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Antinociceptive pharmacology of N-(4-chlorobenzyl)-N'-(4-hydroxy-3-iodo-5-methoxybenzyl) thiourea (IBTU), a high-affinity competitive antagonist of the transient receptor potential vanilloid 1 receptor

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#### List of abbreviations

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

BCTC: N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide

CFA: complete Freund's adjuvant

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

IBTU: N-(4-chlorobenzyl)-N'-(4-hydroxy-3-iodo-5-methoxybenzyl) thiourea

I-RTX: 5-iodoresiniferatoxin

RTX: resiniferatoxin

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TRPV1: transient receptor potential vanilloid type 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 a considerable interest to develop TRPV1 antagonists for the treatment of pain. Recently, IBTU (N-(4-chlorobenzyl)-N'-(4-hydroxy-3-iodo-5-methoxybenzyl) thiourea) was synthesized and 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 as 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 CFA-induced persistent hyperalgesia. Taken together, these data demonstrate that IBTU acts as a TRPV1 antagonist *in vivo* and suggest that it may be of therapeutic use for the treatment of pain.

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## Introduction

The transient receptor potential vanilloid 1 receptor (TRPV1, formerly called VR1) is a non selective 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 transmit nociceptive information to the spinal cord (Szallasi and Blumberg, 1999). TRPV1 is activated by a wide 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; Tominaga et al., 1998; Szallasi and Blumberg, 1999; Caterina et al., 2000; 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-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

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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 wide diversity of structures with competitive TRPV1 antagonism and good potency. Examples include I-resiniferatoxin(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 compound's affinity 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  $\text{Ca}^{2+}$  levels and  $^{45}\text{Ca}^{2+}$  uptake induced by capsaicin or resiniferatoxin. However, IBTU was less effective in displacing the binding of [ $^3\text{H}$ ]resiniferatoxin to TRPV1 heterologously expressed in CHO-TRPV1 cells and appeared to block resiniferatoxin-induced  $\text{Ca}^{2+}$  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  $^{45}\text{Ca}^{2+}$  uptake. In contrast, IBTU does not appear to interact effectively with the predominant intracellular fraction of TRPV1 that dominates the [ $^3\text{H}$ ]resiniferatoxin binding measurements. How this unique *in vitro* antagonism profile translates into a

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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 Laboratories, Indianapolis, IN) were maintained on a 12/12 h light/dark cycle and provided food and water *ad libitum* before experimental procedures. All experiments were performed in accordance with the NIH guidelines after approval by the Animal Care and Use committee of the University of Illinois at Chicago. Capsaicin, capsazepine, acetic acid, formalin, complete Freund's adjuvant (CFA), hexadecyl-trimethylammonium bromide, *o*-diansidine dihydrochloride, and other chemicals were purchased from Sigma (St Louis, MO).

### ***Capsaicin-induced nociception***

Intraplantar injection of capsaicin (3 $\mu$ g/20 $\mu$ L) 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.

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### ***Tail-flick Test***

Antinociception was determined using a tail-flick test as previously described (Tang et al., 2006). Briefly, the distal 1/3 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 6 mice were treated with IBTU, capsazepine, or an equal volume of vehicle. The antinociception response was presented as %MPE (maximal possible effect) as defined by  $\%MPE = 100 \times \frac{(\text{drug response time} - \text{basal response time})}{(\text{cut-off time} - \text{basal response time})}$ . A cut-off time of 12 sec was applied to avoid tissue damage.

### ***Abdominal constriction test of visceral pain***

Testing for inhibition of abdominal constriction was performed as previously described (Yang et al., 2003; Porreca et al., 2006). Mice were placed in individual glass cylinders for a 30-min acclimatization period. Separate groups of 9 mice were injected with 0.6% acetic acid (0.1mL/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 (40mg/kg, *i.p.*) 30 min before the injection of acetic acid.

### ***Formalin test***

Tonic inflammatory pain was induced in separate groups of 6 mice by a subcutaneous injection of formalin (20 µL of 2% solution/mouse) into the dorsal surface



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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 $\mu$ g, *i.pl.*) was co-administered with formalin via the same route.

### ***Complete Freund's adjuvant (CFA)-induced hyperalgesia***

Complete Freund's adjuvant (CFA, *Mycobacterium tuberculosis*, suspended in an oil:saline (1:1) emulsion, 0.5 mg/mL Mycobacterium, Sigma, MO) was injected into the dorsal surface of the left hindpaw of mice (20  $\mu$ g/paw) (Iadarola et al., 1988; Malmberg et al., 2003). Control mice received 20  $\mu$ L 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  $\mu$ g/paw, *i. pl.*) was co-administered with CFA. Antihyperalgesia was calculated as follows: % 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: % 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**

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Thermal and mechanical sensitivity was assessed as previously described (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 a ~10 s response in naïve 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).

***Complete Freund’s adjuvant (CFA)-induced edema and myeloperoxidase (MPO) 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 were 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 Polytron (setting 6 for 30s) in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl-trimethylammonium bromide, and quickly frozen and thawed for three cycles. The homogenate was then centrifuged for 15 min at 40,000 x g at 4 °C. Protein content in the supernatant was determined by a modified Bradford method (Bio-Rad, CA). Supernatant (100 µL) was mixed with 50 µL potassium phosphate buffer (50 mM, pH 6.0) and 25 µL of freshly mixed reaction mixture containing *O*-diansidine

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dihydrochloride (0.167 mg/mL final concentration) and H<sub>2</sub>O<sub>2</sub> (0.0005% final concentration). The absorbance at 450 nm was determined by a microplate reader (Labsystems Multiskan Plus, Waltham, MA). The relative MPO activity was expressed in OD450/mg protein.

### ***Statistical analysis***

All data are presented as Mean  $\pm$  S.E.M. (standard error of mean). Differences in responses between the treatment groups were determined using *Student's t* (two groups) or ANOVA followed by *Dunnnett's t* test (multiple groups). Statistical significance was established at 95%.

## **Results**

### ***Effect of IBTU on Capsaicin induced nocifensive response***

Since 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 intraplantarly (*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<sub>50</sub> = 43  $\pm$  10 mg/kg, Fig. 1A) and the total time spent licking the paw (ED<sub>50</sub> = 58  $\pm$  10 mg/kg, Fig. 1B). IBTU (30 $\mu$ g-90 $\mu$ g/20 $\mu$ L) was also effective when injected intraplantarly, completely blocking the action of capsaicin at the highest dose used (Fig. 2). ED 50 was estimated to be 27  $\pm$  10  $\mu$ g and 21  $\pm$  10  $\mu$ g for suppressing the number of licks and total licking time, respectively. For comparison, capsazepine (30 $\mu$ g/20 $\mu$ L, or 78 pmol/dose,

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*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 appeared to be less effective than 30 $\mu$ g/20 $\mu$ L (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, as the drug exhibited significant antinociception within 5 - 15 min, and reached its peak action in 30 min. IBTU-produced antinociception lasted for at least 2 - 3 h (Fig. 3A) and was dose dependent (ED<sub>50</sub> = 37  $\pm$  10 mg/kg, Fig. 3B). As a positive control, another group of mice 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.

### ***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.,

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2003). Acetic acid (*i.p.*)-administered mice exhibited writhing behavior in naïve 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. Similarly, 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 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). Co-administration of IBTU (30µg/20µL, *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 complete Freund's adjuvant (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 (30µg/20µL, *i.pl.*) was co-

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administered with CFA, thermal hyperalgesia and tactile allodynia were significantly reduced when tested on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day post CFA (Fig. 6 A & B).

### ***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 $\mu$ g/20 $\mu$ L, *i.pl*) co-administered 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 myeloperoxidase (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, co-administration with IBTU (30 $\mu$ g/20 $\mu$ L, *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 wide variety of stimuli including noxious heat, protons (e.g., tissue inflammation-induced low pH environment), specific ligands such as capsaicin or RTX (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

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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 RTX-induced  $\text{Ca}^{2+}$  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 if 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-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  $\mu\text{g}$ ) 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-

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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, as 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 - 3 h, which was longer than that of capsazepine, suggesting that 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°C 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 (*pKa*: 4.74), but also induced by other organic acids such as propionic acid (*pKa*: 4.87) or lactic acid (*pKa*: 3.86). Since TRPV1 responds to proton-induced nociception, it was desirable to test IBTU in the acetic acid model. In fact, 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,



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capsazepine (100  $\mu\text{mol/kg}$ ) is as effective as IBTU (130  $\mu\text{mol/kg}$ ) 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 1990's, its *in vivo* pharmacology was only thoroughly described recently (Walker et al., 2003). In addition to its species-dependent *in vivo* properties, we found 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 effectively 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, which are thought to be mediated by different mechanisms. The early phase is proposed to be caused by direct activation of primary afferent nociceptors and may involve A $\beta$ , A $\delta$ , and high-threshold C fibers (Puig and Sorkin, 1996). The late phase is related to the subsequent inflammatory response and most likely also to spinal cord sensitization (Hunskar 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

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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 experiment paradigms (chemical vs 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). Since CFA-induced persistent hyperalgesia may involve sensitization in the spinal cord dorsal horn and changes at the peripheral terminal (Malmberg et al., 2003), it appears 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 (co-administered 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 appear 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

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was most likely 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, 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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### Footnotes

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## Legends for Figures

Figure 1. Attenuation of nocifensive response to capsaicin by systemically administered IBTU. Separate groups of 9 mice were pretreated with IBTU or an equal volume of vehicle (*i.p.*) 30 minutes before the administration of capsaicin (3 $\mu$ g/20 $\mu$ L, *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  $\pm$  S.E.M. \*\*\**p* <0.001 compared with the vehicle group (BL).

Figure 2. Attenuation of nocifensive response to capsaicin by locally administered IBTU. Separate groups of 6 mice were co-administered with capsaicin (3 $\mu$ g/20 $\mu$ L, *i.pl.*) and different doses of IBTU (30  $\mu$ g, 60  $\mu$ g, 90  $\mu$ g, *i.pl.*), or capsazepine (30 $\mu$ g, *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. Inserts: Dose-response curves of IBTU. Data are expressed as Mean  $\pm$  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.

Figure 3. Inhibition of thermal nociception by IBTU. (A) Time course of antinociception response after a single dose of IBTU (30mg/kg, *i.p.*) or capsazepine (40mg/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. Insert: A dose-response curve of IBTU. Data are expressed in Mean  $\pm$  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

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the vehicle group.

Figure 4. Suppression of acetic acid-induced writhing response by IBTU. Separate groups of 9 mice were pretreatment with IBTU (*i.p.*) or capsazepine (40mg/kg, *i.p.*) 30 min before the injection of acetic acid (0.6%, 0.1mL/10g, *i.p.*). The number of writhes was recorded for 15 min. Insert: A dose-response curve of IBTU. Data are expressed as Mean  $\pm$  S.E.M. \*\*\* $p < 0.001$  compared with the vehicle group.

Figure 5. Inhibition of formalin-induced paw flinching response by IBTU. Separate groups of 6 mice were treated with formalin (2%, 20 $\mu$ L, *i.pl.*) alone or together with IBTU (30 $\mu$ g, *i.pl.*). IBTU significantly attenuated both the early phase and late phase of formalin nocifensive response. Data were expressed as Mean  $\pm$  S.E.M. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared with the formalin group.

Figure 6. Attenuation of CFA-induced (A) thermal hyperalgesia and (B) tactile allodynia. Separate groups of 8 mice were injected with complete Freund's adjuvant (CFA, 20 $\mu$ L, *i.pl.*) alone or together with IBTU (30 $\mu$ g, *i.pl.*). Thermal and mechanical sensitivity was determined before and on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day post CFA injection. Data were expressed as Mean  $\pm$  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.

Figure 7. Effects of IBTU on CFA-induced paw edema.

(A) Separate groups of 8 mice were injected with complete Freund's adjuvant (CFA,

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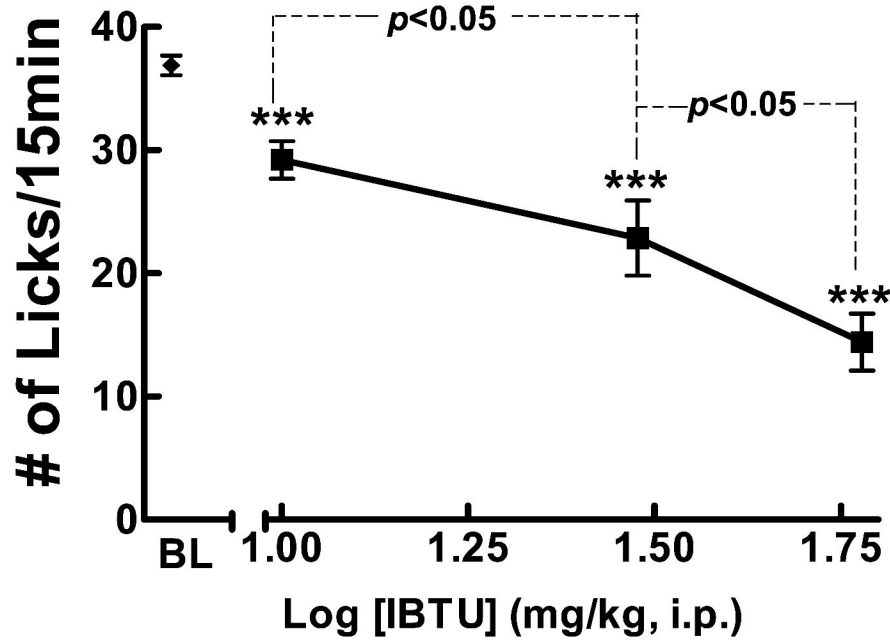
20 $\mu$ L, *i.pl.*) alone or together with IBTU (30 $\mu$ g, *i.pl.*). Paw thickness was determined before and on the 1<sup>st</sup>, 3<sup>rd</sup>, and 7<sup>th</sup> day post CFA injection. Data were expressed as Mean  $\pm$  S.E.M. \* $p$  < 0.05, \*\*  $p$  < 0.01, \*\*\* $p$  < 0.001 compared with the saline group. †  $p$  < 0.05, ††  $p$  < 0.01, ††† $p$  < 0.001 compared with the CFA group.

(B) Separate groups of 8 mice were treated with IBTU (60mg/kg, *i.p.*) 30 min before the injection of complete Freund's adjuvant (CFA, 20 $\mu$ L, *i.pl.*). Paw thickness was determined before and 24 h post CFA injection. Data were expressed as Mean  $\pm$  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.

Figure 8. Effects of IBTU on CFA-induced neutrophil accumulation. Separate groups of 6 mice were co-administered with CFA (20 $\mu$ L, *i.pl.*) and IBTU (30 $\mu$ g, *i.pl.*) or vehicle. Control mice received saline (20 $\mu$ L, *i.pl.*). Twenty-four hours later, myeloperoxidase (MPO) activity in the injected paws was determined. Data are expressed as Mean  $\pm$  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.

Fig. 1

A.



B.

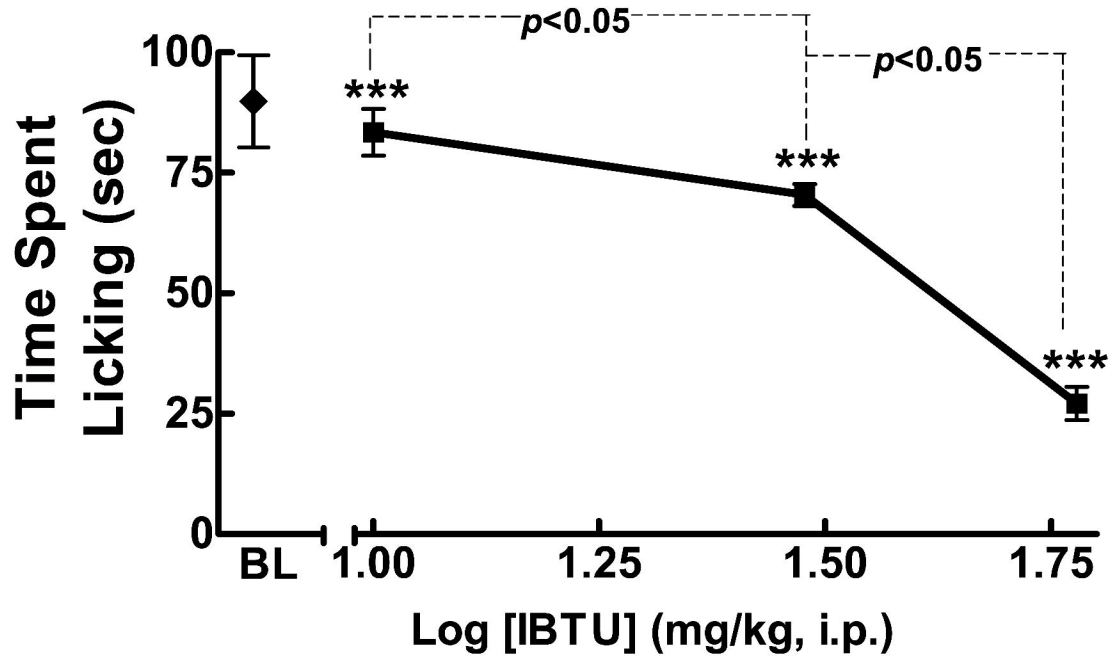
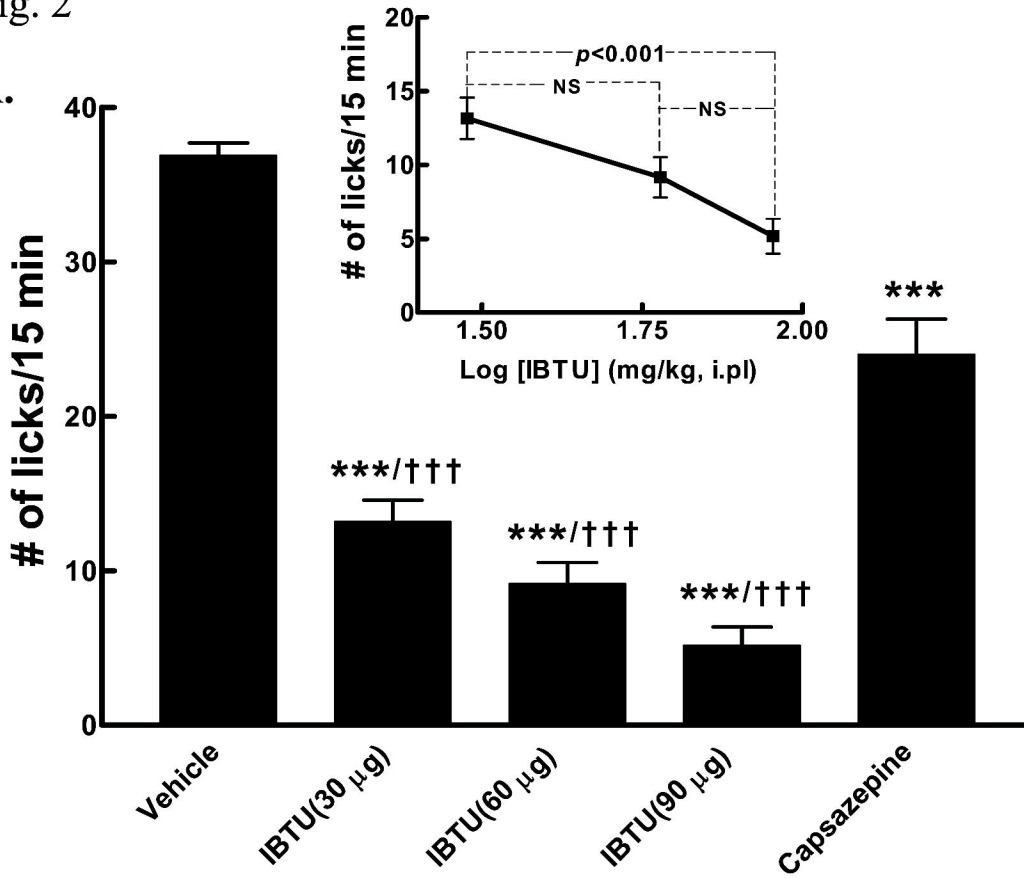


Fig. 2

A.



B.

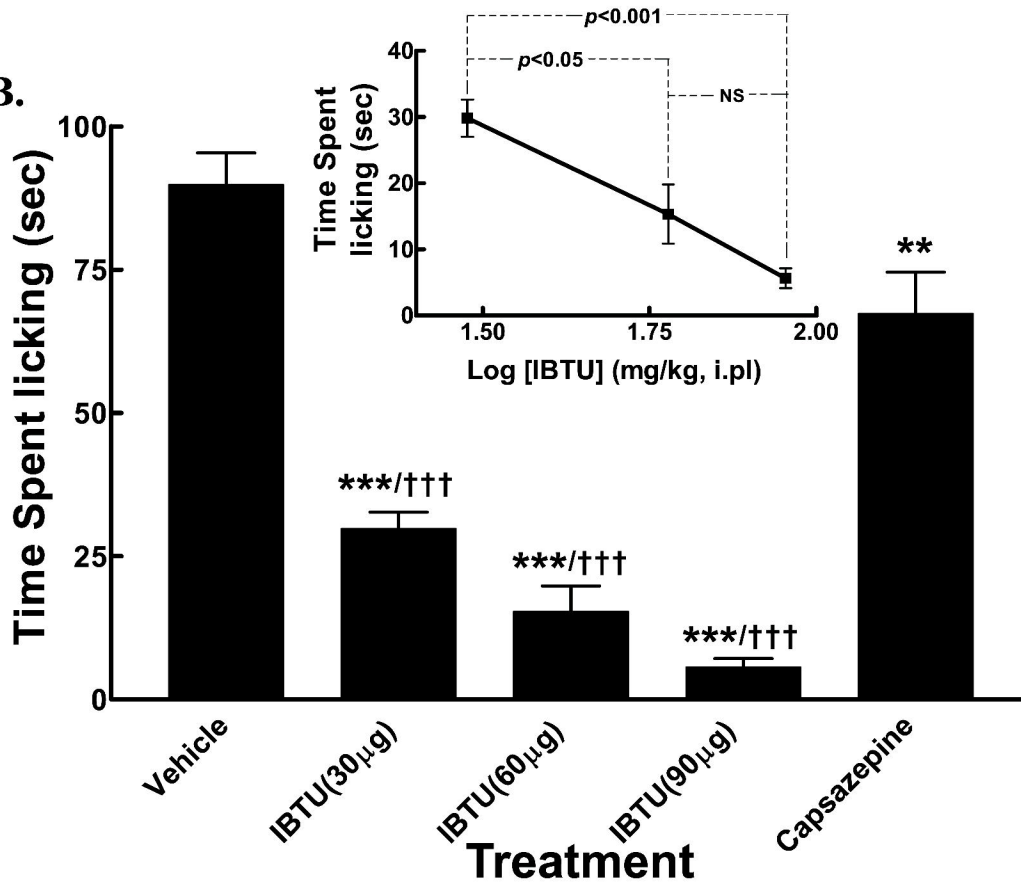
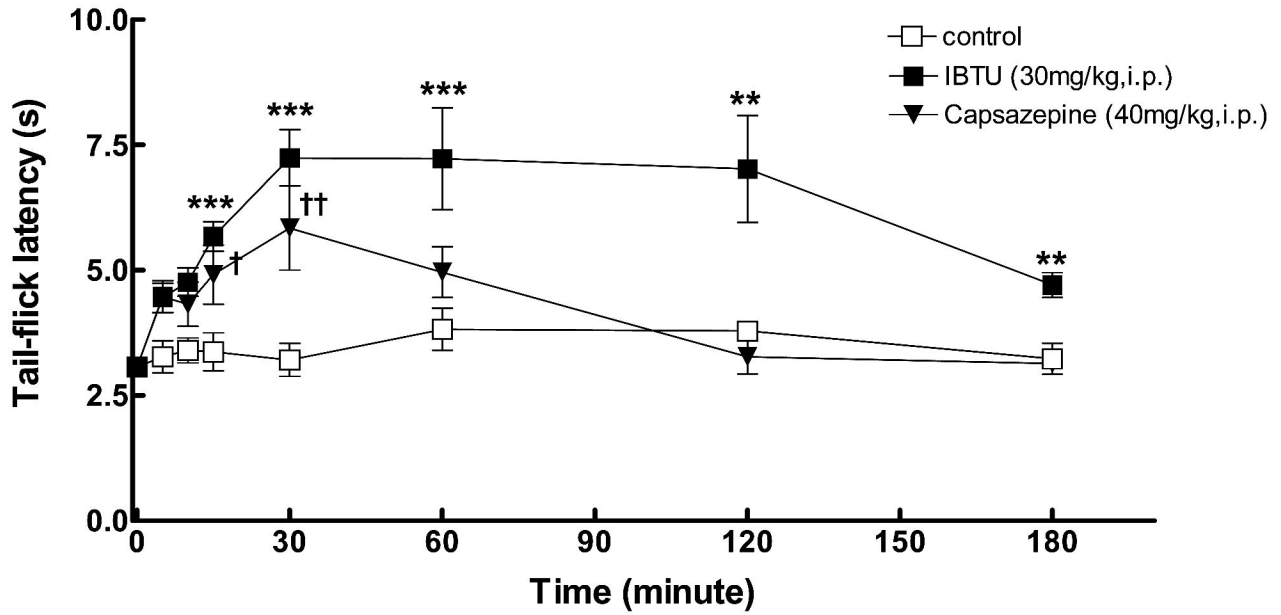


Fig. 3

A.



B.

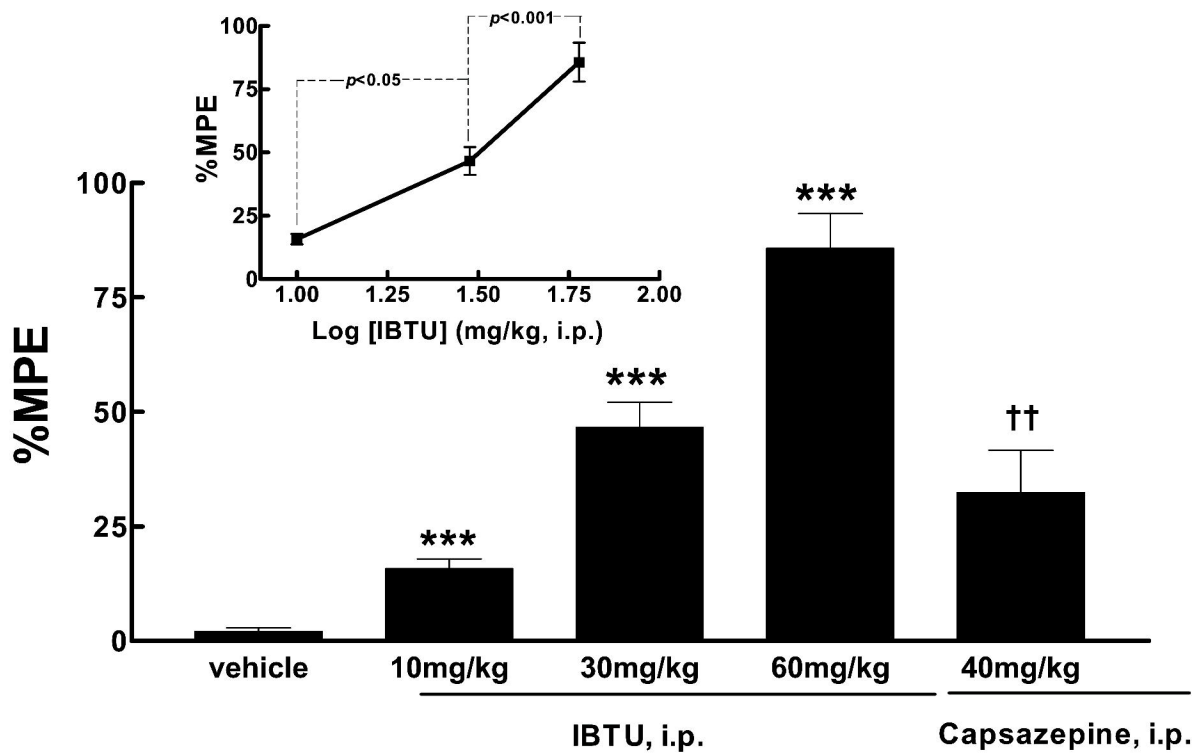




Fig. 4

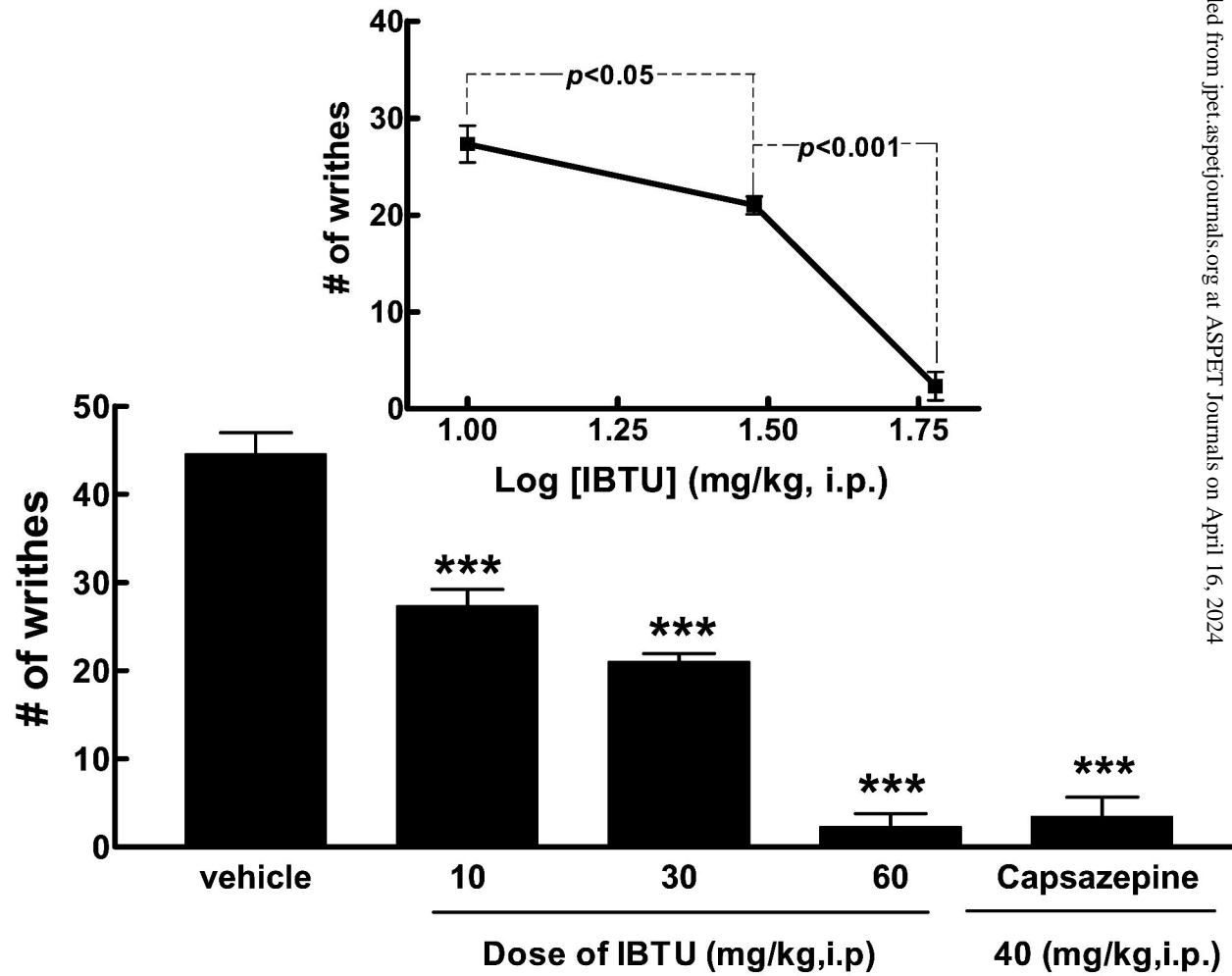
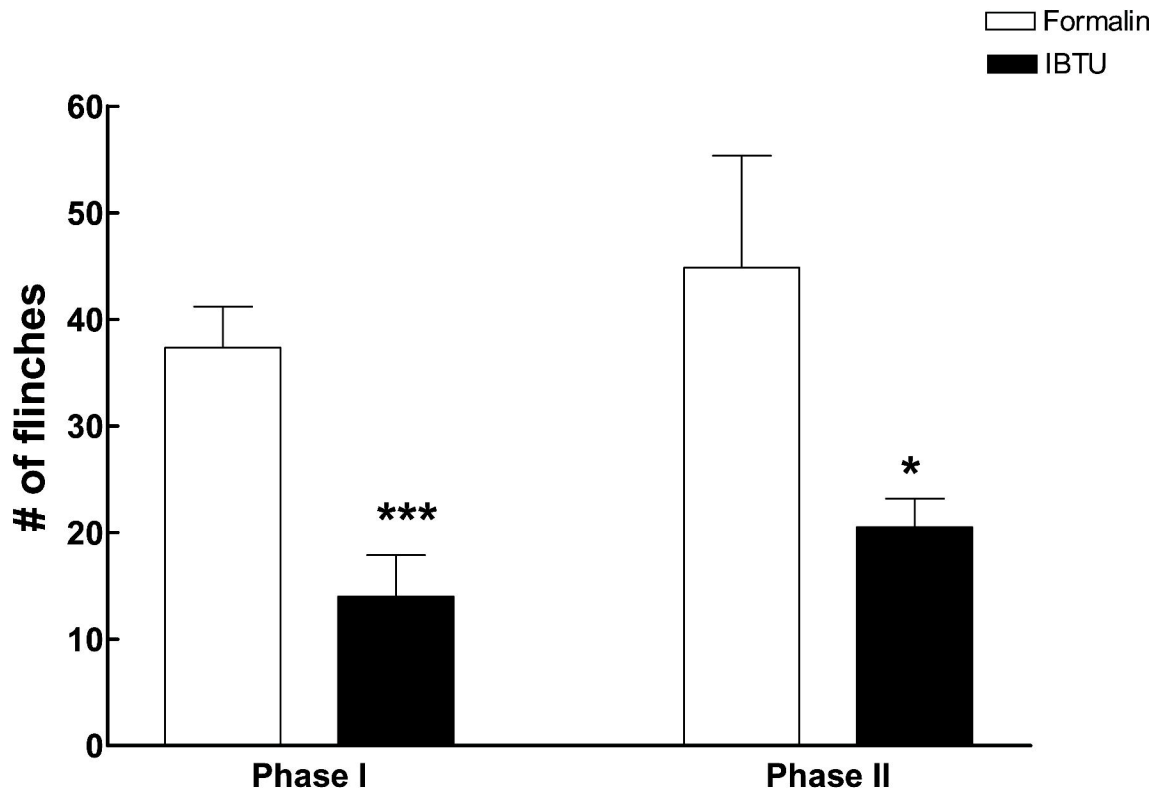


Fig. 5

A.



B.

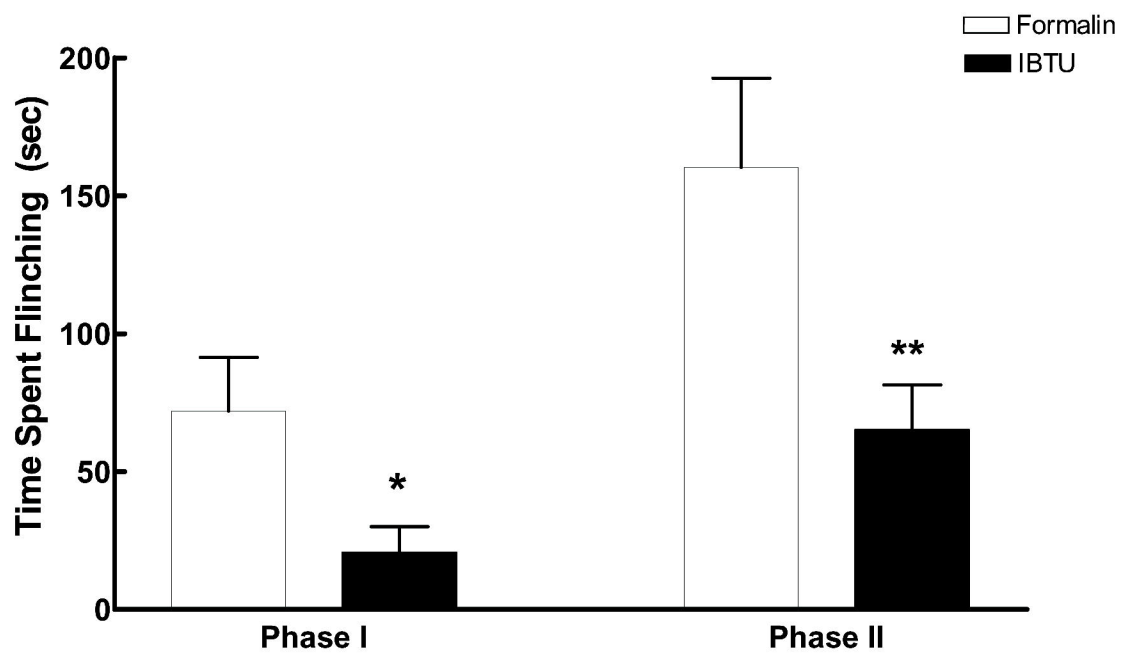


Fig. 6

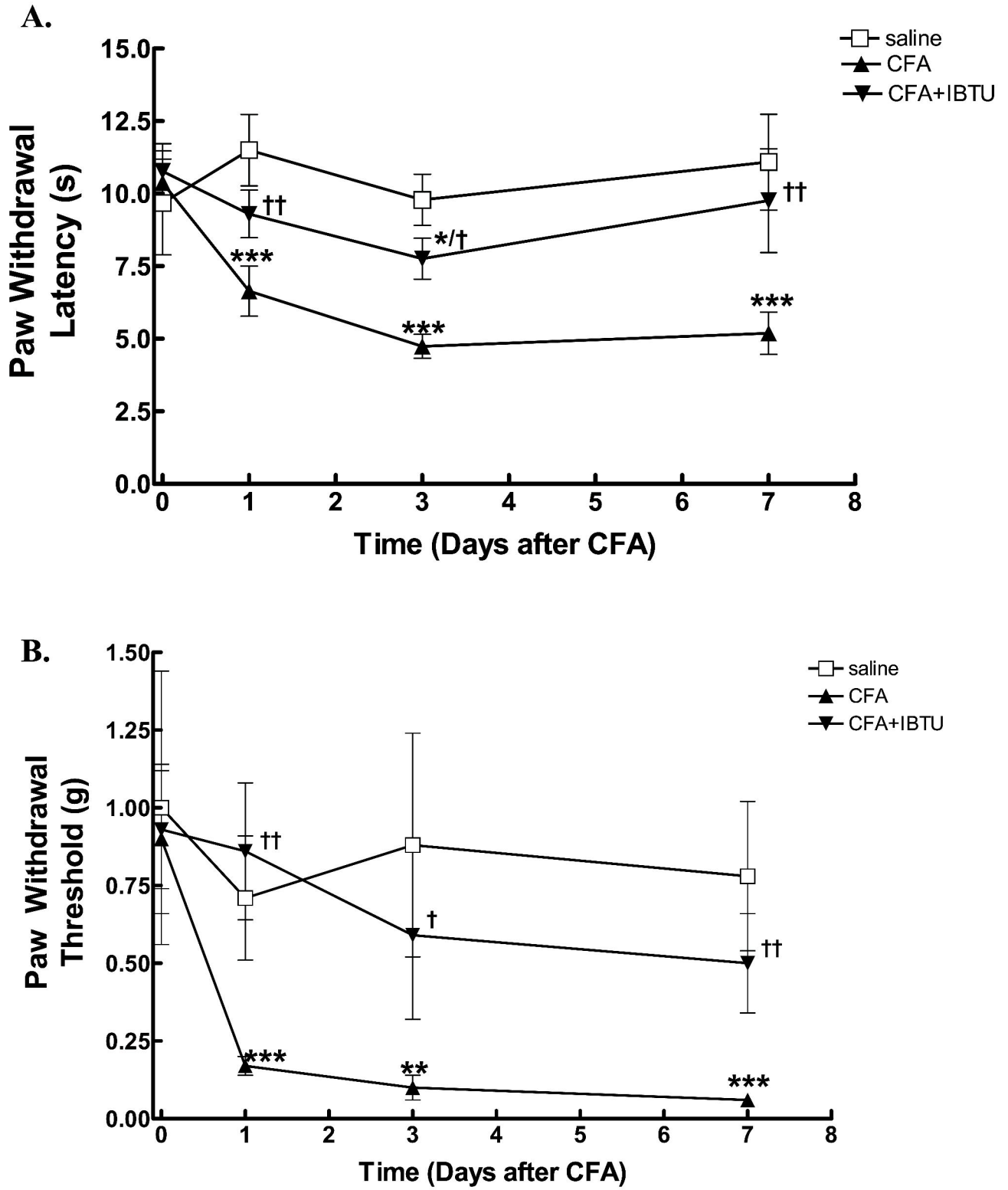
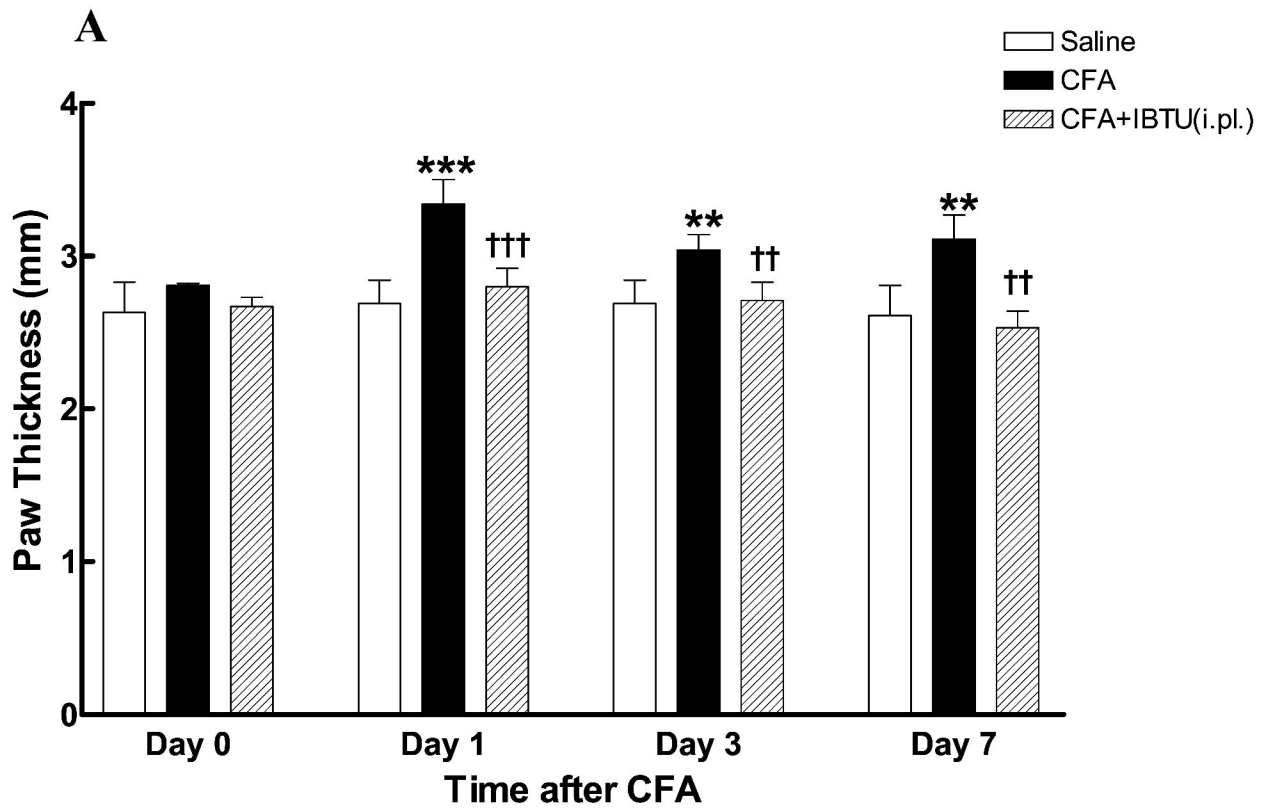


Fig. 7



**B**

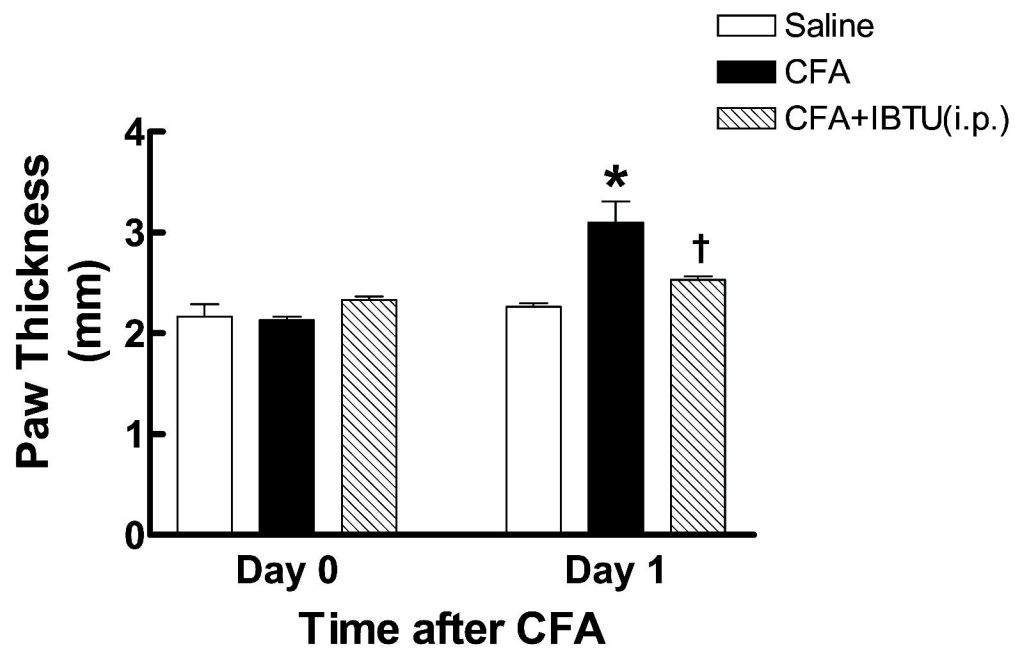


Fig. 8

