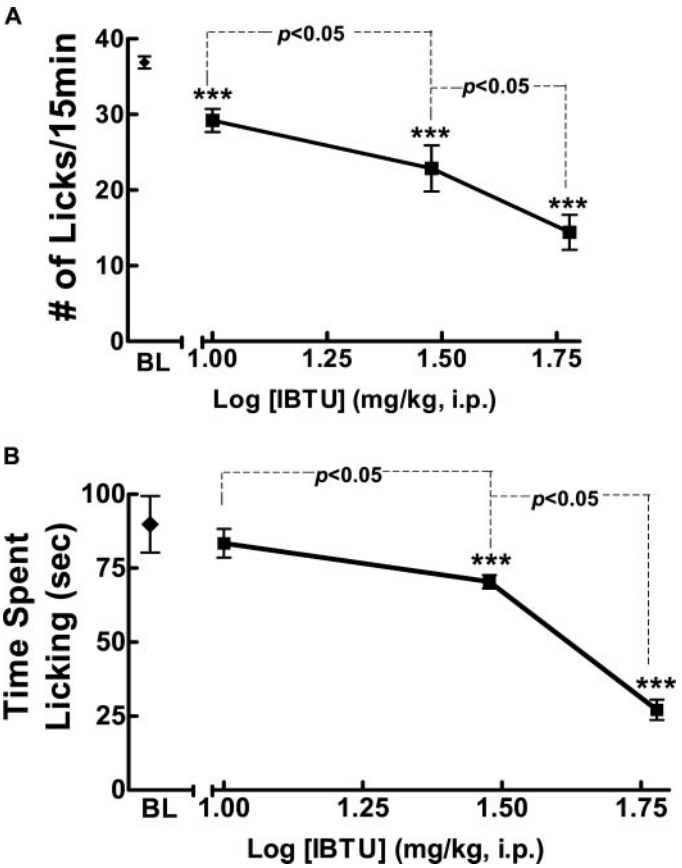

Fig. 1. Attenuation of nocifensive response to capsaicin by systemically administered IBTU. Separate groups of nine mice were pretreated with IBTU or an equal volume of vehicle (i.p.) 30 min before the administration of capsaicin (3 μg/20 μl i.pl.). Both the number of licks (A) and the total time spent licking the injected hindpaw (B) were recorded for 15 min. Data are expressed as mean ± S.E.M. ***, $p < 0.001$ compared with the vehicle group (BL).

Effect of IBTU on Acetic Acid-Induced Writhing. We also tested the effect of IBTU in a visceral pain model using the acetic acid-induced writhing assay. TRPV1 has been previously shown to mediate at least in part acid-induced abdominal constrictive writhing behavior (Ikeda et al., 2001; Rigoni et al., 2003). Acetic acid (i.p.)-administered mice exhibited writhing behavior in naive or vehicle-treated mice (Fig. 4). Pretreatment with IBTU (i.p., 30 min before the injection of acetic acid) dose-dependently reduced the number of writhes. Likewise, capsazepine (40 mg/kg i.p.) was found to be highly effective in attenuating the acetic acid-induced writhing response (Fig. 4). Iodo-resiniferatoxin, another TRPV1 antagonist, was also reported to block acetic acid-induced writhing (Rigoni et al., 2003).

Effect of IBTU in Formalin Test. TRPV1 responds to protons that are found in a low-pH environment, which can be caused by inflammation of surrounding tissues (Caterina et al., 1997). We further tested IBTU in formalin- and CFA-induced inflammatory pain models. First, tonic inflammatory pain was induced by formalin (2% i.pl.), which produced a

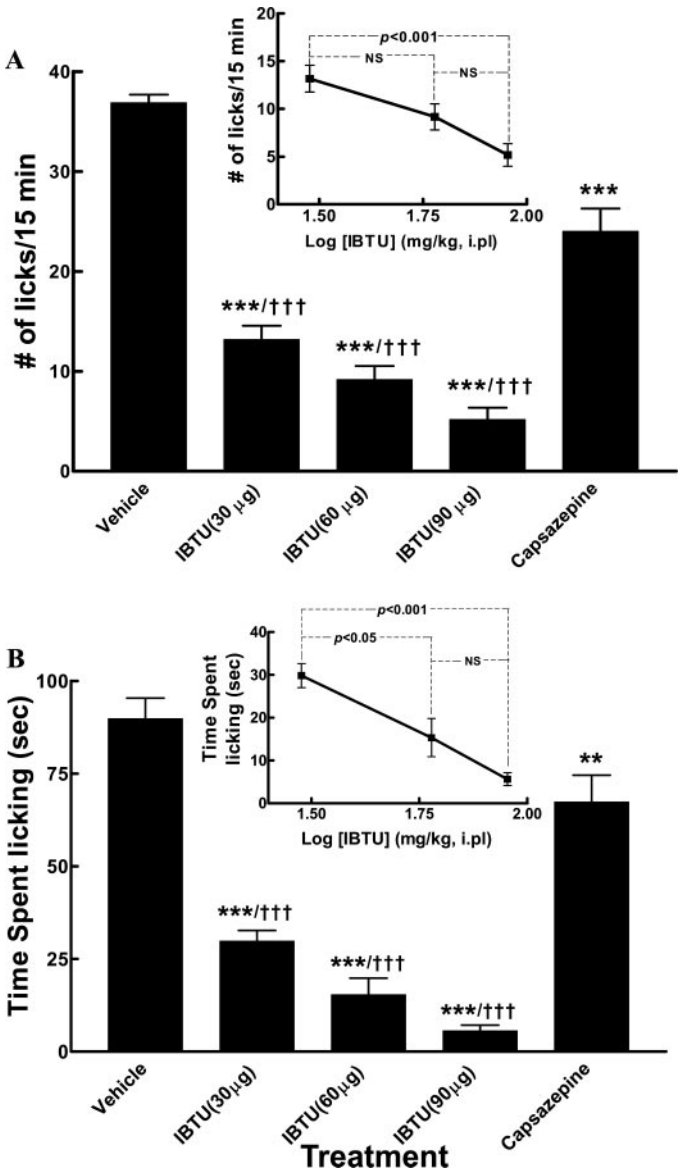

Fig. 2. Attenuation of nocifensive response to capsaicin by locally administered IBTU. Separate groups of six mice were coadministered with capsaicin (3 μg/20 μl i.pl.) and different doses of IBTU (30, 60, and 90 μg i.pl.) or capsazepine (30 μg i.pl.) or vehicle. Both the number of licks (A) and the total time spent licking the injected hindpaw (B) were recorded for 15 min. Insets, dose-response curves of IBTU. Data are expressed as mean ± S.E.M. **, $p < 0.01$; ***, $p < 0.001$ compared with the vehicle group; †††, $p < 0.001$ for the comparison between the capsazepine group and the IBTU group.

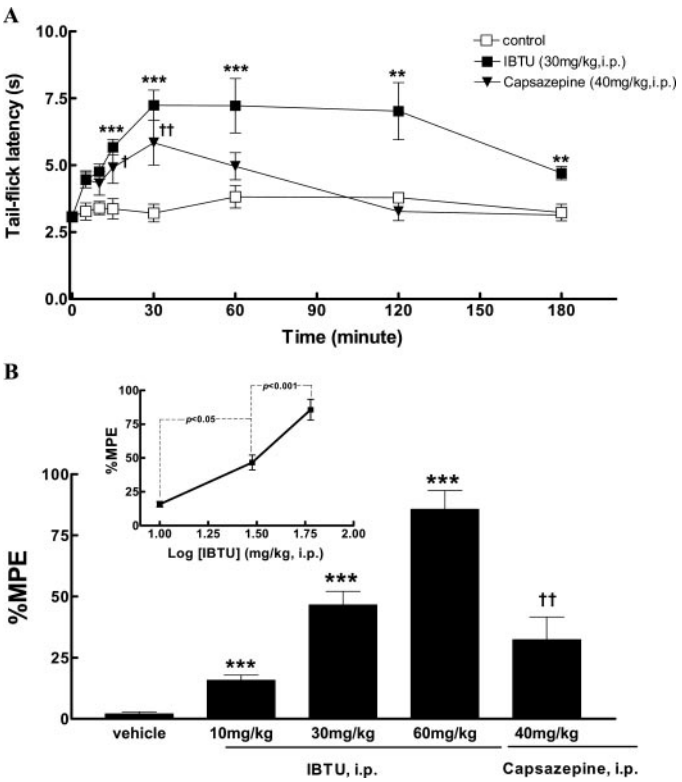

Fig. 3. Inhibition of thermal nociception by IBTU. A, time course of antinociception response after a singe dose of IBTU (30 mg/kg i.p.) or capsazepine (40 mg/kg i.p.). The tail-flick latencies to 52°C water were measured and plotted over 3 h. B, dose-dependent inhibition of thermal nociception by IBTU in comparison with capsazepine. Antinociception was tested 30 min after drug treatment. Inset, a dose-response curve of IBTU. Data are expressed as mean ± S.E.M ($n = 6$). For IBTU, **, $p < 0.01$; ***, $p < 0.001$ compared with the vehicle group. For capsazepine, †, $p < 0.05$; ††, $p < 0.01$ compared with the vehicle group.

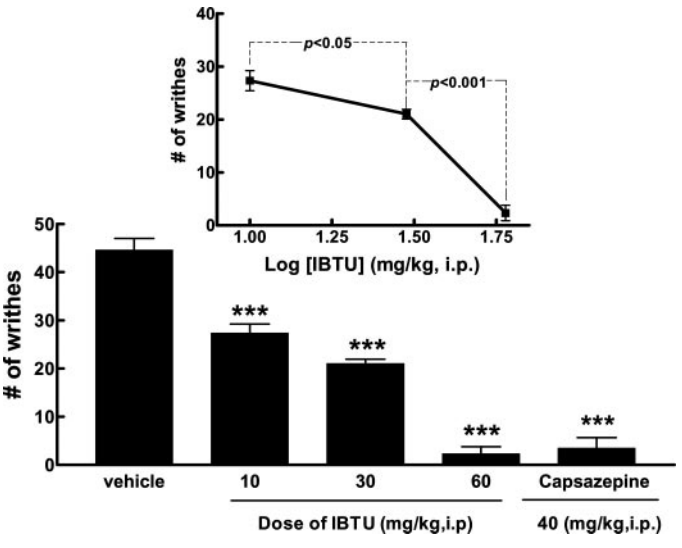

Fig. 4. Suppression of acetic acid-induced writhing response by IBTU. Separate groups of nine mice were pretreated with IBTU (i.p.) or capsazepine (40 mg/kg i.p.) 30 min before the injection of acetic acid (0.6%; 0.1 ml/10g i.p.). The number of writhes was recorded for 15 min. Inset, a dose-response curve of IBTU. Data are expressed as mean ± S.E.M. ***, $p < 0.001$ compared with the vehicle group.

distinct biphasic paw-flinching response that can be divided into an early phase (0–10 min) and a late phase (10–60 min) (Wang et al., 2001; Fig. 5). Coadministration of IBTU (30 μg/20 μl i.pl.) with formalin significantly inhibited both the early phase and late phase of the formalin response (Fig. 5). Both the total number of hindpaw flinches and the total time spent in paw flinching were significantly reduced.

Effect of IBTU on CFA-Induced Persistent Inflammatory Pain. The efficacy of IBTU against hyperalgesia associated with inflammation was further investigated in the CFA-induced inflammatory pain model. Intraplantar injection of CFA produced persistent thermal hyperalgesia and tactile allodynia that lasted for at least 7 days (Fig. 6). When IBTU (30 μg/20 μl i.pl.) was coadministered with CFA, thermal hyperalgesia and tactile allodynia were significantly reduced when tested on the first, third, and seventh day post-CFA (Fig. 6, A and B).

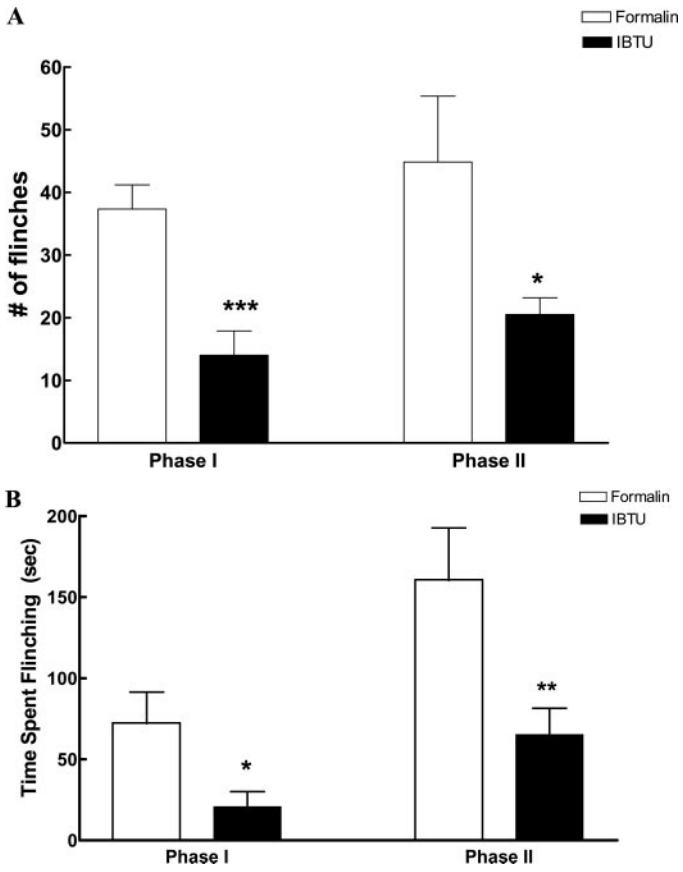

Fig. 5. Inhibition of formalin-induced paw-flinching response by IBTU. Separate groups of six mice were treated with formalin (2%; 20 μl i.pl.) alone or together with IBTU (30 μg i.pl.). IBTU significantly attenuated both the early phase and late phase of formalin nocifensive response. Data are expressed as mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the formalin group.

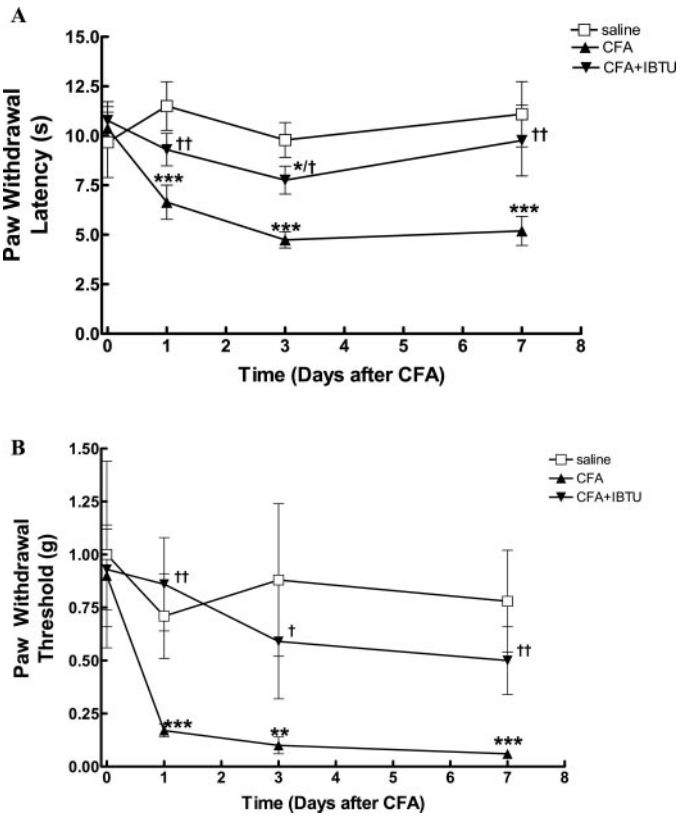

Fig. 6. Attenuation of CFA-induced (A) thermal hyperalgesia and (B) tactile allodynia. Separate groups of eight mice were injected with CFA (20 μl i.pl.) alone or together with IBTU (30 μg i.pl.). Thermal and mechanical sensitivity was determined before and on the first, third, and seventh day post-CFA injection. Data are expressed as mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the saline group. †, $p < 0.05$; ††, $p < 0.01$; and †††, $p < 0.001$ compared with the CFA group.

Effect of IBTU on CFA-Induced Paw Edema and Neutrophil Accumulation. CFA induced persistent swelling of the injected paws, indicative of tissue inflammation (Fig. 7A). Surprisingly, IBTU (30 μg/20 μl i.pl.) coadministered with CFA significantly reduced CFA-induced paw edema (Fig. 7A). In another experiment, IBTU was systemically administered (60 mg/kg i.p.) 30 min before the injection of CFA. Twenty-four hours later, only CFA-injected mice, but not those treated with IBTU and CFA, showed paw edema. To address the potential mechanism of action for this effect by IBTU, we determined the MPO activity in the ipsilateral hindpaws. MPO activity reflects the neutrophil accumulation at the site of inflammation (Kowaluk et al., 2000). Whereas CFA enhanced the MPO activity within 24 h after the injection, coadministration with IBTU (30 μg/20 μl i.pl.) did not alter CFA-induced MPO activity (Fig. 8), suggesting that the action of IBTU was not due to a direct inhibitory action on the accumulation of neutrophils.

Discussion

TRPV1 is a unique polymodal integrator of a variety of stimuli, including noxious heat, protons (e.g., tissue inflammation-induced low-pH environment), and specific ligands such as capsaicin or resiniferatoxin (Caterina et al., 1997; Szallasi and Blumberg, 1999; Hayes et al., 2000; McIntyre et al., 2001). Physiological and pharmacological studies have suggested that TRPV1 is expressed in nociceptors and mediates both acute and chronic pain (Szallasi and Blumberg, 1999). These unique properties of TRPV1 make it a very exciting target for the study and treatment of pain. Agents blocking the receptor are expected to be useful for the treatment of pain as well as other conditions, such as bladder hyperreflexia, detrusor instability, and bony fractures (Szallasi and Appendino, 2004).

IBTU was reported to be a potent and high-affinity competitive TRPV1 antagonist in vitro (Toth et al., 2004). IBTU lacks the ability to bind to intracellular TRPV1 receptors, yet IBTU is 5-fold more potent than capsazepine in blocking capsaicin- or resiniferatoxin-induced Ca^{2+} uptake from extracellular sources (Toth et al., 2004). In this study, we examined the antinociceptive properties of IBTU in several rodent models of acute and inflammatory pain.

In the first series of experiments, we directly tested whether IBTU was capable of blocking capsaicin-induced nociception. We found IBTU (i.p. or i.pl.) dose-dependently suppressed the capsaicin-induced paw-licking nociceptive behavior. These data are in agreement with the in vitro finding that IBTU is a TRPV1 antagonist. Other TRPV1 antagonists, such as capsazepine (a positive control in our experiments), ruthenium red, BCTC, I-RTX, DD161515, and DD191515 have also been reported to block capsaicin-induced paw licking (Santos and Calixto, 1997; Wahl et al., 2001; Garcia-

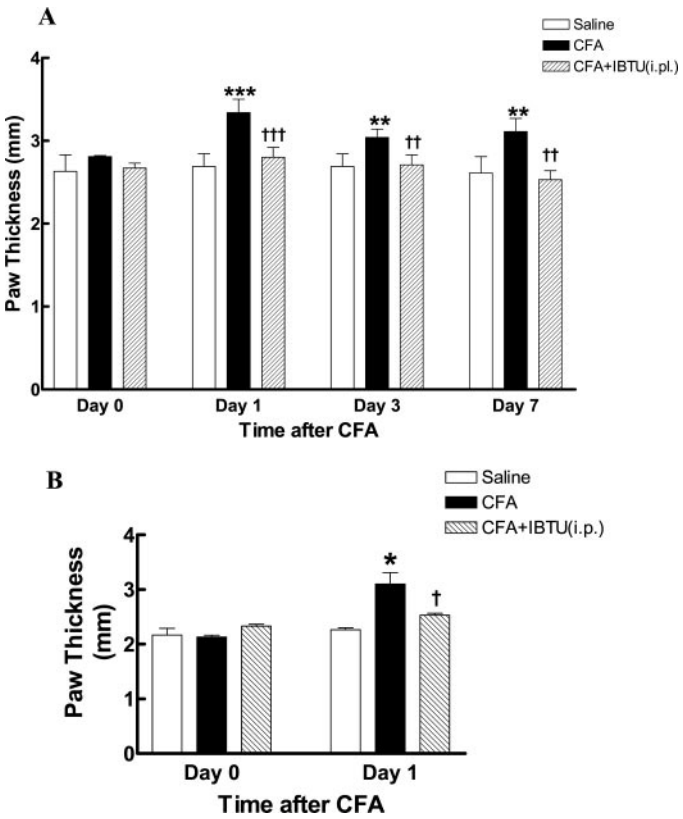

Fig. 7. Effects of IBTU on CFA-induced paw edema. A, separate groups of eight mice were injected with CFA (20 μl i.pl.) alone or together with IBTU (30 μg i.pl.). Paw thickness was determined before and on the first, third, and seventh day post-CFA injection. Data are expressed as mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the saline group. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ compared with the CFA group. B, separate groups of eight mice were treated with IBTU (60 mg/kg i.p.) 30 min before the injection of CFA (20 μl i.pl.). Paw thickness was determined before and 24-h post-CFA injection. Data are expressed as mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with the saline group. †, $p < 0.05$; ††, $p < 0.01$; and †††, $p < 0.001$ compared with the CFA group.

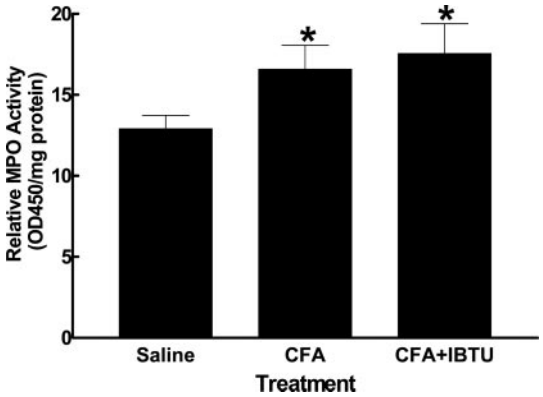

Fig. 8. Effects of IBTU on CFA-induced neutrophil accumulation. Separate groups of six mice were coadministered with CFA (20 μl i.pl.) and IBTU (30 μg i.pl.) or vehicle. Control mice received saline (20 μl i.pl.). Twenty-four hours later, MPO activity in the injected paws was determined. Data are expressed as mean ± S.E.M. *, $p < 0.05$ compared with the saline group. No statistical difference was found between the CFA group and the CFA + IBTU group.

Martinez et al., 2002; Pomonis et al., 2003). For the systemic administration, we were limited by the drug solubility; therefore, the highest dose used was 60 mg/kg, which did not completely block the action of capsaicin. When given intraplantarly, IBTU (90 μg) was able to completely block the action of capsaicin. The residual paw-licking response may be caused by the injection of vehicle or may not be mediated by TRPV1, since even TRPV1-null mice showed slight paw-licking behavior after the injection of capsaicin (i.pl.) (Caterina et al., 2000).

IBTU showed significant antinociception to noxious thermal stimuli, because it dose-dependently prolonged the latencies of tail-withdrawal in a 52°C tail-flick test. This effect was consistent with the report that TRPV1-null mice exhibited marked deficits in their responses to noxious thermal stimuli (Caterina et al., 2000; Davis et al., 2000). Two other TRPV1 antagonists, DD161515 and DD191515, have been reported to exhibit antinociception in a hot-plate test (Garcia-Martinez et al., 2002). The antinociception lasted for at least 2 to 3 h, which was longer than that of capsazepine, suggesting that IBTU may have a longer pharmacokinetic half-life. These data support the notion that TRPV1 is critical in mediating nociceptive perception to noxious thermal stimuli (Caterina et al., 2000). In mice lacking TRPV1, tail-flick latencies to 50 and 52°C water were significantly prolonged (Caterina et al., 2000). Agents blocking TRPV1 are expected to be useful for the treatment of noxious thermal pain.

The acetic acid-induced writhing reaction is often used as a model for visceral pain. Mechanistically, the cause of the pain is induced by an acid. The writhing response is not limited to acetic acid ($pK_a = 4.74$); it also is induced by other organic acids, such as propionic acid ($pK_a = 4.87$) or lactic acid ($pK_a = 3.86$). Because TRPV1 responds to proton-induced nociception, it was desirable to test IBTU in the acetic acid model. Moreover, the acid-induced writhing response has been suggested to be mediated, at least in part, by TRPV1 (Ikeda et al., 2001). Capsazepine was able to block acetic acid-, propionic acid-, or lactic acid-, but not phenylbenzoquinone-induced writhing response (Ikeda et al., 2001). We found that IBTU significantly reduced acid-induced writhing behavior. Interestingly, 100 μmol/kg capsazepine is as effective as 130 μmol/kg IBTU in the acetic acid-induced writhing test. In comparison, IBTU was found to be more effective than capsazepine in capsaicin-induced nociception and tail-flick tests. Although capsazepine was initially reported in the 1990s, its in vivo pharmacology was only thoroughly described recently (Walker et al., 2003). In addition to its species-dependent in vivo properties, its relative efficacy seemed to be assay-related. Our data are consistent with a previous report that capsazepine (40 mg/kg i.p.) was fully effective in inhibiting acetic acid-induced writhing in mice (Ikeda et al., 2001).

Tissue inflammation often results in reduced pH in the surrounding area. We therefore tested the pharmacological action of IBTU in two models of inflammatory pain. Injection of diluted formalin solution into the hindpaw produces two distinct phases of nocifensive response, each of which is thought to be mediated by a different mechanism. The early phase is proposed to be caused by direct activation of primary afferent nociceptors, and it may involve Aβ, Aδ, and high-threshold C-fibers (Puig and Sorkin, 1996). The late phase is related to the subsequent inflammatory response and most probably also to spinal cord sensitization (Hunskaar and Hole, 1987; Puig and Sorkin, 1996). IBTU suppressed formalin-induced paw-flinching and -licking pain behavior in both phases.

A more persistent inflammatory pain was produced by the CFA injection. CFA induced an intense and lasting tissue inflammation of the injected hindpaw that is characterized by edema, erythema, and hyperalgesia (Iadarola et al., 1988; Martin et al., 1999). Several lines of evidence suggest that TRPV1 is involved in CFA-induced hyperalgesia. CFA treatment increases the expression of TRPV1 (Luo et al., 2004) and capsaicin sensitivity in dorsal root ganglion neurons (Nicholas et al., 1999). A more recent study found that the CFA-induced increase in capsaicin and proton responsiveness was mediated by TRPV1 in IB4-positive primary afferent neurons (Breese et al., 2005). IBTU (i.pl.) was found to completely prevent CFA-induced thermal hyperalgesia and tactile allodynia. The latter effect, although not as robust as its action on thermal hyperalgesia, was somewhat surprising. TRPV1-null mice showed a loss of thermal, but not mechanical, hyperalgesia caused by CFA (Caterina et al., 2000). The discrepancy in the role of TRPV1 in mechanical hyperalgesia may be due to different experimental paradigms (chemical versus genetic inhibition), developmental adaptation associated with gene deletion, or genetic background of experimental animals. Capsazepine (up to 100 mg/kg s.c.) did not affect mechanical hyperalgesia induced by CFA in the rat or mouse, although it produced a partial reversal in the guinea pig (Walker et al., 2003). Other TRPV1 antagonists, such as AMG 9810 and BCTC, have also been reported to inhibit both thermal and mechanical hyperalgesia induced by CFA in rats (Pomonis et al., 2003; Gavva et al., 2005). Because CFA-induced persistent hyperalgesia may involve sensitization in the spinal cord dorsal horn and changes at the peripheral terminals (Malmberg et al., 2003), it seems that both peripheral and central mechanisms can be blocked by TRPV1 antagonists. We also found that IBTU was capable of attenuating CFA-induced paw edema. Since both locally (coadministered with CFA via i.pl.) and systemically (i.p.) administered IBTU was able to prevent CFA-induced paw edema, it would argue against the possibility that IBTU may directly interfere with the bioavailability or clearance of CFA. Furthermore, the action of IBTU on CFA paw edema did not seem to be related to the inhibition of neutrophil accumulation. This lack of direct effect on local inflammation would suggest that the action of IBTU on paw edema was most probably due to its action on sensory nerve fibers. Robust activation of TRPV1, by injecting TRPV1 agonists into hindpaws, is known to induce neurogenic inflammation that results in paw edema (Siemens et al., 2006). On the other hand, it is possible that this action of IBTU may not be related to TRPV1.

In summary, the present study demonstrated that IBTU is a TRPV1 antagonist in vivo. IBTU was found to be capable of blocking capsaicin-induced nociception, noxious thermal nociception, the acetic acid-induced writhing response, early and late phase responses to formalin, and CFA-induced thermal and mechanical hyperalgesia and paw edema in mice. IBTU may be of use as a drug lead for the further studies of a new class of TRPV1 antagonists that selectively block the cytoplasmic membrane TRPV1.

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