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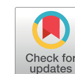
Pharmacological and molecular docking assessment of cryptotanshinone as natural-derived analgesic compound



De Caro Carmen[a,1], Raucci Federica[a,1], Saviano Anella[a], Cristiano Claudia[a], Casillo Gian Marco[a], Di Lorenzo Ritamaria[a], Sacchi Antonia[a], Laneri Sonia[a], Dini Irene[a], De Vita Simona[b], Chini Maria Giovanna[b,c], Bifulco Giuseppe[b,*], Calignano Antonio[a], Maione Francesco[a,*], Mascolo Nicola[a]

[a] Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, Via Domenico Montesano 49, 80131, Naples, Italy
[b] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, 84084, Fisciano, Salerno, Italy
[c] Department of Biosciences and Territory, University of Molise, Contrada Fonte Lappone, Pesche, Isernia, I-86090, Italy

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ABSTRACT

Medicinal plants from traditional chinese medicine are used increasingly worldwide for their benefits to health and quality of life for the relevant clinical symptoms related to pain. Among them, *Salvia miltiorrhiza* Bunge is traditionally used in asian countries as antioxidant, anticancer, anti-inflammatory and analgesic agent. In this context, several evidences support the hypothesis that some tanshinones, in particular cryptotanshinone (CRY), extracted from the roots (Danshen) of this plant exhibit analgesic actions. However, it is surprisingly noted that no pharmacological studies have been carried out to explore the possible analgesic action of this compound in terms of modulation of peripheral and/or central pain.

Therefore, in the present study, by using peripheral and central pain models of nociception, such as tail flick and hot plate test, the analgesic effect of CRY in mice was evaluated. Successively, by the aim of a computational approach, we have evaluated the interaction mode of this diterpenoid on opioid and cannabinoid system. Finally, CRY was dosed in mice serum by an HPLC method validated according to European Medicines Agency guidelines validation rules.

Here, we report that CRY displayed anti-nociceptive activity on both hot plate and tail flick test, with a prominent long-lasting peripheral analgesic effect. These evidences were indirectly confirmed after the daily administration of the tanshinone for 7 and 14 days. In addition, the analgesic effect of CRY was reverted by naloxone and cannabinoid antagonists and amplified by arginine administration. These findings were finally supported by HPLC and docking studies, that revealed a noteworthy presence of CRY on mice serum 1 h after its intraperitoneal administration and a possible interaction of tested compound on μ and k receptors.

Taken together, these results provide a new line of evidences showing that CRY can produce analgesia against various phenotypes of nociception with a mechanism that seems to be related to an agonistic activity on opioid system.

1. Introduction

Pain is a global health problem with a high impact on life quality, becoming one of the most adversaries of modern medicine [1,2]. Despite the large number of available drugs, there are no curative conventional treatments for some forms of pain and nowadays, in the field of drug discovery more attention has been focused on the herbal formulations [3]. The use of medicinal plants as antinociceptive drugs

Abbreviations: ARG, arginine; CB, cannabinoids; COX, cyclooxygenase; CRY, cryptotanshinone; Ctrl, control; CV, coefficient of variation; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; ICV, intracerebroventricular; IP, intraperitoneal; LLOD, lower limit of detection; LLOQ, lower limit of quantifications; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLX, naloxone; PBS, phosphate-buffered saline; RIM, rimonabant; RT, retention time; TLR, toll-like receptor

* Corresponding authors.
E-mail addresses: bifulco@unisa.it (B. Giuseppe), francesco.maione@unina.it (M. Francesco).

[1] These authors share first co-authorship.

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in traditional therapy is estimated to be about 80 % in the world population [4,5]. This aspect has prompted investigators, in the last decades, to pay a lot of attention to the active compounds present in medicinal plants, and to their effects on inflammation and pain [6–8].

Dried roots and rhizomes of *Salvia miltiorrhiza* (commonly known as Danshen) are among the most commonly used traditional chinese medicines in clinic. The material basis for Danshen efficacy mainly includes the presence of hydrophobic tanshinones and hydrophilic acids that can "removing blood stasis and relieving pain" [9,10]. Cryptotanshinone (CRY) is one of the most active components of Danshen, which is popularly used in the treatment of cerebrovascular and cardiovascular diseases [10,11]. In asian countries, the safety of this diterpenoid-like compound has been well-established, and it is widely used for the treatment of arrhythmia, acute ischemic stroke and angina pectoris [12].

Recently, it has been also recognized that tanshinones play a critical role in the alleviation of inflammatory and neuropathic pain by inhibiting High Mobility Group Box 1 (HMGB1) expression [13]. In addition, it has been reported their ability in inducing nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activation, a major regulatory pathway of cytoprotective gene expression against oxidative stress [14]. Contextually, a recent report from Cao and colleagues [15] has highlighted the potential of CRY to inhibit prostaglandin E_2 (PGE_2) production and COX-2 expression via suppression of TLR4/NF-κB signalling pathway. These findings are in accordance with our recent research group investigation, that has demonstrated the long-lasting pain-relieving effects of Danshen and of its related bioactive constituent in animal models of neuropathic pain [16].

Although it has been reported that tanshinones possess a significant antinociceptive effect in different *in vivo* models of nociception, it is surprisingly noted that no pharmacological studies have been carried out to explore the potential analgesic action of CRY on peripheral and central pain. Therefore, in the present study, we further investigated the antinociceptive effect of CRY by an integrated and multidisciplinary approach in order to demonstrate the precise contribution of this tanshinone on both peripheral and central nociceptive state.

2. Materials and methods

2.1. Reagents

CRY (≥ 97 %, HPLC) was obtained from Sigma–Aldrich Co. (Milan, Italy). Stock solutions of tested compound were stored in dimethyl sulfoxide (DMSO). Fresh suspensions were prepared in warm (37 °C) physiological saline (0.9 % NaCl in distilled water) to a final DMSO concentration of 10 %. AM630 (AM; a CB2 receptor selective antagonist) and rimonabant (RIM; a CB1 receptor selective antagonist) were obtained from Sigma–Aldrich Co. (Milan, Italy) and dissolved in DMSO (20 %), Tween-20 (20 %) and physiological saline. Naloxone hydrochloride (NLX; an opioids receptor non-selective antagonist), L-NAME (L-NAME; an inhibitor of nitric oxide synthase) and arginine (ARG) were purchased from R&D System (Milan, Italy) and dissolved in physiological saline. Unless otherwise stated, all the other reagents were from Carlo Erba (Milan, Italy).

2.2. Animals

Experiments were carried out in 8–12-week-old male CD-1 mice according to the guidelines for the safe use and care of experimental animals in accordance with the italian D.L. no. 116 of 27 January 1992 and associated guidelines in the European Communities Council (86/609/ECC and 2010/63/UE) including the 3Rs concept [17,18]. Animals were housed with *ad libitum* access to food and water and maintained on a 12 h light/dark cycle. Experimental study groups were randomized and blinded. All procedures were carried out to minimize the number of animals used (n = 7 per group) and their suffering.

2.3. Models of nociception: hot plate and tail flick test

In the case of hot plate test, the thermal nociception was assessed with a commercially available apparatus consisting of a metal plate 25 cm × 25 cm (Ugo Basile, Italy) heated to a constant temperature of 48.5 ± 0.1 °C, on which a plastic cylinder (20 cm diameter, 18 cm high) was placed. The time of latency (s) was recorded from the moment the animal was placed inside the cylinder up to when it licked its paws or jerked them off the hot plate, jumped off the hot plate, or the latency exceeded the cut-off time of 60 s. The baseline was calculated as the mean of 3 readings recorded before testing, at precise intervals. The time course of latency was then determined at 1, 3, 5 and 24 h after single CRY treatment (10 mg/kg; i.p.). In another set of experiments, the tested compound was administrated daily for 7 and 14 days followed by hot plate readings. The tail-flick latency was obtained using a commercial unit (Ugo Basile, Italy), consisting of an infrared radiant light source (100 W, 15 V bulb) focused onto a photocell utilizing an aluminium parabolic mirror. During the trials, the mice were gently hand-restrained using leather gloves. Radiant heat was focused 3–4 cm from the tip of the tail, and the latency (s) of the tail withdrawal recorded (tail flick time). The measurement was interrupted if the latency exceeded the cut-off time (15 s at 15 V). The baseline and readings were calculated as described for the hot plate test [19,20]. For the antagonism experiments, NLX (1 mg/kg; i.p.), AM (10 mg/kg; i.p.), RIM (0.1 mg/kg; i.p.), L-NAME (30 mg/kg; i.p.) and ARG (1 g/kg; i.p.) were given 30 min before CRY administration.

2.4. Upper gastrointestinal transit

Gastrointestinal transit was studied as previously described [21]. Mice received orally 0.1 ml/10 g/ mouse of a black marker (10 % charcoal suspension in 5 % gum arabic). After 20 min the mice were killed by asphyxiation with CO_2 and the gastrointestinal tract removed. The distance travelled by the marker was measured and expressed as a percentage of the total length of the small intestine from pylorus to caecum. CRY (10 mg/kg) was given i.p. 20 min before charcoal administration. In experiments investigating the mode of action of tested compound, NLX was given at dose of 1 μg/3 μl and 1 mg/kg i.c.v. and i.p. respectively, 30 min before CRY administration. The i.c.v. microinjections were performed using a micro-syringe (10 μl, Hamilton) with a 26-gauge stainless-steel needle that was inserted perpendicularly 3 mm deep through the skull according to the procedure described by Maione and coll. [11] and Cristiano and coll. [22]. The needle was removed after 3 min using three intermediate steps with a 1-min inter-step delay to minimize backflow. After the surgery and NLX injection, mice were placed on a thermal pad until they awakened. All procedures were performed with strict aseptic manipulations and procedures.

2.5. Chromatographic assay

Determination of CRY in mice serum (obtained from whole blood collected by intracardiac puncture) was evaluated by the method proposed by Gao and coll. [23] with some modification. Briefly, 20 μl of serum obtained at both 1 h and 7 days post CRY treatment, was filtered through 0.22 μm filter (Titan Membrane, Millipore) and successively chromatographed on HPLC Jasco LC-Net II/ADC (JASCO International Co., Ltd. Tokyo, Japan) with a 20 μl Rheodyne 8125 injector (Rheodyne, Ronhert Park, California, USA), an UV detector "Jasco MD-2010 plus" (JASCO International Co., Ltd. Tokyo, Japan) (λ254 nm) and a reversed phase Jupiter C-18 5 μm (150 × 4.6 mm) column (Phenomenex, California, USA). Using a gradient elution 0–45 min, 55–90 % methanol and water (with 0.1 % acetic acid) as mobile phase and 1.0 ml/min flow rate. The related standard for CRY was prepared at three concentration levels: 53.16, 83.27 and 171.27 g/l in CH_3OH. The method was validated, considering linearity, LLOD, LLOQ, precision and accuracy, according to European Medicines Agency guidelines

validation rules [24]. The method of constant addition was used for the construction of calibration curves (Supplementary Fig. 1). The assay selectivity was defined by analysis of mice serum, with and without internal standard. We did not observe interfering peaks present at the retention time for CRY (Supplementary Fig. 2).

2.6. Docking analysis: input file preparation

The crystal structures of opioid receptors μ and κ in their active state bound to agonists (PDB: 5C1M [25] and 6B73 [26]) were obtained from the Protein Data Bank [27,28]. Because the experimental structure of the activated opioid receptor δ was not available in the database, it was generated using the homology modelling tool of the software Prime [29–31] with the crystal structure of the μ receptor in its active state used as template. Afterwards, the three target proteins were prepared using the Protein Preparation Wizard tool available in the Schrodinger Suite [32,33]. In detail, this procedure adds missing hydrogens in the structure, creates disulfide bonds, and regulates the bond orders. Then the protonation state at physiological pH is assigned. At the same time, the ligand structures were built with the Maestro's Build Panel [34] and prepared with the LigPrep protocol [35]. Like the Protein Preparation Wizard, this protocol generates the ionization states at physiological pH and generate tautomers unless otherwise indicated.

2.7. Docking experiments

The molecular docking calculations were carried out with the software Glide [36–39] in the Extra Precision Mode. The selection of the initial poses was performed using the expanded sampling and keeping 10.000 poses for each ligand. Out of those, 800 were submitted for energy minimization setting the Van der Waals radii scaling factor at 0.8 and the partial charge cut-off at 0.15. Only 20 poses with an energy lower than 0.5 kcal/mol were returned for each ligand after the procedure was completed. The resulting poses were visually inspected.

2.8. Statistical analysis

The analysis was performed with Graph-Pad Prism (Graph-Pad Software 8.0). All data are presented as means ± S.E.M. and were analyzed using Student's *t*-test (two groups) or one-way ANOVA followed by Bonferroni's multiple comparison test (more than two groups). Differences between means were considered statistically significant with $P \leq 0.05$. Sample size was chosen to ensure α 0.05 and power 0.8. Animal weight was used for randomization and group allocation. No animals were excluded from analysis. The data and statistical analysis comply with the recommendations on experimental design, analysis [40] and data sharing and presentation in preclinical pharmacology [41,42].

3. Results

3.1. Effect of CRY on thermal nociception

We performed a first set of experiments to evaluate the effects of acute i.p. administration of CRY (10 mg/kg) on thermal nociception. As shown in Fig. 1A, the diterpenoid reached its pick of nociceptive response between 1 and 3 h on the hot plate test with a progressive reduction in the following 24 h. Tested compound displayed the same profile on the tail flick test (Fig. 1B). However, CRY possessed a different analgesic profile when administrated chronically (7 and 14 days). Specifically, CRY reached a significant nociceptive threshold in mice on hot plate test after 7 days treatment (with subsequent loss of its analgesic profile after 2 weeks of treatment) (Fig. 1C), whereas on tail flick test CRY possessed a significant thermal latency at both 7 and 14 days (Fig. 1D).

The acute antinociceptive effect induced by the tested compound was completely reverted by pre-treatment with NLX in both hot plate (Fig. 2A) and tail flick (Fig. 2B) test. A different inhibitory profile it was observed with cannabinoid antagonists. In particular, the pre-treatment with a selective CB1 (RIM) and CB2 (AM630) antagonist was able to revert CRY nociceptive response only in the tail flick test (Fig. 2C and D). Although higher than those effects observed after CBs antagonist, it was interesting to observe that pre-treatment with L-NAME was able to evoke the same pharmacological profile. (Fig. 3A and B). L-NAME inhibitory effect was confirmed by arginine pre-treatment that amplified CRY nociceptive threshold on tail flick, but also hot plate, test (Fig. 3A

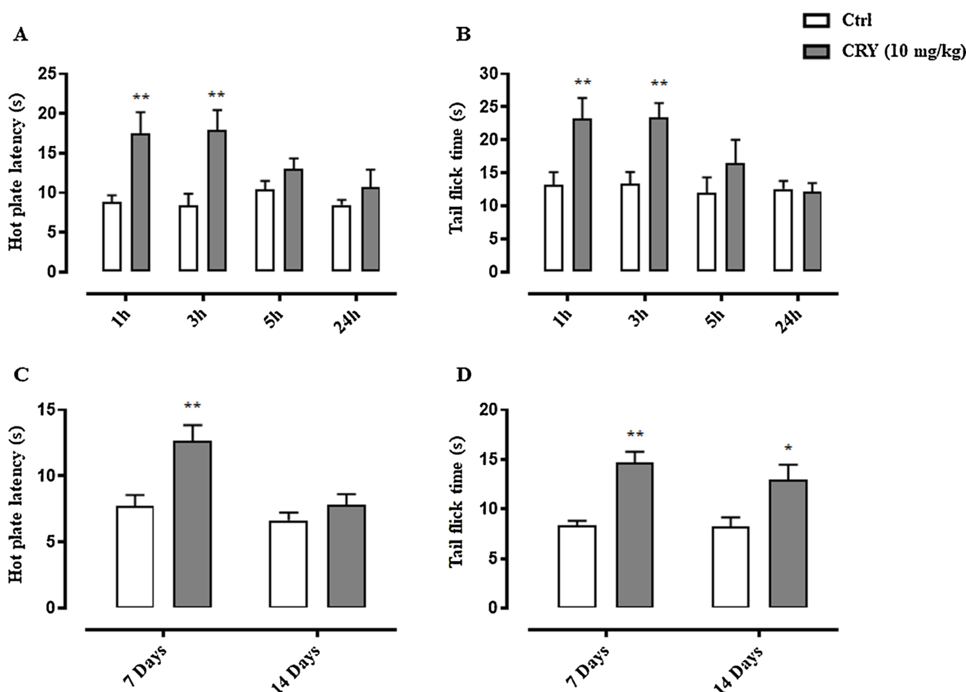

Fig. 1. Time-dependent (from 1 to 24 h) analgesic effect of cryptotanshinone (CRY; i.p., 10 mg/kg) in mice assessed by the hot plate **(A)** and tail flick **(B)** test. In another set of experiment CRY (i.p., 10 mg/kg) was administrated for 7 and 14 days and, 1 h after the last administration, its potential anti-nociceptive profile was evaluated by the aim of hot plate **(C)** and tail flick **(D)** test. Each column represents the mean ± S.E.M. of n = 7. *$P \leq 0.05$, **$P \leq 0.01$ compared to control (Ctrl) group.

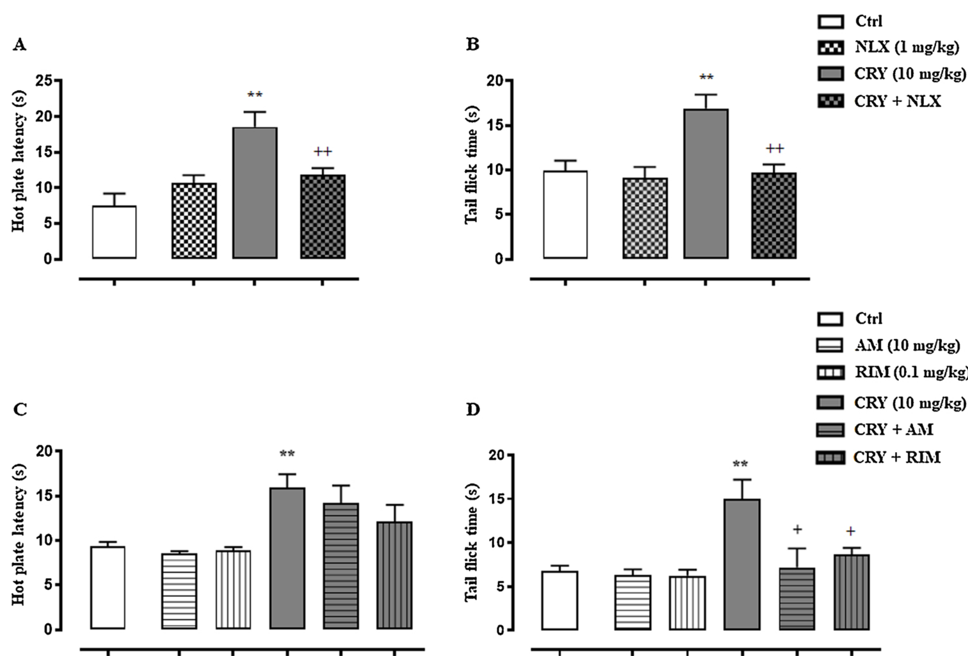

Fig. 2. Effect of naloxone (NLX; i.p., 1 mg/kg), rimonabant (RIM; i.p., 0.1 mg/kg) and AM630 (AM; i.p., 10 mg/kg) on CRY-induced antinociception in mice assessed by hot plate (**A** and **C**) and tail flick (**B** and **D**) test. Antagonists were given 30 min before CRY (i.p., 10 mg/kg) administration. Each column represents the mean ± S.E.M. of n = 7. $^{**}P \leq 0.01$ compared to control (Ctrl) group and $^{+}P \leq 0.05$, $^{++}P \leq 0.01$ compared to CRY-treated mice.

and B). Injection of NLX, AM, RIM, L-NAME and ARG alone did not show any significant effects on both central and peripheral nociceptive threshold.

3.2. CRY and intestinal transit

Next, we tested the capacity of CRY to modulate the upper gastrointestinal transit in CD-1 mice (Fig. 4). 20 min after the administration of the charcoal meal, the percentage of the gastrointestinal transit in Ctrl mice was 64.4 ± 5.3 %. Intraperitoneal injection of CRY (10 mg/kg) significantly reduced the gastrointestinal motility (30.7 ± 5.4 %). The i.p. (but not i.c.v.) administration of NLX (1 mg/kg) completely reverted the reduction of the gastrointestinal transit induced by CRY. Either the i.c.v. or i.p. injection of NLX alone did not show any significant effects in terms of modulation of intestinal transit (Fig. 4).

3.3. Quantitative analysis of CRY on mice serum

Quantitative analysis of samples for the determination of CRY in mice serum revealed a concentration of 0.0158 ± 0.002 mg/ml and 0.0623 ± 0.002 mg/ml respectively after 1 h and two weeks of administration. The method detection limit (method sensitivity) was tested by repeated analysis of blank samples. The detection limits were determined as the concentration giving a peak height three times the noise background. Lower limit of detection (LLOD) = $3*\frac{\text{standard deviation}}{\textit{angular coefficient}}$, LLOD CRY = $3*\frac{61.37394}{2744.6} = 0.067\,\text{g/L}$; Lower limit of quantitation (LLOQ) = $10*\frac{\text{standard deviation}}{\textit{angular coefficient}}$, LLOQ CRY = $10*\frac{61.37394}{2744.6} = 0.224\,\text{g/L}$. The accuracy of the method was tested by relative error calculation. The accuracy values in intra-day variation studies at low, medium and high concentrations were within acceptable limits (n = 5, Table 1). The method precision was expressed by the coefficient of variation of intra- and inter-day variations of the assay under the same operating conditions. The acceptance criterion was set at 15 % (data not shown). Intraday repeatability was evaluated by injecting seven different concentrations of CRY three times. The inter-day assay variations were obtained by repeating after 7 days the same operating conditions.

3.4. Docking results

The opioid receptors system is the main effector of analgesia and sedation [43]. The molecular docking methodology was used to elucidate the binding between cryptotanshinone and the opioid receptors μ, κ, and δ. The crystal structure used for μ and κ receptors were obtained from the Protein Data Bank [27,28] (PDB: 5C1M [25] and 6B73 [26]), the active structure of δ isoform, on the other hand, was created with

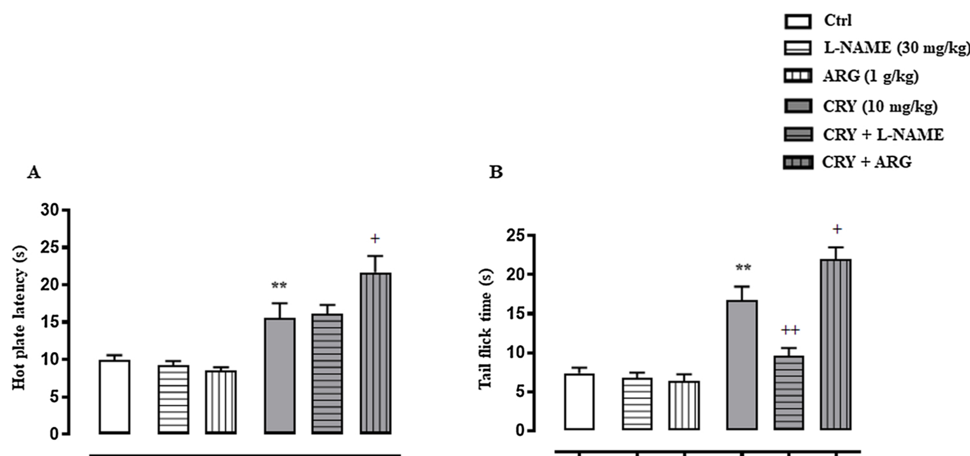

Fig. 3. Effect of L-NAME (L-NAME; i.p., 30 mg/kg) and arginine (ARG; i.p., 1 g/kg) on CRY-induced antinociception in mice assessed by hot plate (**A**) and tail flick (**B**) test. Both compounds were given 30 min before CRY (i.p., 10 mg/kg) administration. Each column represents the mean ± S.E.M. of n = 7. $^{**}P \leq 0.01$ compared to control (Ctrl) group and $^{+}P \leq 0.05$, $^{++}P \leq 0.01$ compared to CRY-treated mice.

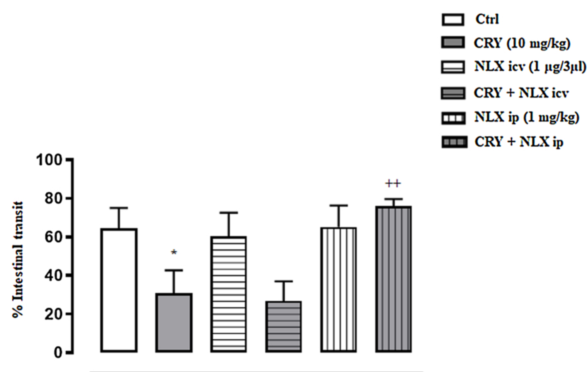

Fig. 4. Effect of cryptotanshinone (CRY; i.p., 10 mg/kg), on mouse intestinal transit, given 20 min before charcoal administration. In experiments investigating the mode of action of tested compound, NLX was given at dose of 1 μg/3 μl and 1 mg/kg i.c.v. and i.p. respectively 30 min before CRY. Each column represents the mean ± S.E.M. of n = 7. *P ≤ 0.05 compared to control (Ctrl) group and ++P ≤ 0.01 compared to CRY-treated mice.

Table 1
Relative error and relative standard deviation of regression line slope (acceptable limits: < 15 %).

	Nominal value (g/L)	Fitted value (g/L)	RE%	RSD%
Cryptotanshinone	0.10	0.09 ± 0.02	2.22	1.16
	0.50	0.52 ± 0.01	1.88	0.58
	1.00	1.03 ± 0.01	0.91	0.58
	2.00	1.09 ± 0.01	0.9	0.58
	5.00	4.99 ± 0.02	0.4	1.16

homology modelling procedure. For what concerns the μ receptor, the binding mode of co-crystallized morphinan agonist BU72 (PDB: 5C1M [25]) (Fig. 5A and B) was used as reference during the comparative experimental-computational analysis. From the structural point of view, CRY was able to occupy the active site of μ-receptor in a manner that is like BU72 (Supplementary Fig. 3). Thus, CRY reproduced the well-known agonist interaction pattern [25,44], making hydrophobic contacts with several amino acids that play a critical role in the biological activation of this kind of receptor (e.g. Asp147, Tyr148, His297, Fig. 5C). Furthermore, the ligand poses resulting from the calculation showed a peculiar π-π stacking interaction with His54 (Fig. 5C and D).

Following the same computational procedure reported above, the crystal structure of the active opioid κ-receptor, in complex with the agonist MP1104 (PDB: 6B73 [26]), was used for the docking studies. Even if, CRY is well inserted in the binding pocket, making hydrophobic interactions with (e.g. Asp138, Tyr139, His291, Leu294, Ala298, Tyr312, Ile316) [26,44], this secondary metabolite showed fewer interactions with respect to the co-crystallized agonist (Fig. 6A and C). Furthermore, CRY showed a hydrogen bond with Gln155 in the binding pocket (Fig. 6C and D) and its spatial orientation resembled that of MP1104 (Supplementary Fig. 4).

Due to the lack of crystal structure of the opioid δ receptor in the active state, for the rationalization of the binding mode between CRY and this receptor, we used the homology modelling procedure to obtain the three-dimensional protein structure. Considering the high sequence identity between μ and δ receptors (56 % of sequence identity), the primary sequence of this receptor was modelled onto the active form of the μ receptor (PDB: 5C1M [25]). In this case, because no co-crystallized ligand could be used as a benchmark, several accurate insights of the opioid ligand-receptor interactions reported to date [44] were used for computational analysis. In more details, CRY was able to establish several hydrophobic contacts with the region of pharmacological interest and made a hydrogen bond with Lys233 (Fig. 7).

4. Discussion

The present study demonstrates that the main hydrophobic constituent, CRY, of *Salvia miltiorrhiza* Bunge, is capable to modulate, in a short time, both central and peripheral thermal nociceptive response. Moreover, CRY is active in terms of long-lasting nociceptive threshold on peripheral nociception. We also report, by *in vivo* and *in silico*

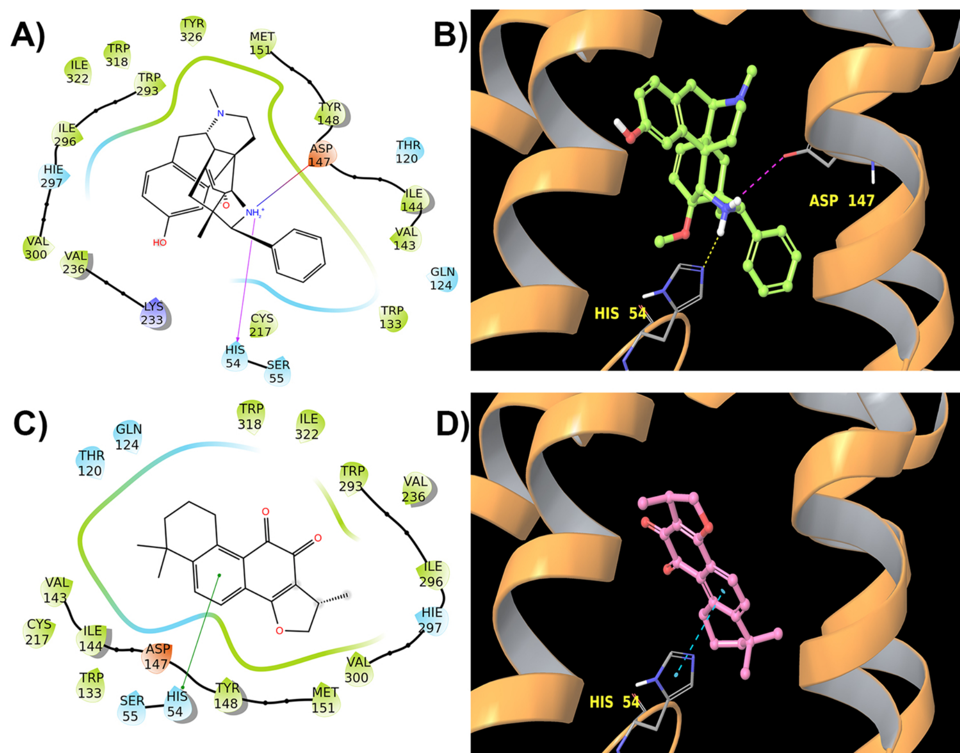

Fig. 5. Protein-ligand interactions with μ-opioid receptor. **A-C**) 2D schematic view for BU72 (**A**) and CRY (**C**). Hydrogen bonds are depicted as pink arrows, π-π stackings are depicted as green lines, and red-to-blue lines indicate salt bridges. Hydrophobic amino acids are represented in green, polar one in cyan, positively charged residues are in blue, negatively charged ones are colored in red, and glycine residues are in white. **B-D**) 3D view of the binding mode for BU72 (**B**) and CRY (**D**). Yellow dotted lines are hydrogen bonds, pink ones are salt bridges, and cyan ones are π-π stackings. Interacting residues are labelled in yellow.

Fig. 6. Protein-ligand interactions with κ-opioid receptor. **A-C**) 2D schematic view for MP1104 (**A**) and CRY (**C**). Hydrogen bonds are depicted as pink arrows, red lines are π-cation interactions, and red-to-blue lines indicate salt bridges. Hydrophobic amino acids are represented in green, polar ones in cyan, positively charged residues are in blue, negatively charged ones are colored in red, glycine residues are represented in white, and CYX indicates a cysteine involved in a disulfide bond. **B-D**) 3D view of the binding mode for MP1104 (**B**) and CRY (**D**). Yellow dotted lines are hydrogen bonds, pink ones are salt bridges, green ones are π-cation interactions, and cyan ones are π-π stackings. Interacting residues are labelled in yellow.

Fig. 7. Protein-ligand interactions between CRY and δ-opioid receptor. **A**) 2D schematic view for CRY. The hydrogen bond is indicated by a pink arrow, hydrophobic amino acids are represented in green, polar one in cyan, positively charged residues are in blue, negatively charged ones are colored in red, and CYX indicates a cysteine involved in a disulfide bond. **B**) 3D view of the binding mode. The hydrogen bond is depicted as a yellow dotted line and the corresponding amino acid is labelled in yellow.

evidence, that CRY interacts with opioids peripheral circuits to modulate nociceptive response.

Pain is an essential protective mechanism meant to prevent tissue damages in organisms. On the other hand, chronic or persistent pain are responsible for long-term disability in patients [45]. Therefore, chronic nociceptive process (that involves many receptor classes, enzymes and signalling pathways) represents one of the major public health problems [46]. Hence, it is critical to better understand chronic pain molecular mechanisms to develop innovative and/or alternative drugs [47].

From the first recorders of opium poppy (*Papaver somniferum*), over 7000 years ago, various forms of natural products have been utilized to treat pain disorders and the known sources of these substances have been thoroughly studied [48]. This has prompted ethnobotanical and ethnopharmacological researchers, over the last decades, to investigate more potential analgesic natural substances resulting in novel structural classes of analgesic compounds and/or discovery of novel mechanisms of actions [49].

Natural products and compounds still hold great promise for the future of drug development especially in the treatment of inflammation and pain [6,50] and in this context, *Salvia* spp. represent one of the most representative examples. Several findings, in fact, have demonstrated the anti-inflammatory and long-lasting pain-relieving effects of Danshen (the dry root of *Salvia miltiorrhiza*) and of its main bioactive constituent (CRY) in different animal models of peripheral [14,15] and neuropathic pain [16].

However, to our knowledge, there are few available reports concerning the antinociceptive effect of systemically administered tanshinones and on the potential interaction/s with cannabinoid and opioid system. Here, in a continue effort to expand our knowledge on the pharmacological profile of tanshinones, we have demonstrated that, acutely, CRY was active on thermal nociception, even if it displayed a more prominent long-lasting analgesic effect in the tail flick test. These evidences where indirectly confirmed after the daily administration of tested compound for 7 and 14 days. In this case, CRY continued to possess an analgesic profile on tail flick. This could be explained by the results obtained by our pharmacokinetics studies performed by high liquid chromatography on mice serum, that evidenced a marked level of administrated diterpenoid even after 7 days treatment. Moreover, these findings are in agreement with data obtained from other groups that revealed a 10 % of bioavailability [51] and detectable fraction [52] of CRY in urine and faeces of mice after its systemic administration. The same authors have also highlighted the possibility that CRY, *in vivo*, could be metabolized by dehydrogenation in tanshinone IIA (another active hydrophobic constituent of Danshen) [53].

The antinociceptive effects of CRY were abolished by NLX pre-treatment in both tail flick and hot plate test, whereas the selective CB1 (RIM) and CB2 (AM) antagonists had inhibitory effects only on peripheral thermal nociception. These results suggest that CRY could produce anti-nociceptive effects centrally via activation of opioids receptors and, peripherally through CB receptors activation, which may lead to the release of endogenous endorphins to activate opioid receptors in the peripheral nerve terminals of sensory neurons. This is in accordance with renowned demonstrations of the interaction between opioids and cannabinoids in the peripheral pain control system to induce analgesia [54,55].

L-Arginine is a substrate for NO synthase, the enzyme that through the systemic releases of NO represents the pathway for the regulation of cell function and communication [56] and behavioural pain responses [57]. It is widely accepted that the release of NO has an opposing effect on nociceptive transmission; however, its interaction on opioids-induced antinociception and their molecular interaction have been extensively demonstrated [58,59]. Since L-NAME reverted the peripheral effect of CRY (also increased by arginine pre-treatment), we also hypothesise that CRY reduction of peripheral thermal nociception was also dependent on NO release.

Opioid analgesics act via the μ-, δ-, and κ-opioid receptors distributed widely in the central and peripheral nervous system. μ-opioid receptors are expressed throughout the gastrointestinal tract and, upon opioids binding, decrease neural activity in the enteric nervous system resulting in opioid-induced constipation [60]. Correspondingly, morphine-induced gastrointestinal transit inhibition is blocked by μ-opioid antagonists, but not by δ and κ antagonists [61] although all three opioid receptors are localized to the enteric nervous system [62]. Although morphine-induced gastrointestinal transit inhibition is selectively blocked by a μ-opioid antagonist, there is a possibility that κ- or δ-opioid antagonists (located adjacent to myenteric plexus structures [63,64]) have combined effect in terms of gastrointestinal transit inhibition [65].

The results obtained on mice intestinal transit reveal a significant inhibition mediated by CRY administration, reverted by i.p., but not i.c.v., NLX pre-treatment. These data, in accordance with the literature [66–68] suggest that inhibition of charcoal meal transit in mice can be mediated by a peripheral, but not central, μ-mediated mechanism and that a peripheral δ and/or k component may also produce a partial inhibition of intestinal motility.

Finally, *in silico* studies carried out using both the experimental X-ray structures of μ and k receptors and the predicted three dimensional model of δ isoform, together with the comparative analysis with respect to known agonists, suggest that CRY (in line with the biological data) exert an interesting interaction on opioid receptors.

5. Conclusion

Considering our *in vivo* evidences and the molecular docking study on CRY that highlighted a good interaction with the opioid receptors, our conclusion hints to a plausible relationship between CRY nociceptive effect and the peripheral interplay of opioids, cannabinoids and NO pathways.

Author contributions

DC, RF, SA, CC, CGM, DLR, SA, LS, DI, DVS and CMG performed the experiments. BG, CA, MF and MN designed the study, drafted and wrote the manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110042.

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