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Analgesic effect of TT-232, a heptapeptide somatostatin analogue, in acute pain models of the rat and the mouse and in streptozotocin-induced diabetic mechanical allodynia

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Abstract

Somatostatin released from capsaicin-sensitive sensory nerves exerts systemic anti-inflammatory and antinociceptive actions. TT-232 is a stable, peripherally acting heptapeptide (D-Phe-Cys-Tyr-D-Trp-Lys-Cys-Thr-NH_2) somatostatin analogue with highest binding affinity for somatostatin sst_4 receptors. It has been shown to inhibit acute and chronic inflammatory responses and sensory neuropeptide release from capsaicin-sensitive nociceptors. In the present study the antinociceptive effects of TT-232 were analysed using both acute and chronic models of nociception. Formalin-induced pain behaviour, noxious heat threshold and streptozotocin-induced diabetic neuropathic mechanical allodynia were examined in rats and phenylquinone-evoked abdominal constrictions were tested in mice. TT-232 (80 μg/kg i.p.) inhibited both early (0–5 min) and late phases (25–45 min) of formalin-induced nociception as revealed by determination of the composite pain score. The minimum effective dose to elevate the noxious heat threshold and diminish the heat threshold drop (heat allodynia) evoked by resiniferatoxin (0.05 nmol intraplantarly) was 20 and 10 μg/kg i.p., respectively, as measured by an increasing-temperature hot plate. TT-232 (10–200 μg/kg s.c.) significantly inhibited phenylquinone-evoked writhing movements in mice, but within this dose range no clear dose–response correlation was found. Five weeks after streptozotocin administration (50 mg/kg i.v.) the diabetes-induced decrease in the mechanonociceptive threshold was inhibited by 10–100 μg/kg i.p. TT-232. These findings show that TT-232 potently inhibits acute chemical somatic/visceral and thermal nociception and diminishes chronic mechanical allodynia associated with diabetic neuropathy, thereby it could open new perspectives in the treatment of various pain syndromes.

Keywords: Somatostatin; Analgesic effect; Formalin test; Writhing test; Thermal nociception; Diabetic neuropathy

1. Introduction

In the peripheral sensory nervous system somatostatin is stored in the capsaicin-sensitive subpopulation of nociceptive afferents which express the transient receptor potential vanilloid 1 (TRPV1) capsaicin receptor (Caterina and Julius, 2001; Szolcsányi, 2002; Clapham et al., 2003; Szolcsányi et al., in press). It has been recently discovered that in response to various stimuli somatostatin is released from these capsaicin-sensitive nociceptors, reaches the circulation and exerts a systemic "sensocrine function" which manifests itself throughout the body in antinociceptive and anti-inflammatory actions (Szolcsányi et al., 1998a,b, in press;

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Helyes et al., 2000, 2001, 2004; Than et al., 2000; Carlton et al., 2001a,b).

On the basis of this mechanism a potential target on somatostatin sst_4 receptor on nociceptive nerve terminals (Hoyer et al., 1995) for novel anti-inflammatory and analgesic drugs was suggested. This was supported by the potent anti-inflammatory and antihyperalgesic effects of a stable somatostatin analogue, TT-232 (Helyes et al., 2000, 2001; Pintér et al., 2002). This heptapeptide with a cyclopentane ring (D-Phe-Cys-Tyr-D-Trp-Lys-Cys-Thr-NH_2) synthesized in our laboratories is a rather selective somatostatin sst_4 receptor agonist (H. Schmidt and D. Hoyer, unpublished observation) devoid of endocrine effects. It failed to diminish growth hormone or gastrin secretion in vivo and it had been initially introduced as an antitumour drug with tyrosine kinase inhibitory action (Kéri et al., 1993, 1996). Whole-body autoradiography in female Wistar rats showed low concentrations in the brain (about 0.1 μg/g) 30 min after a single 2 mg/kg i.v. administration of ^{14}C-TT-232 indicating a very weak penetration of the compound through the blood–brain barrier, central nervous system effects have not been observed even after 5 mg/kg i.v. administration (unpublished data, obtained from the preclinical documentation of the compound).

The broad spectrum of anti-inflammatory actions of TT-232 was shown in several acute and chronic inflammatory models in rats and mice and these effects include potent inhibition of neurogenic inflammation and the articular cartilage and bone destruction in Freund's adjuvant-induced chronic arthritis (Pintér et al., 2002; Helyes et al., 2001, 2004).

The present study aimed at analysing the antinociceptive effect of TT-232 in several conventional and two novel nociceptive tests in rodents.

2. Materials and methods

2.1. Animals

Experiments were performed on Wistar rats of both sexes and male Balb/c mice (Charles Rivers, Budapest, Hungary), which were kept in the Laboratory Animal Centre of the University of Pécs under pathogen free conditions at 24–25 °C and provided with standard rat chow and water ad libitum.

2.2. Drugs and chemicals

TT-232 was synthesized by the Peptide-biochemistry Research Group of Hungarian Academy of Sciences in the Department of Medicinal Chemistry, Semmelweis University, Budapest, Hungary. Formalin (Formaldehydum solutum 37%; Ph.Hg. VII.) was purchased from the Pharmacy of the University of Pécs. Resiniferatoxin, streptozotocin and phenylquinone were purchased from Sigma (St. Louis, MO, USA). Pentobarbital sodium (Nembutal) was bought from May and Baker (UK) and diclofenac sodium from RBI (Natick, MA, USA).

2.3. Investigation of formalin-induced acute nociception

Formalin (50 μl, 2.5%) injected s.c. into the plantar surface of the left hindpaw induced nocifensive reactions in two phases, the first of which (0–5 min) is thought to be due to a direct chemonociceptive effect of formalin, while the second one (20–45 min) is mainly mediated by inflammatory reactions (for review Tjolsen et al., 1992). Nocifensive behaviour of male Wistar rats (180–220 g) was quantitatively evaluated by the duration of paw liftings and the duration of paw lickings, and "Composite Pain Score" (CPS=(1×duration of paw liftings+2×duration of paw lickings in s)/duration of examination period) was calculated (Watson et al., 1997). TT-232 (20–80 μg/kg) or its solvent was injected i.p. 30 min before formalin administration. For determining statistically significant differences the Mann–Whitney *U*-test was used (n=9–10/group; $^*P<0.05$, $^{**}P<0.01$ compared to the solvent-treated control group).

2.4. Phenylquinone-evoked abdominal writhing test

Phenylquinone (0.02% dissolved in distilled water, 0.2 ml) was injected i.p. to elicit abdominal constriction responses in Balb/c mice. After challenge, the animals were placed in a transparent plastic box and their responses were counted during continuous observation for 20 min. TT-232 (5–200 μg/kg) or its solvent was administered s.c. 30 min before phenylquinone treatment. For determining statistically significant differences the Mann–Whitney *U*-test was used (n=9–10/group; $^*P<0.05$, $^{**}P<0.01$ compared to the solvent-treated control group).

2.5. Measurement of the noxious heat threshold and resiniferatoxin-induced heat allodynia

The noxious heat threshold of female Wistar rats (140–180 g) was determined by a computer-controlled increasing-temperature hot plate (Supertech, Pécs, Hungary) which has recently been validated (Almási et al., 2003). The animal was placed onto the plate the temperature of which was linearly increased from 30 °C until the animal showed nocifensive behaviour confined to either hindpaw (licking or lifting). The corresponding plate temperature was regarded as the noxious heat threshold. After conditioning and control measurements, TT-232 (10–200 μg/kg) or its solvent was administered i.p. and threshold determination was repeated 30 min later. The heat thresholds before and after TT-232 or its solvent administration were compared using the Student's *t*-test for paired samples.

Heat allodynia was evoked by intraplantar injection of resiniferatoxin (0.05 nmol), a potent agonist of the capsaicin TRPV1 receptor. Resiniferatoxin induced a 8–10 °C drop of

heat threshold 5 min after administration, which gradually disappeared in 20 min (Almási et al., 2003). One half of the group of animals was pretreated with TT-232 and the other with solvent i.p. 10 min before resiniferatoxin application. For statistical comparison of the resiniferatoxin-induced threshold drops at 5 min in the TT-232- and solvent-treated animals, the Student's *t*-test for unpaired samples was used (n=8–12/group; *P<0.05, **P<0.01). The inhibitory effect of TT-232 on the resiniferatoxin-induced heat allodynia was expressed as percentage inhibition according to the following formula: $(Drop_{solv}-Drop_{drug})/Drop_{solv}\times 100$ where $Drop_{solv}$ and $Drop_{drug}$ refer to the average of the resiniferatoxin-induced threshold drop at 5 min measured in the solvent-and TT-232-treated animals, respectively.

2.6. Examination of neuropathic mechanical allodynia in diabetic rats

Experimental diabetes was induced in male Wistar rats (180–210 g) by a single i.v. injection of 50 mg/kg streptozotocin (*N*-[methylnitrosocarbamoyl]-D-glucosamine, streptozotocin; Courteix et al., 1993; Miki et al., 2001; Khan et al., 2002). Streptozotocin was prepared freshly by dissolving in 0.9% sterile saline. Glucose levels of blood samples taken from the tail vein were measured by Accu-Check glucometer (Roche) and plasma insulin concentrations were determined with ^{125}I-insulin radioimmunoassay kit (Institute of Isotopes, Budapest) 2 weeks later to confirm the development of diabetes. Rats with plasma glucose concentration above 15 mM were considered diabetic (Chen and Levine, 2001).

The mechanonociceptive threshold of the hindpaw was measured with the Ugo Basile Dynamic Plantar Aesthesiometer and the drop of mechanical threshold (allodynia) was expressed in % compared to the initial control values. For determining statistically significant differences the Mann–Whitney *U*-test was used (n=6–9/group; *P<0.05, **P<0.01 compared to the solvent-treated control group).

2.7. Ethics

All experimental procedures were carried out according to the Animals (Scientific Procedures) Act 1998 (Hungary) and complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983). The studies were approved by the Ethics Committee on Animal Research of the University of Pécs.

3. Results

3.1. Effect of TT-232 on formalin-induced acute chemical nociception

The antinociceptive effect of TT-232 (20–160 μg/kg i.p.) and diclofenac (10 and 50 mg/kg i.p.) as a reference drug were assessed on the characteristic two phases (Tjolsen et al., 1992) of the formalin test. In the first phase the nocifensive behaviour as expressed in CPS was slightly and nonsignificantly inhibited except for the dose of 80 μg/kg which induced a significant, 55% inhibition. Diclofenac at the applied doses failed to diminish the first phase of formalin-induced nociceptive behaviour (Fig. 1).

In the second phase TT-232 induced an inhibition with a bell shaped dose–response relationship. The highest antinociceptive effect (66% inhibition) was achieved at the dose of 80 μg/kg. Antinociception induced by TT-232 at a dose of 40 μg/kg was similar to that produced by 50 mg/kg diclofenac, so the compound was about 1000 times more potent than the reference drug (Fig. 1).

3.2. Effect of TT-232 on phenylquinone-evoked abdominal writhing motion

TT-232 (10–200 μg/kg) administered s.c. 30 min before phenylquinone exposure significantly diminished the number of writhes compared to the solvent-treated control group, but a clear dose–response relationship could not be detected in this model (Fig. 2). The highest antinociception was achieved at doses of 20 and 200 μg/kg (70% and 75% inhibition, respectively), but similarly to the formalin test an inhibition with a bell-shaped dose–response correlation occurred in the dose range of 5–100 μg/kg. (Fig. 2).

3.3. Effect of TT-232 on the noxious heat theshold and resiniferatoxin-induced heat allodynia

The control noxious heat threshold of female rats was 44.5±0.2 °C. TT-232 induced a significant elevation of the heat threshold in the dose range of 20–200 μg/kg without

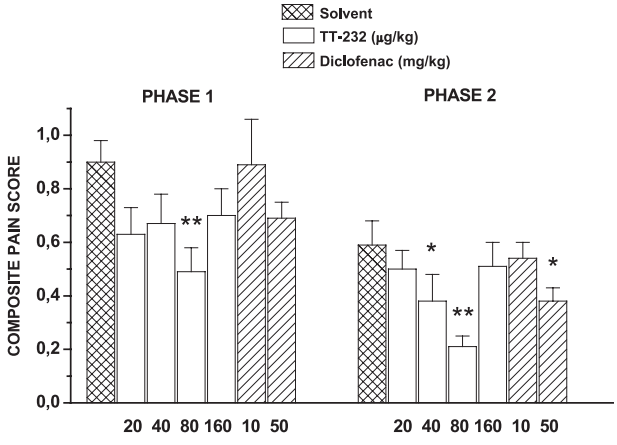

Fig. 1. Effect of i.p. injection of TT-232 and diclofenac as a reference drug on formalin-induced acute nociception in the rat. Results are shown as mean composite pain scores (CPS=(1× duration of paw liftings+2× duration of paw lickings in s)/duration of examination period) with S.E.M. of n=9–10 experiments/group. The Mann–Whitney *U*-test was used for statistical analysis (*P<0.05, **P<0.01 compared to the solvent-treated control group).

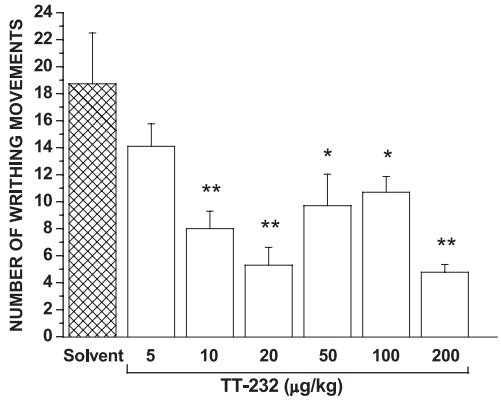

Fig. 2. Effect of TT-232 (s.c.) on the phenylquinone-evoked writhing movements of the mouse. Results are shown as means of the numbers of abdominal contractions with S.E.M. of n=9–10 experiments/group. The Mann–Whitney U-test was used for statistical analysis (*P<0.05, **P<0.01 compared to the solvent-treated control group).

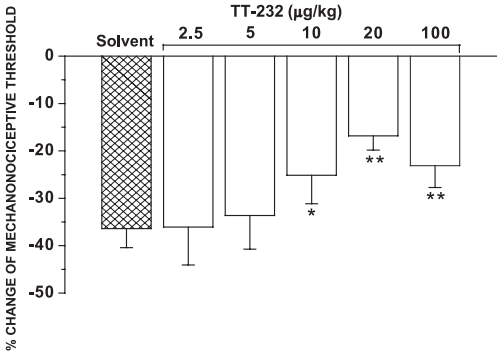

Fig. 4. Effect of TT-232 (i.p.) on diabetic mechanical allodynia, which is expressed as percentage change of the mechanonociceptive threshold of the hindpaws compared to the values measured before diabetes induction. Results are means with S.E.M. of n=6–9 experiments/group. The Mann–Whitney U-test was used for statistical analysis (*P<0.05, **P<0.01 compared to the solvent-treated control group).

any clear dose-dependent relationship. The solvent failed to alter the noxious heat threshold (Fig. 3A).

In the resiniferatoxin-induced heat allodynia model (Almási et al., 2003) intraplantar injection of resiniferatoxin evoked a 7.39±1.3 °C decrease in the heat threshold 5 min after administration. By pretreatment of the rats with TT-232 30 min before the resiniferatoxin injection, this drop of noxious heat threshold was markedly and significantly diminished in the dose range of 10–50 µg/kg i.p. A smaller dose (5 µg/kg) was ineffective and the highest dose of 100 µg/kg induced an about 50%, but non-significant inhibition (Fig. 3B).

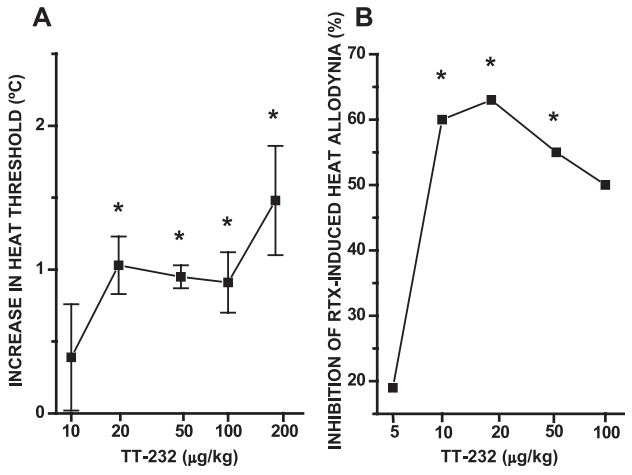

Fig. 3. Effect of TT-232 (i.p.) on the noxious heat threshold (A) and the resiniferatoxin (RTX)-induced drop of heat threshold (heat allodynia, B). Data are means with S.E.M. of 8–12 animals. As the solvent failed to sigificantly alter the heat threshold (data not shown) statistical analysis was performed by comparing thresholds before and after TT-232 administration using the Student's t-test for paired samples. Percentage inhibition of heat allodynia was calculated by comparison of heat threshold values at 5 min after resiniferatoxin injection in the TT-232- and solvent-treated groups (see Methods). For statistical comparison of the resiniferatoxin-induced threshold drop at 5 min in the TT-232-and solvent-treated animals the Student's t-test for unpaired samples was used; *P<0.05, **P<0.01.

3.4. Effect of TT-232 on neuropathic mechanical allodynia in diabetic rats

In non-diabetic rats i.p. administration of 20 µg/kg TT-232 failed to alter the mechanonociceptive thresholds of the hindpaw (data not shown).

After streptozotocin pretreatment (50 mg/kg i.v.) diabetic rats displayed polyuria, reduced growth rate, and a marked increase in water and food intake, but otherwise appeared normal. All the animals treated with streptozotocin developed diabetes in this study, the blood glucose level was 23.6±2.8 mmol/l and the insulin concentration was 8.48±0.2 µIU/ml 2 weeks after streptozotocin administration. No rats were excluded from the experiments due to bad general condition. Five weeks after the induction of diabetes the mechanonociceptive threshold decreased by 28.6±3.1%. Injection of the solvent of TT-232 failed to significantly influence this allodynia. TT-232 at doses of 10–100 µg/kg induced a pronounced inhibition of mechanical allodynia, while lower doses were ineffective. The minimum effective dose of TT-232 was 10 µg/kg and the maximum effect was achieved at 20 µg/kg (Fig. 4).

4. Discussion

The present findings clearly show that the stable heptapeptide somatostatin analogue TT-232 acting at the periphery (Kéri et al., 1993, 1996; Helyes et al., 2001; Pintér et al., 2002) elicits pronounced antinociception. The minimum effective dose of 10–40 µg/kg in two conventional acute nociceptive tests (Le Bars et al., 2001) as the formalin test in the rat (Tjolsen et al., 1992) and phenylquinone writhing test of mice (Hendershot and Forsaith, 1959) as well as in two novel noxious heat threshold tests (Almási et al., 2003) indicates its high potency in two species. In the formalin test TT-232 was about 1000 times more potent than diclofenac. Furthermore, the compound

inhibited and reversed the streptozotocin-induced chronic diabetic mechanical allodynia.

In earlier studies the anti-inflammatory effect of TT-232 was analysed in detail with particular attention to its remarkable inhibitory effect on neurogenic inflammation which is mediated by capsaicin-sensitive nerve endings and which could not be inhibited by cyclooxygenase inhibitors (Helyes et al., 2001, 2004; Pintér et al., 2002). These results revealed that neurogenic inflammation elicited by mustard oil or capsaicin in the rat and mice, respectively, and non-neurogenic dextran oedema in denervated paw of the rat were dose-dependently diminished by TT-232. Acute inflammatory oedema and plasma extravasation evoked by carrageenan, bradykinin, as well as cutaneous neutrophil accumulation after injection of carrageenan or interleukin-1β were dose-dependently inhibited. Freund's adjuvant-induced inflammatory oedema, damage of the arthritic tissues and the accompanying allodynia all were diminished by TT-232. Furthermore, particularly striking was the reversal of mechanical allodynia induced by partial sciatic nerve injury (Helyes et al., 2001, 2004; Pintér et al., 2002). In vitro evidence has been presented that the compound inhibits the release of sensory neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) from capsaicin-sensitive nerve terminals (Pintér et al., 2002).

The following data support the conclusion that the anti-inflammatory effect of the compound is due to an agonistic action on the G-protein-coupled somatostatin sst_4 receptor which inhibits the function of the capsaicin-sensitive nerve terminals. (1) Binding studies with TT-232 to displace somatostatin (somatotropin release inhibiting factor-28; SRIF-28) from somatostatin sst_{1-5} receptor binding sites indicated that the highest affinity of the compound was for the somatostatin sst_4 receptor (H. Schmidt and D. Hoyer, unpublished observation). (2) Involvement of G-proteins in its inhibitory action on the release of sensory neuropeptides was shown by blocking this effect by pertussis toxin (Pintér et al., 2002). (3) TT-232 failed to bind to pituitary membrane preparation, to inhibit growth hormone release and gastric acid secretion indicating that its actions are not mediated by somatostatin sst_2, sst_3 and sst_5 receptors (Kéri et al., 1996). (4) TT-232 can mimic the inhibitory action of somatostatin (10 μg/kg i.p.) on mustard oil-induced neurogenic inflammation (Helyes et al., 2001).

TT-232 has been shown to diminish neurogenic inflammation (Helyes et al., 2001, 2004; Pintér et al., 2002; Szolcsányi et al., in press) mediated by capsaicin-sensitive sensory nerve endings which express the TRPV1 receptor/cation channel (Caterina and Julius, 2001; Szolcsányi, 2002; Clapham et al., 2003). The TRPV1 capsaicin receptor/ion channel is gated by noxious heat, protons and sensitized or activated by various endogenous pain-producing substances (bradykinin, lipoxgenase products) through an intracellular signal transduction pathway (Cesare et al., 1999; Hwang et al., 2000; Liang et al., 2001). These noxious heat-activated peptidergic capsaicin-sensitive nociceptors in the rat and rabbit correspond to the polymodal nociceptors supplied by both C and A-delta fibers (Szolcsányi, 1987; Szolcsányi et al., 1988). Inhibition of the depolarization of these nociceptive nerve endings diminishes both the release of sensory neuropeptides and spike generation needed for nociception. The present study revealed that TT-232 elevated the noxious heat threshold and inhibited the heat allodynia induced by the potent TRPV1 receptor agonist resiniferatoxin (Caterina and Julius, 2001; Szolcsányi, 2002).

The heat threshold-elevating action of TT-232 is in full accord with the studies of Carlton et al. (2001a,b) in which somatostatin receptor agonism inhibited discharge activity of cutaneous polymodal nociceptors evoked by noxious heat stimulation. In their work activation of somatostatin receptors also inhibited the heat sensitization of cutaneous nociceptors evoked by bradykinin superfusion (Carlton et al., 2001a,b) which is paralelled by the ability of TT-232 to diminish resiniferatoxin-induced heat allodynia revealed in the present experiments. The broad spectrum of the antinociceptive actions of TT-232 could theoretically be due to a local anaesthetic-like action, i.e. a direct inhibitory effect on axonal conduction. However, it failed to elevate the mechanonociceptive threshold of untreated rats, which strongly argues against this possibility.

At the cellular level somatostatin can open various K^+ channels and inhibit voltage-gated Ca^{2+} channels which result in inhibition of both spike generation and release of neurotransmitters (Weckbecker et al., 2003). In addition somatostatin sst_4 receptor agonists enhance signalling through mitogen-activated protein kinase (MAPK), phospholipase C and phospholipase A_2 and activate/inhibit phosphotyrosine phosphatases (Weckbecker et al., 2003). The effect of TT-232 on intracellular signal transduction was analysed in several tumour cell lines and the slowly developing tyrosine kinase inhibition seemed to play a pivotal role in its antitumour effect (Kéri et al., 1993, 1996). Early responses of these cells resembled somatostatin sst_4 receptor agonism, since the compound activated both the phosphotyrosine phosphatases and some protein kinases like the extracellular signal-regulated kinase (ERK2/MAPK) (Vantus et al., 1995, 2001). Attenuation of neurogenic inflammation by TT-232 is unlikely to involve tyrosine kinase inhibition, because the release of its mediators, substance P and CGRP, was not diminished by the tyrosine kinase inhibitor genistein (Pintér et al., 2002). In the antinociceptive action of TT-232, however, tyrosine kinase inhibition or dephosphorylation of the TRPV1 receptor might play a role. Tyrosine kinase A signals the nerve growth factor-induced sensitization of nociceptors to heat within minutes (Lewin et al., 1993) and dephosphorylation of the TRPV1 receptor induces antinociception (Caterina and Julius, 2001; Szolcsányi, 2002).

The anti-inflammatory effect of TT-232 increased by elevating the dose (Helyes et al., 2001; Pintér et al., 2002), but in the case of its antinociceptive action a bell shaped

dose–response relationship was observed in the formalin test and resiniferatoxin-induced heat allodynia. This unusual dose–response relationship was less pronounced when examining heat threshold, writhing behaviour and diabetic allodynia. Further experiments are in progress to shed light on the possible contribution of protein dephosphorylation in sensory nerve endings to the analgesic effect of TT-232 and to reveal how these mechanisms contribute to its lower antinociceptive effect at higher doses in several experimental models. Nevertheless, the broad antinociceptive spectrum and high potency of the compound combined with an anti-inflammatory action in a large scale of acute and chronic inflammatory conditions makes this lead molecule very promising for drug development, particularly in the treatment of the pain associated with diabetic neuropathy (Duby et al., 2004) in which the analgesic effect of opiates is often compromised (Dellemijn, 1999).

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