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Tanshinones from *Salvia miltiorrhiza* Bunge revert chemotherapy-induced neuropathic pain and reduce glioblastoma cells malignancy

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ABSTRACT

Medicinal plants and herbal extracts from traditional Chinese medicine are used increasingly commonly worldwide for their benefits to health and quality of life as dietary supplements or as ingredients in functional foods. Among them, *Salvia miltiorrhiza* Bunge (a natural strong remedy for the treatment of a variety of conditions) is traditionally used for centuries in Asian countries as antioxidant, anticancer, and anti-inflammatory agent. In this context, several evidences support the hypothesis that some tanshinones (in particular tanshinone IIA and cryptotanshinone) extracted from the roots (Danshen) of *Salvia miltiorrhiza* exert neuroprotective and analgesic activities.

Oxaliplatin (OXA), a platinum-based drug used for the treatment of solid tumors, induces neuropathic pain which hampers the chemotherapy success. While several attempts were made to prevent oxaliplatin-induced painful neuropathy, a growing number of evidences look to natural sources as an effective remedy to counterbalance the OXA-mediated side effects.

The aim of the present study was to investigate the pain-relieving profile of Danshen and its active constituents tanshinone IIA (TIIA) and cryptotanshinone (CRY) in animal models of neuropathic pain induced by OXA, anticancer drug characterized by a dose-limiting neurotoxicity. Contextually, the neuroprotective and anticancer activities of the selected compounds were tested in different cells lines. A single administration per os of CRY (30 mg mg/kg) significantly, in a dose dependent manner, attenuated chemotherapy-induced pain. A 7 days repeated administrations highlighted the effectiveness and potency of both CRY and TIIA (10 mg/kg). On the other hand, Danshen showed a painkiller profile against oxaliplatin-induced neuropathy. Contextually, Danshen and its active constituents showed remarkable and selective inhibitory activities on glioblastoma cells lines LN-229 (IC_{50}: 50.0 ± 4.0, 48.2 ± 4.9 and 51.9 ± 2.3 μM respectively for Danshen standardized extract, TIIA and CRY) next to healthy but high proliferative cell lines enterocytes (IC_{50}: > 250 μM for TIIA and CRY) and keratinocytes (IC_{50}: > 100 and 97 ± 2 μM respectively for TIIA and CRY).

Taken together the results reported here demonstrated the long-lasting pain-relieving effects of Danshen and its related bioactive constituents in animal models of neuropathic pain and their selective *in vitro* neuroprotective properties on certain central malignancy cells lines. Thus, suggest that *S. miltiorrhiza* roots could be considered as a new potential source of active diterpenoidic compounds useful for pharmaceutical or nutraceutical industries and beneficial as food complements.

1. Introduction

Patients treated with platinum-based chemotherapy in different forms of solid tumors (colorectal, lung, breast and ovarian) frequently experience neurotoxic symptoms such as a distal dose-dependent symmetrical sensory neuropathy, which may lead to premature discontinuation of therapy [1,2]. These symptoms become evident when cumulative oxaliplatin-based dose has administrated [3], even if it has

Abbreviations: CRY, cryptotanshinone; CTRL, control; DMSO, dimethylsulfoxide; OXA, oxaliplatin; PBS, phosphate buffered saline; TIIA, tanshinone IIA

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been observed that in the 90% of patients in treatment with oxaliplatin (OXA) it also causes acute dose-independent neurotoxicity [4]. The acute and chronic forms display specific symptom profile. Acute OXA neurotoxicity is cold-induced or cold-aggravated, characterized by specific distal sensory symptoms such as paresthesias and dysesthesias [5], whereas the chronic form is characterized by distal sensory ataxia, and occurs at a level sufficient to cause patients functional impairment [6].

Therefore, developing treatment regimens ideally with superior effectiveness and minimal adverse effects for these conditions remain a priority in cancer research pushing researchers to investigate new and more reliable potential therapeutic strategies. Contextually, investigation on traditional medicinal plants as ingredients in functional and health care foods routinely used and/or prescribed together with conventional therapy in clinical practice is another deal of international scientific community aimed to improve the effectiveness of chemotherapeutics in the prevention and/or treatment of cancer and cancer-related side effects [7,8].

In this contest, one of the potential alternative could be represented by *Salvia miltiorrhiza* Bunge, a perennial herb belongs to Labiatae family [9]. Its dry root and/or rhizome, commonly known as Danshen in Chinese, is officially listed in the Chines Pharmacopoeia [10]. Due to its diverse pharmacologic properties, is categorized as one of the main commercial herbs in this country. In line, pharmacological research's indicate that diterpenoidic like compounds such as TIIA and CRY from Danshen exhibited remarkable properties for the treatment of cardiovascular [11,12,13,14], cerebrovascular [15,16], inflammatory diseases [17,18] and cancer [19,20,21].

Recent clinical studies have also demonstrated that Danshen improves survival of patients by inhibiting the proliferation of colon cancer cells [22]. Lately, the efficacy of TIIA in chemotherapy-induced painful neuropathy was suggested since Xu et al. [23] demonstrated the efficacy of this Danshen-derived compound in relieving pain and improving neurophysiological functions in patients (II-III phase) with malignant tumor of digestive tract undergoing chemotherapy program with OXA. Furthermore, Danshen and tanshinones prevent neurotoxicity and neurodegeneration in PC12 cells, human neuroblastoma and nigrostriatal dopaminergic neurons [24,25,26]. It has also been shown that TIIA rescues the impairments of astrocytes and spinal cord neurons after ischemic insults and inflammatory traumatic injury [27,28] and that attenuates neuropathic pain via inhibiting glial activation and immune response [29].

Despite the potential of Danshen-related tanshinones as a neuroprotective agent, no experimental study has been conducted to determine their effects in animal models of neuropathic pain induced by OXA. In addition, the impact on their selective antitumor activity remains unknown. These evidences prompted us to investigate the pain-relieving profile of standardized extract of Danshen and its active main constituents (TIIA and CRY) in OXA-related peripheral neuropathy alongside with the examination of their neuroprotective and anticancer activities on neuronal and peripheral high-dividing cell lines.

2. Materials and Methods

2.1. Reagents

Cryptotanshinone and tanshinone IIA (≥ 97%, HPLC) were obtained from Sigma–Aldrich Co. (Milan, Italy) whereas *Salvia miltiorrhiza* Bunge root dried extract (E.S. 1% tanshinones) from New Phargam S.R.L. (Milan, Italy). Oxaliplatin was obtained from Carbosynth (Berkshire, UK).

2.2. Animals

Male CD-1 albino mice (Envigo, Varese, Italy) weighing approximately 22–25 g at the beginning of the experimental procedure, were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least 1 week after their arrival. Ten mice were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (September 22, 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines [30]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Oxaliplatin-induced neuropathic pain

Mice were administered intraperitoneally (i.p.) with 2.4 mg/kg oxaliplatin (dissolved in 5% glucose solution) on days 1-3, 5-9, and 12-13, to develop neuropathic pain [31,32]. Control animals received an equivalent volume of vehicle.

2.4. Compounds administrations

The effects of cryptotanshinone (CRY), tanshinone IIA (TIIA) and *Salvia miltiorrhiza* root extract (Danshen) were evaluated acutely after a single administration *per os* (p.o.) on day 14 of oxaliplatin treatment. CRY and TIIA were analyzed also after a repeated treatment: starting from the first day of oxaliplatin treatment, TIIA and CRY were daily administered p.o. for 14 days, behavioral measurements were performed on days 7 and/or 14, 24 h after the last administration of compounds.

2.5. Cold plate test

Each animal was placed in a stainless-steel box (12 cm × 20 cm × 10 cm) with a cold plate as floor. The temperature of the cold plate was kept constant at 4 °C ± 1 °C. Pain-related behavior (licking of the hind paw) was observed and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 s [32].

2.6. Rota-rod test

The rota-rod test was used to determine the integrity of motor coordination in mice after drug treatment [33]. The apparatus used for the rota-rod tests consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the time the animals kept their balance on the rotating rod for a maximum of 10 min (600 s). After a maximum of 6 falls from the rod the test was suspended and the time was recorded.

2.7. Hole board test

The hole board test was used to determine the spontaneous mobility (board) and the exploratory activity (hole) of mice after drug treatment. The apparatus consisted of a 40 cm square plane with 16 flush mounted cylindrical holes (3 cm diameter) distributed 4 × 4 in an equidistant, grid-like manner. Mice were placed on the center of the board one by

one and allowed to move about freely for a period of 5 min each. Two photobeams, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice.

2.8. Cell cultures

Human cancer cell lines have been a useful tool for the study of the molecular biology and therapy of cancer in many tumour types. Human brain glioblastoma cancer cell, epithelial-like type as LN-229; human brain likely glioblastoma cancer cell, epithelial-like type as U-87 MG, were respectively grown in DMEM (Invitrogen, Paisley, UK) supplemented with 5% fetal bovine serum (FBS, Cambrex, Verviers, Belgium) and in EMEM (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), and both with L-glutamine (2 mM, Sigma, Milan, Italy), penicillin (100 units/ml, Sigma) and streptomycin (100 µg/ml, Sigma), and cultured in a humidified 5% carbon dioxide atmosphere at 37 °C, while human colon adenocarcinoma cells Caco-2, was grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 20% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), and cultured in a humidified 5% carbon dioxide atmosphere at 37 °C, according to ATCC recommendations. Human HaCaT keratinocytes, used as healthy control cell lines, were grown in DMEM (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (FBS, Cambrex, Verviers, Belgium), L-glutamine (2 mM, Sigma, Milan, Italy), penicillin (100 units/ml, Sigma) and streptomycin (100 µg/ml, Sigma) and cultured in the same experimental conditions. Moreover, Caco-2 cells, led to differentiation, were used at post-confluence stage as a model of human enterocytes [34].

2.9. Bioscreens in vitro

The activity of tanshinone IIA, cryptotanshinone and the extract of *Salvia miltiorrhiza* was investigated through the estimation of a “cell survival index” [35], obtained from the combination of the automated cell count and the percentage values derived from the metabolic MTT assay, as the arithmetic mean, providing a more accurate parameter of the actual number of cells that survive after a preclinical study in vitro. Cells were inoculated in 96-microwell culture plates at a density of 10^4 cells × well and allowed to grow for 24 h. The medium was then replaced with fresh medium and cells were treated for further 48 h with a range of concentrations from 10 to 100 µM of TIIA and CRY, while a range of concentrations from 10 to 100 µg/ml of *Salvia miltiorrhiza* extract. Using the same experimental procedure, cell cultures were also incubated with the vehicle DMSO as negative control. Cell viability was evaluated using the MTT assay procedure, which measures the level of mitochondrial dehydrogenase activity - using the yellow 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) as substrate - to convert dissolved MTT into insoluble purple formazan. In short, after the treatments, the medium was removed and the cells were incubated with 20 µl × well of a MTT solution (5 mg/ml) for 1 h in a humidified 5% CO_2 incubator at 37 °C. The incubation was stopped by removing the MTT solution and by adding 100 µl/well of DMSO to solubilize the obtained formazan. Finally, the absorbance was monitored at 550 nm using a microplate reader (iMark microplate reader, Bio-Rad, Milan, Italy). Cell number was determined by TC10 automated cell counter (Bio-Rad, Milan, Italy), providing an exact and reproducible total count of cells and a live/dead ratio in one step by a specific dye (trypan blue) exclusion assay. Bio-Rad’s TC10 automated cell counter uses disposable slides, TC10 trypan blue dye (0.4% trypan blue dye w/v in 0.81% sodium chloride and 0.06% potassium phosphate dibasic solution) and a CCD camera to count cells based on the analyses of captured images. Once the loaded slide is inserted into the slide port, the TC10 automatically focuses on the cells, detects the presence of trypan blue dye and provides the count. When cells are damaged or dead, trypan blue can enter the cell allowing living cells to be counted. Operationally, after treatments in 96-microwell culture plates, the medium was removed and the cells were collected. Ten microliters of cell suspension, mixed with 0.4% trypan blue solution at 1:1 ratio, were loaded into the chambers of disposable slides. The results are expressed in terms of total cell count (number of cells per ml). If trypan blue is detected, the instrument also accounts for the dilution and shows live cell count and percent viability. Total counts and live/dead ratio from random samples for each cell line were subjected to comparisons with manual haemocytometers in control experiments. The calculation of the concentration required to inhibit the net increase in the cell number and viability by 50% (IC_{50}) is based on plots of data (n = 6 for each experiment) and repeated five times (total n = 30). IC_{50} values were obtained by means of a concentration response curve by nonlinear regression using a curve fitting program, GraphPad Prism 5.0, and are expressed as mean values ± SEM (n = 30) of five independent experiments.

2.10. Statistical analysis

The analysis of variance of the behavioural data was performed by one way ANOVA, and a Bonferroni's significant difference procedure was used as post-hoc comparison, by using the “Origin 9” software (OriginLab, Northampton, USA). In vitro data were analyzed using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA) and ANOVA test for multiple comparisons was performed followed by Bonferroni’s test. All data were presented as mean values ± SEM. Values of $P \leq 0.05$ were considered statistically significant. Investigators were blind to all experimental procedures.

3. Results

3.1. Pain relief, acute treatments

The effects of *Salvia miltiorrhiza* products against neuropathic pain were studied in a mouse model of chemotherapy-induced neuropathy. The neurotoxicity of oxaliplatin (OXA) was used to develop a neuropathic syndrome characterized by thermal hypersensitivity after repeated (7 or 14 days) administrations (2.4 mg/kg, intraperitoneally) [31,32]. On day 14, the response to a cold non-noxious stimulus (allodynia-like symptom) was measured by the Cold plate test. The pain threshold of OXA-treated animals decreased to 9.5 ± 0.2 s in comparison to control, vehicle-treated animals (18.7 ± 0.2 s). A single administration per os (p.o.) of cryptotanshinone (CRY) at 30 mg/kg was able to significantly increase the pain threshold of OXA-treated mice. The onset was 15 min after treatment lasting for 45 min, at 30 min neuropathic pain was fully reverted (Fig. 1a). The lower dose (10 mg/kg) was ineffective. In the same condition, tanshinone IIA (TIIA; 10 and 30 mg/kg p.o.) did not show effect against pain (Fig. 1b). As shown in Fig. 1c, the extract from *Salvia miltiorrhiza* roots, Danshen, induced a dose-dependent pain-relieving effect. Danshen (titled as 1% tanshinones) dosed at 300 and 600 mg/kg significantly increased the pain threshold. The higher dose was active between 15 and 45 min peaking at 30 min when the hypersensitivity was completely abolished (Fig. 1c).

3.2. Pain relief, repeated treatments

The pain-relieving profiles of tanshinones were also studied after a repeated administration. CRY and TIIA (10 mg/kg) were administered daily p.o. in OXA-treated mice starting from the first day of OXA injection. On day 7, 24 h after the last administration, both CRY- and TIIA-treated mice showed a significantly increased pain threshold (Fig. 2). A new administration of compounds to the repeatedly treated animals did not improve pain relief (data not shown). In conclusion, a

subchronic treatment was able to increase potency and efficacy of both compounds. In particular, the effects of TIIA were unmasked. Importantly, the pain-relieving effect after a repeated treatment was long lasting and the possibility of tolerance development was excluded.

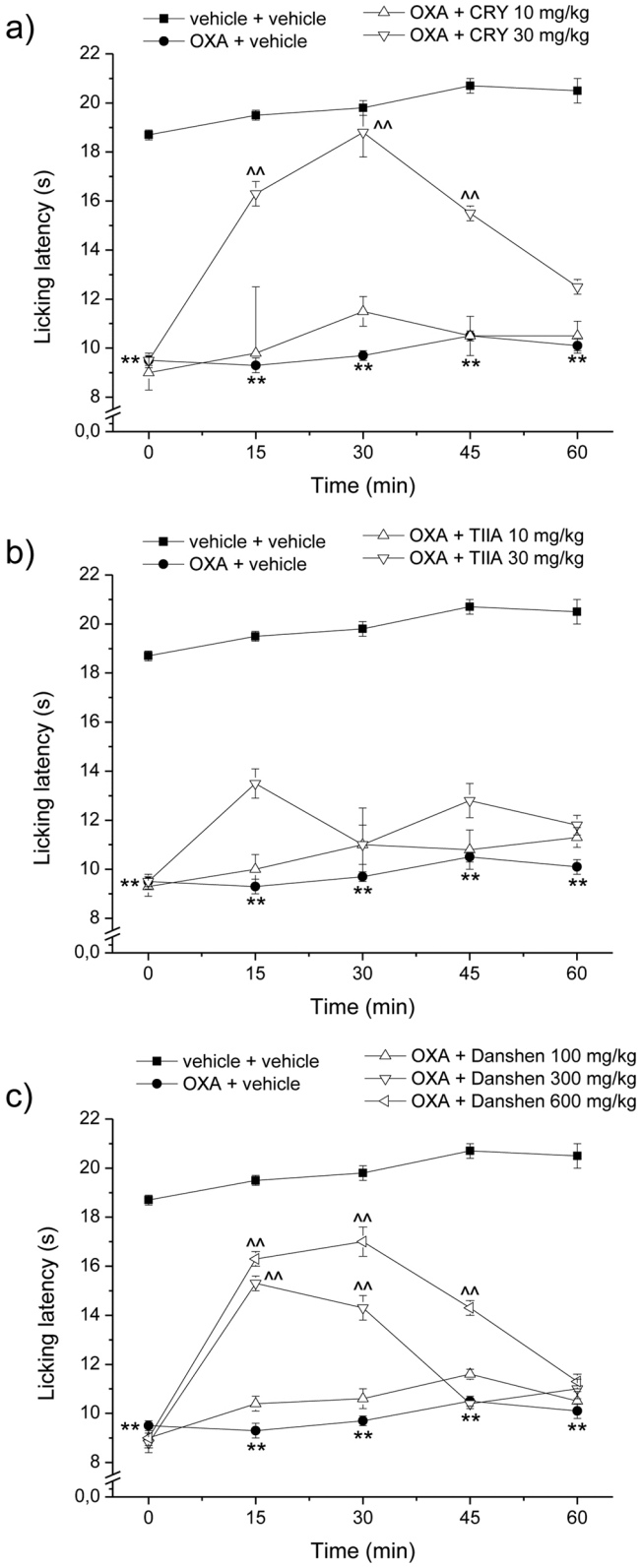

Fig. 1. Effect of cryptotanshinone (CRY), tanshinone (TIIA), and *Salvia miltiorrhiza* root extract (Danshen) on oxaliplatin-induced neuropathic pain in mice. Oxaliplatin (2.4 mg/kg) was administered (i.p.) during days 1-3, 5-9, and 12-13, and the subsequent neuropathic pain determined by using the cold plate test. On day 14, a) CRY, b) TIIA, and c) Danshen were administered (p.o.), and their anti-neuropathic pain activity subsequently determined. The time latency (in seconds) for the first signs of pain-related behavior (i.e., lifting and licking of the hind paw) was recorded after 15, 30, 45, and 60 min. The plots are representative of 10 mice analyzed in 2 different experimental sets, the error bars are the SEM. The post hoc Bonferroni's was performed. **P < 0.01 vs vehicle + vehicle treated animals; ^^P < 0.01 vs OXA + vehicle treated animals.

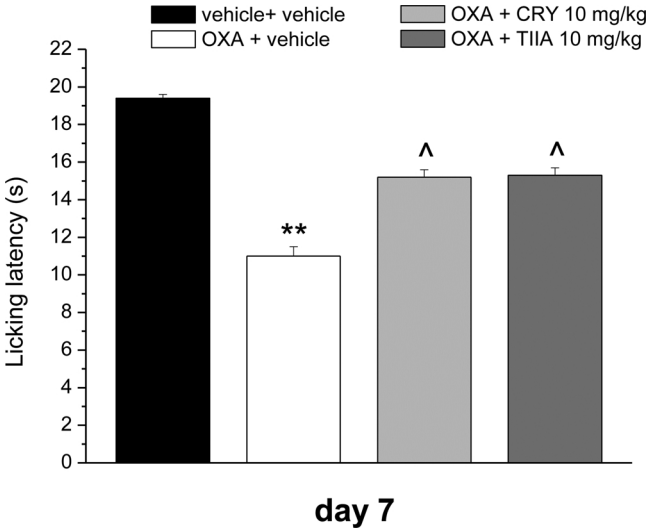

Fig. 2. Anti-neuropathic pain activity of cryptotanshinone (CRY) and tanshinone (TIIA) after repeated treatment. Mice were treated (i.p.) with 2.4 mg/kg oxaliplatin following the described protocol. Starting from the first day of oxaliplatin treatment, CRY and TIIA were daily administered p.o.. On day 7, 24 hours after the last injection, pain threshold was determined by the cold plate test and compared to that for vehicle + vehicle treated animals. Results are the mean of 10 animals analyzed in 2 different experimental sets, the error bars are the SEM. The post hoc Bonferroni's was performed. **P < 0.01 vs vehicle + vehicle treated animals; ^^P < 0.01 vs OXA + vehicle treated animals.

3.3. Motor and neurological evaluations

The safety of tanshinones was evaluated measuring the locomotor and exploratory activities (Hole board test) as well as the motor coordination (Rota rod test) in OXA-treated mice. As shown in Table 1, OXA did not alter nor locomotor neither exploratory activities. On day 7, both CRY and TIIA did not modify these parameters 30 min after a single administration to OXA-treated animals. Moreover, measurements were performed also after a repeated treatment (daily starting from the first day of OXA treatment). On day 7, 24 h after the last administration of compounds, CRY and TIIA did not modify the performances in comparison to OXA *per se* and vehicle. On day 14, the acute and repeated administrations of TIIA and the acute CRY were safe whereas the repeated administration of CRY significantly decreased the locomotor and exploratory activities (Table 1). Motor coordination skills after repeated treatment are shown in Table 2. On day 7, the time spent on a rotating rod was not modified nor by OXA neither by CRY or TIIA. On day 14, OXA reduced the coordination (150.8 ± 20.3 s) in comparison to control (278.5 ± 45.0 s). The co-administration with TIIA did not alter OXA effect, CRY basically (P = 0.066) worse OXA effect (Table 2).

3.4. Cytotoxic activity of tanshinones

Tanshinone IIA (TIIA) and cryptotanshinone (CRY) were examined

Table 1
Effect of acute (a) and repeated (c) administration of *cryptotanshinone* and *tanshinone IIA* on locomotor and exploratory activities in mice. Hole board test.

Treatments	Dose mg/ kg	Day 7		Day 14	
		Hole	Board	Hole	Board
vehicle + vehicle		40.3 ± 5.6	75.6 ± 6.4	42.0 ± 4.0	80.7 ± 7.3
OXA + vehicle		50.2 ± 10.2	82.3 ± 4.6	44.8 ± 5.0	70.3 ± 8.6
OXA + CRY (a)	30	42.6 ± 4.4	70.9 ± 8.9	39.9 ± 3.7	85.3 ± 12.9
OXA + CRY (r)	10	40.8 ± 5.2	84.3 ± 7.8	18.5 ± 3.7*	27.0 ± 4.9*
OXA + TIIA (a)	30	45.8 ± 3.2	75.6 ± 7.4	42.6 ± 5.8	67.9 ± 8.1
OXA + TIIA (r)	10	42.9 ± 2.9	70.6 ± 5.5	33.2 ± 8.3	67.8 ± 7.6

Oxaliplatin (2.4 mg/kg) was repeatedly administered over 2 weeks. The effects of tanshinone IIA (TIIA) and cryptotanshinone (CRY) were evaluated acutely (a) 30 min after a single administration p.o. on days 7 and 14 of oxaliplatin treatment. Measurements were performed also after a repeated (r) treatment; starting from the first day of oxaliplatin treatment, TIIA and CRY were daily administered p.o. for 14 days, behavioral measurements were performed 24 h after the last administration of compounds. The Hole board test was performed measuring the spontaneous mobility (board) and the exploratory activity (hole) of mice were automatically recorded. Each value represents the mean of 10 mice analyzed in 2 different experimental sets, ± SEM. *P < 0.05 *vs* vehicle + vehicle treated animals.

Table 2
Effect of repeated administration of cryptotanshinone and tanshinone on motor coordination in mice. Rota rod test.

Treatments	Dose mg kg	Day 7	Day 14
		Time (s)	Time (s)
vehicle + vehicle		296.4 ± 29.2	278.5 ± 45.0
OXA + vehicle		249.8 ± 30.7	150.8 ± 20.3*
OXA + CRY	10	275.4 ± 33.8	95.7 ± 10.7*
OXA + TIIA	10	267.9 ± 26.5	186.8 ± 20.1*

Oxaliplatin (2.4 mg/kg) was repeatedly administered over 2 weeks. The effects of tanshinone IIA (TIIA) and cryptotanshinone (CRY) were evaluated after a repeated treatment. Starting from the first day of oxaliplatin treatment, CRY and TIIA were daily administered p.o. for 14 days, behavioral measurements were performed on days 7 and 14, 24 h after the last administration. The integrity of motor coordination was assessed on the basis of the time the animals kept their balance on the rotating rod for a maximum of 10 min (600 s). After a maximum of 6 falls from the rod the test was suspended and the time was recorded. Each value represents the mean of 10 mice analyzed in 2 different experimental sets, ± SEM. *P < 0.05 *vs* vehicle + vehicle treated animals.

for their cytotoxic activities against several tumor and normal human cell lines (LN-229; U-87 MG, Caco-2, HaCaT and human enterocytic cell lines). Both TIIA and CRY revealed a strong and broadly similar antiproliferative/cytotoxic activity on all cancer cell lines used, in a dose-dependent way (Fig. 3). In particular, this effect was very significant on LN-229 and U-87 MG glioblastoma cells. The concentration-effect curves (Fig. 3)- here reported in terms of a “cell survival index” - show a typical concentration-dependent sigmoid trend achieving IC_{50} values in the low micromolar range. Indeed, according to the IC_{50} values (see Table 3), the tanshinone IIA and cryptotanshinone reduce the proliferation of all the used cancer cell lines, while they are not so cytotoxic against the used healthy cell lines.

3.5. Cytotoxic activity of Salvia extract

The activity of the extract of *Salvia miltiorrhiza* (rich in tanshinones) [14] was studied for the cell growth-inhibitory activity against cancer and normal human cell lines LN-229, U-87 MG and HaCaT. As shown by graphs (Fig. 4), Salvia extract revealed a very strong antiproliferative activity on both cancer cell lines used, in a dose-dependent way and in particular by using the two highest concentrations. Noteworthy, the antiproliferative activity is not so prominent on the used healthy cells HaCaT, as demonstrated by IC_{50} values (Table 3). Overall, these results suggest that tanshinones display a high antiproliferative activity *in vitro* assays against human tumor cells.

4. Discussion

Oxaliplatin, a third-generation platinum derivative, is one of the most effective chemotherapeutic drugs used to treat advanced (especially colorectal) cancer [36]. However, it is associated with the development of neurotoxicity, which is one of the most prevalent and dose-limiting effects that includes mechanical allodynia and hypersensitivity to cold [37,38]. Although oxaliplatin-induced neuropathy has been studied for several years, no effective therapy against its side effects are currently available.

As a result, some patients have utilized or expressed interest in complementary medicine (in particular to phytomedicine and nutraceutical) for disease prevention and/or enhancing quality of life. In this context the search on phytomedicine-based therapy could represent a steady coadjuvant approach [8,39,40]. Even if there is a rigorous debate about the real effect of these compounds on human health it has been difficult to reliably show beneficial effects of specific phytocomplex or nutraceuticals on cancer treatment [41].

Our study reveals that *Salvia miltiorrhiza* root extract (Danshen) and its main active lipophilic components TIIA and CRY attenuate *in vivo* the oxaliplatin-induced nociceptive hypersensitivity and proposes their selective inhibitory activities on glioblastoma cells lines. We speculate that these findings may provide a beneficial choice for oxaliplatin neuropathy treatment in the continuing expanding context of phytomedicine.

In order to test whether Danshen and its main constituents were effective in attenuating oxaliplatin-induced peripheral neuropathy, we designed a neurotoxicity model using male CD-1 albino mice. Consistent with our previous reports [42,32], hypersensitivity to mechanical and thermal stimuli was progressively increasing over time reaching significant values after 1 and 2 weeks of treatment when the cumulative dose of oxaliplatin injected is clinically relevant for neuropathy development [43]. On day 14, oxaliplatin-dependent cold allodynia was dose-dependently prevented by a single oral administration of Danshen and its related active component CRY. On the contrary, TIIA was inactive after a single administration. Nevertheless, a repeated daily administration was able to unmask the activity of TIIA and to show the maintenance of CRY efficacy. The presence of significant pain relieving effects after several days of treatment suggest the possibility of a long-term treatment without decrease of efficacy.This is of pivotal importance considering the need of chronic treatments for this kind of persistent pain, a reason why the classical analgesic compounds like opioid are unsatisfactory [44].

To validate the data, the safety profile of tanshinones was evaluated by measuring the locomotor and exploratory activities as well as the motor coordination. Both compounds after a single treatment did not induce motor or neurological alterations. The complete absence of behavioral alterations was confirmed also on day 7, after 6 days of repeated administration. To note, on day 14 after 13 days of treatment a

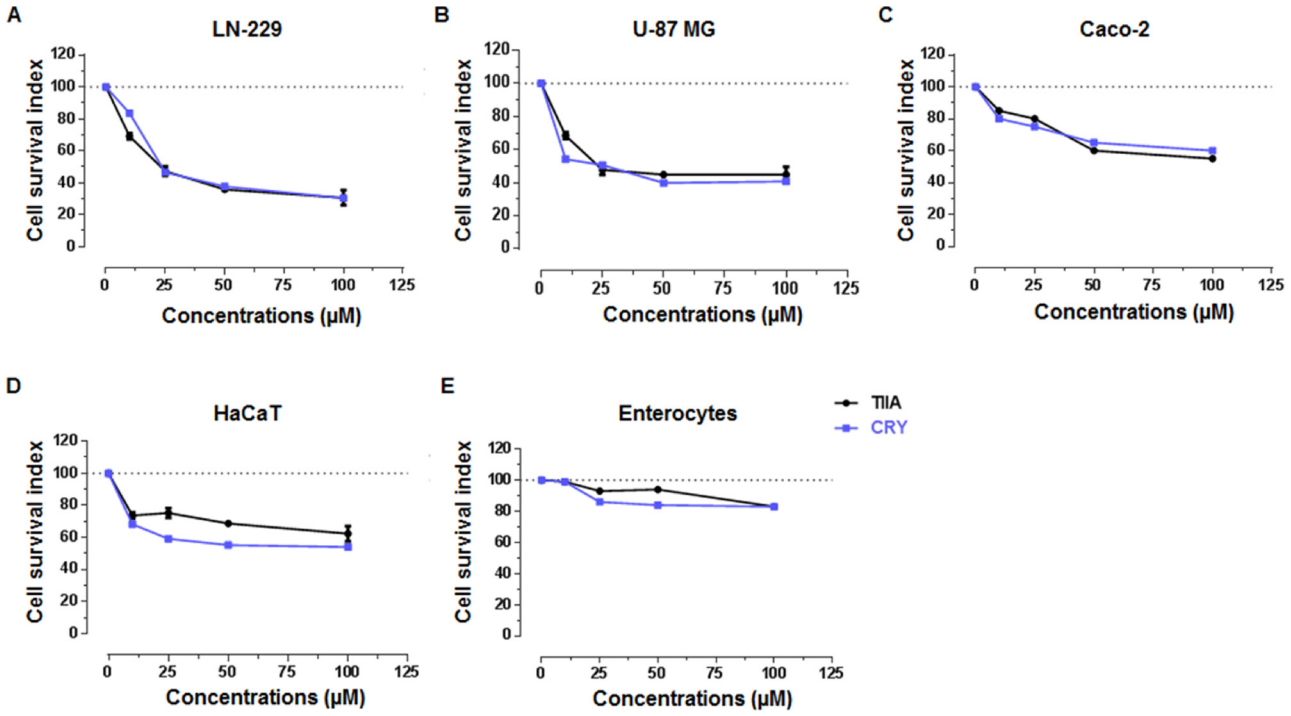

Fig. 3. Cell survival index: evaluated by the MTT assay and monitoring of live/dead cell ratio for A human brain glioblastoma cancer cell, LN-229; B human brain likely glioblastoma cancer cell, U-87 MG; C human colon adenocarcinoma cancer cell, Caco-2, D human HaCaT keratinocytes and E human enterocytic cells, following 48 h of incubation with a specific range of concentration (10 – 100 μM) of Tanshinone IIA (TIIA) and Cryptotanshinone (CRY), as indicated in the legend. Data are expressed as percentage of untreated control cells and are reported as mean of five independent experiments ± SEM (n = 30).

Table 3
IC50 values relative to the TIIA, CRY (μM) and Salvia miltiorrhiza extract (μg/mL) in the cancer and healthy cell lines.

	TIIA	CRY	Salvia
LN-229	48.2 ± 4.9	51.9 ± 2.3	50.0 ± 4.0
U-87 MG	48.6 ± 3.1	50.0 ± 4.2	53.8 ± 2.0
Caco-2	98.0 ± 1.5	< 100	n.a.
HaCaT	< 100	97.2 ± 2.0	86.0 ± 4.0
Enterocytes	< 250	< 250	n.a.

IC50 values relative to the TIIA, CRY (μM) and Salvia miltiorrhiza extract (μg/mL) in the cancer cell lines (LN-229, U-87 MG and Caco-2) and human healthy cells (HaCaT and enterocytes) following 48 h of incubation. The values are reported as mean values ± SEM (n = 30). (n.a. = not assessed).

cumulative toxicity of CRY (when added to OXA dosing) appeared. TIIA was safe at both time point.

The efficacy of Danshen and its constituents, tanshinones, against neuropathic pain is consistent with previously published data that describe tanshinones (in particular TIIA) as potent monoacylglycerol lipase (MAGL) inhibitor [45]. MAGL is the main metabolic enzyme involved in the inactivation of the endocannabinoid 2-arachidonoylglycerol (2-AG), the inhibition of this serine hydrolase increases endocannabinoid levels evoking pain relief [46,47]. Accordingly, TIIA was also effective in reducing visceral and somatic persistent pain in rats [48]. Of particular interest is also the obtained results on our bioscreens *in vitro* aimed to investigate the tanshinone's cytotoxic effect on different tumor cell lines. Our results showed that both TIIA and CRY possess a strong cytotoxic activity, particularly on LN-229 and U-87 MG glioblastoma cells. This would suggest a possible selectivity of action. These observations have been summarized also by analyzing the IC_{50} values shown in Table 3, where both TIIA and CRY

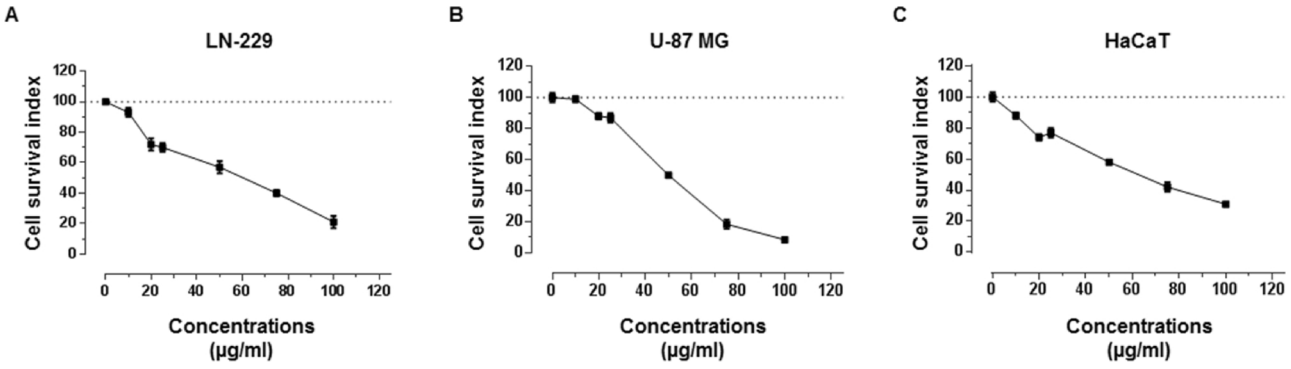

Fig. 4. Salvia extract activity. Cell survival index, evaluated by the MTT assay and monitoring of live/dead cell ratio for A human brain glioblastoma cancer cell, LN-229; B human brain likely glioblastoma cancer cell, U-87 MG and C human HaCaT keratinocytes, following 48 h of incubation with a specific range of concentration (10 – 100 μg/ml) of extract of *Salvia miltiorrhiza*. Data are expressed as percentage of untreated control cells and are reported as mean of five independent experiments ± SEM (n = 30).

present higher IC_{50} values on human colon adenocarcinoma cells Caco-2 than on LN-229 and U-87 MG glioblastoma cancer cell lines.

Our results are in accordance with related studies that have indicated a role of tanshinones in the inhibition of cancer growth by induction of apoptosis [49,50]. Thus, it was reasonable to speculate if tanshinones would play an important role in the inhibition of glioblastoma cell lines. The cellular and molecular changes underlying neuroblastoma development and progression remain poorly understood; however, the nuclear factor-κB (NFκB) transcription factor seem to influence both neuroblastoma survival and cell death [51,52]. Accordingly, we and other have recently demonstrated that tanshinones TIIA and CRY selectively interfere with NFκBp65 expression (with a specular and significant modulation of IKBα) in the CNS [16] and that these compounds seem to be similar as anticancer agents to emodin in terms of inhibition of neuroectodermal tumors [53,54,55,56].

On the other hand, there were still many details yet unknown about the primary mechanism of the suppression of glioblastoma by tanshinones, and for this reason it was necessary to validate whether these compounds exhibited toxicity and if there was the presence of any by-effects on normal human cells. To this aim, we have tested the effect of Danshen, CRY and TIIA on human HaCaT keratinocytes and human enterocytic cell lines. Our results showed that they do not significantly reduce the proliferation of tested healthy cell lines.

5. Conclusion

As a whole, we demonstrated for the first time that herbal medicine Danshen ameliorated the oxaliplatin-induced neuropathy in the mouse model of oxaliplatin-induced nociceptive hypersensitivity. Moreover, we have also shown its selective inhibitory activities on glioblastoma cells lines. Based on our results, we contribute a plausible explanation and the opportunity for the evaluation of Danshen (and its active constituents TIIA and CRY), in the treatment or combination therapy of oxaliplatin–associated neuropathy. Undoubtedly, there were some limitations in our study. The exact mechanisms responsible for the neuroprotective effects of these natural compounds are still unknown and also a long-term study is needed to further confirm our results. Thus, there is an urgent need to carry out further experiments to establish a precise foundation for the application of this plant extract as a new potential source of active diterpenoidic compounds useful for pharmaceutical and/or nutraceutical industries.

Author contributions

All listed authors have contributed substantially to the research or manuscript preparation. In particular, LDCM carried out the *in vivo* experiments whereas MP, MGF and FM the cell culture and *in vitro* experiments. LDCM, FM, CI, VDF, CG and NM conceived the study, planned its design, and drafted the paper. All authors read and approved the final paper.

Conflicts of interest

The authors declare no conflict of interest. The work described has not been submitted elsewhere for publication, in whole or in part.

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