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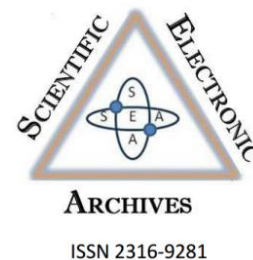
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## Intrathecal administration of a novel pyrazolyl-thiazole derivative induces delayed antinociception in mice

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**Abstract.** In this study we investigated whether the intrathecal administration (i.t.) of the novel pyrazolyl-thiazole derivative 2-[5-trichloromethyl-5-hidroxy-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl]-4-(4-bromophenyl)-5-methylthiazole (B50) caused antinociception in adult male mice, using the hot plate and acetic acid writhing assays. B50 (200 nmol/ 5  $\mu$ l, i.t.) caused antinociception 90-120 minutes after its administration. Naloxone (8.25  $\mu$ mol/ kg, s.c.) reverted the antinociceptive action of B50 (200 nmol/ 5  $\mu$ l, i.t.), in the acetic acid writhing assay, suggesting that opioid mechanisms are involved in the antinociception caused by B50. B50 had no effect on spontaneous locomotion or rotarod performance, indicating that the currently reported antinociceptive effect of B50 is not related to unspecific motor effects.

**Keywords:** Intrathecal; antinociception; thiazole derivative; writhing test; hot-plate test; nonsteroidal anti-inflammatory drugs.

### Introduction

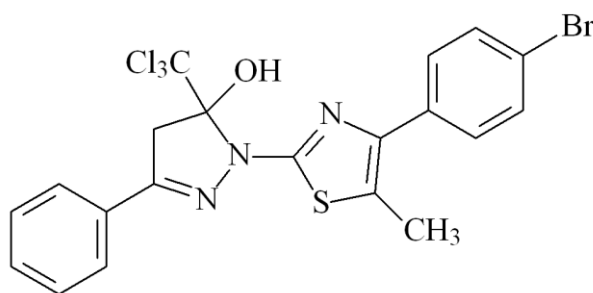
In pain therapy, there are two main classes of analgesic drugs (1), namely opiates, which directly abolish the nociceptive transmission in the central nervous system (CNS) by binding to opioid receptors (2-3), and non-steroidal anti-inflammatory drugs (NSAIDs), which prevent the sensitization of pain receptors in peripheral sites by inhibiting cyclooxygenase (4).

Since pain and fever are the most common complaints in clinical practice and the arsenal of effective analgesics and antipyretics is relatively small, we have been investigating the antinociceptive effects and antipyretic potential of new pyrazole derivatives (5-7). We have described that the pyrazole derivative, 3-methyl-5-hydroxy-5-trichloromethyl-1H-1-pyrazolcarboxamide induces antinociception in the neurogenic and inflammatory phases of the formalin test, and that such an effect

does not involve opioid receptors (5). This compound and its 3-phenyl substituted derivative also causes antinociception in the acetic acid writhing test in mice which is prevented by the spinal administration of nonspecific serotonergic and alpha-2-adrenoceptor antagonists. The antinociceptive effect of two novel pyrazole derivatives have been recently described using thermal tests in mice, 3-ethoxymethyl-5-ethoxycarbonyl-1H-1-methylpyrazole (Pz 2) and 3-ethoxymethyl-5-ethoxycarbonyl-1-phenyl-1H-pyrazole (Pz 3) using in mice. Interestingly, naloxone prevents Pz 3- but not Pz 2-induced antinociception suggesting the involvement of

opioid mechanisms in the antinociceptive effect of Pz 3 (7).

It has been recently described that it pyrazolyl-thiazole compound 2-[5-trichloromethyl-5-hydroxy-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl]-4-(4-bromophenyl)-5-methylthiazole (B50) (Figure 1) causes antinociception that is prevented by naloxone (8). Therefore, we evaluated the antinociceptive effect of intrathecal injection (i.t.) of B50 and the involvement of the opioid mechanisms in such an effect in adult male mice. The effects of this compound on spontaneous and forced locomotion (rotarod test) were also evaluated.



**Figure 1.** Chemical structure of 2-[5-trichloromethyl-5-hydroxy-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl]-4-(4-bromophenyl)-5-methylthiazole (B50).

## Methods

The present study was conducted in accordance with the ethical guidelines established for investigations of experimental pain in conscious animals (9) and it was carried out under licenses issued by Ethics Committee for the Use of Animals in Research of the Federal University of Santa Maria, RS, Brazil.

### Animals

Three month-old male albino Swiss mice (30-40 g) bred in our animal house were used. The animals were housed in groups of 20 to a cage at controlled temperature ( $23 \pm 1^\circ\text{C}$ ) with a 12-h light/dark cycle and with standard lab chow and tap water *ad libitum*. Each animal was used only once. The experiments were approved by the Committee on the Use and Care of Laboratory Animals from Federal University of Santa Maria, RS, Brazil.

### Drugs

Naloxone, morphine sulfate, and Tween 80 were purchased from Sigma (St. Louis, MO). The

2-[5-trichloromethyl-5-hydroxy-3-phenyl-4,5-dihydro-1H-pyrazol-1-yl]-4-(4-bromophenyl)-5-methylthiazole (B50, Figure 1), was synthesized as reported elsewhere (10) and was suspended in 2.5% Tween 80 in 0.9% sodium chloride (vehicle). All other reagents were of analytical grade and were purchased from local suppliers.

### Intrathecal administration of B50

I.t. injections were made in anaesthetized mice at the L5, L6 intervertebral space as described by Hylden and Wilcox (11). Briefly, a volume of 5  $\mu\text{l}$  was administered i.t. with a 28-gauge needle connected to a 10  $\mu\text{l}$  Hamilton micro syringe, while the animal was lightly restrained to maintain the position of the needle. Puncture of the dura was indicated behaviourally by a slight flick of the tail.

### Writhing test

Animals were injected with 5  $\mu\text{l}$  (i.t.) of vehicle, morphine (6.5 nmol/ 5  $\mu\text{l}$ ) or B50 (200 nmol/ 5  $\mu\text{l}$ ) 0, 30, 60, 120 or 240 minutes before of

acetic acid injection (0.8% in distilled water - 10 ml/kg body weight, i.p.) (12), and 5 min later were transferred to an open field (28x18x12 cm) with a floor divided into 15 equal areas. The number of writhes was counted over a period of 10 min by an observer who was not aware of the animals' treatment. In order to determine a dose-response curve, the animals were injected with 5  $\mu$ l (i.t.) of vehicle or B50 (100, 200 or 400 nmol/ 5 $\mu$ l) 120 minutes before of acetic acid and were observed as described above. In those experiments designed to evaluate the participation of spinal opioid mechanisms in the antinociceptive effect of this pyrazoline, the animals were injected with vehicle or B50 (200 nmol/ 5  $\mu$ l, i.t.) (pre-treatment) 90 min before saline or naloxone (8.25  $\mu$ l/ kg, s.c.). Thirty minutes thereafter the animals were injected with acetic acid and were observed as described above.

#### *Rotarod*

Twenty-four hours before the experiments, all animals were trained in the rotarod (3.7 cm in diameter, 8 rpm) until they could remain in the apparatus for 60 s without falling. On the day of the experiment, the animals were injected with 5  $\mu$ l (i.t.) of vehicle or B50 (200 nmol/ 5  $\mu$ l, i.t.) and subjected to the rotarod 120 minutes thereafter. The latency to fall from the apparatus was recorded with a stopwatch up to 240 s (13).

#### *Hot-plate test*

In the hot-plate test, an animal was placed on a metal plate maintained at  $50 \pm 0.1^\circ\text{C}$ . The latency to the nociceptive responses hind paw licking or jumping was measured according to the method described by Hunskaar et al. (14), and expressed as the hot-plate latency. To minimize tissue damage, a cut-off time of 90 sec was established. Animals were administered with 5  $\mu$ L (i.t.) vehicle, B50 (200 nmol/ 5  $\mu$ l) or morphine (6.5 nmol/ 5  $\mu$ l), 90, 120 or 150 minutes before the hot-plate test.

#### *Open field*

Immediately after the 90 minutes hot plate test measure, the mice were transferred to an open field (28x18x12 cm) with a floor divided into 15 equal areas. The number of rearing responses and areas crossed with the four paws was counted over a period of 5 minutes by an observer who was not aware of the animals' treatment.

#### *Statistical analysis*

The number of writhes and ambulation scores were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Data from the experiment that aimed to evaluate the role of spinal opiate receptors in B50-induced antinociception were analyzed by a two-way ANOVA, followed by the Student-Newman-Keuls test. Hot-plate test data were analyzed by a two-way ANOVA, followed by the Student-Newman-Keuls test. All data were expressed as mean  $\pm$  S.E.M. The level of significance was set as  $P < 0.05$  and F values are presented in the text only when  $P < 0.05$ .

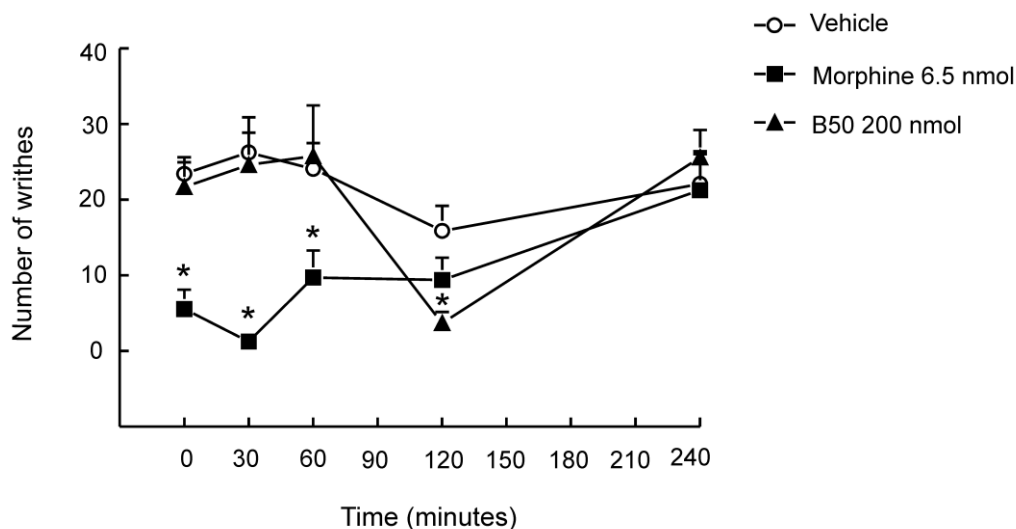
### **Results and discussion**

Figure 2 shows the effect of morphine (6.5 nmol, i.t.) or B50 (200 nmol, i.t.) on the number of writhes induced by acetic acid 0, 30, 60, 120 and 240 min after administration of B50 or morphine. Statistical analysis (one-way ANOVA followed by SNK test) revealed that B50 (120 min after its administration) and morphine (0-60 min after its administration) decreased the number of abdominal writhes [ $F(2,24) = 8.6$ ;  $P < 0.05$ ].

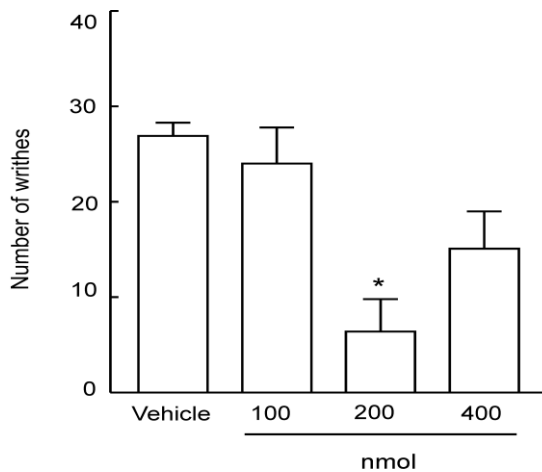
Figure 3 shows the effect of increasing doses of B50 (100; 200; 400 nmol/ 5  $\mu$ l, i.t.) on the number of writhes induced by acetic acid. Statistical analysis revealed a significant effect of treatment [ $F(3,42) = 7.1$ ;  $P < 0.05$ ]. *Post hoc* comparison showed that only the dose of 200 nmol of B50 decreased the number of writhes, as compared to the vehicle-treated group. This dose of B50 did not alter rotarod performance or exploratory activity in an open field, suggesting that B50 does not cause motor impairment, which might be misinterpreted as antinociception (Table 1).

Figure 4 shows the effect of naloxone (8.25  $\mu$ mol/kg, s.c.), a nonselective opioid antagonist, on the antinociception induced by B50 (200 nmol, i.t.) in the acetic acid writhing test. Statistical analysis (two-way ANOVA) revealed a significant pre-treatment (vehicle or B50) by treatment (saline or naloxone) interaction [ $F(1,45) = 7.2$ ;  $P < 0.05$ ], indicating that naloxone reverted B50-induced antinociception.

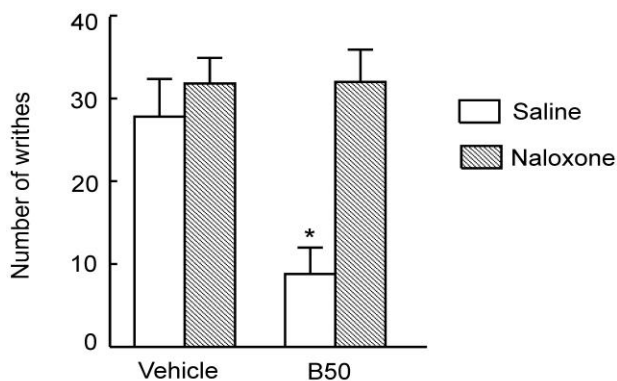
Figure 5 shows the effect of morphine (6.5 nmol, i.t.) or B50 (200 nmol, i.t.) on paw withdrawal or licking latencies in the hot-plate test. Statistical analysis (two-way ANOVA, followed by SNK test) revealed that B50 (90 min after its administration) significantly prolonged hot-plate latencies, compared to the vehicle-treated group [ $F(4,88) = 2.54$   $P < 0.05$ ].



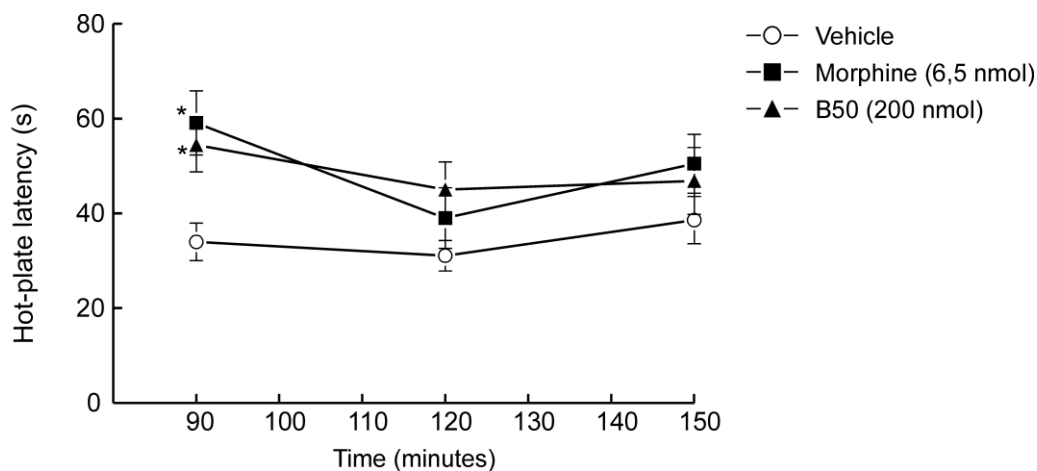
**Figure 2.** Effect of morphine (6.5 nmol/5  $\mu$ l, i.t.) or B50 (200 nmol/5  $\mu$ l, i.t.) on the number of writhes induced by acetic acid at 0, 30, 60, 120 and 240 min after treatment with B50 or morphine. Data are reported as mean  $\pm$  S.E.M., n=8-15 per group. \* $P$ <0.05 compared with vehicle (2.5% Tween 80 in 0.9% NaCl).



**Figure 3.** Effect of B50 (100, 200 or 400 nmol/5  $\mu$ l, i.t.) on the number of writhes induced by acetic acid at 120 min after treatment with B50. Data are reported as mean  $\pm$  S.E.M., n = 10-12 per group. \* $P$ <0.05 compared with vehicle (2.5% Tween 80 in 0.9% NaCl).



**Figure 4.** Effect of naloxone (8.25  $\mu\text{mol/kg}$ , s.c.) on the antinociception induced by B50 (200 nmol/5  $\mu\text{l}$ , i.t.). Data are reported as mean  $\pm$  S.E.M., n =10-14 per group. \* $P$ <0.05 compared with vehicle (2.5% Tween 80 in 0.9% NaCl).



**Figure 5.** Effects of morphine (6.5 nmol/5  $\mu\text{l}$ , i.t.), or B50 (200 nmol/5  $\mu\text{l}$ , i.t.) on the latencies to hind paw lick or jumping in the constant temperature ( $50 \pm 0.1^\circ\text{C}$ ). Data are reported as mean  $\pm$  S.E.M., n = 14-16 per group. \* $P$ <0.05 compared with vehicle (2.5% Tween 80 in 0.9 % NaCl).

**Table 1.** Effect of B50 (200 nmol/5  $\mu\text{l}$ , i.t.) and morphine (6.5 nmol/5  $\mu\text{l}$ , i.t.) on the latency for the first fall and number of falls in the rotarod and on the crossing and rearing in the open-field.

	Vehicle	B50	Morphine
Latency for the first fall	79.74 $\pm$ 22.15	102.27 $\pm$ 27.34	-----
Number of falls	1.12 $\pm$ 0.31	1.74 $\pm$ 0.46	-----
Crossing	58.28 $\pm$ 4.50	59.86 $\pm$ 7.11	68.56 $\pm$ 4.11
Rearing	17.86 $\pm$ 2.50	19.06 $\pm$ 2.69	18.00 $\pm$ 2.23

Data are expressed as mean  $\pm$  SEM for n=10-14 animals in each group. Vehicle (2,5% Tween 80 plus saline/5  $\mu\text{l}$  i.t.), B50 (200nmol/5 $\mu\text{l}$ ) and morphine(6.5 nmol/i.t.).

In the current study we show that the pyrazole-thiazole derivative B50, at the dose of 200 nmol (i.t.), causes antinociception in the acetic acid abdominal writhing assay and in the hot plate test, 120 and 90 minutes after its administration, respectively.

It is interesting that the dose-response curve for B50 was biphasic, with the larger dose (400 nmol) showing less response, and that the antinociceptive effect of this pyrazole derivative appeared only 90 minutes after its administration. The currently reported biphasic dose-effect curve is, to some extent, similar to those reported elsewhere (15-17). In those studies, the authors propose that drug doses larger than the effective dose might allow the interaction with other binding sites, which could counteract the antinociceptive effect caused by small doses of the compound. In this respect, are particularly remarkable the studies that presented ultra-low doses of nonselective opioid antagonists (by systemic and i.t. route) induce antinociception and increase systemic morphine analgesia (15,18,19). It has been suggested that ultra-low doses (picomolar to nanomolar) of an agonist activate a  $G_s$ -coupled mode of the opioid receptor to activate adenylate cyclase (AC) and increase neuronal excitability. These effects produce behavioral hyperalgesia, and are prevented by ultra-low doses of opioid receptor antagonists (15,18,19). On the other hand, opioids at doses within the micromolar range activate a  $G_i/G_o$ -coupled mode of the receptor to inhibit AC activity and reduce neuronal excitability, effects that produce antinociception and are reverted by higher doses of antagonists. Therefore, considering its biphasic antinociceptive effect, B50 seems to be similar to opioid antagonists or, like nalbuphine, produces agonist effects at one receptor subpopulation and antagonist effects at other (20). In fact, the finding that naloxone reverts the antinociceptive effect of B50 suggests that it activates opioid mechanisms, and agrees with this view.

An interesting finding of the present study was that the i.t. injection of B50 caused antinociception only after a 90-120 minutes lag in both antinociceptive tests, while its systemic injection causes antinociception in 30 minutes (8). This pharmacologic profile suggests that B50 is a pro-drug, which has to be metabolized to cause antinociception.

A major concern in experiments designed to assess the antinociceptive action of novel compounds is whether pharmacological treatment causes other behavioral alterations, such as motor or coordination impairment and sedation. In the

present study we showed that B50 does not alter rotarod performance or open field exploratory activity measures. Therefore, the possibility that the currently reported B50-induced antinociception is due to motor effects or sedation sounds unlikely.

In summary, in this study we described that the intrathecal injection of the novel pyrazolyl-thiazole derivative B50 causes delayed antinociception in chemically and in thermally motivated tests. B50-induced antinociception was reverted by the nonselective opioid antagonist naloxone, suggesting that opioid mechanisms are involved in such an effect.

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