



## Eriodictyol: A flavonoid antagonist of the TRPV1 receptor with antioxidant activity

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### ABSTRACT

The transient potential vanilloid 1 receptor (TRPV1) is a calcium-permeable channel responsible for the transduction and modulation of acute and chronic pain signaling. As such, this receptor is a potential target for the treatment of a number of pain disorders. However, AMG517, a TRPV1 antagonist, presents several clinical limitations that include the induction of severe hyperthermia. The aim of this study was to investigate the possible interaction of the flavonoid eriodictyol with the TRPV1 receptor and to determine its putative antinociceptive and hyperthermic effects. Eriodictyol was able to displace [<sup>3</sup>H]-resiniferatoxin binding (IC<sub>50</sub> = 47; 21–119 nM) and to inhibit calcium influx mediated by capsaicin (IC<sub>50</sub> = 44; 16–125 nM), suggesting that eriodictyol acts as a TRPV1 antagonist. Moreover, eriodictyol induced antinociception in the intraplantar capsaicin test, with maximal inhibition of 49 ± 10 and 64 ± 4% for oral (ID<sub>50</sub> = 2.3; 1.1–5.7 mg/kg) and intrathecal (ID<sub>50</sub> = 2.2; 1.7–2.9 nmol/site) administration, respectively. Eriodictyol did not induce any change in body temperature or locomotor activity. Orally administered eriodictyol (4.5 mg/kg) prevented the nociception induced by intrathecal injections of capsaicin, as well as the non-protein thiol loss and 3-nitrotyrosine (3-NT) formation induced by capsaicin in spinal cord. Eriodictyol also reduced the thermal hyperalgesia and mechanical allodynia elicited by complete Freund's adjuvant (CFA) paw injection. In conclusion, eriodictyol acts as an antagonist of the TRPV1 receptor and as an antioxidant; it induces antinociception without some of the side effects and limitations such as hyperthermia that are expected for TRPV1 antagonists.

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### 1. Introduction

Transient receptor potential (TRP) channels represent a superfamily of six-transmembrane-domain ion channels that are permeable to cations, primarily calcium [1]. TRPV1 is a member of the vanilloid TRP subfamily and is activated by harmful stimuli such as heat and acidification as well as by chemical irritants such as capsaicin. The TRPV1 receptor is activated by several endogenous substances, including anandamide and leukotriene B<sub>4</sub>, and has been implicated in the development of certain inflammatory diseases and neuropathic pain [2,3].

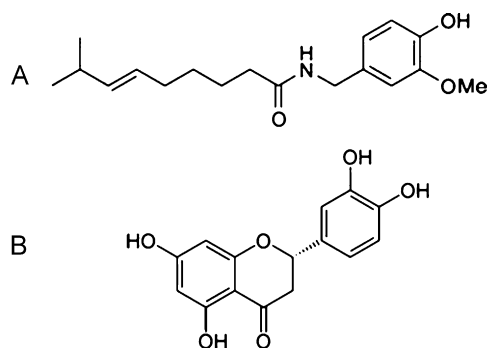
It has been demonstrated that antagonism of TRPV1 leads to antinociception in several models of inflammatory and neuro-

pathic pain [4,5]. Moreover, the genetic ablation of TRPV1 receptors has also been used to demonstrate its importance in pain transmission [6–8]. However, a recent clinical trial of AMG517, a TRPV1 antagonist, showed this compound to be unsatisfactory for pain treatment due to some severe side effects, primarily the development of hyperthermia [9]. Thus, a search for novel compounds that modulate TRPV1 channel activity and have safe and efficient analgesic effects is necessary.

To address this question, our research group has been examining several natural compounds that are structurally similar to known TRPV1 ligands. In these tests, we selected the flavonoid eriodictyol (3',4',5,7-tetrahydroxyflavanone), which presents some characteristics commonly found in TRPV1 ligands (Fig. 1), such as regions A and B that are present in capsaicin, a TRPV1 agonist [10,11]. Furthermore, eriodictyol also shows antioxidant effects [12], and some studies have indicated that antioxidant compounds, such as N-acetylcysteine (NAC), induce antinociception in several pain models [13,14]. These findings suggest that eriodictyol has the potential to exert antinociceptive action.

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**Fig. 1.** Chemical structures of the TRPV1 receptor ligands (A) capsaicin and (B) the flavonoid eriodictyol (3',4',5,7-tetrahydroxyflavanone).

The main objective of the present study was to characterize the interaction of eriodictyol with the TRPV1 receptor and to evaluate its possible antinociceptive effect.

## 2. Materials and methods

### 2.1. Animals

Three-month-old male albino Swiss mice (25–35 g) and male Wistar rats (200–300 g) bred in our animal house were used in this study. Animals were housed at a controlled temperature ( $22 \pm 2^\circ\text{C}$ ) with a 12 h light/dark cycle and were provided standard lab chow and tap water *ad libitum*. The animals were habituated to the experimental room for at least 1 h before experiments. Each animal was used for only one experiment. The experiments reported in this study were carried out in accordance with current ethical guidelines for the investigation of experimental pain in conscious animals [15] and were approved by the Ethics Committee of the Federal University of Santa Maria (process number 23081.001086/2009-87).

### 2.2. Drug treatments

To determine the possible systemic antinociceptive effect of eriodictyol, eriodictyol dissolved in 5% Tween 80, 20% polyethylene glycol and 75% saline (0.9% NaCl) was orally administered to the animals. For intrathecal treatment, the eriodictyol stock solution was dissolved in the same vehicle and diluted in phosphate-buffered saline (PBS, composition in mmol/L: 137 NaCl, 2.7 KCl and 10 phosphate buffer) to the desired concentration (final concentration: 0.05% Tween 80 and 0.2% polyethylene glycol). The intrathecal injection of vehicle alone had no effect. The TRPV1 antagonists AMG9810 and SB366791 [16,17] were dissolved in the same vehicle and administered orally. For *in vitro* assays, stock solutions of all compounds were dissolved in ethanol and diluted in PBS. The final concentration of ethanol was not higher than 0.1%.

The procedure for intrathecal (i.t.) administration was based on a previously described technique [18]. Briefly, the intervertebral space between L5 and L6 was punctured directly using a 28-gauge needle attached to a Hamilton micro syringe. A total volume of 5  $\mu\text{L}$  was administered. Mice were not anesthetized during these procedures. A tail flick was used as an indication that the needle had penetrated the dura.

### 2.3. [ $^3\text{H}$ ]-Resiniferatoxin binding assay

Because eriodictyol is structurally similar to some known TRPV1 ligands, we investigated the possibility that it binds to the TRPV1 receptor. We tested eriodictyol using the [ $^3\text{H}$ ]-resinifer-

atoxin ([ $^3\text{H}$ ]-RTX) binding assay as described previously [19]. Briefly, spinal cord tissue was homogenized in buffer A (pH 7.4, 5 mM KCl, 5.8 mM NaCl, 2 mM  $\text{MgCl}_2$ , 0.75 mM  $\text{CaCl}_2$ , 137 mM sucrose, and 10 mM HEPES) and centrifuged for 10 min at  $1000 \times g$  at  $4^\circ\text{C}$ . The supernatant was then centrifuged for 30 min at  $35,000 \times g$  at  $4^\circ\text{C}$ . The resulting pellets were resuspended in buffer A and frozen until the day of assay. The binding mixture (final volume 500  $\mu\text{L}$ ) contained buffer A plus 0.25 mg/mL bovine serum albumin (BSA), membranes (0.5 mg/mL), and 2 nM [ $^3\text{H}$ ]-RTX in the presence or absence of eriodictyol (3–300 nM). For the measurement of nonspecific binding, 100  $\mu\text{M}$  non-radioactive RTX was used. The reaction was initiated by incubating tubes at  $37^\circ\text{C}$  for 60 min and was stopped by transferring the tubes to an ice bath and adding 100  $\mu\text{g}$  of bovine  $\alpha_1$ -acid glycoprotein to reduce non-specific binding. Free [ $^3\text{H}$ ]-RTX was separated from membrane-bound [ $^3\text{H}$ ]-RTX by centrifugation for 30 min at  $35,000 \times g$  at  $4^\circ\text{C}$ . Scintillation counting was used to quantify binding. Specific binding, which represented 60–70% of the total binding, was calculated as the difference between total and non-specific binding. The results are reported as % of specific binding.

### 2.4. Calcium influx assay

After evaluating the ability of eriodictyol to bind to the TRPV1 receptor, we measured its capacity to change calcium influx. Synaptosomes were prepared from rat spinal cord samples and incubated with Fura 2-AM (10  $\mu\text{M}$ ) for 30 min at  $37^\circ\text{C}$ . The reaction was diluted to 1.5 mL (5 mg/mL of protein) with Krebs-Ringer medium ( $\text{Ca}^{2+}$  free) and incubated for 30 min at  $37^\circ\text{C}$ . The reaction was stopped by centrifugation (30 s at  $12,000 \times g$ ), and the final pellet was resuspended in 1.5 mL Krebs-Ringer medium ( $\text{Ca}^{2+}$  free). To start the reaction, 1.5  $\mu\text{L}$  of 1 M  $\text{CaCl}_2$  (1 mM) plus different concentrations of eriodictyol were added followed by the addition of capsaicin (20  $\mu\text{M}$ ). We measured  $\text{Ca}^{2+}$  influx by monitoring fluorescence at 505 nm from excitation at 382 nm in a spectrofluorimeter (RF-5301 PC, Shimadzu). Background fluorescence from an equivalent sample of synaptosomes not loaded with fura 2-AM was recorded at the beginning of the experiment. At the end of each experiment, calibration was performed by recording maximum fluorescence values after the addition of 15  $\mu\text{L}$  of 10% Triton X100. Results are expressed as percentage of the maximum response obtained with Triton [20].

### 2.5. Capsaicin-induced nociception

The peripheral capsaicin test was carried out as previously described [21,22]. Animals were habituated to the observation location, which consisted of a glass chamber, for at least 30 min before the experiment. Following habituation, 20  $\mu\text{L}$  of capsaicin (1 nmol/paw) was injected intraplantarly (i.pl) into the right hind paw, and the total time spent licking and flinching the injected paw was measured for 5 min as a nociception index. For the spinal capsaicin test, 0.2 nmol/site of capsaicin was injected i.t. as previously described [22]. In this test, the total time spent licking the hind paws and tail during the 5 min following injection was used as a measure of nociception. The vehicle used in each test (0.15% ethanol in saline for i.pl. test and PBS for the i.t. test) was prepared and used as a control treatment; it did not evoke nociceptive behavior (data not shown).

### 2.6. CFA-induced inflammation

To induce the development of inflammatory thermal hyperalgesia and mechanical allodynia, we used the CFA-induced inflammatory pain model. Animals were anesthetized with

halothane and 20  $\mu$ L of CFA (1 mg/mL suspension of heat-killed *Mycobacterium tuberculosis* in liquid paraffin) was injected into the right hind paw. Forty-eight hours later, the nociceptive alterations were observed [22,23].

## 2.7. Thermal hyperalgesia measurement

Thermal hyperalgesia was recorded using previously described methods [24] with minor modifications. Briefly, animals were habituated in a Plexiglas chamber for 30 min; a radiant light beam generated by a 60 W light bulb was then directed onto the right hind paw (Plantar test, Ugo Basile, Italy). The time between the onset of the stimulus and manifestation of the paw withdrawal response was automatically measured and taken as an index of the thermal nociceptive threshold.

## 2.8. Mechanical allodynia measurement

Mechanical allodynia was evaluated with the up-and-down method [25] using von Frey filaments. Briefly, mice were placed in cages with a wire mesh bottom that allowed full access to the paws. The paw was touched with a series of 7 von Frey hairs with logarithmic increments (0.02, 0.07, 0.16, 0.4, 1.4, 4.0 and 10.0 g). The von Frey hairs were applied perpendicular to the plantar surface with sufficient force to cause slight buckling against the paw and held for approximately 2–4 s. Stimuli were presented at intervals of several seconds, allowing for the resolution of any behavioral responses to previous stimuli.

## 2.9. Locomotor activity

To evaluate possible non-specific muscle relaxant or sedative effects of the administered compound, mice were subjected to motor impairment evaluation [26]. We first examined spontaneous motor coordination in the open-field test. The apparatus consisted of a Plexiglas box measuring 40 cm  $\times$  60 cm  $\times$  50 cm, the floor of which was divided into 12 equal squares. The number of squares crossed with all paws, as well as the animal's hearing behavior, was measured in a 5-min session. Forced motor activity was also evaluated using the rota-rod test. Twenty-four hours before the experiment, all animals were trained on the rota-rod (3.7 cm in diameter; 8 r.p.m.) until they could remain on the apparatus for 60 s without falling. On the day of the experiment, animals were subjected to the rota-rod test 1 h after the administration of eriodictyol (4.5 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.). The total number of falls that occurred over a 240 s period and the latency for the first fall from the apparatus was recorded.

## 2.10. Body temperature

Because some TRPV1 ligands and antioxidants may modulate body temperature, we investigated the effect of eriodictyol on body temperature. The animals' rectal temperature was determined; they were then given vehicle, eriodictyol (4.5 mg/kg), SB366791 (10 mg/kg) or AMG9810 (10 mg/kg) orally. New temperature measurements were taken at various time points following drug administration, and the difference ( $\Delta$ °C) between pre-injection and post-injection values was calculated as previously described [27].

## 2.11. Antioxidant activity

### 2.11.1. ABTS assay

To determine whether eriodictyol has antioxidant activity, we performed the ABTS radical reduction test [28,29]. Briefly, 1 mL of ABTS solution (7 mM) was incubated in the dark with potassium persulfate (140 mM) for 16–18 h at room temperature. The

solution was then diluted (1:88) in phosphate buffer (10 mM, pH 7.0). To perform the reaction, 1 mL of diluted ABTS solution was incubated with 0.1 mL of sample for 30 min in the dark. Rutin was used as a positive control, and water was used as a blank (100% of absorbance).

### 2.11.2. Estimations of thiol levels

Following nociception testing, animals were sacrificed using halothane, and the lumbar portion of the spinal cord was collected for the estimation of non-protein thiol content [30]. Tissue was homogenized in 1.0 mL of 0.02 M EDTA and the homogenate was centrifuged at 4000  $\times$  g for 15 min at 4 °C. The supernatant (300  $\mu$ L) was incubated with 50  $\mu$ L of 50% TCA at 4 °C for 60 min and then centrifuged at 12,000  $\times$  g for 5 min at 4 °C; the resulting supernatant was used for determination of the non-protein thiol content of the sample. For this, 200  $\mu$ L of Tris/HCl (200 mM, pH 8.9) and 20  $\mu$ L of DTNB (2.5 mM) were added to a 100- $\mu$ L sample in a microplate and incubated at room temperature for 5 min. The resulting color was measured at 405 nm using a FisherBiotech Microkinetics Reader BT 2000. Cysteine was used as a standard to determine the thiol (SH) content in the samples, and thiol levels were corrected for its protein contents. Protein was measured using Coomassie Blue dye with BSA as a standard [31].

### 2.11.3. Slot-blot assay for 3-nitrotyrosine

Peroxonitrite is one of the most reactive nitrogen species formed during oxidative stress; it can attack proteins, leading to the production of 3-nitrotyrosine (3-NT) and altered functioning. Thus, we measured the level of 3-NT in lumbar spinal cord samples after injection of capsaicin or vehicle and used it as a marker of reactive species production [22]. Briefly, 5  $\mu$ L of sample was added to 5  $\mu$ L of 12% SDS and 5  $\mu$ L of Laemmli buffer (pH 6.8; 0.125 M Tris, 4% SDS and 20% glycerol) and incubated for 20 min at room temperature. The resulting solution was applied to a nitrocellulose membrane under vacuum using a slot-blot apparatus. The membrane was blocked in blocking buffer (3% BSA) for 1 h and incubated for 1 h with an anti-3-NT polyclonal antibody diluted 1:2000 in PBS (0.05 mM, pH 7.4) containing 0.01% sodium azide and 0.2% Tween 20. The membrane was washed three times in PBS and incubated for 1 h with an alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody diluted in PBS (1:8000). Blots were dried, scanned using Adobe Photoshop, and quantified using Scion Image (PC version of Macintosh-compatible NIH image). The 3-NT blot displayed a faint background that was corrected for in image analysis.

## 2.12. Drugs and reagents

Eriodictyol was either isolated from the ethyl acetate fraction obtained from the leaves of *Vernonia tweedieana* Baker by column chromatography on silica gel or purchased from Sigma (St. Louis, USA). The structure was identified by spectroscopic methods, primarily  $^1$ H NMR,  $^{13}$ C NMR and Dept 135 spectra, and by comparison with published data. Eriodictyol purity (>95%) was verified by HPLC–DAD. No difference in biological effect or purity was observed in the eriodictyol from the two sources in *in vitro* and *in vivo* experiments. Capsaicin, sodium dodecyl sulfate (SDS), glycerol, polyethylene glycol 400, PBS, bovine serum albumin (BSA), Tween 20, Tween 80, anti-rabbit IgG alkaline phosphatase secondary antibody, anti-3-NT polyclonal antibody,  $\alpha_1$ -acid glycoprotein, DTNB (5,5'-dithiobis(2-nitro-benzoic acid)), CFA, SB366791, AMG9810, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, rutin and Fura 2-AM (stock solution 1 mM in dimethyl sulfoxide, DMSO) were purchased from Sigma (St. Louis, USA). Trichloroacetic acid (TCA) and cysteine were purchased from Vetec (Rio de Janeiro,

Brazil) and diluted in water prior to use. [ $^3\text{H}$ ]-RTX was purchased from PerkinElmer (Boston, USA) and diluted in buffer A before use.

### 2.13. Statistical analysis

The results are presented as mean  $\pm$  S.E.M. except in the case of  $\text{ID}_{50}$  (the dose of compound that inhibits the nociceptive effect by 50% relative to the control value) and  $\text{IC}_{50}$  (the concentration of compound that inhibited calcium influx or binding by 50% relative to the control value) values, which are reported as geometric means accompanied by respective 95% confidence limits. The  $\text{ID}_{50}$  and  $\text{IC}_{50}$  values were determined by non-linear regression analysis with a sigmoid dose-response equation using GraphPad Software 5.0 (Graph Pad, USA). The percentages of maximal inhibition ( $I_{\text{max}}$ ) are reported as the mean  $\pm$  S.E.M. of the inhibition obtained in each individual experiment in relation to the control values (vehicle for the *in vivo* results; 100% of specific binding for the [ $^3\text{H}$ ]-RTX binding assay; 100% of maximum response obtained with triton for the calcium influx assay). The level of significance was set at  $p < 0.05$ . Data were analyzed using Student's *t*-test, one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls's (SNK) *post-hoc* test or two-way ANOVA followed by Bonferroni's *post-hoc* test.

## 3. Results

### 3.1. Characterization of TRPV1 antagonism

#### 3.1.1. [ $^3\text{H}$ ]-Resiniferatoxin binding

Initially, we determined the possible interaction of eriodictyol with the TRPV1 receptor. A binding assay was performed using [ $^3\text{H}$ ]-RTX, which is a ligand of the vanilloid site of the TRPV1 receptor. Eriodictyol was able to displace [ $^3\text{H}$ ]-RTX-specific binding from rat spinal cord membranes with an  $\text{IC}_{50}$  of 47 (21–119) nM and an  $I_{\text{max}}$  of  $68.9 \pm 9.0\%$  (Fig. 2A).

#### 3.1.2. Effects of eriodictyol on intrasynaptosomal calcium influx

To determine the functional effects of eriodictyol binding, we investigated its capacity to alter calcium influx mediated by capsaicin. In this study, eriodictyol inhibited the calcium influx elicited by capsaicin in spinal cord synaptosomes with an  $\text{IC}_{50}$  of 44 nM (Fig. 2B). Eriodictyol (300 nM) in the absence of capsaicin did not alter the calcium influx (results not shown).

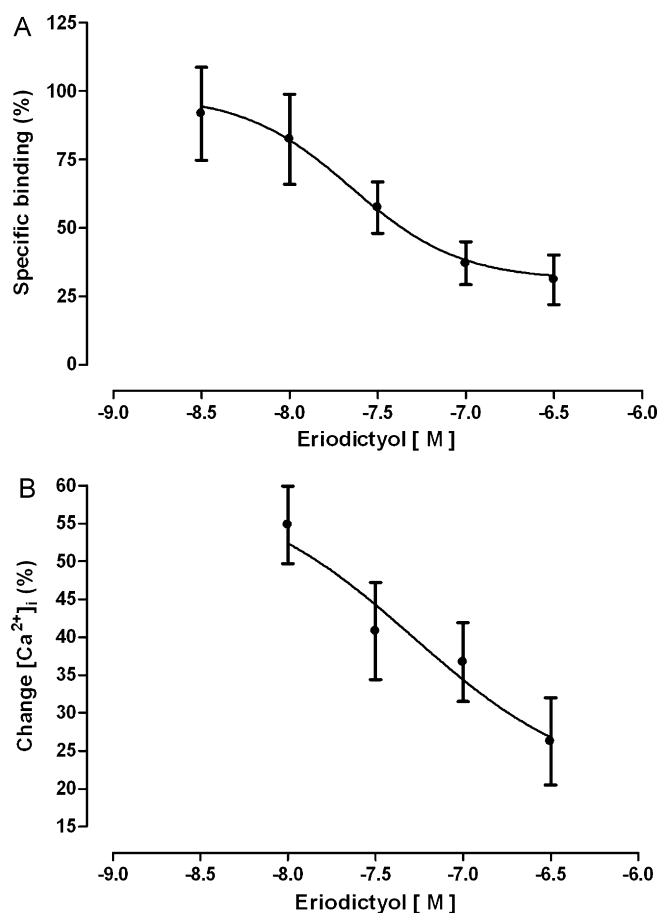
### 3.2. Characterization of eriodictyol effects *in vivo*

#### 3.2.1. Antinociceptive effect of eriodictyol

We investigated the possible antinociceptive effect of eriodictyol using a capsaicin-induced overt nociception test. In mice, orally administered eriodictyol had an antinociceptive effect with an  $\text{ID}_{50}$  of 2.3 (1.1–5.7) mg/kg and an  $I_{\text{max}}$  of  $49 \pm 10\%$ . The eriodictyol-induced antinociceptive effect (4.5 mg/kg, *p.o.*) started 1 h after treatment and lasted for up to 2 h (Fig. 3A and B).

Eriodictyol was also administered intrathecally (1–10 nmol/site). With this route of administration, we observed a greater antinociceptive effect that began 15 min and lasted until 4 h after administration and that displayed an  $\text{ID}_{50}$  of 2.2 (1.7–2.9) nmol/site and an  $I_{\text{max}}$  of  $64 \pm 4\%$  (Fig. 3C and D). We then performed the intrathecal capsaicin test and administered eriodictyol orally (4.5 mg/kg) to determine whether oral eriodictyol was able to reach the spinal cord and inhibit central noxious stimulation. In this protocol, a  $71 \pm 5\%$  inhibition of nociception was observed (Fig. 3E).

We did not observe any change in locomotor activity after eriodictyol administration in either the rota-rod or open-field tests. Thus, the antinociceptive effect of eriodictyol is not due to any alteration in motor performance (Table 1).



**Fig. 2.** Effects of eriodictyol on the TRPV1 receptor. Capacity of eriodictyol to (A) bind to the TRPV1 receptor and displace [ $^3\text{H}$ ]-RTX binding and (B) to inhibit the calcium influx elicited by capsaicin (20  $\mu\text{M}$ ). Each point represents the mean of the specific binding (%), (A) or the change in  $[\text{Ca}^{2+}]_i$  (%), (B)  $\pm$  SEM of four experiments conducted in duplicate.

#### 3.2.2. CFA-induced nociception

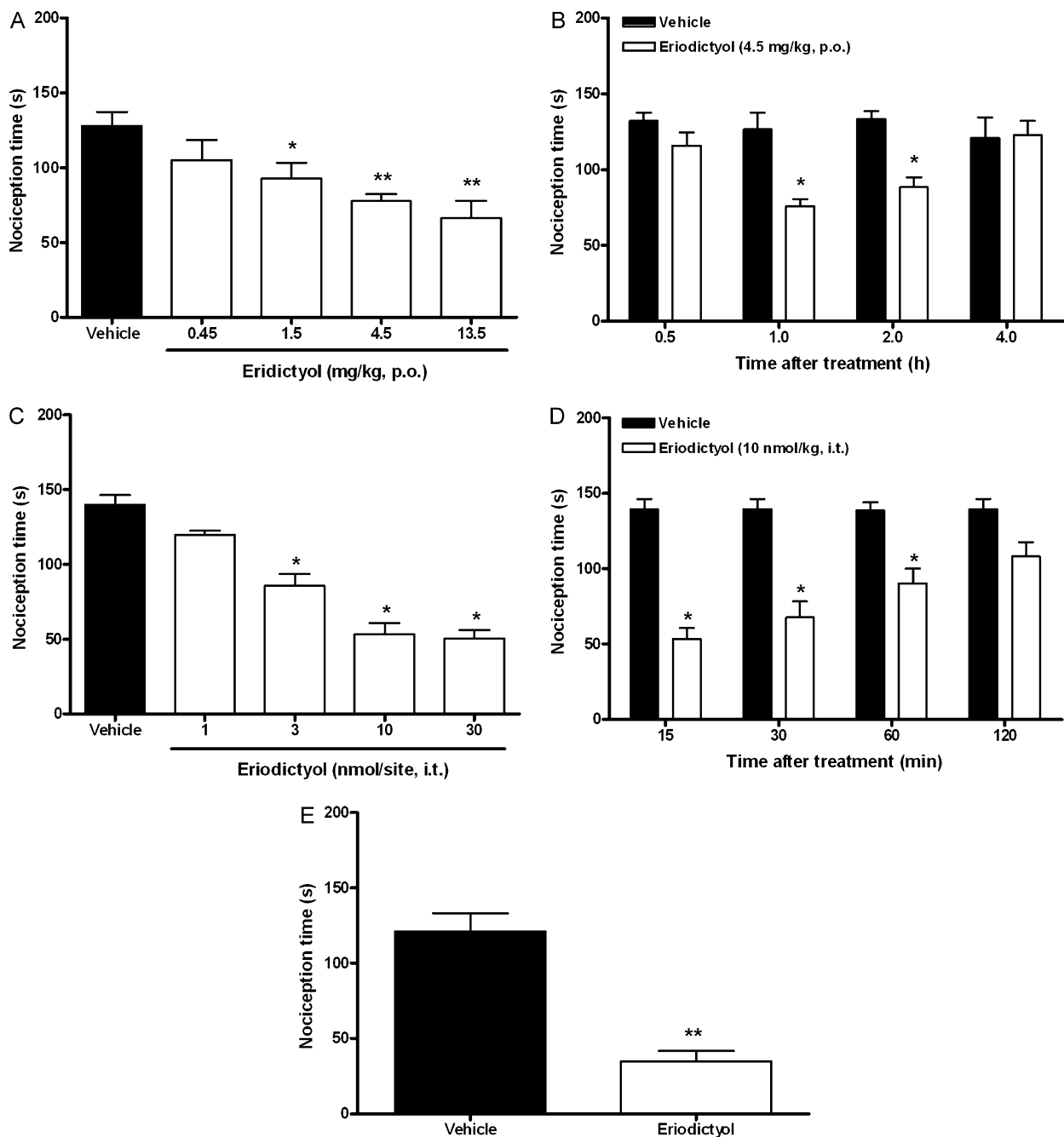
Because the TRPV1 receptor may be activated during the inflammatory process at the site of inflammation and in the spinal cord, we evaluated the effect of eriodictyol and SB366791 or AMG9810, which were used as positive controls for the effects of compounds that antagonize TRPV1 receptor activation, on the thermal hyperalgesia and mechanical allodynia induced by CFA. One hour after administration, eriodictyol (4.5 mg/kg, *p.o.*) caused an anti-hyperalgesic and anti-allodynic effect, fully reversing hyperalgesia (100%) and partially reducing the allodynia ( $64 \pm 7\%$ ). At the same time, AMG9810 (10 mg/kg, *p.o.*) reduced only  $92 \pm 3\%$  of the hyperalgesia and  $55 \pm 26\%$  of the allodynia induced by CFA, while SB366791 (10 mg/kg, *p.o.*) reduced only the thermal hyperalgesia induced by CFA ( $89 \pm 10\%$ ) without altering the mechanical allodynia (Fig. 4).

**Table 1**

Side effects evaluated 1 h after vehicle or eriodictyol (4.5 mg/kg, *p.o.*) administration.

| Treatment ( <i>p.o.</i> ) | Rota-rod        |               | Open field     |                |
|---------------------------|-----------------|---------------|----------------|----------------|
|                           | First fall (s)  | Falls         | Crossing       | Rearing        |
| Vehicle                   | 93.2 $\pm$ 12.0 | 2.3 $\pm$ 0.8 | 50.8 $\pm$ 7.2 | 13.6 $\pm$ 1.9 |
| Eriodictyol               | 64.6 $\pm$ 32.2 | 1.3 $\pm$ 0.9 | 33.5 $\pm$ 6.6 | 8.5 $\pm$ 2.6  |

No significant differences were observed between groups, Student's *t* test. Results are expressed as the mean  $\pm$  S.E.M. ( $n = 5$ ).



**Fig. 3.** Antinociceptive effect of eriodictyol measured in the capsaicin test. Dose-response effect of eriodictyol administered (A) orally or (C) intrathecally 1 h before the intraplantar capsaicin test. The time course of eriodictyol administered orally (B; 4.5 mg/kg) or intrathecally (D; 10 nmol/site) in the intraplantar capsaicin test. (E) Effect of eriodictyol (4.5 mg/kg, p.o.) on the nociception induced by intrathecal capsaicin (0.2 nmol/site). Data are expressed as the mean  $\pm$  SEM ( $n = 6-7$ ) of the nociception time (s). \* $p < 0.05$ , \*\* $p < 0.01$ , based on Student's *t*-test (E), one-way ANOVA followed by SNK *post-hoc* test (A and C) or two-way ANOVA followed by Bonferroni's *post-hoc* test (B and D).

### 3.2.3. Body temperature

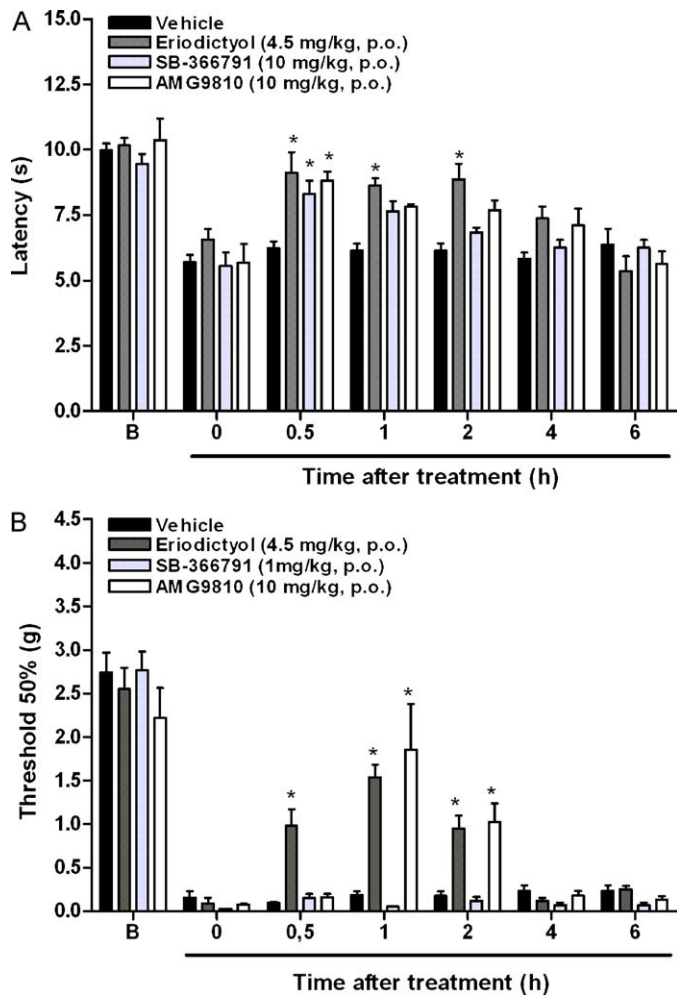
The development of marked hyperthermia is a major limitation in the use of TRPV1 antagonists in clinical trials. We therefore assessed the effect of eriodictyol (4.5 mg/kg, p.o.), SB366791 (10 mg/kg, p.o.) and AMG9810 (10 mg/kg, p.o.) on rectal temperature in mice. Neither eriodictyol nor SB366791 significantly altered basal temperature, while AMG9810 induced a  $2.48 \pm 0.15\%$  increase in the rectal temperature (Fig. 5).

### 3.3. Antioxidant activity

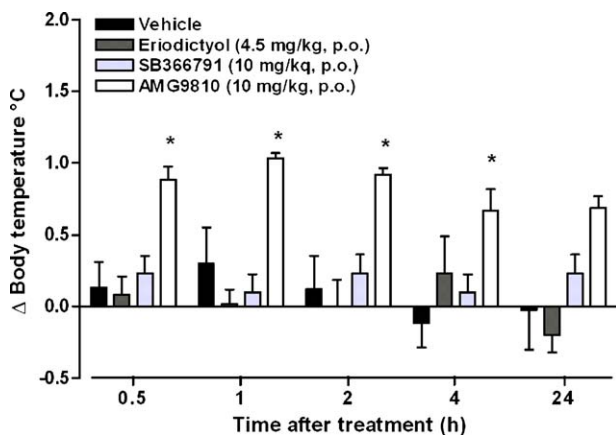
It is known that TRPV1 activation not only elicits nociception but also causes oxidative stress, which seems to contribute to

central pain signaling [22]. Use of the intraplantar capsaicin test demonstrated a decrease in non-protein thiol levels 5 min after capsaicin injection into the lumbar spinal cord, and pretreatment with eriodictyol (4.5 mg/kg) prevented this loss (Fig. 6A).

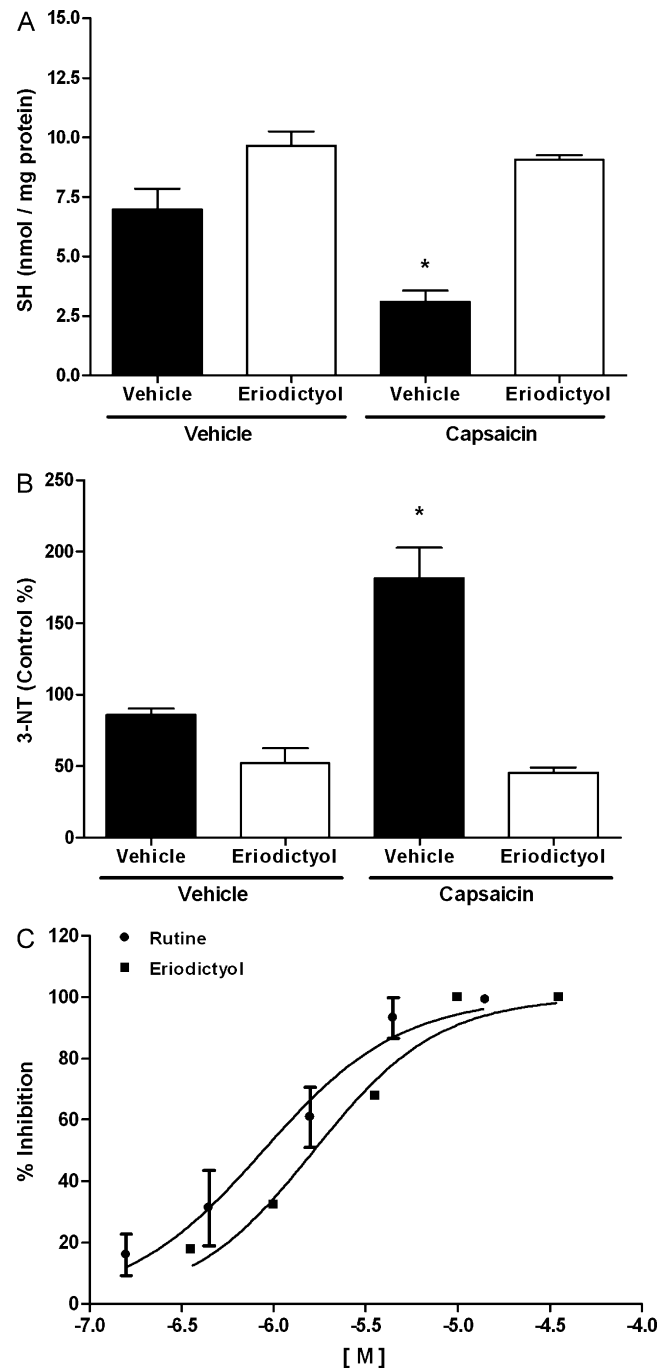
Another event associated with peripheral noxious stimulation is an increase in the rate of formation of peroxynitrite; this compound attacks tyrosine residues leading to the formation of 3-nitrotyrosine (3-NT) [32]. We measured 3-NT levels in the lumbar spinal cords of mice treated with vehicle or with eriodictyol (4.5 mg/kg, p.o.). As previously described [22], an increase in 3-NT levels in the lumbar spinal cord was seen 5 min after intraplantar capsaicin injection and this increase was prevented by pretreatment with eriodictyol. Eriodictyol did not



**Fig. 4.** Effect of eriodictyol (4.5 mg/kg, p.o.), SB366791 (10 mg/kg, p.o.) and AMG9810 (10 mg/kg, p.o.) on the nociception induced by complete Freund's adjuvant (CFA). Effect of the treatments on (A) hyperalgesia and (B) allodynia elicited by intraplantar CFA. Data are expressed as the mean  $\pm$  SEM of the latency (s, A) or the mechanical threshold 50% (g, B). \* $p < 0.05$ , compared with vehicle group, two-way ANOVA followed by Bonferroni's *post-hoc* test ( $n = 6-7$ ).



**Fig. 5.** Body temperature measured after treatment with eriodictyol (4.5 mg/kg, p.o.), SB-366791 (10 mg/kg, p.o.) or AMG9810 (10 mg/kg, p.o.). Data are expressed as the mean  $\pm$  SEM of the change in body temperature ( $\Delta$ °C) in comparison to the basal level. \* $p < 0.05$  compared with vehicle pretreated mice, two-way ANOVA followed by Bonferroni's *post-hoc* test ( $n = 6$ ).



**Fig. 6.** Levels of oxidative stress markers measured in a lumbar spinal cord sample 5 min after intraplantar injection of capsaicin (1 nmol/paw). Non-protein thiol content (SH; A) or 3-nitrotyrosine (3-NT; B) after eriodictyol (4.5 mg/kg, p.o.) treatment ( $n = 4-6$ ). (C) Antioxidant effect of eriodictyol or rutin in the ABTS scavenger assay. Data are expressed as the mean  $\pm$  SEM of the SH and 3-NT levels (A and B,  $n = 6-7$ ) or the mean  $\pm$  SEM of the % inhibition of ABTS oxidation (C, 3 experiments carried out in duplicate). \* $p < 0.05$  compared with vehicle pretreated mice (black bars), one-way ANOVA followed by SNK *post-hoc* test. Non-protein thiol levels are represented as free SH levels and 3-NT levels as % of control (vehicle-vehicle) treated group.

change basal 3-NT levels in mice that received intraplantar vehicle injections (Fig. 6B).

The prevention of the oxidative stress that normally occurs following intraplantar capsaicin injection may result in part from the ability of eriodictyol to antagonize the TRPV1 receptor and in part from an associated antioxidant activity. To evaluate this possibility, we performed the ABTS radical test used to screen

antioxidant compounds. In this experiment, eriodictyol showed antioxidant activity similar to that of rutin (positive control) with an  $IC_{50}$  of 1.7 (1.3–2.1)  $\mu$ M (Fig. 6C).

#### 4. Discussion

The transient receptor potential (TRP) family represents a superfamily of ion channels formed by six transmembrane domains that are capable of permeating cations, primarily calcium. These channels, particularly the subtype vanilloid 1 (TRPV1), are involved in the detection and transmission of painful stimuli [1]. As a result, these receptors have been implicated as a possible target for pain treatment [33]. To find new molecules that interact with this receptor, we tested the flavonoid eriodictyol. This compound inhibited the binding of a known modulator to the TRPV1 receptor and inhibited the calcium influx mediated by capsaicin. Eriodictyol also had an antinociceptive effect in the intraplantar and intrathecal capsaicin tests and antihyperalgesic and anti-allodynic effects in the CFA test. Furthermore, we observed that eriodictyol fully prevented the oxidative stress induced by capsaicin in the spinal cord without altering motor activity or body temperature.

To begin the search for new molecules that may interact with the TRPV1 receptor, we focused on compounds found in medicinal plants with known antinociceptive effects. We tested several compounds in the [ $^3$ H]-RTX binding assay to determine which compounds could interact with the vanilloid site of TRPV1. Based on preliminary findings, we selected the flavonoid eriodictyol, which displaces [ $^3$ H]-RTX binding with greater potency (approximately 47 nM) than the classical TRPV1 agonist capsaicin (approximately 3200 nM) [34]. We then used a capsaicin-mediated calcium influx assay to determine whether eriodictyol binding leads to functional modulation of the TRPV1 receptor. Eriodictyol inhibited calcium influx with potency similar to that observed in the binding assay and had no effect on calcium influx in the absence of capsaicin. This indicates that eriodictyol acts as an antagonist of the TRPV1 receptor and that it inhibits calcium influx with greater potency than some classical TRPV1 antagonists, including SB366791 (651.9 nM, [35]), 5-iodoresineferatoxin (56.7 nM, [36]) and capsazepine (7.7  $\mu$ M, [37]).

The TRPV1 receptor is currently the most promising target for the development of new analgesic drugs [38]. The vanilloid receptor is distributed both in the periphery, where it acts as a sensor of noxious stimuli, and in the spinal cord, where it participates in the transmission of pain [39]. After determining that eriodictyol acts as an antagonist of the TRPV1 receptor *in vitro*, our next step was to determine whether it could attenuate the nociception induced by capsaicin. Eriodictyol had antinociceptive effects with high efficacy and potency when administered either orally or intrathecally. Furthermore, oral eriodictyol administration attenuated the nociception induced by intrathecal capsaicin, suggesting that orally administered eriodictyol may reach the spinal cord. This is a critical feature for new drug development, because most compounds used to treat pain are administered orally [40].

Despite the fact that the capsaicin pain model is useful for screening antinociceptive compounds that may act on the TRPV1 receptor, it does not have a good correlation with clinical pain [41]. We therefore tested the effects of eriodictyol, SB366791 and AMG9810 on CFA-induced arthritis. Eriodictyol and AMG9810 had both anti-allodynic and anti-hyperalgesic effects, while SB366791 showed only an anti-hyperalgesic effect. In several disease processes, TRPV1 receptor levels may also be modulated, resulting in increased sensitivity to pain. In rodents, an increase in the expression of TRPV1 protein at the site of the chronic inflammation induced by CFA has been observed, and it has been shown that this leads to increased sensitivity to thermal stimulation [42]. Similar increases in TRPV1 expression have also been reported in humans

with local inflammation [43]. In chronic painful processes, increases in TRPV1 function mediated by post-transcriptional events such as phosphorylation and nitration at distinct sites of the TRPV1 receptor have been observed [44,45]. These facts clearly indicate a critical role for this receptor in the ontogeny of pain. The observation that eriodictyol effectively reversed the nociception caused by CFA has potential clinical implications for this compound in light of the fact that patients are usually treated for well-established painful conditions.

Although hyperthermia is a common side effect of TRPV1 antagonists, we found that eriodictyol had no effect on body temperature, while AMG9810 induced hyperthermia in mice. Administration of AMG517, an analog of AMG9810, was recently shown to cause marked and persistent hyperthermia (approximately 40 °C) in humans at doses smaller than those necessary to elicit an analgesic effect. The hyperthermia elicited by AMG517 has been found to be resistant to classical treatments for fever, such as acetaminophen. This hyperthermia was attributed to an increase in peripheral vasoconstriction, which decreases heat loss through the skin, increasing the body temperature [9]. Eriodictyol has been shown to cause vasorelaxation [46]; this could explain why eriodictyol did not induce hyperthermia. These data suggest that eriodictyol may be a safer treatment than other currently known TRPV1 antagonists.

Our research group recently demonstrated that capsaicin-induced nociception occurs concomitantly with spinal oxidative stress. Treatment with NAC, an ROS scavenger, attenuated both the oxidative stress and the nociception induced by capsaicin. Thus, ROS production appears to be an important mechanism of pain induction/transmission in mice [22]. The TRPV1 receptor seems to play a critical role in oxidative stress-mediated nociception. The stimulation of TRPV1 receptors may induce oxidative stress in sensory neurons and the spinal cord, and some oxidants may modulate TRPV1 receptor function [22,44,47]. Here we found that eriodictyol prevented the development of oxidative stress in the lumbar spinal cord. This effect may be due either to direct antagonism of the TRPV1 receptor or to an antioxidant effect. To distinguish between these alternatives, we performed the ABTS test and confirmed that eriodictyol also has an antioxidant effect [47]. Even though eriodictyol has an approximately 38-fold greater potency for antagonism of the TRPV1 receptor than for antioxidant activity, a direct antioxidant effect could contribute, at least in part, to its antinociceptive effect.

The occurrence of oxidative stress has been described in several animal models of pain, and oxidative stress has been implicated as a mechanism in the induction and maintenance of pain [48]. This hypothesis is strengthened by the fact that some antioxidant substances present antinociceptive effects on both mechanical allodynia and thermal hyperalgesia, as observed for eriodictyol [22,48]. Some molecular targets involved in nociception, such as the NMDA receptor and the excitatory amino acid transporter 1 (EAAT<sub>1</sub>), have also been implicated in oxidative stress [31]. Thus, eriodictyol could induce antinociception by directly antagonizing the TRPV1 receptor as well as by protecting other targets of reactive species.

In summary, the flavonoid eriodictyol induced antinociceptive, anti-allodynic and anti-hyperalgesic effects in mice without altering locomotor activity or body temperature. The antinociceptive effects of eriodictyol seem to be due to a combination of TRPV1 antagonism and an antioxidant effect. Thus, eriodictyol may be a good prototype for the development of more effective and/or more potent agents for the treatment of pain.

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## References

- [1] Nilius B, Owsianik G, Voets T, Peters JA. Transient receptor potential cation channels in disease. *Physiol Rev* 2007;87(January (1)):165–217.
- [2] Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997;389:816–24.
- [3] Montell C. The TRP superfamily of cation channels. *Sci STKE* 2005;272.
- [4] Honore P, Wismer CT, Mikusa J, Zhu CZ, Zhong C, Gauvin DM. A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethylbenzyl)-urea], a novel transient receptor potential type V1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. *J Pharmacol Exp Ther* 2005;314:410–21.
- [5] Pomonis JD, Harrison JE, Mark L, Bristol DR, Valenzano KJ, Walker K. N-(4-Tertiarybutylphenyl)-4-(3-cholorophyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide (BCTC), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties: II. In vivo characterization in rat models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther* 2003;306:387–93.
- [6] Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 2000;288:306–13.
- [7] Bölskei K, Helyes Z, Szabó A, Sándor K, Elekes K, Németh J, et al. Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. *Pain* 2005;117:368–76.
- [8] Ta LE, Bieber AJ, Carlton SM, Loprinzi CL, Low PA, Windebank AJ. Transient Receptor Potential Vanilloid 1 is essential for cisplatin-induced heat hyperalgesia in mice. *Mol Pain* 2010;6:1–15.
- [9] Gavva NR, Treanor JJ, Garami A, Fang L, Surapaneni S, Akrami A, et al. Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain* 2008;136:202–10.
- [10] Walpole CS, Wrigglesworth R, Bevan S, Campbell EA, Dray A, James IF, et al. Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 1. The aromatic “A-region”. *J Med Chem* 1993;36:2362–72.
- [11] Walpole CS, Wrigglesworth R, Bevan S, Campbell EA, Dray A, James IF, et al. Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 2. The amide bond “B-region”. *J Med Chem* 1993;36:2373–80.
- [12] Koleckar V, Opletal L, Brojerova E, Rehakova Z, Cervenka F, Kubikova K, et al. Evaluation of natural antioxidants of *Leuzea carthamoides* as a result of a screening study of 88 plant extracts from the European Asteraceae and Cichoriaceae. *J Enzyme Inhib Med Chem* 2008;23:218–24.
- [13] Perez RS, Zuurmond WW, Bezemer PD, Kuik DJ, van Loenen AC, de Lange JJ, et al. The treatment of complex regional pain syndrome type I with free radical scavengers: a randomized controlled study. *Pain* 2003;102:297–307.
- [14] Hacimuftuoglu A, Handy CR, Goettl VM, Lin CG, Dane S, Stephens Jr RL. Antioxidants attenuate multiple phases of formalin-induced nociceptive response in mice. *Behav Brain Res* 2006;173:211–6.
- [15] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983;16:109–10.
- [16] Gavva NR, Tamir R, Qu Y, Klionsky L, Zhang TJ, Immke D, et al. AMG 9810 [(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide], a novel vanilloid receptor 1 (TRPV1) antagonist with antihyperalgesic properties. *J Pharmacol Exp Ther* 2004;313:474–84.
- [17] Koczyńska B. Role of VR1 and CB1 receptors in modeling of cardio-respiratory response to arvanil, an endocannabinoid and vanilloid hybrid, in rats. *Life Sci* 2008;83:85–91.
- [18] Hylden JL, Wilcox GL. Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* 1980;67:313–6.
- [19] Szallasi A, Biro T, Modarres S, Garlaschelli L, Petersen M, Klusch A, et al. Dialdehyde sesquiterpenes and other terpenoids as vanilloids. *Eur J Pharmacol* 1998;356:81–9.
- [20] Bergsman JB, Tsien RW. Syntaxin modulation of calcium channels in cortical synaptosomes as revealed by botulinum toxin C1. *J Neurosci* 2000;20:4368–78.
- [21] Sakurada T, Katsumata K, Tan-No K, Sakurada S, Kisara K. The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord. *Neuropharmacology* 1992;31:1279–85.
- [22] Rossato MF, Velloso NA, de Oliveira Ferreira AP, de Mello CF, Ferreira J. Spinal levels of nonprotein thiols are related to nociception in mice. *J Pain* 2010;11:545–54.
- [23] Ferreira J, Campos MM, Pesquero JB, Araújo RC, Bader M, Calixto JB. Evidence for the participation of kinins in Freund's adjuvant-induced inflammatory and nociceptive responses in kinin B1 and B2 receptor knockout mice. *Neuropharmacology* 2001;41:1006–12.
- [24] Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988;32:77–88.
- [25] Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol* 1980;20:441–62.
- [26] Walker CIB, Trevisan G, Rossato MF, Franciscato C, Pereira ME, Ferreira J, et al. Antinociceptive activity of *Mirabilis jalapa* in mice. *J Ethnopharmacol* 2008;120:169–75.
- [27] Otuki MF, Lima FV, Malheiros A, Cechinel-Filho V, Delle Monache F, Yunes RA, et al. Evaluation of the antinociceptive action caused by ether fraction and a triterpene isolated from resin of *Protium kleinii*. *Life Sci* 2001;69:2225–36.
- [28] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol Med* 1999;26:1231–7.
- [29] Klafke JZ, da Silva MA, Panigás TF, Belli KC, de Oliveira MF, Barichello MM, et al. Effects of *Campomanesia xanthocarpa* on biochemical, hematological and oxidative stress parameters in hypercholesterolemic patients. *J Ethnopharmacol* 2010;127:299–305.
- [30] Sedlak J, Lindsay RH. Estimation of total, protein-bound and sulphidril groups in tissue with Elman's reagent. *Anal Biochem* 1968;25:192–205.
- [31] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- [32] Chen Z, Muscoli C, Doyle T, Bryant L, Cuzzocrea S, Mollace V, et al. NMDA-receptor activation and nitroxidative regulation of the glutamatergic pathway during nociceptive processing. *Pain* 2010;149:100–6.
- [33] Pal M, Angaru S, Kodimuthali A, Dhingra N. Vanilloid receptor antagonists: emerging class of novel anti-inflammatory agents for pain management. *Curr Pharm Des* 2009;15:1008–26.
- [34] Szallasi A, Szabó T, Bíró T, Modarres S, Blumberg PM, Krause JE, et al. Resiniferatoxin-type phorboid vanilloids display capsaicin-like selectivity at native vanilloid receptors on rat DRG neurons and at the cloned vanilloid receptor VR1. *Br J Pharmacol* 1999;128:428–34.
- [35] Varga A, Németh J, Szabó A, McDougall JJ, Zhang C, Elekes K, et al. Effects of the novel TRPV1 receptor antagonist SB366791 in vitro and in vivo in the rat. *Neurosci Lett* 2005;385:137–42.
- [36] Shimizu I, Iida T, Horiuchi N, Caterina MJ. 5-Iodoresiniferatoxin evokes hypothermia in mice and is a partial transient receptor potential vanilloid 1 agonist in vitro. *J Pharmacol Exp Ther* 2005;314:1378–85.
- [37] Docherty RJ, Yeats JC, Piper AS. Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurones in culture. *Br J Pharmacol* 1997;121:1461–7.
- [38] Wong GY, Gavva NR. Therapeutic potential of vanilloid receptor TRPV1 agonists and antagonists as analgesics: Recent advances and setbacks. *Brain Res Rev* 2009 Apr;60(1):267–77.
- [39] Patapoutian A, Tate S, Woolf CJ. Transient receptor potential channels: targeting pain at the source. *Nat Rev Drug Discov* 2009;8:55–68.
- [40] Buxton ILO. Pharmacokinetics and pharmacodynamics: the dynamics of drug absorption, distribution, action and elimination. In: Brunton L, Lazo JS, Parker KLP, editors. Goodman and Gilman's The Pharmacological Basis of Therapeutics. The McGraw-Hill; 2006. p. 1–40.
- [41] Haanpää ML, Backonja MM, Bennett MI, Bouhassira D, Cruccu G, Hansson PT, et al. Assessment of neuropathic pain in primary care. *Am J Med* 2009;122(10 (Suppl.)):S13–21.
- [42] Yu L, Yang F, Luo H, Liu FY, Han JS, Xing GG, et al. The role of TRPV1 in different subtypes of dorsal root ganglion neurons in rat chronic inflammatory nociception induced by complete Freund's adjuvant. *Mol Pain* 2008;4:61.
- [43] Shieh KR, Yi CH, Liu TT, Tseng HL, Ho HC, Hsieh HT, et al. Evidence for neurotrophic factors associating with TRPV1 gene expression in the inflamed human esophagus. *Neurogastroenterol Motil* 2010;22:971–7.
- [44] Yoshida T, Inoue R, Morii T, Takahashi N, Yamamoto S, Hara Y, et al. Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat Chem Biol* 2006;2:596–607.
- [45] Chuang HH, Lin S. Oxidative challenges sensitize the capsaicin receptor by covalent cysteine modification. *Proc Natl Acad Sci USA* 2009;106:20097–102.
- [46] Ma F, Zhang L, Westlund KN. Reactive oxygen species mediate TNFR1 increase after TRPV1 activation in mouse DRG neurons. *Mol Pain* 2009;5:31.
- [47] Ramón Sánchez de Rojas V, Somoza B, Ortega T, Villar AM, Tejerina T. Vasodilatory effect in rat aorta of eriodictyol obtained from *Satureja obovata*. *Planta Med* 1999;65:234–8.
- [48] Kim HK, Park SK, Zhou JL, Taghialatela G, Chung K, Coggeshall RE, et al. Reactive oxygen species (ROS) play an important role in a rat model of neuropathic pain. *Pain* 2004;111:116–24.