ORIGINAL ARTICLE

Blockade of TRPA1 with HC-030031 attenuates visceral nociception by a mechanism independent of inflammatory resident cells, nitric oxide and the opioid system

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Abstract

Background: Some studies have shown a somatic nociceptive response due to the activation of transient receptor potential A1 channels (TRPA1), which is modulated by the TRPA1 antagonist HC-030031. However, a few studies report the role of TRPA1 in visceral pain. Therefore, we investigated the participation of TRPA1 in visceral nociception and the involvement of nitric oxide, the opioid system and resident cells in the modulation of these channels.
Methods: Mice were treated with vehicle or HC-030031 (18.75–300 mg/kg) before ifosfamide (400 mg/kg), 0.75% mustard oil (50 μL/colon), acetic acid 0.6% (10 mL/kg), zymosan (1 mg/cavity) or misoprostol (1 μg/cavity) injection. Visceral nociception was assessed through the electronic von Frey test or the writhing response. Ifosfamide-administered mice were euthanized for bladder analysis. The involvement of nitric oxide and the opioid system were investigated in mice injected with ifosfamide and mustard oil, respectively. The participation of resident peritoneal cells in acetic acid-, zymosan- or misoprostol-induced nociception was also evaluated.
Results: HC-030031 failed to protect animals against ifosfamide-induced bladder injury ($p > 0.05$). However, a marked antinociceptive effect against ifosfamide, mustard oil, acetic acid, zymosan and misoprostol was observed ($p < 0.05$). Neither L-arginine (600 mg/kg) nor naloxone (2 mg/kg) could reverse the antinociceptive effect of HC-030031. The reduction of the peritoneal cell population inhibited the acetic acid and zymosan-related writhes without interfering with the misoprostol effect.
Conclusions: Our findings suggest that the blockade of TRPA1 attenuates visceral nociception by a mechanism independent of the modulation of resident cells, nitric oxide and opioid pathways.

1. Introduction

Visceral pain is one of the most frequent reasons why patients seek medical attention (Cervero and Laird, 1999) and the most common form of pain produced by diseases such as, gastric distention (Kondo et al., 2010), inflammatory bowel disease (Bueno et al.,

2007) and haemorrhagic cystitis (Lima-Júnior et al., 2007).

Considering the diverse nature of diseases, it is extremely difficult to design a model of visceral pain in laboratory animals that can exactly mimic all the characteristics of functional visceral pain (Sengupta, 2009). Experimental pain models for assessment of analgesic

What's already known about this topic?
- TRPA1 is involved in visceral pain.
- However, the precise mechanism by which TRPA1 contributes to visceral pain is still unknown.

What does this study add?
- This study provides a pharmacological approach regarding the participation of TRPA1 in visceral nociception and the possible mechanisms involved.

effect needs to be reproducible, valid and responding in a uniform way to changes in pain level (Staahl et al., 2006). Some pain models provide meaningful results that are directly applicable to the interpretation of clinical data (Giamberardino, 1999).

The role of transient receptor potential (TRP) channels in visceral pain has been broadly investigated (Cattaruzza et al., 2010). TRP channels are excitatory ion channels mainly expressed in nociceptive neurons and contribute to the detection of noxious mechanical, thermal and pro-inflammatory stimuli (McNamara et al., 2007; Eid et al., 2008; Wang et al., 2008; Kerstein et al., 2009). Currently, they have been considered as potential targets in the management of visceral and inflammatory pain. Members of the TRP vanilloid family, such as Transiente Receptor Potential Vanilloid 1 (TRPV1), are widely involved in visceral hyperalgesia (Hobson and Aziz, 2007; Sipe et al., 2008). However, there are only a few reports regarding the participation of TRPA1 on visceral nociception (Yang et al., 2008; Cattaruzza et al., 2010; Kondo et al., 2010).

Transient receptor potential ankyrin-1 (TRPA1) is expressed in sensory nerves and mediates cold, mechanical and chemical nociception (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004). It is directly activated by exogenous alimentary (e.g., mustard oil) and environmental (e.g., acrolein) irritants (Bautista et al., 2006).

Notably, TRPA1 is expressed in visceral afferent neurons and is known to participate in inflammatory responses and the establishment of hypersensitivity (Cattaruzza et al., 2010). It has been reported that inflammatory agents can directly activate or indirectly sensitize TRPA1 to induce somatic pain and hyperalgesia (Petrus et al., 2007; Andrade et al., 2008; Wang et al., 2008; Liang et al., 2010; Fernandes et al., 2011). Wang et al. (2008) revealed that bradykinin might trigger the sensation of pain through TRPA1 activation by a phospholipase C-dependent mechanism-

.However, the precise mechanism by which TRPA1 contributes to visceral pain and inflammation is still unknown. Some hypothesis have been tested, including the role of TRPA1 on substance P release from the primary sensory neurons through phosphorylation of p38 (Nakamura et al., 2012), and the release of histamine by mast cells (Andrade et al., 2008). Furthermore, non-neuronal cells on which TRPV1 and TRPA1 are expressed vary from vascular smooth muscle to keratinocytes and endothelium (Fernandes et al., 2012), which suggests a broader role of TRPA1.

Therefore, the present research investigated the role of TRPA1 in visceral nociception and the participation of nitric oxide (NO), the opioid system and resident cells in the modulation of these channels.

2. Methods

2.1 Animals

Male Swiss mice weighing 20–25 g were kept in a temperature-controlled room (22 ± 2°C) under 12-h dark–light cycles with free access to food and water. The animals were fasted 18 h before the experiments. The Local Ethics Committee for Animal Experiments approved the entire protocol (number 92/10), complying to IASP's guidelines for pain research in animals (Zimmermann, 1983). All the experiments performed in this research were conducted by a blinded person who was unaware of the treatments and group divisions.

2.2 Drugs

HC-030031 was kindly provided by Hydra Biosciences, Inc. (Cambridge, MA, USA). For all experiments, HC-030031 was suspended in 0.5% carboximethylcellulose (0.5% CMC). Diazepam (Sanofi, São Paulo, SP, Brazil), indomethacin (Sigma-Aldrich, São Paulo, SP, Brazil), morphine (Cristália, São Paulo, SP, Brazil), naloxone (Cristália, São Paulo, SP, Brazil), mesna (Eurofarma, São Paulo, SP, Brazil), Ifosfamide (Eurofarma, São Paulo, SP, Brazil), misoprostol (Herbron, Caruaru, PE, Brazil), L-NAME (Sigma-Aldrich, São Paulo, SP, Brazil), L-Arginine (Sigma-Aldrich, São Paulo, SP, Brazil), mustard oil (Sigma-Aldrich, São Paulo, SP, Brazil), acetic acid and zymosan (Sigma-Aldrich, São Paulo, SP, Brazil) were dissolved in sterile saline (0.9%).

2.3 Induction and cystitis quantification

To study the anti-inflammatory and the antinociceptive effects of HC-030031 on ifosfamide-induced

haemorrhagic cystitis, groups of mice ($n = 8$) were treated with HC-030031 (75, 150 or 300 mg/kg, p.o.) or vehicle (0.5% CMC in distilled water) 60 min before the intraperitoneal injection of ifosfamide (IFO, 400 mg/kg). The animals were euthanized 12 h later. The bladder was removed by careful dissection, emptied of urine, weighed to quantify vesical oedema (increase in bladder wet weight expressed as mg/20 g body weight) and evaluated for the macroscopic and microscopic parameters described previously (Gray et al., 1986). Briefly, bladders were macroscopically examined for oedema and haemorrhage in a blinded manner. According to Gray's scoring criteria (Gray et al., 1986), oedema was considered severe (3+) when fluid was seen externally and internally in the bladder walls, moderate (2+) when confined to the internal mucosa, mild (1+) between normal and moderate and normal (0) when no oedema was observed. Haemorrhage was scored as follows: 3+, intravesical clots; 2+, mucosal haematomas; 1+, telangiectasia or dilatation of bladder vessels; and 0, normal.

Bladders were fixed in formalin for 12 h, dehydrated in alcohol and embedded in paraffin. Serial 5-μm sections were stained with haematoxylin and eosin for analysis of the lesions. Histopathological analysis was performed by a blinded person who was unaware of the treatments and group divisions, and scored as follows: (0) normal epithelium and absence of inflammatory cell infiltration and ulceration; (1) mild changes involving reduction of urothelial cells, flattening with submucosal oedema, mild haemorrhage and few ulcerations; (2) moderate changes including mucosal erosion, inflammatory cell infiltration, fibrin deposition, haemorrhage and multiple ulcerations; and (3) severe changes of previously described parameters.

2.4 Evaluation of sensitivity to mechanical stimulation of the abdomen

Electronic von Frey test was performed to assess visceral nociception after injection of ifosfamide. The apparatus consists of acrylic boxes ($12 \times 20 \times 17$ cm) with a floor grid, which is viewed through a mirror positioned below, allowing observation of animals. A digital analgesy meter connected to a force transducer with a 0.7 mm² polypropylene tip (electronic von Frey) detects the intensity of stimulation (grams) applied to animals. We considered the appearance of any of the following behaviours on application of the tip as a withdrawal response: (1) sharp retraction of abdomen; (2) immediate licking or scratching of site of application of the tip; (3) jumping; or (4) flinching the hind paw (Laird et al., 2001). Care was taken not to stimulate the same point in succession to avoid 'wind-up' effects or desensitization. We also avoided stimulation in the area of the external genitalia and concentrated on the lower to mid abdomen (Laird et al., 2001). We obtained the readings of nociceptive responses before animal treatment with HC-030031 (baseline or T0) and 12 h (T1) after the intraperitoneal administration of saline or ifosfamide. The results were obtained in grams for the difference (T0-T1) between the applied forces [mean ± standard error of mean (SEM)].

2.5 Mustard oil-induced colonic nociception

To assess the antinociceptive effect HC-030031 on mustard oil-induced colonic nociception, groups of mice groups ($n = 8$) were treated with HC-030031 (18.75, 37.0 or 75 mg/kg, p.o.) or vehicle (0.5% CMC in distilled water) 60 min before the intracolonic administration of mustard oil (Sigma-Aldrich, São Paulo, SP, Brazil; 0.75% in 0.9% saline, 50 μL/animal). A group of controls received a similar volume of saline (50 μL/colon). The sensitivity to mechanical stimulation of the abdomen was measured before animal treatment with HC-030031 (baseline or T0) 10 (T1) min after mustard oil instillation.

2.6 Writhing test

The writhing test was performed as previously described (Collier et al., 1968). Briefly, mice treated with 0.9% sterile saline (10 mL/kg, i.p.) or HC-030031 (18.75; 37.5 or 75.0 mg/kg, p.o.) were administered acetic acid 0.6% (10 mL/kg, i.p.), zymosan (1 mg/mL/cavity, i.p.) or misoprostol (1 μg/0.2 mL/cavity, i.p.) into the peritoneal cavity. The intensity of nociception was quantified by counting the cumulative number of writhes occurring between 0 and 30 min after stimulus injection.

2.7 Depletion of total peritoneal resident cells by peritoneal lavage

Swiss mice were anaesthetized with tribromoethanol and three hypodermic needles were inserted into the abdominal cavity. Sterile saline 10 mL was injected through the needle placed near the sternum. The abdominal cavity was then gently massaged for 1 min, and the peritoneal fluid was collected via the two needles inserted into the inguinal region. This operation was repeated three times. Control (sham) mice underwent insertion of the three needles and were

Table 1 HC-030031 does not protect against ifosfamide-induced haemorrhagic cystitis.

Experimental groups	Bladder wet weight	Macroscopic analysis		Microscopic analysis
		Oedema	Haemorrhage	
Saline (10 mL/kg)	20.3 ± 0,8	0 (0–0)	0 (0-0)	0 (0-0)
Ifosfamide (IFO 400 mg/kg)	44.0 ± 3,2*	2 (1–3)*	3 (1–3)*	2,5 (1–3)*
HC-030031 (75 mg/kg) + IFO	55.6 ± 5,1	2 (1–3)	2,5 (0–3)	3 (2–3)
HC-030031 (150 mg/kg) + IFO	39.1 ± 4,7	1 (0–3)	1,5 (0–3)	3 (2–3)
HC-030031 (300 mg/kg) + IFO	50.5 ± 7,7	1,5 (1–3)	2 (0–3)	3 (3-3)
Mesna (80 mg/kg) + IFO	27.0 ± 2,6**	0 (0–2) **	0 (0–2)**	1 (0–2)**

Data are presented as mean ± SEM for bladder wet weight or median values (minimum and maximum) for macroscopic and microscopic scores. Analyses were performed 12 h after ifosfamide injection (ANOVA and Student Newman–Keul's test – parametric data; Kruskal–Wallis and Dunn's test – non-parametric data).

*$p < 0.05$ versus saline.

**$p < 0.05$ versus IFO.

manipulated similarly, but no fluid was injected or withdrawn. Thirty minutes after recovering from sedation, the animals were injected with acetic acid 0.6% (10 mL/kg), zymosan (1 mg/cavity) or misoprostol (1 μg/cavity),and the number of writhes was counted in the interval between 0 and 30 min after injection of the stimuli (Souza et al., 1988).

2.8 Prostaglandin E₂ (PGE₂)-induced licking paw

PGE_2 (3 nmol/20 μL per paw) or saline (vehicle per paw) was injected under the plantar surface of the right hind paw (i.pl. injection) 60 min after HC-030031 (75 mg/kg, p.o.). The animals were allowed to individually adapt to the experimental area (transparent glass cylinders, 20 cm in diameter) for at least 20 min before algogen or vehicle injection. After injection, the mice were observed individually for 15 min, and the total time spent licking the injected paw recorded (Kassuya et al., 2007).

2.9 Open-field test

Mice were observed for spontaneous locomotion in an open-field apparatus for a period of 4 min. This test was performed 60 min after of oral administration of HC-030031 (75 mg/kg), vehicle (0.5% CMC in distilled water) or diazepam (5 mg/kg; Capaz et al., 1981).

2.10 Statistical analysis

Data were subjected to Student's t-test for unpaired values, One-way analysis of variance followed by Student Newman–Keul's test or Kruskal–Wallis followed by Dunn's test, as appropriate. Values were expressed as mean ± SEM (parametric data) or median (minimum-maximum) for non-parametric data. The level of significance was set at $p < 0.05$.

3. Results

3.1 HC-030031 is devoid of anti-inflammatory effect

Table 1 shows that ifosfamide induced a significant increase in bladder wet weight, macroscopic (oedema and haemorrhage) and microscopic scores when compared with saline ($p < 0.05$). HC-030031, in all doses tested (75, 150 and 300 mg/kg, p.o), failed to protect the animals against ifosfamide-induced damage ($p > 0.05$). In addition, the standard control, mesna (80 mg/kg, i.p.), significantly protected mice from the increase in bladder wet weight and the macroscopic (oedema and haemorrhage) and microscopic scores compared to the ifosfamide-injected group ($p < 0.05$).

3.2 Visceral antinociceptive effect of HC-030031

Fig. 1A shows that ifosfamide induced a significant increase in visceral nociception when compared with saline ($p < 0.05$). Additionally, HC-030031, in all doses tested (75, 150 and 300 mg/kg, p.o), and the standard control, mesna (80 mg/kg, i.p.), significantly protected mice from visceral nociception development ($p < 0.05$). Similar nociceptive response was found when mustard oil was administered into the colon of mice compared to saline ($p < 0.05$, Fig. 1C). Such response was significantly inhibited when mice were administered HC-030031 (37.5 and 75 mg/kg). We also observed that pretreatment with L-NAME (10 mg/kg, Fig. 1B) and morphine (5 mg/kg, Fig. 1D) prevented ifosfamide and mustard oil-induced nocice-
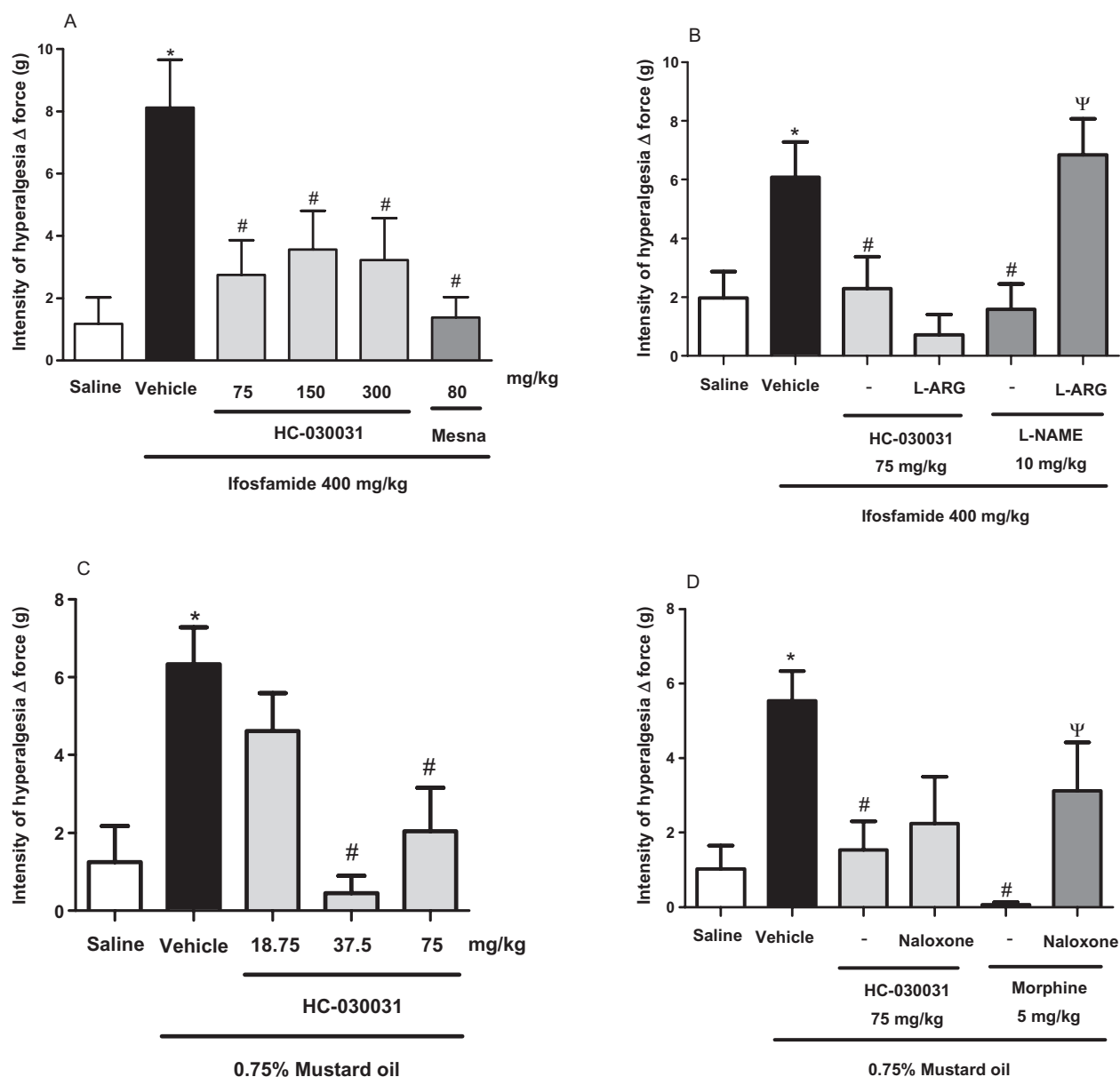
Figure 1 Effect of HC-030031 on visceral nociception: study of nitric oxide and opioid system involvement. The nociceptive response was assessed by the electronic von Frey test before animal treatments and 12 h after ifosfamide administration (A, B) or 10 min following mustard oil injection (C, D). Visceral nociception was prevented by HC-030031, which was unaffected by L-arginine (B) or naloxone pretreatment (D). The bars represent mean ± SEM of 6–8 mice. Intensity of hyperalgesia was expressed in grams (g). *$p < 0.01$ compared to saline group, #$p < 0.05$ versus ifosfamide or mustard oil group, Ψ $p < 0.05$ in comparison with L-NAME or morphine (ANOVA and Student Newman–Keul's test).

ption, respectively, which was markedly reverted ($p < 0.05$) by L-arginine (600 mg/kg) and naloxone (2 mg/kg). However, neither L-arginine nor naloxone affected the antinociceptive effect of HC-030031 ($p > 0.05$).

We further investigated the protective effect of HC-030031 in three viscero-somatic models of nociception using the writhing test initiated by different algogens. We found (Fig. 2A–C) that acetic acid, zymosan and misoprostol evoked pain-related behaviours (42.43 ± 3.07; 8.66 ± 2.17; and 5.57 ± 1.19, respectively), which were significantly inhibited by HC-030031 ($p < 0.05$) in the doses tested (18.75, 37.5 and 75 mg/kg). The maximal inhibitory potential varied according to the algogenic substance used and was higher against zymosan (84.1%, Fig. 2B) and
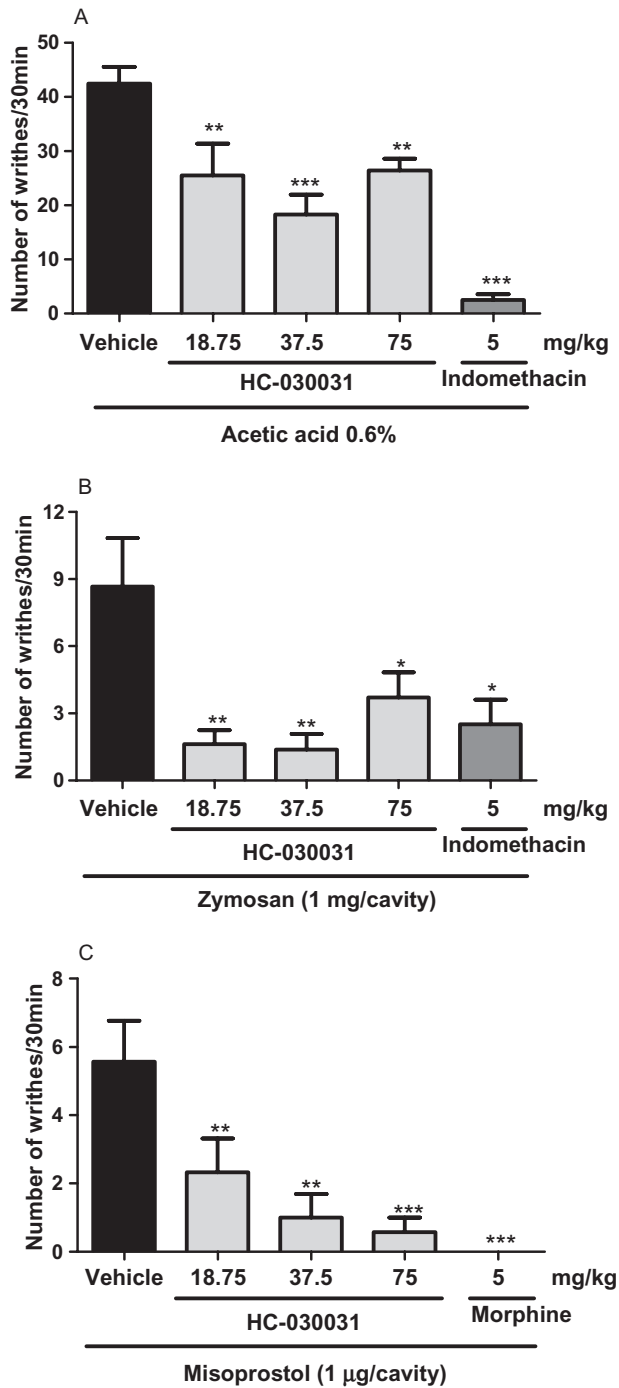
Figure 2 Antinociceptive effect of HC-030031 on acetic acid, zymosan and misoprostol-induced writhing behaviour. Swiss mice were pretreated with CMC, HC-030031, indomethacin or morphine 60 min previously the i.p. instillation of acetic acid (A), zymosan (B) or misoprostol (C). The bars represent mean ± SEM of the writhing behaviours expressed during 30 min ($n = 8$). $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ versus vehicle group (ANOVA and Student Newman–Keul's test).
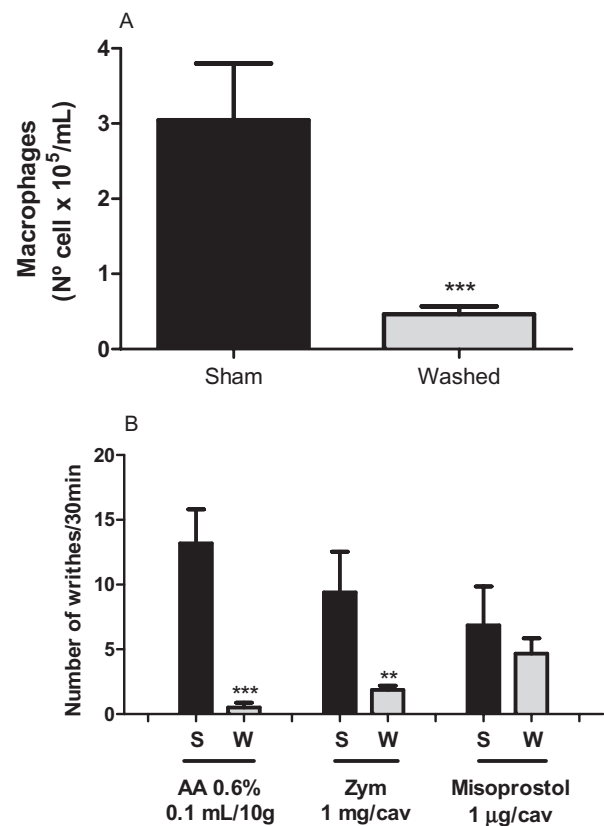

Figure 3 Effect of resident peritoneal cell depletion on acetic acid, zymosan or misoprostol-induced writhing behaviour. Anaesthetized mice had the peritoneal cavity perfused (W) with sterile saline. A no-washed group was included (sham). Following this procedure, heparinized PBS was instilled i.p. to perform the peritoneal cell count (A). Sham (S) and washed (W) groups ($n = 8$) were injected with acetic acid, zymosan or misoprostol. The number of writhes was recorded for 30 min (B). The bars represent mean ± SEM. $**p < 0.01$ and $***p < 0.001$ compared to sham group (unpaired Student's t-test).

misoprostol (89.7%, Fig. 2C) than acetic acid (57%, Fig. 2A). As positive controls for inhibition of nociception, indomethacin (in acetic acid- and zymosan-induced writhes) and morphine (misoprostol-induced nociception) showed markedly ($p < 0.05$) protective effects of 94%, 71.1% and 100%, respectively (Fig. 2A–C).

The role of resident macrophages in the writhing response was evaluated by washing the peritoneal cavity. To test the efficacy of this procedure, we first counted the number of cells in the peritoneum lavage. Fig. 3A shows a pronounced reduction ($p < 0.0004$) in peritoneal macrophage population (84.87%) when compared to non-washed cavities. Fig. 3 also shows that peritoneal-washed mice are less responsive ($p < 0.05$) to acetic acid (0.50 ± 0.38) and zymosan (1.86 ± 0.34) stimuli compared to sham animals
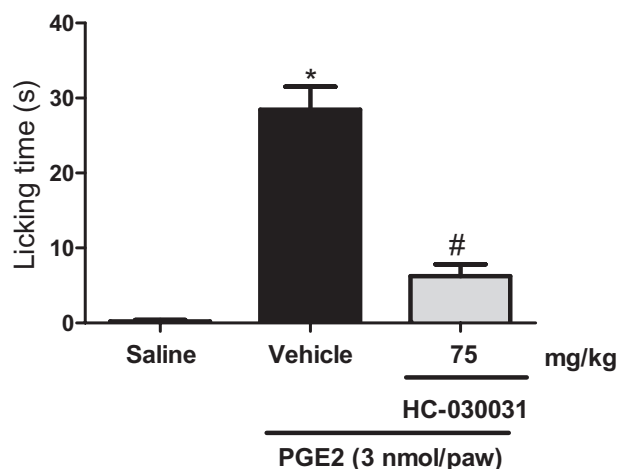
Figure 4 Effect of HC-030031 on paw PGE$_2$-induced nociception. Vehicle or HC-030031 treated animals were injected with saline or PGE$_2$ into the hind paw. Bars represent mean ± SEM time in seconds mice spent licking the injected paws. *$p < 0.001$ versus saline group and #$p < 0.01$ versus PGE$_2$ group (ANOVA and Student Newman–Keul's test).

(acetic acid: 13.17 ± 2.64; zymosan: 9.40 ± 3.14, Fig. 3B). However, in spite of the peritoneal lavage procedure, misoprostol was able to elicit similar nociceptive responses compared to the sham group ($p > 0.05$, Fig. 3B).

We further investigated whether HC-030031 would have an effect against a known direct nociceptor activator. As observed in Fig. 4, intraplantar PGE$_2$ caused a significant nociceptive behaviour compared to saline-injected mice ($p < 0.001$). Pretreatment with HC-030031 (75 mg/kg) significantly decreased the total time mice spent licking the PGE$_2$-injected paw (a 78.02% inhibition, $p < 0.001$).

3.3 Effect on locomotor activity in open-field test

To rule out the possible locomotor impairment due to HC-030031 treatment, we performed the open-field test. HC-030031 administration did not affect locomotion (Fig. 5) compared to vehicle-treated mice ($p > 0.05$). However, diazepam, a known sedative drug used as a positive control, significantly reduced ($p < 0.01$) the number of fields explored by the animals (41.74% inhibition, Fig. 5).

4. Discussion and conclusions

In this study, HC-030031, a TRPA1 receptor antagonist, was shown to have antinociceptive effects on

different models of visceral pain. In addition, no anti-inflammatory activity was observed for that substance.

Haemorrhagic cystitis is a common inflammatory condition due to bladder accumulation of acrolein, an urotoxic substance produced by liver metabolism of the anticancer agents, cyclophosphamide and ifosfamide (Cox, 1979). Some studies report that acrolein is a potential activator of TRPA1 receptors (Bautista et al., 2006). Thus, we initially investigated whether the ifosfamide-induced inflammatory response would be dependent on TRPA1 receptors. We observed that HC-030031, even at doses as high as 300 mg/kg, did not inhibit bladder damage. However, mesna, the only clinically available drug used to prevent haemorrhagic cystitis, afforded a clear anti-inflammatory effect. Therefore, haemorrhagic cystitis-associated inflammation is unlikely related to TRPA1 activation by acrolein, but it is probably related to direct tissue damage and resident cell stimulation by this metabolite. However, the role of TRPA1 in inflammation is still possible since it is expressed in capsaicin-sensitive primary afferent neurons known to contribute to neurogenic inflammation that result in increased vascular permeability and oedema formation (Silva et al., 2011).

Patients with bladder injury due to cancer treatment may also exhibit abdominal pain. Then, we further tested the antinociceptive activity of HC-030031 in the model of haemorrhagic cystitis-induced nociception (Olivar and Laird, 1999). We found that HC-030031 markedly inhibited ifosfamide-induced nociception, possibly suggesting that acrolein would be responsible for TRPA1 activation (Bautista et al., 2006).
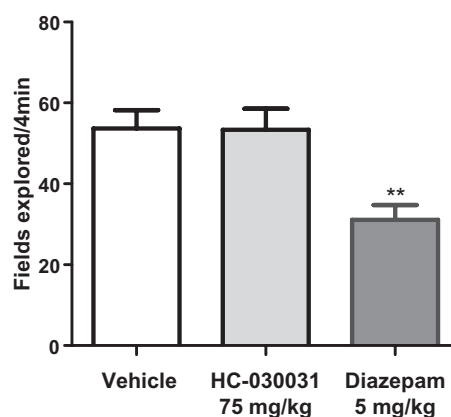

Figure 5 HC-030031 does not cause ambulation impairment. Vehicle, HC-030031 or diazepam treated mice ($n = 8$) were observed for 4 min regarding the ambulation in the open-field arena. **$p < 0.01$ compared to vehicle group. The bars represent the mean ± SEM number of fields explored (ANOVA and Student Newman–Keul's test).

Considering the well-known participation of NO in the haemorrhagic cystitis animal model (Souza-Filho et al., 1997), we decided to use such animal model to study the involvement of this mediator in the anti-nociceptive effect of HC-030031. It has been suggested that endogenous NO is involved in the inflammatory events leading to haemorrhagic cystitis after cyclophosphamide administration. Additionally, the role for peripherally acting NO in hyperalgesia is supported by pharmacological data (reviewed by Miyamoto et al., 2009). Furthermore, Miyamoto et al. (2009) provided genetic and pharmacological evidence that NO donors activate TRPV1 and TRPA1 both in heterologous systems and in naïve dorsal root ganglion (DRG) neurons. Thus, we investigated whether L-arginine, a NO synthesis precursor, would be able to reverse the effect of HC-030031 upon noxious stimulation due to ifosfamide injection. We found that a high dose of L-arginine failed in that regard. However, L-NAME administration led to a prominent antinociceptive activity, which was prevented by L-arginine pretreatment. The present finding is the first report regarding the role of NO in ifosfamide-induced visceral nociception. Notwithstanding previous results, we cannot rule out the modulation of TRPA1 function by inflammatory mediators. A recent published article suggests that endogenous activation of TRPA1 plays a critical role in the development of tumour necrosis factor-alpha (TNF-α)-induced mechanical hyperalgesia (Fernandes et al., 2011). Wang et al. (2008) also suggested that bradykinin released in response to tissue inflammation might trigger the sensation of pain by TRPA1 activation. It is also proposed that TRPA1 is involved in ET-1-induced spontaneous pain-like behaviour in mice (Liang et al., 2010). Therefore, the pathways by which TRPA1 is activated in haemorrhagic cystitis merit further investigation.

We further evaluated the protective effect of HC-030031 in the model of mustard oil-induced visceral nociception (Laird et al., 2001). Mustard oil is a known TRPA1 activator (Macpherson et al., 2007). Cattaruzza et al. (2010) reported that TRPA1 is expressed by DRG neurons that innervate the colon and that TRPA1 mediates inflammatory hyperalgesia of the colon. In our study, we showed that even low doses of the TRPA1 blocker, HC-030031 (37.5 and 75 mg/kg), significantly inhibited the noxious response elicited by the intracolonic instillation of mustard oil. These low doses, which are in a 1:2 dilution scale, were chosen considering the pronounced result found in the haemorrhagic cystitis model of nociception. Our results are corroborated by previous results, which demonstrated that higher doses of

HC-030031 (300 mg/kg) reduce the spinal c-Fos response to intracolonic allyl isothiocyanate (AITC), the active compound of mustard oil (Mitrovic et al., 2010). This indicates that the effect of AITC is mediated by TRPA1. Kondo et al. (2010) suggested that in response to noxious gastric distension, generator potentials mediated via TRPA1 may promote peripheral action potentials, which in turn result in the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK 1/2) in DRG and/or spinal neurons leading to visceral pain. In a review published by Engel et al. (2011), it is reported that the continuous sensitization of TRPA1 by inflammatory mediators is found to be essential for colitis induction in the model of TNBS colitis through the release of neuropeptides. These neuronal mediators could be responsible for maintaining the inflammatory process (Engel et al., 2011) and hyperalgesia (Kondo et al., 2010).

It is currently unknown whether the antinociceptive action of HC-030031 would partially involve the opioid system. Taking into account that morphine effectively inhibits the noxious response due to the intracolonic instillation of mustard oil (Laird et al., 2001), we used this animal model to evaluate the participation of the opioid system on the antinociceptive effect of HC-030031. To test this hypothesis, we treated animals with the opioid receptor antagonist, naloxone, before TRPA1 blocker administration. As observed, no reversal effect was detected. However, the effect of the positive control, morphine, was prevented by the opioid receptor antagonist. These findings possibly exclude the participation of the opioid system in the antinociceptive action of HC-030031.

We also evaluated the antinociceptive effect of HC-030031 in a viscero-somatic model of nociception (Laird et al., 2001) induced by three different algogens: acetic acid, zymosan and misoprostol, a PGE_1 stable analogue. We found that HC-030031 in all doses tested markedly inhibited the noxious response elicited by these agents. The literature reports that the release of TNF-α initiates the release of interleukin-1β and interleukin-8, which in turn liberate cyclooxygenase metabolites and sympathomimetic amines, respectively (Ferreira, 1993). Both cyclooxygenase products and sympathomimetic amines play a role in the development of the persistent nociceptor hypersensitivity induced by TNF-α (Ferreira, 1993; Sachs et al., 2002).

In a paper published by Ribeiro et al. (2000), the authors clearly suggested that the nociceptive activity of zymosan and acetic acid in the writing model may be due to the release of TNF-α, interleukin-1β and interleukin-8 by resident peritoneal macrophages and

mast cells. Such conclusions were partially achieved by reducing or enhancing the peritoneal cell population. We similarly verified the mild nociceptive activities of zymosan and acetic acid in animals depleted of peritoneal resident cells. In addition, indomethacin, a non-specific cyclooxygenase inhibitor, markedly prevented the zymosan and acetic acid-related writhes, suggesting that neosynthesized prostaglandins are involved in this nociceptive effect. We also evaluated the contribution of peritoneal cells to misoprostol-induced nociception. Accordingly fitting with results reported by Ribeiro et al. (2000) using iloprost, a stable analogue of prostacyclin, we showed that lavage of the cavities did not affect the nociceptive response to misoprostol. Therefore, these results suggest that misoprostol, like iloprost, may activate the nociceptor directly (Ferreira et al., 1978). Besides, morphine, a potent nociceptor stabilizer, was able to prevent neuron activation due to misoprostol injection. To support the suggestion that HC-030031 may stabilize the nociceptor directly, we further investigated its effect in the PGE$_2$-induced paw nociception (Kassuya et al., 2007). In accordance, HC-030031 significantly prevented PGE$_2$-induced nociceptive licking paw behaviour.

Taking into account that the sensitization of primary sensory neurons involves an inflammatory cascade, the modulation of nociception could be reached by the inhibition of any intermediate mediator, including TNF-α, or by stabilising the nociceptor directly. In our opinion, HC-030031 would be acting through the later mechanism due to two main reasons: (1) The nociceptive effect of PGE$_2$ and of a PGE$_1$ stable analogue, misoprostol, was both abolished by HC-030031. Therefore, the antinociceptive activity of HC-030031 is not due to an inhibitory effect on prostaglandin release; (2) peritoneal lavage did not prevent the nociceptive capacity of misoprostol. Thence, in this experimental condition, misoprostol did not induce the release of inflammatory mediators by resident peritoneal cells. Altogether, these data point out that the antinociceptive action of HC-030031 results from direct nociceptor stabilization and seems to be independent of the inhibition of inflammatory resident cells.

In addition, our study showed that HC-030031 did not impair locomotion performance in open-field tests with mice. However, diazepam, a sedative drug adopted as a positive control, markedly inhibited mice locomotion. Eid et al. (2008) also demonstrated that HC-030031, even at higher doses (300 mg/kg), failed to have any effect on rotarod response. These results indicate that the antinociceptive effect of this TRPA1 antagonist is not due to animal sedation.

In summary, our results suggest that the antinociceptive effect of HC-030031 in various animal models of visceral pain seems to be independent of the inhibition of inflammatory resident cells, NO and opioid pathways. We also showed that HC-030031 likely acts as a direct nociceptor stabilizer. This study provides perspective for the effective management of visceral pain through the modulation of TRPA1 channels. However, the mechanisms that underlie the involvement of TRPA1 receptors and its regulation by inflammatory mediators need further investigation.

Author contributions

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