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Tanshinone IIA Exhibits Anticonvulsant Activity in Zebrafish and Mouse Seizure Models

Olivia Erin Buenafe,[†] Adriana Orellana-Paucar,[†,‡] Jan Maes,[†] Hao Huang,[§] Xuhui Ying,[¶,○] Wim De Borggraeve,[⊥] Alexander D. Crawford,[†,∥] Walter Luyten,[▽] Camila V. Esguerra,*[,†] and Peter de Witte*[,†]

[†]Laboratory for Molecular Biodiscovery, Department of Pharmaceutical & Pharmacological Sciences, KU Leuven - University of Leuven, 3000 Leuven, Belgium

[‡]Facultad de Ciencias Quimicas, Escuela de Bioquimica y Farmacia, Universidad de Cuenca, Cuenca 101168, Ecuador

[§]College of Pharmacy, Gannan Medical University, Ganzhou 341000, China

[¶]College of Pharmacy, Nankai University, Tianjin 300071, China

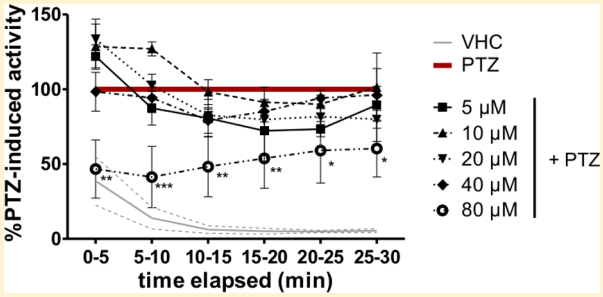
[○]Analysis Center, Tsinghua University, Beijing 100084, China

[⊥]Molecular Design and Synthesis, Department of Chemistry, KU Leuven - University of Leuven, 3001 Leuven, Belgium

[∥]Luxembourg Centre for Systems Biomedicine, University of Luxembourg, 4362 Esch-sur-Alzette, Luxembourg

[▽]Department of Biology, KU Leuven - University of Leuven, 3000 Leuven, Belgium

ABSTRACT: Danshen or Chinese red sage (*Salvia miltiorrhiza*, Bunge) is used by traditional Chinese medicine (TCM) practitioners to treat neurological, cardiovascular, and cerebrovascular disorders and is included in some TCM formulations to control epileptic seizures. In this study, acetonic crude extracts of danshen inhibited pentylenetetrazol (PTZ)-induced seizure activity in zebrafish larvae. Subsequent zebrafish bioassay-guided fractionation of the extract resulted in the isolation of four major tanshinones, which suppressed PTZ-induced activity to varying degrees. One of the active tanshinones, tanshinone IIA, also reduced *c-fos* expression in the brains of PTZ-exposed zebrafish larvae. In rodent seizure models, tanshinone IIA showed anticonvulsive activity in the mouse 6-Hz psychomotor seizure test in a biphasic manner and modified seizure thresholds in a complex manner for the mouse i.v. PTZ seizure assay. Interestingly, tanshinone IIA is used as a prescription drug in China to address cerebral ischemia in patients. Here, we provide the first *in vivo* evidence demonstrating that tanshinone IIA has anticonvulsant properties as well.



KEYWORDS: *Tanshinone IIA, Salvia miltiorrhiza, zebrafish PTZ model, mouse seizure models, pentylenetetrazol*

Epilepsy affects approximately 60 million people worldwide, of whom 30% suffer from pharmacoresistant seizures. The economics of maintaining a cost-effective therapeutic regimen hinge on its long-term efficacy and the probability of patients developing treatment-resistant seizures or possible side-effects ranging from sedation to Steven–Johnson syndrome.[1] To address such issues, there is an ongoing hunt for new antiepileptic drugs (AEDs) with novel mechanisms of action and with minimal or no side-effects.

There is a resurgence of interest in exploring plant, microbial, and marine resources used in traditional medicine for potential drug leads. According to the World Health Organization (WHO), 70–80% of the population in developing and developed countries have used, or depend on such therapies, which became the impetus for efforts in bioprospecting and drug development.[2] Despite prevalent skepticism, as many as 25% of pharmaceutical drugs on the market are plant-based and were discovered through investigations on traditional or folk medicine practiced by different cultures.[1,2] The rationale behind this approach in drug discovery is that small molecules isolated or derived from natural sources offer a more diverse set of structures compared with compounds synthesized through medicinal chemistry or combinatorial techniques.[2]

Each plant or marine extract can be treated as a library potential of hits, which can be screened using an appropriate medium- to high-throughput *in vivo* model such as zebrafish (*Danio rerio*), a freshwater teleost of the Cyprinidae family.[3,4] In screening for potential hits and leads for AED development, we have previously described the use of larval zebrafish as a platform for pinpointing AED-like activity of small molecules isolated from plant sources.[3,4] In a study conducted by Baraban *et al.*, 7-days post-fertilization (dpf) zebrafish larvae display

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seizure-like behavior (e.g., "whirlpool" swimming, loss of posture) upon exposure to the chemoconvulsant pentylenetetrazol (PTZ).[5,6] This response to PTZ is comparable to rodent models, with similar outcomes to treatment with known antiepileptic drugs.[6−8] This similarity, as well as other factors such as ease of propagation and sample introduction and the availability of automated scoring or tracking devices, positions zebrafish as a robust model for prescreening extracts (and eventually isolated compounds) from various plant libraries or ethnopharmacopeias, such as the *materia medica* of traditional Chinese medicine (TCM). From a random sampling of 40 plants from the aforementioned *materia medica* used by TCM practitioners to treat various conditions, including epilepsy, we selected Chinese red sage or danshen.

Danshen (*Salvia miltiorrhiza*, Bunge) is a plant from TCM's *materia medica* that is often used in cardio- and cerebrovascular disorders.[9,10] It is commonly prescribed by TCM practitioners to stroke patients and is included in a TCM drug formulation *ding xian wan* to control epileptic seizures.[9,11] The active compounds documented in danshen belong mostly to a specific diterpenoid class referred to as tanshinones and salvianolic acid derivatives. The former are isolated mainly via lipophilic extraction of the dried root powder and are also responsible for the characteristic red color of the herb (*danshen* literally means "red root"). Danshen's predominant bioactive constituent, tanshinone IIA, has been a focus of research for the past decade in the field of cardiovascular and cerebral ischemia. The reported biological activities of tanshinone IIA vary from antiatherosclerotic[12,13] to cardioprotective[9,13] and neuroprotective.[14,15] Because of the extensive preclinical and clinical studies on its cardioprotective and antiatherosclerotic properties, tanshinone IIA and its more water-soluble derivative sodium tanshinone IIA sulfonate are used in China as prescription treatments for angina pectoris and stroke.[13,15] The neuroprotective effects of danshen extracts on cerebral ischemia and Alzheimer's disease models have been elucidated, but scant data are available for its ascribed anticonvulsive effects, making it a potential library of small molecules to be screened in larval zebrafish for antiepileptic activity.

RESULTS AND DISCUSSION

Crude Extract of Danshen and Its Active Components Reduce PTZ-Induced Movement in 7-dpf Larval Zebrafish. Two different exposure times to the crude extract were initially tested in order to determine the incubation period required for optimal activity in zebrafish larvae. Previous empirical data obtained for other crude extracts and compounds tested revealed that shorter exposure times were often sufficient to detect bioactivity, whereas for some compounds a longer overnight incubation was necessary [4] (data not shown). Zebrafish larvae were exposed to different concentrations of the acetone crude extract of danshen for 1 h (for 7-dpf larvae) or 18 h (for 6-dpf larvae) before PTZ treatment and activity tracking. Danshen crude extract reduced PTZ-induced activity in larvae after 1-h exposure time at its maximum tolerated concentration (MTC, 5 μg/mL), but not after 18 h of exposure (Figure 1). Beyond the MTC, larvae displayed bradycardia, loss of posture, and delayed touch response after 3 h of exposure, followed by death after 18 h.

The crude extract of danshen was subjected to analytical HPLC analysis, which revealed the presence of 11 peaks (Figure 2). In order to differentiate the peaks in terms of their intrinsic activity aside from approximate abundance, these were

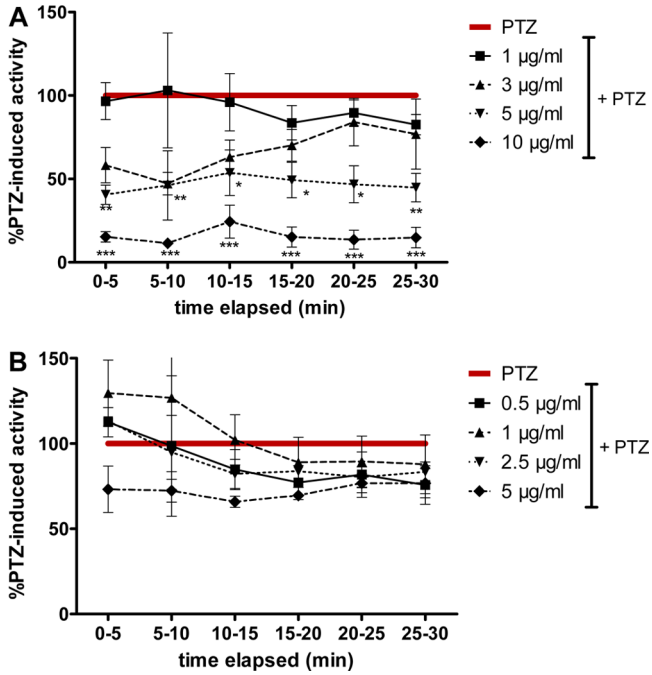

Figure 1. PTZ-induced activity curve of 7-dpf zebrafish larvae after pretreatment with different concentrations of danshen crude extract. Results were normalized against PTZ controls (set at 100%). Exposure to extract during 1 h (A) or 18 h (B). Analysis was done by two-way ANOVA, with *P* values <0.05 (*), <0.01 (**), and <0.001 (***) indicated per time period.

initially dissolved in equivolume amounts (10 μL) of DMSO before exposing 7-dpf larvae in 0.3× Danieau's solution (1% final DMSO concentration) for 18 h before subsequent exposure to PTZ; peaks that showed signs of toxicity in larvae after the pre-exposure period were titered from half to one-tenth of their original unknown concentrations. Four peaks were found to be active in this manner (data not shown) and became the focus of semipreparative isolation for structure elucidation via NMR spectroscopy. One- and two-dimensional NMR analyses of the isolated peaks showed that they are structurally related to each other as tanshinones (Table 1), with the chemical shifts matching those listed in the literature.[16] Active peaks were identified as 15,16-dihydrotanshinone I (**1**), cryptotanshinone (**2**), tanshinone IIA (**3**), and miltirone (**4**) (Figure 3), with average yields of 2.8% for miltirone to 13.1% for cryptotanshinone (Table 1).

Concentration−response curves were obtained for the active tanshinones. The MTC values for **1**, **2**, **3**, and **4** were 2.5, 17, >80 (at maximum solubility), and 7 μM, respectively, with greatest reduction in PTZ-induced activity in 7-dpf larvae at these concentrations (Figure 4A−D). 15,16-Dihydrotanshinone I (**1**) at its active concentration, however, did not display consistent reduction of PTZ-induced activity over the course of 30 min compared with the other tanshinones (Figure 4A). Cryptotanshinone (**2**) displayed significant reduction of PTZ-related activity only at its MTC (Figure 4B), unlike miltirone (**4**) whose effective concentration was lower than its MTC (Figure 4D). Tanshinone IIA (**3**), at 80 μM, also displayed significant reduction of PTZ-induced activity throughout the duration of the assay (Figure 4C).

Taking into account the estimated percent content of these active tanshinones in the crude extract, the crude extract (at 5 μg/mL, see Figure 1) shows equivalent reduction of PTZ-

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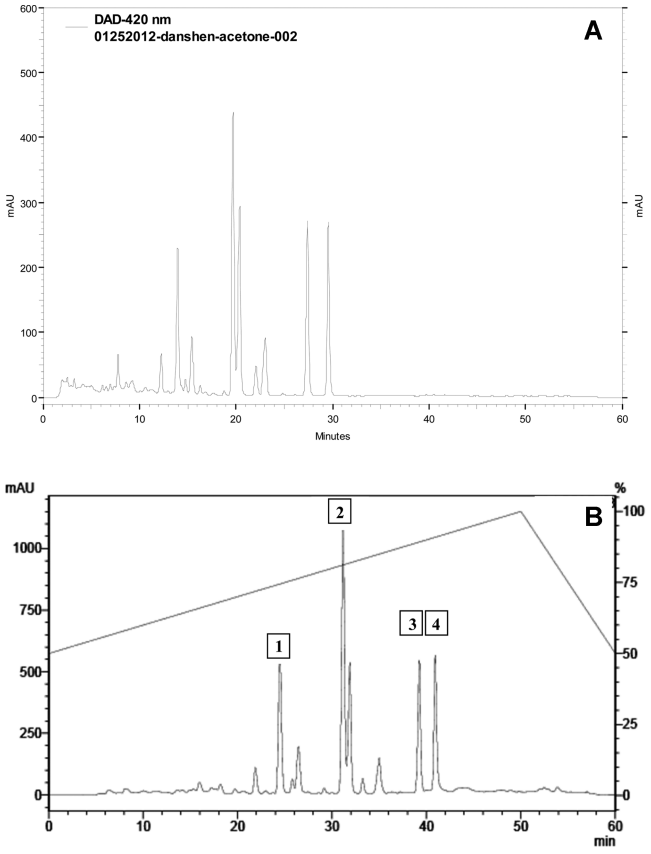

Figure 2. Reverse-phase HPLC chromatograms of danshen crude extract (CE) using the (A) analytical and (B) semipreparative columns. The calculated percentage yields from semipreparative HPLC are listed in Table 1.

Table 1. Fractions Isolated Using Semipreparative Reverse-Phase HPLC Analysis of Danshen Crude Acetone Extract Residue (130.6 mg), with Yields Expressed As Percentage (%) of the Extract or Residue

HPLC peak label	weight of peak residue (mg)	% in CE[a]	estimated concentration in 5 μg/mL CE (μM)	peak identity (via NMR)
1	6.13	4.7	0.84	15,16-dihydrotanshinone I
2	17.07	13.1	2.2	cryptotanshinone
3	10.12	7.7	1.3	tanshinone IIA
4	3.68	2.8	0.50	miltirone

[a]CE, crude extract.

induced activity in larvae at 3−60-fold lower concentrations of total tanshinones compared with individual tanshinones (Figure 4, Table 1). This indicates that there may be a complex interplay of the compounds present in the extract, which act in a synergistic manner, wherein the activity of the whole (or crude) extract is greater than the sum of its active parts.[17] The complexity of this aforementioned interplay within the larva may contribute to the loss of activity upon 18 h of pre-exposure prior to treatment with PTZ. Other less-active or inactive tanshinones may also play a role in either improving the compounds' penetration into the fish's system by modulating the drug-efflux pumps present in cells[18,19] in a similar fashion to those in bacteria[20] and cancer cells[21] or by modifying the solubilities of the active compounds.[17]

Because the lipophilic fraction of danshen has not been fully explored for its potential anticonvulsant effects in earlier literature, the identified active tanshinones bear further analysis, along the lines of structure−activity relationships. In a study conducted by Lee *et al.*, tanshinones isolated from danshen displayed different *in vitro* affinities to the central γ-amino-butyric acid receptor ($GABA_A$ receptor).[22] A danshen diterpenoid, miltirone, was reported to have a sedative activity and was shown *in vitro* to be a partial $GABA_A$ receptor agonist.[22] Tanshinone IIA was also shown to have affinity for the same receptor as miltirone but is approximately 10 times weaker.[22] The results of the *in vivo* larval PTZ assay we performed show this disparity of activity between miltirone and tanshinone IIA, with significant reduction of PTZ-activity at 6.125 and 80 μM, respectively (Figure 4). According to Lee *et al.*, the activity of tanshinones in modulating benzodiazepine receptors is due to the presence of the A ring regardless of the addition of methyl moieties at position 4 (Figure 3), and the replacement of the furan ring D with an isopropyl group.[23] Our results also imply that the activity of the compounds can differ from each other based on the modifications on the A ring and the furan D ring (Figure 3). We observed that the modification of ring D via hydrogenation at $\Delta^{15,16}$ resulted in a 5-fold increase in activity between cryptotanshinone and tanshinone IIA.

Among the four tanshinones that were active in our larval PTZ assays, tanshinone IIA and miltirone possess significant characteristics that need to be considered. Miltirone appears more active than tanshinone IIA. However, its narrow effective-concentration range (Figure 4D) and its sedative activity[22] as a potential side effect make the compound less attractive for further development. As noted earlier,[10,13,24] tanshinone IIA has been identified as a predominant bioactive constituent of danshen and developed as a prescription drug for cardio- and cerebrovascular disorders. We therefore chose to focus on this specific compound for additional experiments.

Intravenous Microinjection of Tanshinone IIA in Zebrafish Larvae. Tanshinone IIA is poorly soluble in water; hence its indicated MTC value is beyond its maximum solubility at 80 μM. In order to observe the compound's further reduction of PTZ-induced movement in larvae as well as to be able to approximate the dosage in milligrams per kilogram, additional locomotor recordings were made on intravenously (i.v.)-injected 3-dpf zebrafish larvae with either vehicle (1:1 DMSO/0.3× Danieau's solution) or 100 mM tanshinone IIA solutions. Intravenously injected tanshinone IIA significantly reduced PTZ-induced movement in 3-dpf larvae (Figure 5). Taking into account the bolus volume (0.5 nL), concentration (100 mM), and average weight of a 3-dpf larva (0.25 mg), the estimated calculated dose was 59 mg/kg larva.

The results from intravenous administration of tanshinone IIA into larvae were comparable to those generated from pre-exposure to the compound in larval medium. Regardless of larval stage or mode of administration, both techniques resulted in a statistically significant 50% reduction of PTZ-induced movement, indicating that the solubility of tanshinone IIA alone cannot fully account for the 50% reduction limit.

Reduction of Brain *c-fos* Expression in Zebrafish Larvae Treated with Tanshinone IIA. Previous studies have shown the robustness of the *in situ* hybridization assay for *c-fos* transcription in the zebrafish larval CNS as a high-throughput indicator of the neural response to chemo-convulsant treatment in lieu of electroencephalographic

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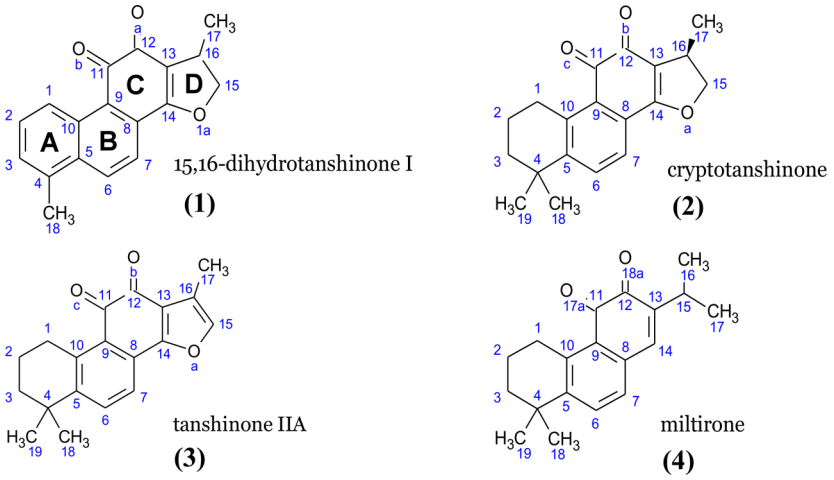

Figure 3. Molecular structures of the tanshinones (**1**−**4**) isolated from danshen crude acetone extract.

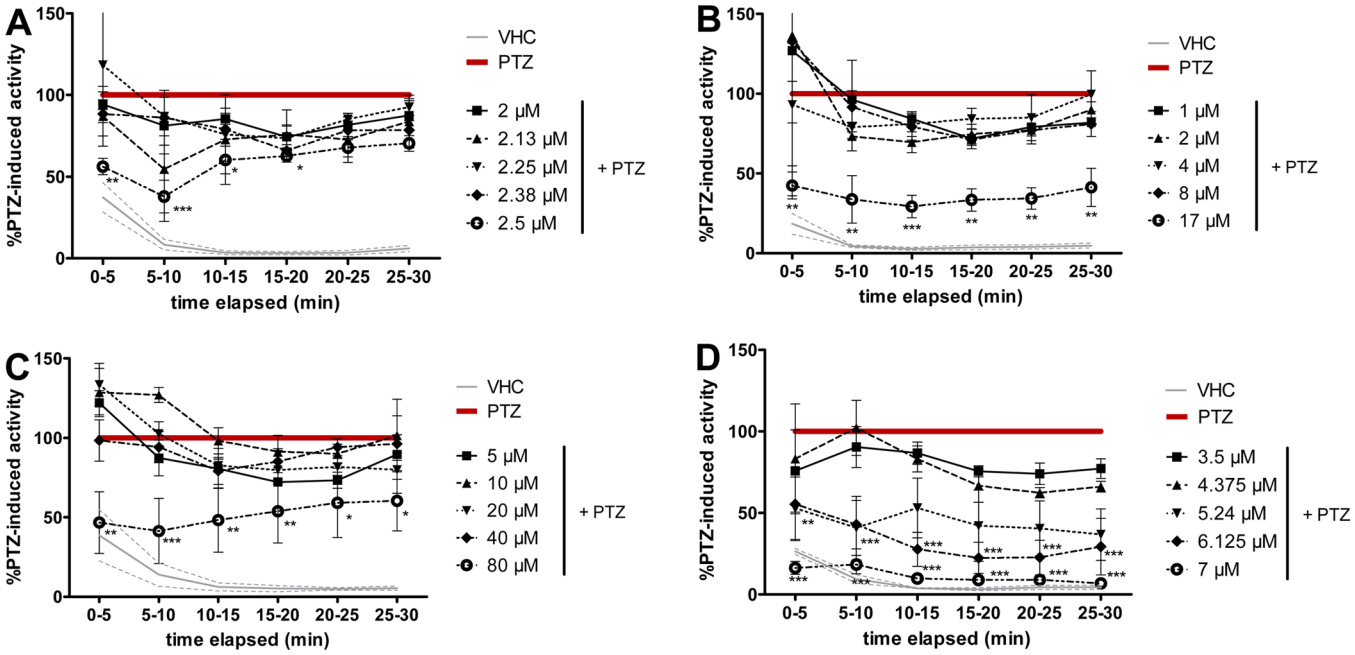

Figure 4. PTZ-normalized concentration−response curves for (A) 15,16-dihydrotanshinone I, (B) cryptotanshinone, (C) tanshinone IIA, and (D) miltirone. Results were normalized against PTZ controls (set at 100%). Analysis was done by two-way ANOVA, with *P* values <0.05 (∗), <0.01 (∗∗), and <0.001 (∗∗∗) indicated per time period.

(EEG) recordings.[25] Confirmation of neuroprotective activity of tanshinone IIA was initially carried out through whole mount *in situ* hybridization (WISH) of 6/7-dpf larvae that were pre-exposed to the test compounds for 18 h before subsequent exposure to PTZ. Overexpression of the oncogene *c-fos* in the brain was scored for sham-, vehicle-, and compound-treated larvae, because the extent of *c-fos* overexpression has been linked to severity of seizures and neuronal insults in the brain.[6,25] Larvae exposed to tanshinone IIA (80 μM) for 18 h before PTZ treatment showed a reduction of *c-fos* expression (Figure 6) compared with larvae pre-exposed to vehicle, with significant reduction of staining in the hindbrain. Quantitative analysis confirms the reduction of *c-fos* staining in the brain by as much as one-third (Figure 7). Subsequent *c-fos* WISH analysis was also performed on 3-dpf larvae i.v.-injected with either vehicle or 100 mM tanshinone IIA before PTZ treatment. Results show a 50% reduction of *c-fos* expression in tanshinone IIA-injected larvae that were exposed to PTZ (Figure 8). This reduction of *c-fos* expression corresponds to the reduction of PTZ-induced movement in the larval behavioral assays (Figures 4C and 5).

Regardless of mode of administration, tanshinone IIA demonstrates significant reduction of *c-fos* expression, indicating the potential anticonvulsant action of the compound, since an increase in brain *c-fos* gene expression is a well-documented marker for seizure induction and brain damage.[6,25] The reduction of *c-fos* expression by tanshinone IIA also implies that the compound is able to penetrate into the brain to a certain extent and thus exert its anticonvulsant properties. Tanshinone IIA is known to play a significant role in the TORC1−CREB pathway, controlling neuronal excitation, proliferation of neuronal cells, and formation of long-term memory and learning.[14] The lowered *c-fos* expression in larval zebrafish that were pretreated with tanshinone IIA prior to PTZ

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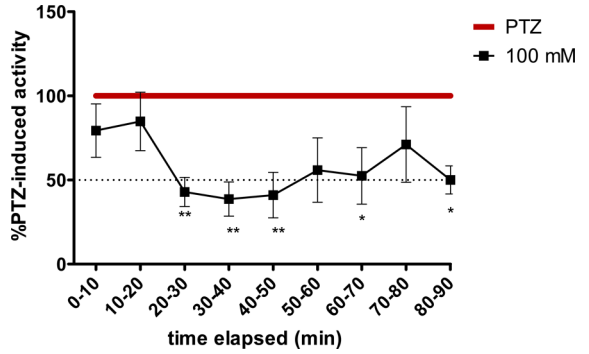

Figure 5. PTZ-normalized concentration−response curves for intravenous injection of vehicle-only and tanshinone IIA in 3-dpf zebrafish larvae that were subsequently exposed to 20 mM PTZ and tracked for 90 min. Results were normalized against PTZ controls (set at 100%). Analysis was done by two-way ANOVA, with P values <0.05 (∗), <0.01 (∗∗), and <0.001 (∗∗∗) indicated per time period.

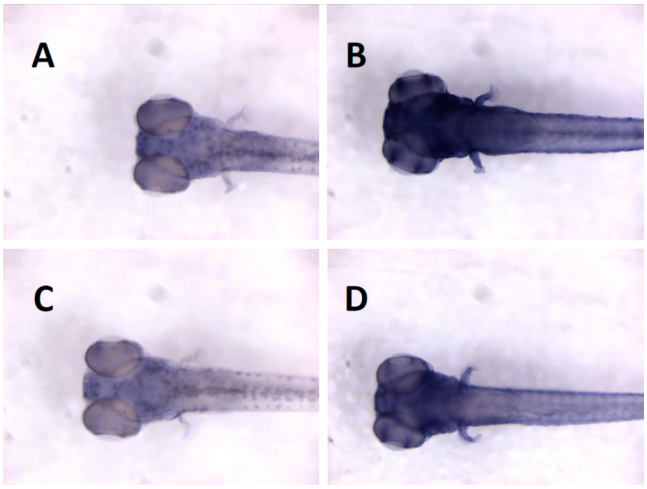

Figure 6. WISH of 7-dpf zebrafish larvae to visualize the modulation of *c-fos* expression: (A) larva with vehicle only pretreatment, unexposed to PTZ before fixation and staining; (B) larva with vehicle only pretreatment, exposed to PTZ for 30 min before fixation and staining; (C) larva pretreated with 80 μM tanshinone IIA for 18 h, unexposed to PTZ before fixation and staining; and (D) larva pretreated with 80 μM tanshinone IIA before PTZ exposure, fixation, and staining.

exposure may be due to the regulation of TORC1−CREB signaling, because *c-fos* expression is located downstream of this pathway.[14] This upstream regulation may be coupled with tanshinone IIA's mild affinity for $GABA_A$ receptors, which counteracts neuronal excitation caused by glutamatergic neurons. Tanshinone IIA has demonstrated inhibitory activity in glutamate release from cortical synaptosomes in rats through the suppression of presynaptic voltage-dependent Ca^{2+} entry and the mitogen-activated protein kinase (MEK) signaling cascade,[26] which may also contribute to the decrease in *c-fos* expression because neuronal damage due to glutamate excitotoxicity[27] may be reduced via this particular mode of suppression.

Evaluation of Tanshinone IIA in Rodent Models. We explored the potential activity of tanshinone IIA in two standard rodent seizure models: the 6-Hz psychomotor seizure assay in mice and the timed i.v. PTZ infusion test.

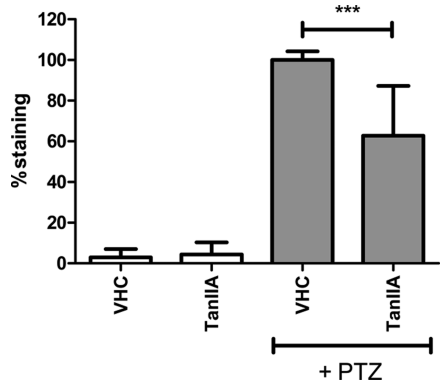

Figure 7. Quantitative analysis of WISH staining of 7-dpf zebrafish larvae to visualize the modulation of *c-fos* expression by vehicle and 80 μM tanshinone IIA (TanIIA) with (gray bars) or without (white bars) additional exposure to PTZ. Student's t test was performed to determine statistical significance with P values <0.05 (∗), <0.01 (∗∗), and <0.001 (∗∗∗).

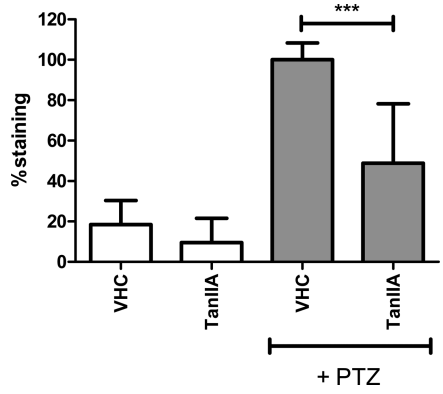

Figure 8. Quantitative analysis of WISH staining of i.v.-injected 3-dpf zebrafish larvae to visualize the modulation of *c-fos* expression by vehicle and 100 mM tanshinone IIA (TanIIA) with (gray bars) or without (white bars) additional exposure to PTZ. Student's t test was performed to determine statistical significance with P values <0.05 (∗), <0.01 (∗∗), and <0.001 (∗∗∗).

The 6-Hz psychomotor seizure test in rodents was reported to be a useful model for screening anticonvulsants with novel properties since it does not completely discriminate compounds based on their mechanism of action,[28] as compared with chemoconvulsants specific to certain excitatory or inhibitory pathways, thus making this assay an effective option in assessing the validity of selected compounds as anticonvulsants. Application of a 6-Hz electrical stimulus through the corneas of mice triggers seizures that are similar to automatisms found in human limbic epilepsy.[28,29] In this assay, doses of 0.5−1 mg/kg tanshinone IIA were successful in protecting 5 out of 6 mice (Figure 9), thus showing potential anticonvulsant effects by protecting the brain against limbic or focal seizures. However, this effect was lost at higher doses of tanshinone IIA, suggesting that the compound acts in a biphasic or hormetic manner, since increasing or decreasing the dose of tanshinone IIA 10-fold lowered its efficacy as an anticonvulsant agent in the 6-Hz seizure model. Such hormetic dose responses have indeed been previously reported in chemically diverse pro- and anticonvulsant agents with different modes of action.[30,31] Another observation is that tanshinone IIA was able to exert an effect in the 6-Hz mouse seizure model at concentrations lower

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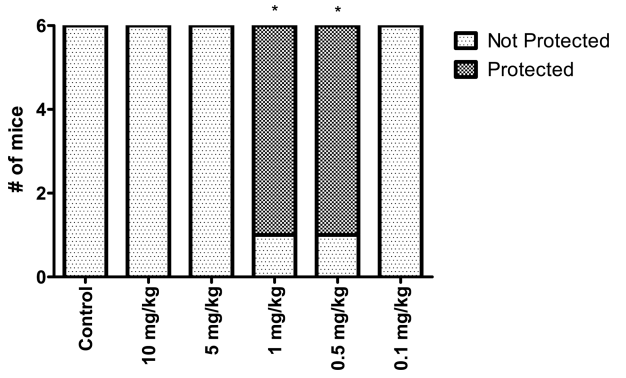

Figure 9. Bar graphs of 6-Hz psychomotor seizure assay on male NMRI mice treated with tanshinone IIA (44 mA; n = 6 mice per treatment group). Analysis was done using Fischer's exact test at 95% confidence level to determine statistical significance (indicated by ∗).

than those used in preclinical cardiovascular research[32] by as much as 40-fold. This 40-fold difference can be interpreted in the following manner: off-target or alternate system effects are avoided, if low doses of the compound are used.

The i.v.-PTZ mouse test serves as a model for acute generalized seizures by determining changes in seizure thresholds. PTZ delivered via an i.v.-infusion system has shown to be more reliable in selecting and describing compounds with pro- or anticonvulsant effects as compared with PTZ delivered subcutaneously, which is prone to false positives and negatives.[33] Tanshinone IIA at 10, 1, and 0.1 mg/kg injected i.v. into mice displayed a complex response upon continuous i.v.-PTZ infusion (Figure 10). Under these

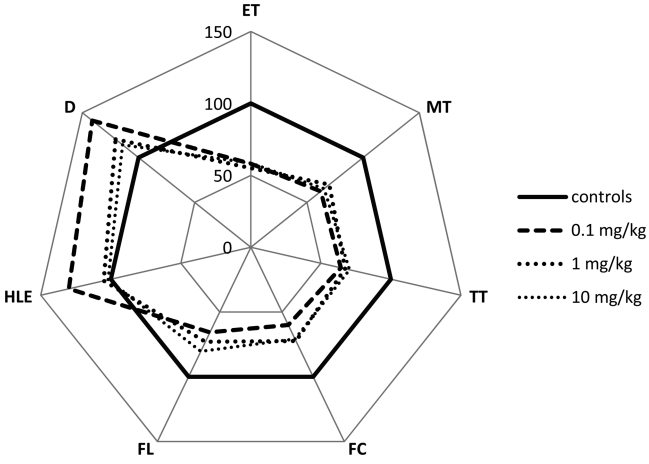

Figure 10. Radar graphs of PTZ-i.v. infused mice treated with tanshinone IIA (n = 6 mice per treatment group). Results for each group were normalized against the controls (set at 100%). Mice were scored according to the PTZ dose used per seizure progression: ear twitch (ET), tail twitch (TT), myoclonic twitch (MT), forelimb clonus (FC), falling (F), tonic hindlimb extension (HLE), and death (D). Standard deviations were not included in the graph but are listed in the corresponding table (Table 2).

conditions, reduction of seizure threshold was observed at the initial stage of PTZ infusion and subsequently coupled with an increase in threshold at latter stages (Figure 10, Table 2).

The early seizure stages triggered by the initial low-dose PTZ correspond to generalized absence seizures,[34] while the later, tonic–clonic stages are due to the action of high-dose PTZ, leading to generalized status epilepticus (SE) and death.[35] Tanshinone IIA reduced the amount of PTZ needed to elicit the early seizure stages (i.e., ear twitch, myoclonic twitch, tail twitch), while it also significantly increased the amount of PTZ needed to reach the final seizure end point (Figure 10, Table 2). The results indicate that the compound may have pro- and anticonvulsant features, which are highly dependent on the amount of PTZ. The failure of tanshinone IIA during the early, low-dose PTZ stage may be due to the compound being a poor competitor to PTZ, a $GABA_A$ antagonist, and could possibly interact with other pathways that may exacerbate the effects of low-dose PTZ. At higher doses of PTZ, this proconvulsant effect is reversed; despite being a strong $GABA_A$ antagonist, high doses of PTZ interact with non-GABA related receptors, upon which tanshinone IIA could compete at a better advantage. Overall, tanshinone IIA has a more complex activity profile in this model compared with the 7-dpf larval PTZ assay. This discrepancy may be due to numerous factors such as the difference between mammalian and teleost brains or the different pharmacokinetic dynamics of zebrafish and mice.[7] In the case of commercially available antiepileptic drugs, which were tested in both zebrafish and rodent behavioral models, a number of compounds like topiramate and zosinamide show a positive response in zebrafish but not in rodent models.[7]

The disparate outcomes of both rodent models, the ambiguity of tanshinone IIA's activity in the i.v. PTZ assay and its positive activity in the 6-Hz mouse seizure model, illustrate the potentially complex mode of action of the compound. Such disparity is not unheard of in small-molecule screening of potential anticonvulsants; mexiletine, a class IB antiarrhythmic drug, has anticonvulsant properties in the maximal electroshock seizure (MES) model in mice but has also failed in i.v. PTZ tests.[36] A major concern in interpreting results based on these animal models is that they are administered to naïve animals in an acute fashion and consequently do not consider the pathophysiological mechanisms of chronic epilepsy. However, since these models address the question of seizure suppression, which is the primary key in the treatment of epilepsy, the relevance of the results generated through their use is not diminished. The National Institutes of Health (NIH) designed a drug-screening scheme for their anticonvulsant screening program (ASP) wherein compounds are initially assessed for their anticonvulsant activity in at least one acute, nonmechanistic model before subjecting them to more discriminatory, mechanistic ones[36] to determine whether these compounds can modify the progression of the disease aside from controlling the critical symptom (i.e., seizures). Clearly, tanshinone IIA should be tested in additional chronic and spontaneous seizure assays such as kindling, GAERS, and genetic epilepsy models (among others) in order to characterize and validate its potential as a disease-modifying antiepileptic compound.

To complement the rodent seizure assays for tanshinone IIA, we wanted to determine whether the selected dose ranges could possibly contribute to neurological effects on their own. NMRI mice were injected i.p. with 10 and 1 mg/kg of the compound and were subjected to the beam-walking assay. Both groups of mice performed as well as mice injected with vehicle only, which fared better than those injected with diazepam (1 mg/kg), which resulted in an increase in the number of foot slips and falls (Figure 11). Based on these results, tanshinone IIA did not elicit sedative or similar neurological effects comparable to diazepam, a potent $GABA_A$ agonist, despite its structural

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Table 2. Mean PTZ Dose Corresponding to Specific Seizure Phenotype Per Treatment Group Correlated to the Radar Graph in Figure 9[a]

	mean PTZ dose corresponding to specific seizure phenotype (mg/kg)			
	control (vehicle only)	0.1 mg/kg TanIIA	1 mg/kg TanIIA	10 mg/kg TanIIA
ear twitch (ET)	77.1 ± 7.5	44.7 ± 5.4*	42.4 ± 2.0*	45.1 ± 1.5*
myoclonic twitch (MT)	83.0 ± 2.9	51.6 ± 8.9*	58.0 ± 6.5*	54.1 ± 5.9*
tail twitch (TT)	86.2 ± 3.8	55.4 ± 10.8*	58.0 ± 6.5*	60.7 ± 4.5*
forelimb clonus (FC)	103.3 ± 20.3	62.0 ± 18.4*	74.8 ± 10.0	73.2 ± 7.6
falling (FL)	101.8 ± 21.8	66.9 ± 21.6*	74.3 ± 16.3	81.8 ± 16.4
tonic hindlimb extension (HLE)	125.6 ± 13.6	163.3 ± 35.2*	132.0 ± 29.0	128.1 ± 10.8
death (D)	134.2 ± 12.5	189.6 ± 36.9*	161.7 ± 22.4*	153.3 ± 8.9*

[a]Statistical analysis was done using one-way ANOVA with Dunnett's test, with significant differences (indicated by *, $P < 0.001$) seen for all tanshinone IIA treatment sets.

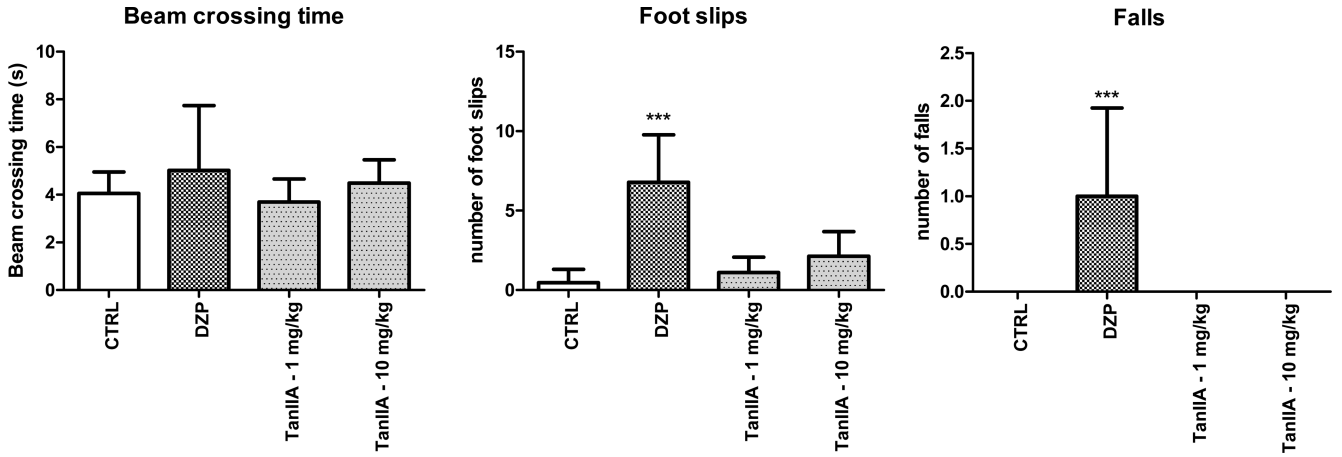

Figure 11. Scores for beam-walking assay of male NMRI mice treated with 1 and 10 mg/kg tanshinone IIA ($n = 6$ mice per treatment group). Statistical analysis was done using one-way ANOVA with Dunnett's test, with P values <0.05 (*), <0.01 (**), and <0.001 (***) indicated per treatment group.

resemblance to another $GABA_A$ agonist, miltirone, thus indicating that tanshinone IIA has a propensity to interact with pathways other than those ascribed to GABA and related agonists in order to exert its anticonvulsant properties.

In conclusion, we have demonstrated that the acetone crude extract of danshen can significantly reduce convulsions induced in our larval zebrafish-PTZ assay and that a number of its hydrophobic tanshinones are responsible for this activity. One such tanshinone, tanshinone IIA, is active in both the zebrafish-PTZ and mouse 6-Hz psychomotor seizure models and has a complex profile in the mouse-PTZ infusion model. The issues regarding its solubility, bioavailability, and multitarget effects could possibly be addressed by exploring more water-soluble derivatives, some of which have readily available pharmacokinetic data,[13,15] making tanshinones a potential source of novel anticonvulsants.

METHODS

Plant Materials and Reagents. Dried danshen root powder was purchased from Tong Ren Tang, Beijing (http://www.tongrentang.com/en/index.php). Tanshinone IIA and dihydrotanshinone I standards (98% purity via HPLC) were sourced from IMAM International Pharmaceuticals, Ltd. (Tianjin, China).

Crude Extract Preparation. Five grams of dried danshen root powder was dispersed in 50 mL of acetone via ultrasonication (Bransonic 5510E-MT: Danbury 06813, USA); the resulting solution was filtered and reduced to dryness (60 mg). The resulting residue (CE) was dissolved in spectroscopic-grade dimethyl sulfoxide (DMSO; Acros Organics, Geel, Belgium) as 20 mg/mL stock solution. For rodent assays, the residue was dissolved in 50/50 DMSO/poly(ethylene glycol)-200 (DMSO/PEG-200) as a 100 mg/mL stock solution. All stock solutions were stored at −20 °C.

High-Performance Liquid Chromatography (HPLC). Approximately 200 μg of the crude extract was dissolved in 20 μL of HPLC-grade acetonitrile (ACN; Acros Organics, Geel, Belgium) and was injected into a Hitachi EZChrom Elite HPLC system with a Luna C-18(2) reversed-phase silica analytical column (Phenomenex, Torrance, CA, USA), a quaternary solvent pump, and a diode array detector. A gradient solvent system (0−40 min, 50% ACN/water to 100% ACN; 41−60 min, 100% ACN to 50% ACN/water) at 0.8 mL/min was used to resolve the components in the extract. For semipreparative work, 10 mg of the extract was injected in a Gilson 712 HPLC system with a Waters 2487 dual-wavelength detector (Waters USA, Milford, MA, USA) and Sun Fire Prep C-18 reversed-phase semipreparative column (10 × 250 mm^2, 5 μm; Waters USA, Milford, MA, USA). A similar gradient solvent system at 2.5 mL/min was used in this scheme to isolate fractions of the components. The isolated fractions were subject to rotary evaporation and purged with dry N_2 gas to obtain residues for further analyses.

Nuclear Magnetic Resonance Spectroscopy (NMR). The residues were dissolved in 2.5 mL of deuterated DMSO (Sigma-Aldrich, St. Louis, MO, USA) in 5-mm inner diameter glass NMR tubes and run in a Bruker Avance 600-MHz NMR spectrometer. Data and structure analyses for proton and carbon signals used ACDLabs NMR Processor Academic Edition 12 (ACDLabs, Cambridge, MA, USA).

Zebrafish. Adult zebrafish (AB strain) were reared at 28 °C on a 14/10 h light/dark cycle according to standard aquaculture conditions. Eggs were collected following natural spawning, sorted, and raised in 0.3× Danieau's solution (1.5 mM HEPES, pH 7.6, 17.4 mM NaCl,

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0.21 mM KCl, 0.12 mM $MgSO_4$, and 0.18 mM $Ca(NO_3)_2$) under constant light conditions in an incubator set at 28 °C until 7 dpf.

Mice. Male 10−12-week-old C57Bl/6 and NMRI mice were kept on a 12/12 h light/dark cycle and were provided with standard rodent diet *ad libitum*. All aspects of animal experiments and husbandry were carried out in strict compliance with the National and European Regulations and were approved by the Animal Care and Use Committee of KU Leuven (Ethische Commissie van de KU Leuven, approval numbers P101/2010 and P061/2013) and in accordance with Belgian and European Laws, guidelines, and policies for animal experimentation, housing, and care (Belgian Royal Decree of April 6, 2010, and European Directive 2010/63/EU on the protection of animals used for scientific purposes).

Automated Larval Zebrafish PTZ Assay. One 7-dpf zebrafish larva was placed in each well of a 96-well plate. Excess larval medium was removed and replaced with 100 μL of either control or sample solution. The prepared plate was placed in a dark box inside an incubator, set at 28 °C, for 1 or 18 h, and each well was subsequently inspected for signs of toxicity (e.g., irregular heart-rate, loss of posture, edema, necrosis, delayed startle or touch response). The maximum tolerated concentration (MTC) was designated as the highest sample concentration that did not elicit any signs of toxicity in 6/7-dpf larvae after 18 h of exposure.[7] Upon addition of 100 μL of 40 mM PTZ per well, the plate was positioned in the zebrafish tracking box (Viewpoint, Lyon, France), and the larvae were allowed to habituate for 5 min before recording for 30 min. Tracking data was exported into Excel format and processed as such before statistical analysis via GraphPad Prism v.5 for Windows. Each tracking data set was normalized against the PTZ-only control values (set at 100%) within each set, with each subsequent replicate set pooled before two-way ANOVA with Bonferroni *post hoc* analysis.

Intravenous Microinjection of Tanshinone IIA into 3-dpf Zebrafish Larvae. In order to overcome potential issues with tanshinone IIA's solubility in the larval medium, i.v. microinjections were performed. Three days post-fertilization zebrafish larvae were anesthetisized with tricaine before securing them on a grooved agarose mold in supine position. One-half nanoliter of tanshinone IIA (100 mM in 50% DMSO) was injected into the circulation through the duct of Cuvier (future common cardinal vein, CCV) [37] using a pulled-glass capillary needle. Injected larvae were left to recover in tricaine-free larval medium in 96-well plates for 30 min before exposure to 20 mM PTZ for automated locomotor assays, with corresponding sets of sham-injected and uninjected larvae for controls.

Whole Mount *in Situ* Hybridization (WISH) of 7-dpf Zebrafish Larvae for Brain *c-fos* Expression. This assay was performed to assess the extent of seizure suppression in zebrafish brains, because increased *c-fos* expression serves as a well-documented indicator for seizure onset in both fish and mammalian models.[6,25] At least 30 6/7-dpf larvae were exposed to 1 mL of vehicle or sample solution 18 h before addition of 1 mL of 40 mM PTZ for 30 min. The treated larvae were fixed in 4% paraformaldehyde overnight and then exposed to 10 μg/mL proteinase K before hybridization with a digoxigenin-labeled *c-fos* RNA probe, according to standard procedures.[6,25,38] The extent of staining was quantified using KS300 software (Carl Zeiss, Bulgaria) and analyzed via unpaired Student's *t* test with GraphPad Prism v.5 for Windows.

Timed PTZ-Infusion Mouse Assay. Twenty-four C57Bl/6J mice were divided into control and treatment groups of six mice each. Mice were restrained, and a lateral caudal vein was catheterized with a 1-cm 29-gauge needle attached to polyethylene tubing, secured with surgical tape. One-hundred microliters of vehicle (50/50 DMSO/PEG200) or different concentrations of tanshinone IIA was i.v.-infused into mice for 2 min at 50 μL/min using an automated i.v. injector pump (World Precision Instruments ALADOIN-1000 11VDC, 0.75 Å) and then incubated for 10 min before shifting to a continuous infusion of 7.5 mg/mL PTZ (0.9% saline water) at 150 μL/min. Mice were scored according to the time latencies of seizure progression: ear twitch (ET), tail twitch (TT), myoclonic twitch (MT), forelimb clonus (FC), falling (F), tonic hindlimb extension (HLE), and death (D). All surviving mice were euthanized immediately at the end of the infusion.

6-Hz Psychomotor Seizure Assay in Mice. NMRI mice of approximately 30−35 g were grouped six per cage. Each mouse was injected i.p. with vehicle (50/50 DMSO/PEG200) or different concentrations of danshen crude extract or tanshinone IIA 30 min before the procedure. Seizures were induced via corneal stimulation (6-Hz, 0.2-ms rectangular pulse width) using a Ugo-Basil device. Prior to the placement of corneal electrodes, a drop of 0.5% xylocaine was applied to the eyes of the animal. Animals were restrained manually and released immediately in a cage of Plexiglas following the stimulation. Then the animal was observed for characteristic signs such as stun, forelimb clonus, twitching of vibrissae, and Straub tail for at least 45 s. Protection was defined as the absence of a seizure within the expected time frame.

Beam-Walking Test. NMRI mice of approximately 30−35 g were grouped six per cage and were trained three times to walk across an 80-cm wooden beam from an open platform. Each mouse was injected i.p. with vehicle (50/50 DMSO/PEG200), tanshinone IIA (10 and 1 mg/kg), or diazepam (1 mg/kg) 30 min before the actual experiment. The duration of the crossings, number of foot slips and falls were recorded for each mouse for three trials.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: camila.esguerra@pharm.kuleuven.be.

*E-mail: peter.dewitte@pharm.kuleuven.be.

Notes

The authors declare no competing financial interest.

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