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Potent nonopioid antinociceptive activity of telocinobufagin in models of acute pain in mice

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Abstract

Introduction: In recent decades, several researches have been conducted in search of new analgesics that do not present the side effects of opioids. In this context, animal venoms contain natural painkillers that have been used for the development of new analgesics.

Objective: The aims of this study were to evaluate the antinociceptive effects of telocinobufagin (TCB), a bufadienolide isolated from *Rhinella jimi* venom, in murine acute pain models, and to verify the participation of the opioid system in these effects.

Methods: TCB was purified from *R. jimi* venom by high-performance liquid chromatography, and its structure was confirmed by spectrometric techniques. TCB was administered intraperitoneally (i.p.) (0.062, 0.125, 0.25, 0.5, and 1 mg·kg^{-1}) and orally (p.o.) (0.625, 1.125, 2.5, 5, and 10 mg·kg^{-1}) in mice, which were then subjected to pain tests: acetic acid–induced writhing, formalin, tail-flick, and hot-plate. Involvement of the opioid system in TCB action was evaluated by naloxone i.p. injected (2.5 mg·kg^{-1}) 20 minutes before TCB administration. In addition, the TCB action on the μ, δ, and κ opioid receptors was performed by radioligand binding assays.

Results: In all the tests used, TCB showed dose-dependent antinociceptive activity with more than 90% inhibition of the nociceptive responses at the doses of 1 mg·kg^{-1} (i.p.) and 10 mg·kg^{-1} (p.o.). Naloxone did not alter the effect of TCB. In addition, TCB did not act on the μ, δ, and κ opioid receptors.

Conclusion: The results suggest that TCB may represent a novel potential nonopioid therapeutic analgesic for treatment of acute pains.

Keywords: Telocinobufagin, Analgesics, Bufadienolides, Animal venoms

1. Introduction

Opioid analgesics have been used for the pain treatment, but their side effects, such as sedation, physical dependence, tolerance, and respiratory depression, may limit their clinical use.[9,11,30] Therefore, research for new analgesics has been performed over the past decades, and in this context, the selectivity and specificity of toxins from venoms of snakes, spiders, scorpions, marine animals, and amphibians have enabled their use as therapeutic analgesics.[12,18,26,28,50,51]

Telocinobufagin (TCB) is a bufadienolide that was first isolated from the *Ch'an Su* tea, a traditional Chinese medicine prepared from toad venoms (*Bufo bufo gargarizans* Cantor and B. *melanostictus* Schneider), that possesses biological activities, such as cardiotonic, blood pressure stimulation, anti-inflammatory, anesthetic, and antineoplastic activities.[26,33,38] Bufadienolides are a group of polyhydroxy C-24 steroids and their glycosides, containing a six-membered lactone (α−pyrone) ring at the C-17β position, which have been isolated not only from amphibians (*Bufo* spp.),[38] but also from plants (*Kalanchoe* sp.),[41] fireflies (*Photinus* sp.),[48] snakes (*Rhabdophis* sp.),[1,3] and more recently from animals (rats[7,23] and dogs[5]) and humans.[4,6,35] Bufadienolides are now recognized as endogenous steroidal hormones and display a large range of activities such as antiangiogenic, immunosuppressant, regulation of cell growth and differentiation, apoptosis, glucose metabolism, antiendometriosis, positive inotropism, natriuresis, and mood control[8,23,26,43,45]

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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However, bufadienolides can also bind to Na^+/K^+-ATPase and activate other signal transduction pathways, which are independent of the ion pumping activity.[37] Thus, recent studies have shown that some bufadienolides, such as TCB, bufalin, and marinobufagin, may exhibit significant diuretic and natriuretic effects in rodent kidneys by downstream phosphorylation cascade through Src kinase-Ras-Raf-ERK1/2 pathway, which forms a signaling complex with Na^+-K^+-ATPase.[2,25]

Furthermore, some bufadienolides may also induce several events that are not related to the Na^+/K^+-ATPase, such as the anti-inflammatory and analgesic effects. A study performed with patients showed that the bufadienolides scilliroside and proscillaridin-A, both isolated from squill bulb (*Urginea maritima*), reduce the musculoskeletal pains when topically applied. However, the mechanism of action of these compounds was not evaluated.[10] Another work shows that bufalin exhibited anti-inflammatory and antinociceptive activities in mice. The anti-inflammatory effect of this compound was showed by reduction of the carrageenan-induced paw oedema and by inhibition of the activation of NF-κB signalling. In addition, the antinociceptive actions were showed by i.p administration of bufalin in models of acute murine pain. Furthermore, these antinociceptive effects were inhibited by naloxone, suggesting that the mechanism of action of bufalin involves the opioid system.[55] In addition, another study showed that bufalin exhibited analgesic effects in patients with hepatic cancerous pain through increase in hepatic blood circulation.[54]

Preliminary results, performed by Carvalho et al., have shown evidence that TCB exhibits antinociceptive effects in model of neuropathic pain in mice.[13–15] Thus, the aims of this study were to evaluate the antinociceptive effects of TCB administered by i.p and p.o routes in 4 classical murine acute pain models (acetic acid–induced writhing, phases 1 and 2 of formalin, tail-flick, and hot-plate tests) and to verify the participation of the opioid system in these effects.

2. Material and methods

2.1. Reagents and venom

Chemicals were purchased from Sigma-Aldrich (St Louis, MO). Morphine and diazepam were obtained from Cristalia (Brazil). The *R. jimi* venom was obtained from the Universidade Estadual do Ceará (UECE).

2.2. Purification and structural analysis of TCB

TCB was purified from *R. jimi* venom by high-performance liquid chromatography. A solution containing 1-g venom dissolved in 4-mL ethanol absolute was centrifuged at 17,000*g* for 60 minutes. The supernatant was injected into a Shim-Pack PREP-ODS column (25 × 250 mm) eluted isocratically (5 mL·min^{-1}) with 40% acetonitrile/water containing 0.05% trifluoracetic acid over a period of 50 minutes and analyzed at 214 nm. The peak eluted at 27 to 28 minutes containing TCB was lyophilized and stored at −25°C. An aliquot of 100 μg of this purified sample was submitted for rechromatography under the same conditions to estimate its purity.

The TCB structure was confirmed by the methodology previously described.[2] Briefly, the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) was performed on a Bruker Avance DRX 500 NMR spectrometer (Bruker, Biospin, Rheinstetten, Germany), at room temperature, using deuterated chloroform ($CDCl_3$) as the solvent, and was internally referenced to the residual non-deuterated solvent signal at δ_H 7.27 ppm for proton, and at δ_C 77.0 ppm for the central peak of the triplet of the deuterated chloroform carbon. Both one- and two-dimensional ^{1}H and ^{13}C NMR spectra were used to confirm the structure of TCB. The 2 methyl groups characteristics of the tetracycle steroidal skeleton were easily characterized by the sharp singlets, integrating for 3 protons each, at δ_H 0.72 and 0.94 (H-18 and H-19, respectively). By the same token, the hexadienolide (C20–C24) side chain was characterized by the doublets at δ_H 6.28 and 7.82 (J = 10 Hz, H-23 and H-22, respectively) and at δ_H 7.24 (br. s, H-21), while the carbinol proton appeared at δ_H 4.20 (H-3). The correlation of each proton with the carbon to which it is attached was achieved through the 2-dimensional Heteronuclear Single-Quantum Coherence NMR spectrum, thus confirming the TCB structure.

2.3. Animals

Male Swiss Webster mice (25–30 g), purchased from the vivarium of UECE, were maintained in a temperature-controlled room

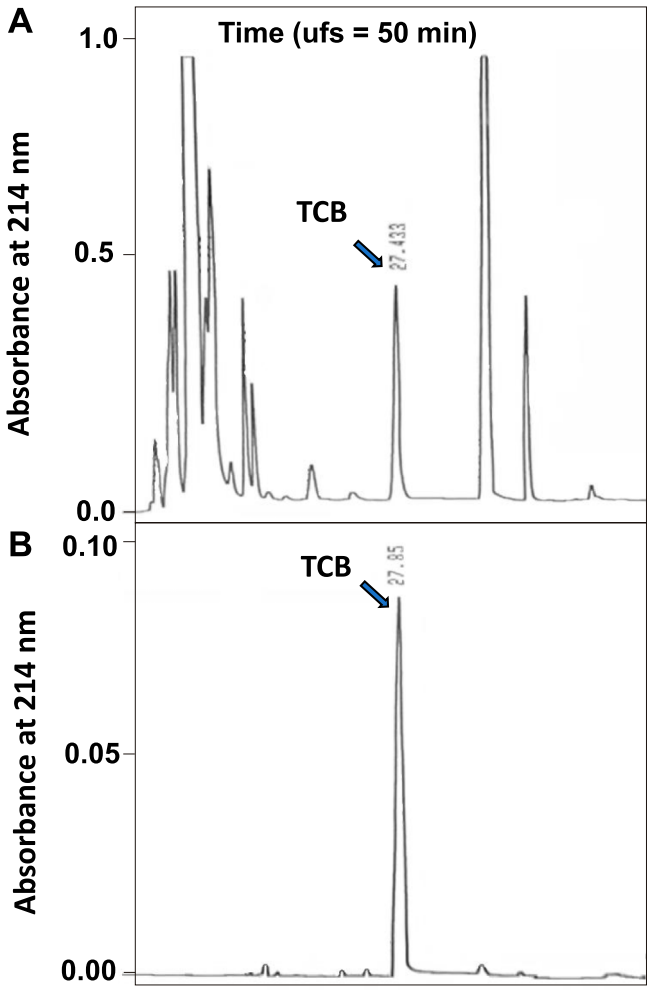

Figure 1. Purification of telocinobufagin (TCB) from *R. jimi* venom by reversed-phase HPLC. (A) A solution containing 1-g venom dissolved in 4-mL absolute ethanol was prepared and centrifuged at 17,000*g* for 60 minutes. The supernatant was injected into a preparative Shim-Pack PREP-ODS column (25 × 250 mm) eluted isocratically (5 mL·min^{-1}) with 40% acetonitrile/water containing 0.05% trifluoracetic acid (TFA) over a period of 50 minutes and analyzed at 214 nm. The peak eluted at 27 to 28 minutes containing TCB was lyophilized and stored at −25°C. (B) An aliquot of 100 μg of this purified sample was submitted to rechromatography under the same conditions to estimate its purity (>95%). HPLC, high-performance liquid chromatography; ufs, unit full scale.

(22 ± 2°C) for 12-hour light/dark cycles with free access to food and water. Before each experiment (12 hours), the animals were limited to a water-only diet. Animal care and research protocols were conducted in accordance with the guidelines adopted by Guide for the Care and Use of Laboratory Animals (NIH Publication 86-23) and by the Colégio Brasileiro de Experimentação Animal (COBEA) and were approved by the Animal Ethical Committee of UECE (protocol 6490035/2017).

2.4. Pharmacological assays

2.4.1. Randomization and blinding of the experiments

The allocation of mice to different groups (n = 8) was at random.[31] The experiments were blinded to diminish the possibility of a subjective effect in collecting data.[24,31] Briefly, although an unblinded group prepared all the experimental conditions (dices, envelopes containing pieces of papers with codes, and tables with random numbers for different doses of drugs), another blinded group performed the experiments.

2.4.2. Doses and routes of drug administration

In this study, a large dose range for TCB and morphine was tested. Thus, for intraperitoneal (i.p.) injection, TCB doses (0.01-, 0.031-, 0.062-, 0.125-, 0.25-, 0.5-, 1-, 2-, and 4mg·kg^{-1} body weight) were prepared by dilution in 2% dimethylsulfoxide in sterile water. For oral (p.o) route, TCB doses (0.1-, 0.312-, 0.625-, 1.25-, 2.5-, 5-, 10-, 20-, and 40 mg·kg^{-1}) were prepared by dilution in labrasol/transcutol solution (4·2^{-1}; vol·vol^{-1})[21] and administered by gavage. Morphine doses were prepared by dilution in sterile water, either for i.p. (0.05-, 0.1-, 0.25-, 0.5-, 1-, 2-, 4-, 8-, and 16 mg·kg^{-1}) or for p.o (0.5-, 1.0-, 2.5-, 5-, 10-, 20-, 40-, 80-, and 120 mg·kg^{-1}) routes.[16,19,29]

However, for the construction of the dose/response curves, the following increasing doses were sequentially selected: the first dose, which was the highest of the doses that had no effect, followed by 2 to 3 doses showing increasing antinociceptive effect, and the last dose, which was the lowest of the doses that induced >90% blocking of the nociceptive effects.

The naloxone dose (2.5 mg·kg^{-1}, i.p.) was prepared by dilution in sterile water.[55]

2.4.3. Acetic acid–induced writhing test

TCB, morphine, and vehicles (controls) were administered by i.p. and p.o routes before i.p. injection of acetic acid (0.6% in sterile water, 10 mL·kg^{-1}). Animals were placed into clear plastic cages for observation and to calculate the number of abdominal writhings, which were defined as an exaggerated extension of the abdomen combined with the outstretching of the hind limbs. The number of writhing was calculated at a start time of 5 minutes after acetic acid injection and continued for 20 minutes. The antinociceptive effects were calculated as the relative decreasing in the number of writhing of the control group.[17]

2.4.4. Formalin test

TCB, morphine, and vehicles (controls) were administered by i.p. and p.o routes before intraplantar injection of formalin (20 μL, 2.5% in sterile water) into the right hind paw. The animals were placed in a glass cylinder, and the time spent licking the injected paw was considered indicative of nociception. Responses were recorded from 0 to 5 minutes (first phase, neurogenic) and from 15 to 30 minutes (second phase, inflammatory) after formalin injection. The antinociceptive effects were calculated as the relative decreasing in the reaction time of the control group.[49]

2.4.5. Tail-flick test

TCB, morphine, and vehicles (controls) were administered by i.p. and p.o routes before immersing the tail in water at 50 ± 0.5°C. The time (seconds) between immersing the tail in water and the withdrawal by a brief vigorous movement was recorded as the response latency. A 20-second cutoff time was used to minimize tissue damage. The antinociceptive effects were calculated as the relative increasing in the reaction time of the control group.[46]

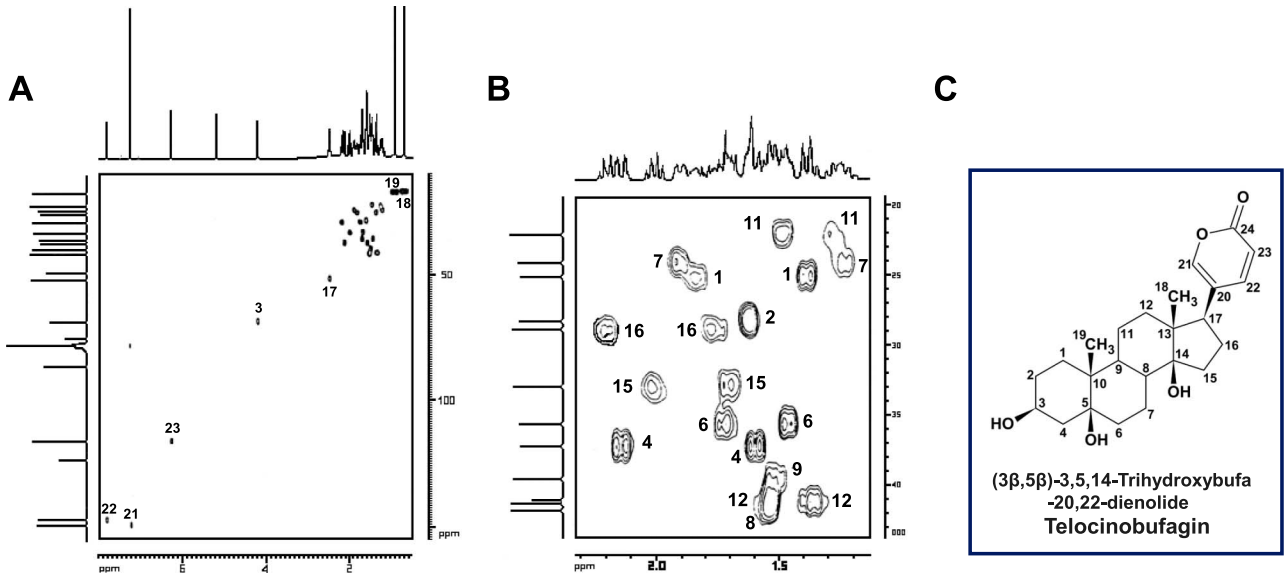

Figure 2. Structural analysis of purified telocinobufagin (TCB). 2D ^{1}H,^{13}C-HSQC NMR spectrum of TCB (500/125 MHz, $CDCl_3$). (A) Full spectrum; (B) expansion (10–50 ppm [δ_C] and 1.0–2.3 ppm [δ_H]). The numbers inside the spectrum indicate the correlation of each proton to the respective carbon of the structure of TCB to which it is connected; (C) structure of purified TCB. HSQC, Heteronuclear Single-Quantum Coherence.

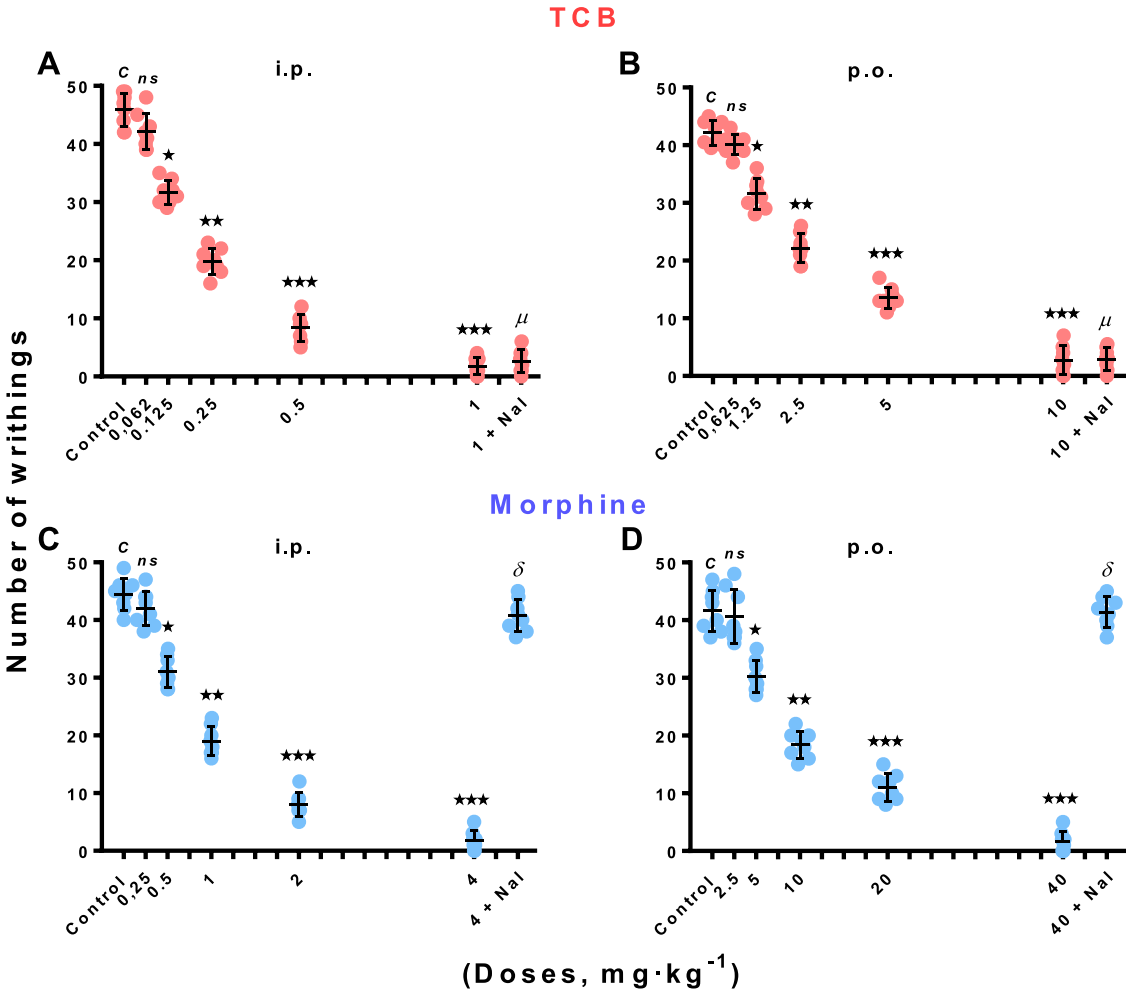

Figure 3. The antinociceptive effect of telocinobufagin (TCB) in the acetic acid–induced writhing test. TCB, morphine, and respective vehicles of dilution (controls) were administered 20 and 60 minutes, respectively, by i.p. (A, C) and p.o (B, D) routes in mice before i.p. injection of 0.6% acetic acid. Naloxone (Nal) was i.p. injected 20 minutes before i.p. and p.o administrations of both TCB and morphine, which in turn were, respectively, administered 20 and 60 minutes before i.p. injection of acetic acid. The number of writhings was calculated at a start time of 5 minutes after acetic acid injection and continued for 20 minutes. Data are expressed as mean ± SD (n = 8). ANOVA followed by the Tukey as post hoc test. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the controls (respective vehicles). $^{\mu}P > 0.05$ compared with the TCB (1 mg·kg^{-1}, i.p. or 10 mg·kg^{-1}, p.o.). $^{\delta}P < 0.001$ compared with the morphine (4 mg·kg^{-1}, i.p. or 40 mg·kg^{-1}, p.o.). ANOVA, analysis of variance.

2.4.6. Hot-plate test

TCB, morphine, and vehicles (controls) were administered by i.p. and p.o routes before placing the animals on heated surface maintained at 55 ± 0.5°C. The time (seconds) between placement and the licking of hind paws or jumping was recorded as the response latency. A 15-second cutoff time was used to minimize tissue damage. The antinociceptive effects were calculated as the relative increasing in the reaction time of the control group.[39]

2.4.7. Open-field test

TCB and vehicles of dilution (controls) were administered 20 and 60 minutes, respectively, by i.p. (1 mg·kg^{-1}) and p.o (10 mg·kg^{-1}) routes in mice before the rotarod test. In addition, diazepam (1 mg·kg^{-1}) and respective vehicle were administered by i.p. route 20 minutes before the open-field test. Each animal was placed into the center of the open-field area and was allowed to ambulate freely for 5 minutes. The number of areas crossed with all paws was recorded.[22]

2.4.8. Rotarod test

TCB and vehicles of dilution (controls) were administered 20 and 60 minutes, respectively, by i.p. (1 mg·kg^{-1}) and p.o (10 mg·kg^{-1}) routes in mice before the rotarod test. After mouse stabilization, the rotation was progressively augmented at a rate of 1 rpm. Mice were subjected to spinning at 4 rpm, and the time that they managed to remain on the rod and the speed at which they fell off were recorded. The average of 3 trials was used for each mouse.[32]

2.4.9. Assessment of opioid system involvement

To evaluate the participation of the opioid system in the TCB action, naloxone (2.5 mg·kg^{-1}), a nonselective opioid receptor antagonist, was i.p. injected 20 minutes before administration of the highest dose of TCB and morphine used in all the tests.[55]

2.5. Radioligand binding assay

This experiment, performed in duplicate, was also used to assess the involvement of TCB in the opioid system, evaluating its binding

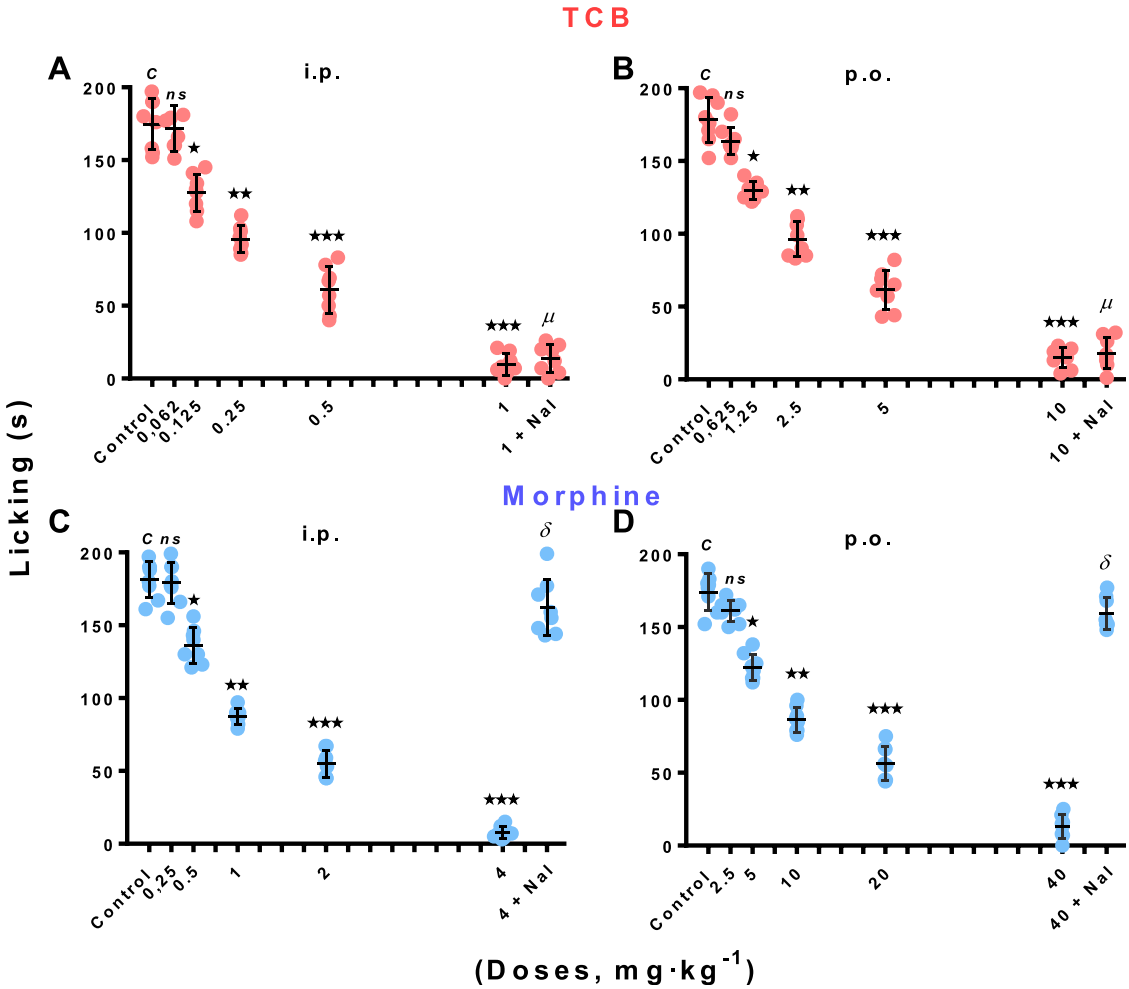

Figure 4. The antinociceptive effect of TCB in phase 1 of the formalin test. TCB, morphine, and respective vehicles of dilution (controls) were administered 20 and 60 minutes, respectively, by i.p. (A, C) and p.o (B, D) routes in mice before intraplantar injection of formalin (20 μL, 2.5% in sterile water) into the right hind paw. Naloxone (Nal) was i.p. injected 20 minutes before i.p. and p.o administrations of both TCB and morphine, which in turn were, respectively, administered 20 and 60 minutes before intraplantar injection of formalin. Responses were recorded from 0 to 5 minutes (first phase, neurogenic) after formalin injection. Data are expressed as mean ± SD (n = 8). ANOVA followed by the Tukey as post hoc test. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the controls (respective vehicles). $^{\mu}P > 0.05$ compared with the TCB (1 mg·kg^{-1}, i.p. or 10 mg·kg^{-1}, p.o.). $^{\delta}P < 0.001$ compared with the morphine (4 mg·kg^{-1}, i.p. or 40 mg·kg^{-1}, p.o.). ANOVA, analysis of variance.

on the μ, δ, and κ opioid receptors present in human cells, which were grown at 37°C in a humidified atmosphere containing 5% CO2.[20] For assay with opioid receptor μ (OP3, MOP), human recombinant CHO-K1 cells were used. The ligand was 0.0619 μM [^{3}H] D-Ala'. NMe-Phe, Gly-oil-enkephalin (DAMGO)-highly selective μ receptor agonist, and the control was 1% DMSO. The nonspecific ligand was 10-μM naloxone (K_D: 0.41 nM; B_{max}: 3.8 pmole·mg^{-1} protein; specific ligation: 90%). The incubation was performed with Tris-HCl 50 mM, pH 7.4 buffer for 60 minutes at 25°C. For assay with receptor opioid δ (OP1, DOP), human recombinant CHO cells were used. The ligand was 1.29 nM [^{3}H] naltrindole δ-highly receptor selective agonist, and the control was 1% DMSO. The nonspecific ligand was 10-μM naloxone (K_D: 0.49 nM; B_{max}: 8.6 pmole·mg^{-1} protein; specific ligation: 90%). The incubation was performed with Tris-HCl 50 mM, MgCl 5 mM, pH 7.4 buffer for 60 minutes at 25°C. For assay with opioid receptor κ (OP2, KOP), human recombinant HEK-293 cells were used. The ligand was 0.0155 μM [^{3}H] N-Methyl-N-7-(1-pyrrollidinyl)-1-oxaspiro4.5dec-8-yl)benzeneacetamide-k-opioid receptor selective agonist (U-69593), and the control was 1% DMSO. The nonspecific ligand was 10-μM naloxone (K_D: 0.4 nM; B_{max}: 1.1 pmole·mg^{-1} protein; specific ligation: 90%). The incubation was performed with Tris-HCl 50 mM, pH 7.4 buffer for 60 minutes at 25°C. The TCB interaction with the different opioid receptors was evaluated through the possible blockade of binding by the specific agonists.

2.6. Statistical analysis and determination of ED_{50}

The data were expressed as the mean ± SD. Significant differences were analyzed by analysis of variance followed by the Tukey post hoc test. $P < 0.05$ was considered statistically significant. ED_{50} values were determined by the *GraphPad Prism 7.03* (https://www.graphpad.com/www/graphpad/assets/File/Prism%206%20-%20Dose-response.pdf). Briefly, for the results in the dose/response columns, the following steps were performed: (1) the X values were transformed to *log* form; (2) the Y values were normalized; (3) nonlinear regression curves were constructed: for the data from writhing and formalin (phases 1, 2) tests were used the *Dose-response-Inhibition* followed by *log(agonist) vs normalized response-Variable slope,* to determine the IC_{50} values (= EC_{50}); for the data from tail-flick and hot-plate tests were used the *Dose-*

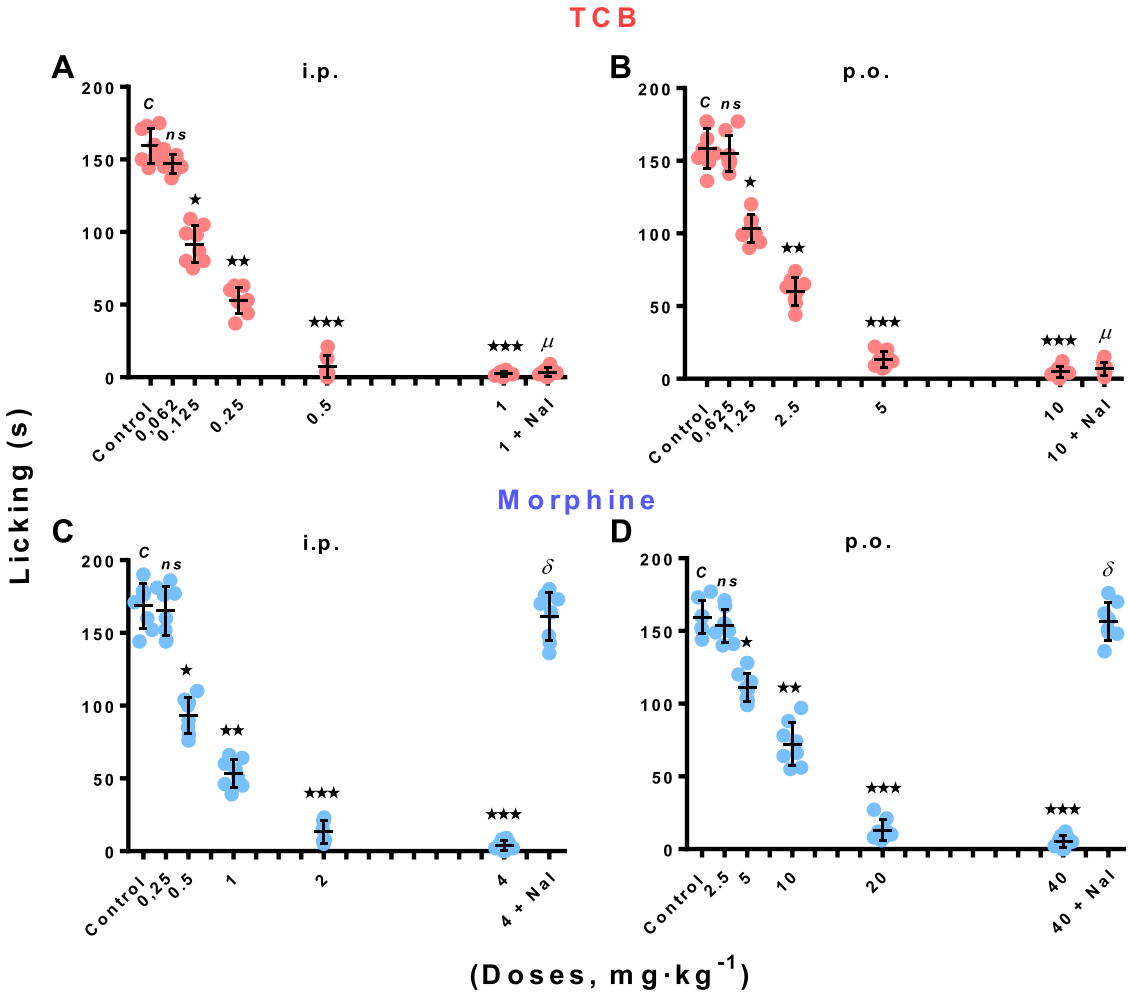

Figure 5. The antinociceptive effect of TCB in phase 2 of the formalin test. TCB, morphine, and respective vehicles of dilution (controls) were administered 20 and 60 minutes, respectively, by i.p. (A, C) and p.o (B, D) routes in mice before intraplantar injection of formalin (20 μL, 2.5% in sterile water) into the right hind paw. Naloxone (Nal) was i.p. injected 20 minutes before i.p. and p.o administrations of both TCB and morphine, which in turn were, respectively, administered 20 and 60 minutes before intraplantar injection of formalin. Responses were recorded from 15 to 30 minutes (inflammatory phase) after formalin injection. Data are expressed as mean ± SD (n = 8). ANOVA followed by the Tukey as post hoc test. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the controls (respective vehicles). $^{\mu}P > 0.05$ compared with the TCB (1 mg·kg^{-1}, i.p. or 10 mg·kg^{-1}, p.o.). $^{\delta}P < 0.001$ compared with the morphine (4 mg·kg^{-1}, i.p. or 40 mg·kg^{-1}, p.o.). ANOVA, analysis of variance.

response-Stimulation followed by *log(agonist) vs. normalized response-variable slope* to determine the EC_{50}."

3. Results

3.1. Purification and structural analysis of TCB

Figure 1A shows the purification of TCB from the venom of *R. jimi* by high-performance liquid chromatography. An aliquot of this purified sample was submitted for rechromatography under the same conditions, and the results shows that the TCB purity was >95% (**Fig. 1B**).

Figure 2A–C shows the structural determination of purified TCB: [(3β,5β)-3,5,14-Trihydroxybufa-20,22-dienolide].

3.2. Antinociceptive activity of TCB in the writhing test

Figure 3A and B shows that the lowest TCB doses administered by i.p (0.062 mg·kg^{-1}) and p.o (0.625 mg·kg^{-1}) routes did not cause significant antinociceptive effects. However, the other 4 doses administered by i.p (0.125, 0.25, 0.5, and 1 mg·kg^{-1}) and by p.o (1.125, 2.5, 5, and 10 mg·kg^{-1}) routes significantly inhibited the number of contortions in mice in a dose-dependent manner. In addition, TCB caused >90% inhibition of the nociceptive effects at doses of 1 mg·kg^{-1} (i.p) and 10 mg·kg^{-1} (p.o).

3.3. Antinociceptive activity of TCB in the formalin test

3.3.1. Phase 1

Figure 4 shows that the lowest TCB doses administered by i.p (0.062 mg·kg^{-1}) and p.o (0.625 mg·kg^{-1}) routes did not cause significant antinociceptive effects. However, the other 4 doses administered by i.p (0.125, 0.25, 0.5, and 1 mg·kg^{-1}) and by p.o (1.125, 2.5, 5, and 10 mg·kg^{-1}) routes significantly inhibited the licking time during the first 5 minutes after intraplantar injection of formalin. Furthermore, TCB caused >90% inhibition of the nociceptive effects at doses of 1 mg·kg^{-1} (i.p) and 10 mg·kg^{-1} (p.o).

3.3.2. Phase 2

Figure 5A, B shows that the lowest TCB doses administered by i.p (0.062 mg·kg^{-1}) and p.o (0.625 mg·kg^{-1}) routes did not cause significant antinociceptive effects. However, the other 4 doses

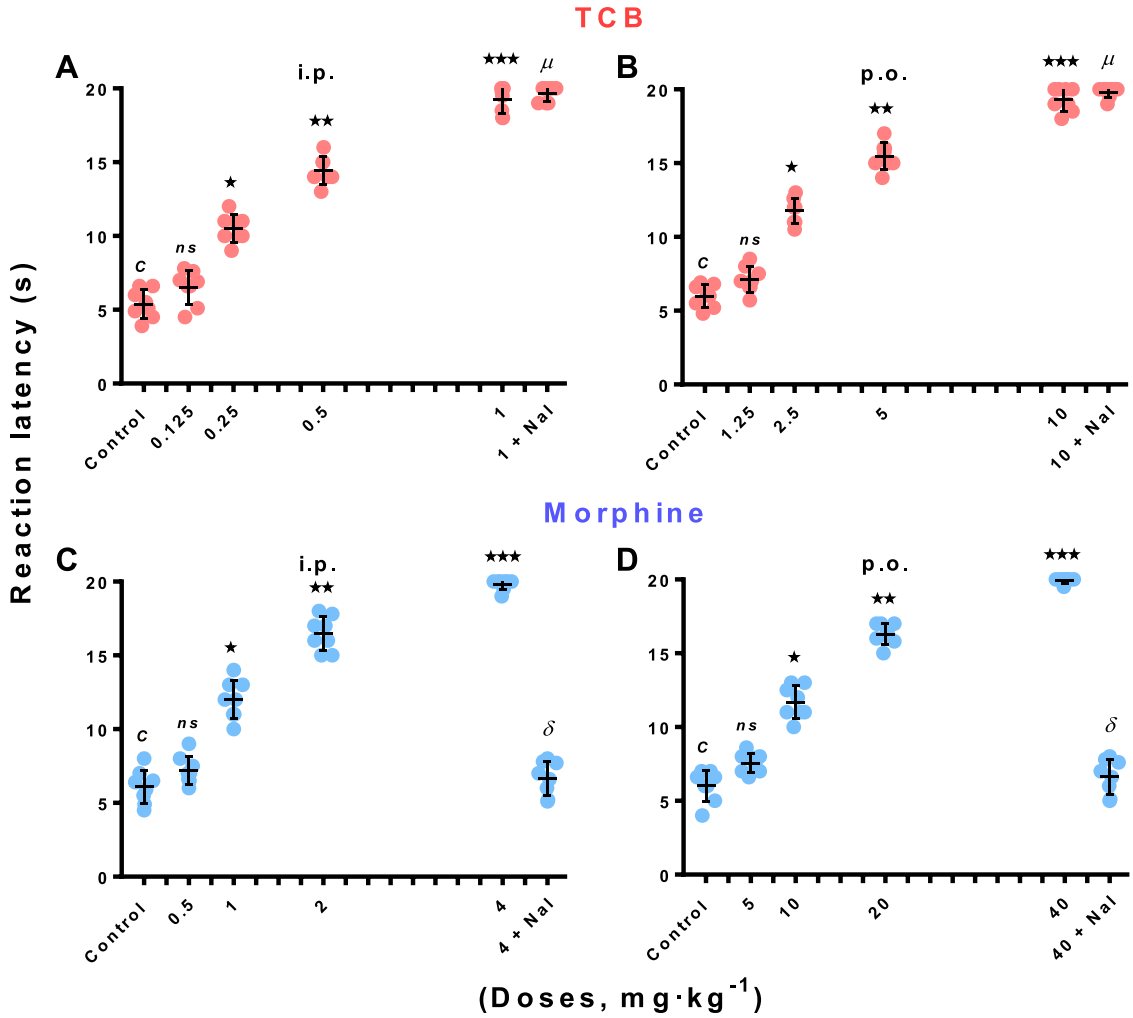

Figure 6. The antinociceptive effect of TCB in the tail-flick test. TCB, morphine, and respective vehicles of dilution (controls) were administered 20 and 60 minutes, respectively, by i.p. (A, C) and p.o (B, D) routes in mice before immersing the tail in water at 47 ± 0.5°C. Naloxone (Nal) was i.p. injected 20 minutes before i.p. and p.o administrations of both TCB and morphine, which in turn were, respectively, administered 20 and 60 minutes before immersing the tail in water. The time (in seconds) between immersing the tail in water and the withdrawal by a brief vigorous movement was recorded as the response latency. Data are expressed as mean ± SD (n = 8). ANOVA followed by the Tukey as post hoc test. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the controls (respective vehicles). $^{\mu}P > 0.05$ compared with the TCB (1 mg·kg^{-1}, i.p. or 10 mg·kg^{-1}, p.o.). $^{\delta}P < 0.001$ compared with the morphine (4 mg·kg^{-1}, i.p. or 40 mg·kg^{-1}, p.o.). ANOVA, analysis of variance.

administered by i.p (0.125, 0.25, 0.5, and 1 mg·kg^{-1}) and by p.o (1.125, 2.5, 5, and 10 mg·kg^{-1}) routes significantly inhibited the licking time from 15 to 30 minutes after intraplantar injection of formalin. In addition, TCB caused >90% inhibition of the nociceptive effects at doses of 1 mg·kg^{-1} (i.p) and 10 mg·kg^{-1} (p.o).

3.4. Antinociceptive activity of TCB in the tail-flick test

Figure 6A, B shows that the lowest TCB doses administered by i.p (0.062 mg·kg^{-1}) and p.o (0.625 mg·kg^{-1}) routes did not cause significant antinociceptive effects. However, the other 4 doses administered by i.p (0.125, 0.25, 0.5, and 1 mg·kg^{-1}) and by p.o (1.125, 2.5, 5, and 10 mg·kg^{-1}) routes significantly increased the reaction time (the withdrawal by a brief vigorous movement) in a dose-dependent manner. Furthermore, TCB caused >90% inhibition of the nociceptive effects at doses of 1 mg·kg^{-1} (i.p) and 10 mg·kg^{-1} (p.o).

3.5. Antinociceptive activity of TCB in the hot-plate test

Figure 7A, B shows that the lowest TCB doses administered by i.p (0.062 mg·kg^{-1}) and p.o (0.625 mg·kg^{-1}) routes did not cause significant antinociceptive effects. However, the other 4 doses administered by i.p (0.125, 0.25, 0.5, and 1 mg·kg^{-1}) and by p.o (1.125, 2.5, 5, and 10 mg·kg^{-1}) routes significantly increased the reaction time (the withdrawal by a brief vigorous movement) in a dose-dependent manner. In addition, TCB caused >90% inhibition of the nociceptive effects at doses of 1 mg·kg^{-1} (i.p) and 10 mg·kg^{-1} (p.o).

3.6. The time–response curves for TCB

The time-responses for TCB were performed in the tail-flick and hot-plate tests. The results show that TCB (1 mg·kg^{-1}, i.p; 10 mg·kg^{-1}, p.o) presented significant reaction latencies for 3 hours after administration (**Fig. 8A, B**).

3.7. Open-field test

TCB administered by i.p. (1 mg·kg^{-1}) and by p.o (10 mg·kg^{-1}) routes did not affect locomotion in mice because the number of areas crossed by all paws in the TCB-treated groups was not significantly different from the control group over a 5-minute

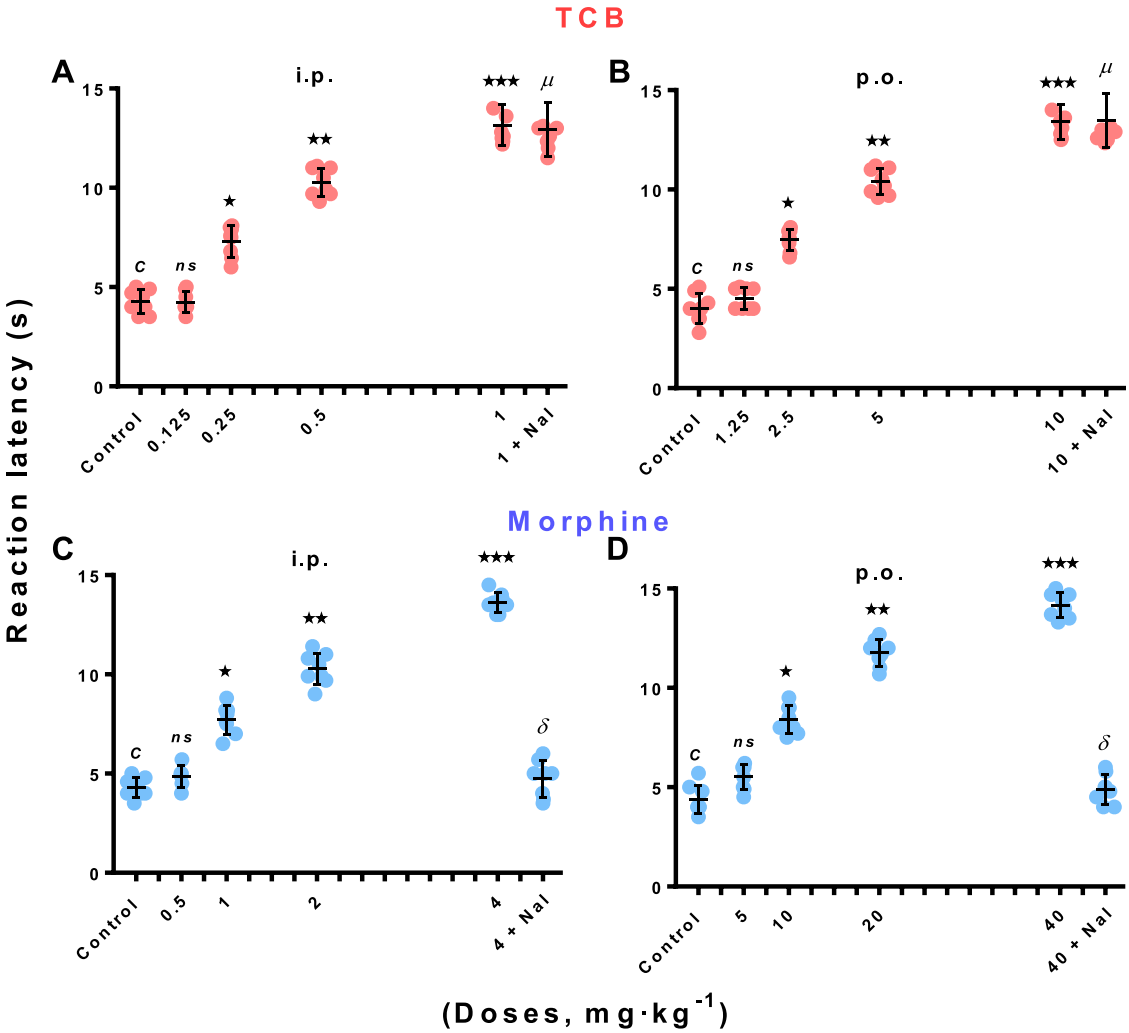

Figure 7. The antinociceptive effect of TCB in the hot-plate test. TCB, morphine, and respective vehicles of dilution (controls) were administered 20 and 60 minutes, respectively, by i.p. (A, C) and p.o (B, D) routes in mice before placing the animals on heated surface maintained at 50 ± 0.5°C. Naloxone (Nal) was i.p. injected 20 minutes before i.p. and p.o administrations of both TCB and morphine, which in turn were, respectively, administered 20 and 60 minutes before placing the animals on heated surface. The time (in seconds) between placement and the licking of hind paws or jumping was recorded as the response latency. Data are expressed as mean ± SD (n = 8). ANOVA followed by the Tukey as post hoc test. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the controls (respective vehicles). $^{\mu}P > 0.05$ compared with the TCB (1 mg·kg^{-1}, i.p. or 10 mg·kg^{-1}, p.o.). $^{\delta}P < 0.001$ compared with the morphine (4 mg·kg^{-1}, i.p. or 40 mg·kg^{-1}, p.o.). ANOVA, analysis of variance.

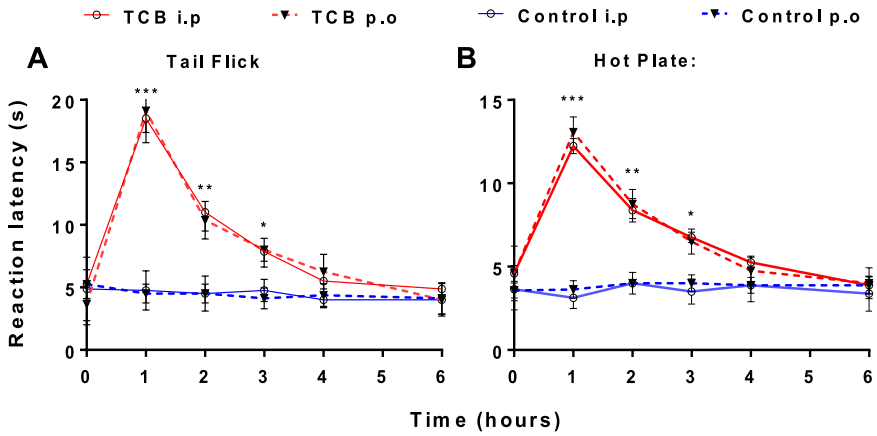

Figure 8. Response latencies of TCB in the tail-flick and hot-plate tests. The response latencies of TCB, administered by i.p (1 mg·kg^{-1}) and p.o (4 mg·kg^{-1}) routes, were measured at 1, 2, 3, 4, 5, and 6 hours in the tail-flick (A) and hot-plate (B) tests. The respective dilution vehicles were used as controls. Data are expressed as mean ± SD (n = 8). ANOVA followed by the Tukey as post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the respective vehicle controls. ANOVA, analysis of variance.

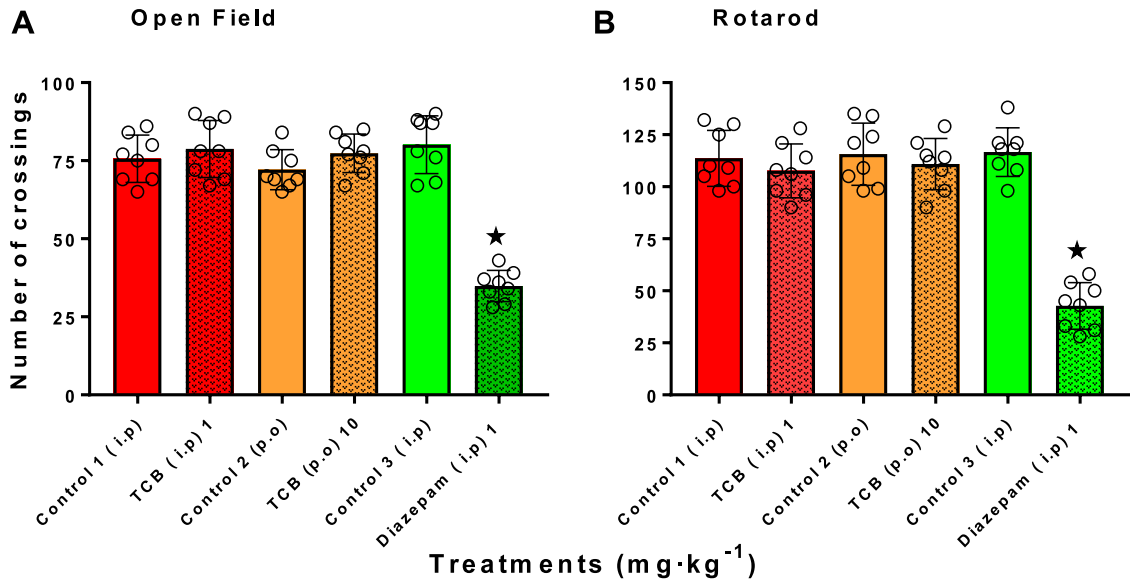

Figure 9. The open-field and rotarod tests. TCB and respective dilution vehicles (controls 1 and 2) were administered 20 and 60 minutes, respectively, by i.p. and p.o routes in mice before the open-field (A) and rotarod (B) tests. Diazepam and the respective dilution vehicle (controls 3) were administered 20 minutes before the both tests. In open-field test, each animal was placed into the center of the open-field area and was allowed to ambulate freely for 5 minutes. The number of areas crossed with all paws was recorded. In rotarod test, after mouse stabilization, the rotation was progressively augmented at a rate of 1 rpm. Mice were subjected to spinning at 4 rpm, and the time that they managed to remain on the rod and the speed at which they fell off were recorded. The average of 3 trials was used for each mouse. Data are expressed as mean ± SD (n = 8). ANOVA followed by the Tukey as post hoc test. *$P < 0.05$, compared with the control (respective vehicle). ANOVA, analysis of variance.

period. By contrast, diazepam (1 mg·kg^{-1}, i.p.) significantly >50% suppressed ambulatory behavior (**Fig. 9A**).

3.8. Rotarod test

TCB administered by i.p. (1 mg·kg^{-1}) and by p.o (10 mg·kg^{-1}) routes did not affect the forced motor coordination, since the time of the TCB-treated mice was not significantly different from the control group. Diazepam (1 mg·kg^{-1}, i.p.) significantly >50% suppressed ambulatory behavior (**Fig. 9B**).

3.9. ED_{50}

Table 1 shows the ED_{50} values for TCB and morphine calculated from the results of the nociceptive tests. All the results from the relation ED_{50} (TCB)/ED_{50} (Mor) were about 0.25, indicating that the TCB potency was about 4 times higher than that of morphine.

3.10. Radioligand binding assays

The results described in **Table 2** show that TCB did not bind to μ, δ, and κ opioid receptors. These results corroborate with those of the in vivo assays, which showed that the antinociceptive effects of TCB were not reverted by naloxone.

Table 1

ED_{50} values of TCB and morphine.

Tests	i.p route			p.o route		
	ED_{50}		$\frac{ED_{50}\ (TCB)}{ED_{50}\ (Mor)}$	ED_{50}		$\frac{ED_{50}\ (TCB)}{ED_{50}\ (Mor)}$
	TCB	Mor		TCB	Mor	
Writhing test	0.20	0.79	0.25	2.62	9.28	0.28
Formalin (phase1)	0.27	1.00	0.27	2.23	8.91	0.25
Formalin (phase2)	0.15	0.64	0.23	1.78	8.05	0.22
Tail-flick	0.32	1.1	0.29	2.71	12.6	0.21
Hot-plate	0.35	1.32	0.27	3.24	10.93	0.29

ED_{50}, 50% of the effective dose; Mor, morphine; Nal, naloxone.

4. Discussion

In this study, 4 classical models of acute murine pain, contortion induced by acetic acid, formalin (phases 1 and 2), tail-flick, and hot-plate tests, were used to evaluate the antinociceptive effects of TCB.[36,52]

The acetic acid–induced writhing test is a model of inflammatory pain used to screen new agents with peripheral analgesic and anti-inflammatory properties.[17] The behaviors are considered to be reflexes and to be evidence of visceral pain.[42] In this test, TCB and morphine, by i.p and p.o route, significantly inhibited, in a dose-dependent manner, the number of writhes in mice (**Fig. 3A, B**). In addition, in this test, the TCB efficacy was similar to that of morphine, since both drugs >90% inhibited the nociceptive effects.

A neurogenic and inflammatory pain model, the formalin test, was also used to further assess the antinociceptive properties of TCB. Formalin elicits a biphasic behavioral response. The first phase (neurogenic phase) occurs during the first 5 minutes after formalin injection, and the behavioral effects are related to the direct chemical stimulation of nociceptors. The second phase (inflammatory phase) occurs during the 15th and 30th minutes after formalin injection, and this phase involves inflammatory pain that is induced by a combination of stimuli, including inflammation of peripheral issues and mechanisms of central sensitization. Centrally acting drugs such as opioids inhibit both phases equally, but peripherally acting drugs, such as nonsteroidal anti-inflammatory drugs and corticosteroids, only inhibit the second phase.[47] In our study, TCB and morphine reduced, in a dose-dependent manner, the pain responses during both phases (**Figs. 4A, B and 5A, B**). Furthermore, in this assay, the TCB efficacy was similar to that of morphine, since both drugs >90% inhibited the nociceptive effects.

Table 2

Test of TBC activity in the opioid receptor biochemical tests.

Receptor	TCB			Reference		
	Species	n	Concentration	% inhibition*	Compound	IC_{50}
μ opioid (OP3, MOP)	Hum	2	10 μM	−1	DAMGO†	0.0619 μM
δ opioid (OP1, DOP)	Hum	2	10 μM	1	Naltrindole	1.29 nM
κ opioid (OP2, KOP)	Hum	2	10 μM	6	U-69593‡	0.0155 μM

* Significance criterion or significant response: >50% maximum inhibition.
† D-Ala'. NMe-Phe, Gly-oil-enkephalin—highly selective peptide agonist.
‡ N-Methyl-N-7-(1-pyrrollidinyl)-1-oxaspiro4.5dec-8-yl)benzeneacetamide-k-opioid receptor selective ligand.
Hum, human.

The tail-flick test was used to evaluate the antinociceptive effect of TCB in the spinal reflex.[46] TCB and morphine significantly increased (in a dose-dependent manner) the reaction time (the withdrawal of the tail) during the tail-flick test (**Fig. 6A, B**). In addition, in this assay, the TCB efficacy was similar to that of morphine, since both drugs >90% inhibited the nociceptive effects.

The hot-plate test is used to distinguish between central and peripheral antinociceptive effects. This test evaluates a possible central action in which agents exert their analgesic effects through supraspinal and spinal receptors.[39] TCB and morphine increased (in a dose-dependent manner) the reaction time (paw licking and/or jumping) during the hot-plate test (**Fig. 7A, B**). Furthermore, in this assay, the TCB efficacy was similar to that of morphine, since both drugs >90% inhibited the nociceptive effects.

The comparison between the ED_{50} values of TCB and morphine was show in **Table 1**. Although the efficacy of TCB and morphine has been shown to be similar (**Figs. 3–7**), the results from the relation ED_{50} (TCB)/ED_{50} (Mor), in all the tests, were around 0.25, indicating that the TCB potency was about 4 times higher than that of morphine.

The time-responses for TCB and morphine, by i.p and p.o routes, in the tail-flick and hot-plate tests, show significant reaction latencies during 3 hours after administration (**Fig. 8A, B**). Furthermore, the TCB time-responses are similar to those of morphine by i.p and p.o routes.[16,19,29]

To evaluate the involvement of the opioid system on the TCB action, naloxone (2.5 $mg{\cdot}kg^{-1}$, i.p) was used before the TCB (1 $mg{\cdot}kg^{-1}$, i.p; 4 $mg{\cdot}kg^{-1}$, p.o). In all the nociceptive tests, naloxone did not inhibit the antinociceptive effects of TCB (**Figs. 3–7**). In addition, these results were corroborated by radioligand binding assay, which showed that TCB did not bind as orthosteric ligands to μ, δ, and κ opioid receptors (**Table 2**).[20] These results indicate that the mechanism of action of TCB is not opioid.

Furthermore, it is also interesting to compare the antinociceptive effects of TCB with those of bufalin that exhibits anti-inflammatory action mediated by inhibition of the NF-κB signaling and potent antinociceptive activity in contortion induced by acetic acid, formalin (phases 1 and 2), and hot-plate tests.[55] However, contrary to TCB, the antinociceptive effects of bufalin were inhibited by naloxone, suggesting the involvement of the opioid system. Furthermore, as the ED_{50} of bufalin has not been determined and as only 2 doses showed significant effects in that work, it is difficult to make comparison of their results with those of TCB.

Two tests were applied to evaluate the possible alterations of locomotor activity in mice after TCB administration: the open-field test, used to assay general locomotor activity levels, anxiety, and willingness to explore in animals,[22,53] and the rotarod, a performance test to evaluate the forced motor coordination.[32] These assays show that TCB did not cause significant sedative and locomotor activity changes in mice (**Fig. 9A, B**). Thus, these results indicate that the analgesic activity of TCB is not influenced by sedative effects or motor disabilities.

Because bufadienolides have been identified in animal and human tissues, several works have suggested that TCB may be an endogenous steroid that exhibits physiological functions,[8,23,26,43,45] but to date, no evidence has been presented that this compound may be an endogenous analgesic. On the other hand, dysregulation of this hormone seems to play a role in a number of disease states.[27,40,41,44] In this context, although TCB may exhibit significant diuretic and natriuretic effects in rat kidneys by downstream phosphorylation cascade through Src kinase-Ras-Raf-ERK1/2 pathway,[2,25] a recent study shows that the chronic infusion of TCB for 4 weeks in mice may promote increased proteinuria and cystatin C.[34] Thus, we cannot exclude that TCB may induce toxic effects in a chronic setting, and to better elucidate these conflicting results, further studies could be performed looking for the same experimental conditions, such as identical animal species, dose range, route of administration, and in vivo and in vitro experiments.

In conclusion, the results taken together suggest that TCB, administered by i.p and p.o routes, possesses central and peripheral nonopioid antinociceptive effects with similar efficacy to that of morphine. In addition, the results also shown that the TCB potency was about 4 times higher than that of morphine. Finally, although further studies are needed to elucidate the mechanism of action of TCB, this compound may represent a novel potential nonopioid therapeutic analgesic for the acute pain treatment.

Disclosures

The authors have no conflicts of interest to declare.

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