Resveratrol attenuates neuropathic pain through balancing pro-inflammatory and anti-inflammatory cytokines release in mice

Lei Tao 1, Qian Ding 1, Changjun Gao *, Xude Sun *

Department of Anesthesiology, Tangdu Hospital, Fourth Military Medical University, Xi’an 710038, China

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A B S T R A C T

Anti-inflammatory activity of resveratrol has been widely studied, while its beneficial effect on the management of neuropathic pain, a refractory chronic syndrome with pro-inflammatory implicated in, is very little investigated. In the present study, the effects of different doses and various time window of administration of resveratrol were explored in a neuropathic mouse model of chronic constriction injury (CCI) of the sciatic nerve. It was demonstrated that pretreatment of resveratrol (5, 10, 20 and 40 mg/kg) for 7 consecutive days before CCI did not alleviate neuropathic pain, while it clearly relieved the pain when administered after CCI and such pain relief effect was more pronounced when administrated right after the peak of pain symptom at day 7 after CCI, as evidenced by the alleviation of thermal hyperalgesia and mechanical allodynia. Such a beneficial effect of resveratrol was in a dose-dependent manner. Mechanistic study showed that resveratrol repressed the expression of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, and promoted the expression of anti-inflammatory cytokine IL-10 at the same time, which was further confirmed in a cell model of microglia. It was also shown that neuropathic pain inversely correlated with pro-inflammatory cytokines release in mice and inflammatory and anti-inflammatory cytokine IL-10 in all experimental mice from Spearman correlation coefficient. Our study reveals that resveratrol displays a significant neuropathic pain relief effect and paved a way for novel treatment of chronic pain.

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1. Introduction

Neuropathic pain is caused by injury or disease to the peripheral or central nervous system [1]. Allodynia (pain evoked by normally non-noxious stimuli) and hyperalgesia (an increased response to a noxious stimuli) are routinely observed in human neuropathic pain condition as well as in animal models, and are often refractory to analgesics and interventional therapeutic methods [1,2]. Neuropathic pain is caused by alteration of signaling pathways in neuronal populations located in the dorsal root ganglion, the spinal cord and cerebral areas [1]. Within the dorsal horn of the spinal cord, the activation of the second order sensory neurons contributed to the development and preservation of neuropathic pain [1], through the activation of many different mechanisms, such as the production of inflammatory cytokines [3,4], the exacerbate activation of N-methyl-D-aspartate (NMDA) receptor [5] and the stimulation of transcriptional factors, such as the nuclear factor-kappa B (NF-κB) cascade [6]. Many different drugs have been tried to control and reduce neuropathic pain, but since the underlying mechanisms are multiple and complex, the treatment and management of this distressing condition is far from satisfactory [1,2].

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenolic compound. It is widely employed to protect against cardiovascular diseases and cancers, as well as to enhance anti-aging effects in numerous organisms [7,8] due to its anti-inflammatory, anti-inflammatory, cell growth-modulatory and anticarcinogenic properties [9]. Resveratrol has been recently reported to be able to attenuate pain in several different researches [10–16]. It was observed that administration of resveratrol attenuated thermal hyperalgesia in a mouse model of diabetic neuropathic pain [12]. Maia et al. reported its advantages for endometriosis-related pain [11]. Also, its promising potential for the treatment of nucleus pulposus-mediated pain was investigated in vitro and in vivo [16]. During its effect on pain relief, different mechanisms were proposed, such as by inhibition of spinal glial activation and CX3CR1 upregulation in bone cancer pain [12], inhibition of nitric oxide and TNF-α for its anti-nociceptive activity in attenuating diabetic neuropathic pain [10], and activation of AMPK to attenuate ERK and mTOR signaling in sensory neurons during its inhibition of incision-induced acute and chronic pain [13].

Regarding neuropathic pain, it has been reported in rats that administration of resveratrol attenuated neuropathic pain through the activation of spinal sirtuin 1 (SIRT1) [16]. Also, it was suggested that resveratrol exhibited an anti-inflammatory and anti-catabolic effect on...
the messenger RNA and protein level of IL-6, IL-8, MMP1, MMP3 and MMP13 [16]. In the present study, we aim to examine the ability of resveratrol to reduce neuropathic pain and explore the underlying molecular mechanisms in a neuropathic mouse model of chronic constriction injury (CCI) of the sciatic nerve.

2. Materials and methods

2.1. Animals

Female Balb-c mice (21–22 g) were obtained from Shanghai Medical Center (Shanghai, China). For dose response and time window of administration study of resveratrol, 8 animals per group were used. The present study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Tangdu Hospital, Fourth Military Medical University. The IACUC committee members at Tangdu Hospital, Fourth Military Medical University approved this study. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Chronic constriction injury (CCI)

CCI was conducted according to routine protocols. Animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal (i.p.) injection), an incision was made in the right thigh, and the sciatic nerve was exposed. Two loose ligatures with 1 mm apart were then made around the nerve using the 6–0 poly-glycolic acid synthetic absorbable sutures. The incision was closed using 5–0 interrupted nylon sutures. A control group was included with sham surgery where the right sciatic nerve was exposed but not further manipulated. The animals did not receive postoperative analgesics in order to preserve the pain associated with CCI and further to avoid undesirable interactions with resveratrol.

2.3. Drug administration

For dose response and time window studies, resveratrol was administered by i.p. injection at 5, 10, 20 and 40 mg/kg (n = 8 mice/compound/dose). The administration was daily repeated for 7 consecutive days. Resveratrol was dissolved in DMSO/saline solution (1:9 v/v), and a stock solution of 5 mM resveratrol (Life Technologies, USA) was prepared and stored at −20 °C until use. DMSO/saline solution was used as vehicle and the final concentration of DMSO is below 0.1%.

2.4. Behavioral measurements

The thermal withdrawal latency and mechanical withdrawal threshold of all mice were measured at the time of 0, 7, 14, 21, 28 and 35 days after CCI. All the measurements were performed by the same observer who was blind to the animal treatments. The Hargreaves test was used to evaluate the thermal withdrawal latency by a plantar algometer (Tes7370, Ugo Basile, Comerio, Italy). Mice were placed in clear plastic cages on an elevated glass plate. A constant intensity radiant heat source was focused underneath the glass and aimed at the plantar surface of the ipsilateral hindpaw. A digital timer automatically read the duration after CCI. All the measurements were performed by the same observer who was blind to the animal treatments. Measurements were repeated three times at intervals of 5 min, and the mean value of the three measurements was taken as the latency. A cutoff time of 25 s of irradiation was used to avoid any tissue damage. The mechanical withdrawal threshold of the ipsilateral hindpaw was measured by an electronic von Frey anesthesiometer (2390 series; IITC Instruments, Woodland Hills, USA). Briefly, the rats were placed in a plastic cage on a metal mesh floor and were allowed to adapt to this set-up prior to testing. A hand-held force transducer fitted with a 0.7 mm² polypropylene tip was applied to the plantar surface of the ipsilateral hindpaw. Measurements were repeated three times at intervals of 5 min, and the mechanical threshold was defined as the force (g) initiating a withdrawal response averaged from the three measurements.

2.5. ELISA

Total RNA was extracted for reverse transcription using TRIzol reagent (Invitrogen, New York, NY, USA) according to the manufacturer’s protocols. The cDNA was synthesized using a PrimeScript 1st Strand cDNA synthesis kit (TaKaRa Biotechnology, Dalian, China). RT-PCR was performed in a single tube using SYBR Premix kit (TaKaRa Biotechnology) with an ABI 7300 real-time PCR machine (Life Technologies, USA). The relative gene expression levels were calculated by normalizing the quantified cDNA transcript level to the GAPDH transcript level and were normalized to those of Sham + veh group. Each value represents the average of at least 3 independent experiments.

2.7. Western blot

Western blotting was performed according to routine protocols. The following primary antibodies were used: anti-IL-10 (1:1000, BD Bioscience, San Jose, CA); anti-GAPDH (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with the corresponding horseradish peroxidase-conjugated antibody (1:2000; Promega, Madison, WI). Immunoreactive bands were visualized using the Western Blotting Luminol Reagent and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4, Media Cybernetics). The experiment was repeated for at least three times independently.

2.8. Primary culture of microglia

Primary hippocampal microglia were isolated from glial cultures prepared from newborn (~24 h old) Balb-c mice according to routine protocols. Glial cells were cultured in 75 cm² flasks for 14 days in DMEM/F12 (Gibco, USA) supplemented with 10% FCS (Hyclone, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. Microglia were then isolated from primary mixed glial cell cultures on day 10 by shaking the flasks overnight at 300 rpm on a rotary shaker at 37 °C. Cells were cultured for 2 days before further experiments. The purity of primary microglia is over 95% as determined by immunocytochemistry with antibody against CD11b.

2.9. Lactate dehydrogenase (LDH) assay

After a cell dies, lactate dehydrogenase (LDH) inside the cell will be released. LDH is stable, and its level is routinely used as an indicator to determine cytotoxicity of an agent. Its detection was conducted with the LDH detection activity assay kit (Sigma) at 450 nm according to the manufacturer’s protocol.

2.10. MTT assay

The cell viability of primary microglia was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For the analysis of cell viability after different treatment, primary
microglia were washed extensively with PBS solution for 3 times and then incubated with 0.5 mg/ml of MTT solution for 4 h at 37 °C. The formed formazan crystals in individual cultures were dissolved in DMSO and the absorbance was measured at 490 nm. All experiments for cell viability assay were repeated three times independently.

2.11. Statistical analysis

All data are expressed as the mean ± SD. A two-way repeated-measure analysis of variance (ANOVA) followed by the Tukey’s post hoc multiple comparisons test was used to examine the behavioral data at different time-points and across all groups. Data of protein and gene levels from each independent group were compared using a one-way ANOVA followed by the Tukey’s post hoc multiple comparisons test was used to examine protein and gene levels from each independent group. Significance was reached at values of \( p < 0.05 \) or \( p < 0.01 \).

3. Results

3.1. Effects of resveratrol treatment on thermal hyperalgesia and mechanical allodynia in neuropathic mice

Resveratrol was previously reported to exhibit pain relief effect in different animal models. To assess its effect on neuropathic pain, it was used to treat neuropathic mice (CCI model) at different dosage at different time windows. Thermal hyperalgesia and mechanical allodynia in pain behavior were evaluated 0, 7, 14, 21, 28 and 35 days after CCI (Fig. 1). In the experiments, resveratrol was introduced at three time windows: pre-treatment before CCI (Fig. 1a & b), post-treatment right after CCI (Fig. 1c & d) and post-treatment right after the peak of pain symptom (Fig. 1e & f). It was observed that the effect of resveratrol on neuropathic pain was dependent on the time window of administration. When resveratrol was administrated for 7 consecutive days prior to sciatic nerve injury, withdrawal latency and threshold in the CCI + Res groups had no significant difference from those in the vehicle group (CCI + veh) (Fig. 1a & b). Interestingly, when mice were...
post-treated with resveratrol right after CCI, resveratrol induced an obvious reduction of thermal hyperalgesia (Fig. 1c) and mechanical allodynia (Fig. 1d) at day 7 and the peak of pain symptom was clearly postponed which otherwise occurred at day 7 (as indicated in Fig 1e & f). Moreover, when mice were post-treated right after the peak of pain symptom at day 7 post-CCI surgery, a similar reduction of hyperalgesia (Fig. 1e) and allodynia (Fig. 1f) was also observed and such reduction was even more obvious (compared Fig. 1e & f with Fig. 1c & d). Meanwhile, it was observed that the beneficial effect of resveratrol against neuropathic pain was in a dose-dependent manner (Fig. 1c–f). Resveratrol relieved hyperalgesia (Fig. 1c & e) and allodynia (Fig. 1d & f) most significantly at 40 mg/kg, compared to 20 or 10 mg/kg, and such relief was not significant at 5 mg/kg (Fig. 1c–f). Besides, long-term treatment of resveratrol from day 7 to day 35 after CCI greatly relieved the neuropathic pain with the best effect at day 21, then reaching to the maximal effect (Supplementary Fig. S1). However, the animals were not able to completely recover.

3.2. Cytokine mRNA expressions in ipsilateral sciatic nerve of experimental mice

To investigate the inflammatory response during resveratrol treatment of mice of sciatic nerve injury, mRNA levels of representative cytokines including TNF-α, IL-1β, IL-6 and IL-10 were measured in ipsilateral sciatic nerve of experimental mice with RT-PCR at day 14 after CCI. The mRNA levels were normalized to those of Sham + veh group. In the experiment, resveratrol at different doses (5, 10, 20 or 40 mg/kg) was administrated by i.p. injection from day 7 to day 14 after CCI since its pain relief effect was more obvious when administrated right after the peak of pain symptom at day 7. As well documented, CCI injury significantly induced TNF-α, IL-1β, IL-6 and IL-10 mRNA expression in ipsilateral sciatic nerve of experimental mice at day 14 after CCI (Fig. 2), compared to the Sham + veh control. Interestingly, it was found that the resveratrol treatment repressed TNF-α (Fig. 2a), IL-1β (Fig. 2b) and IL-6 (Fig. 2c) mRNA expression while further increased IL-10 mRNA expression (Fig. 2d) after CCI surgery, compared to the CCI + veh control. Moreover, such changes were resveratrol dose-dependent: the higher dosage of resveratrol, the more significant changes except that such effect was not significant at a low dosage 5 mg/kg.

3.3. Cytokine protein expressions in ipsilateral sciatic nerve of experimental mice

Meanwhile, the protein levels of different cytokines were measured with corresponding ELISA. Consistently with their mRNA levels, CCI injury significantly induced TNF-α, IL-1β, IL-6 and IL-10 protein expression in ipsilateral sciatic nerve (Fig. 3), compared to the Sham + veh control. Also, resveratrol treatment inhibited TNF-α, IL-1β and IL-6 protein levels while promoted IL-10 protein level, compared to the control CCI + veh group. Such effects of resveratrol were also dose-dependent. Further hematoxylin-eosin (H&E) staining and immunostaining confirmed that resveratrol treatment did alleviate inflammatory responses in CCI model animals, revealed by the decreased infiltrated cells and CD4 immunostained cells (Supplementary Fig. S2).

3.4. Immunomodulatory effects of resveratrol on primary culture of microglia

To further examine the immunomodulatory effect of resveratrol, primary microglia were cultured and used as an in vitro model since they are an important type of cells modulating inflammation in the neurological system [17]. The cytotoxicity of resveratrol was firstly measured with MTT assay and LDH release assay in the culture medium during culture. Preliminary data showed that resveratrol displayed a significant cytotoxicity at 10 μM or higher (Supplementary Fig. S3), and therefore its immunomodulatory effect on primary microglia was investigated at 0.1, 1, 2 and 5 μM. Microglia was stimulated by lipopolysaccharides (LPS, 100 ng/ml, 24 h) and then treated with different doses of resveratrol (0.1, 1, 2 or 5 μM) during the entire culture. ELISA experiments
showed that TNF-α, IL-1β, IL-6 and IL-10 protein content was dramatically elicited by LPS (Fig. 4) as previously reported [18]. However, the increase of TNF-α (Fig. 4a), IL-1β (Fig. 4b) or IL-6 (Fig. 4c) was significantly repressed by the treatment of resveratrol (1, 2 and 5 μM), while the increase of IL-10 (Fig. 4d) was even more promoted. Such immunomodulatory effect of resveratrol was not noticed at a low dosage of 0.1 μM. Also, such change was noticed in intracellular IL-10 protein expression by Western blot analysis (Fig. 4e, f). Worth of mention, when amyloid-beta (Aβ) peptides was used to stimulate the microglia, nitric oxide (NO) level, which is believed to participate in nociception processing [19], in the resveratrol treatment group was greatly reduced (Supplementary Fig. S4).

3.5. Correlations between withdrawal latency or withdrawal threshold and IL-1β, IL-10 TNF-α and IL-6 levels in the ipsilateral sciatic nerve

The correlations between withdrawal latency or withdrawal threshold and cytokines level in the ipsilateral sciatic nerve were examined in all experimental mice (n = 48). It was clearly shown that the protein level of IL-1β, TNF-α or IL-6 inversely correlated with withdrawal latency (Fig. 5a, c and d) or withdrawal threshold (Fig. 5e, g and h). Meanwhile, it was found that the protein level of IL-10 has no obvious correlation with withdrawal latency (Fig. 5b) or withdrawal threshold (Fig. 5f).

4. Discussion

Pro-inflammatory cytokines have been shown to be involved in the genesis, persistence, and severity of neuropathic pain following nerve injury [1,3]. The precise mechanism in neuropathic pain is very complex and it is thought to originate through multiple pathophysiological processes [1]. There is quite evidence implicating the pro-inflammatory cytokines in the induction and facilitation of neuropathic pain [20–25]. Recently, pro-inflammatory substances like bradykinin, prostaglandins and signal molecules like cytokines have been identified as allogenic factors [20]. An early increase of TNF-α was reported 12 h after injury [20]. Neutralizing antibodies to TNF-α were able to reduce hyperalgesia that evolved due to the nerve injury. Neutralizing antibodies to IL-1 receptor or IL-6 receptor also reduced pain related behavior [20]. These results lead to the conclusion that pro-inflammatory cytokines are involved in neuropathic pain. Meanwhile, anti-inflammatory cytokine IL-10 is known to suppress the production of pro-inflammatory cytokines [26,27]. Milligan et al. reported that overexpression of IL-10 proved to be an efficacious treatment for neuropathic pain [21].

Resveratrol is a natural polyphenol found in grapes and wine. It exerts different beneficial antioxidant, anti-inflammatory, and anti-apoptotic effects on many different diseases (injuries) [8]. Specifically, its anti-inflammatory property has been proposed for many different inflammation-associated diseases [9]. Chuang et al. reported that resveratrol attenuated the TNF-α induced expression of inflammatory genes such as IL-6, IL-1β, IL-8, and monocyte chemoattractant protein-1 and the secretion of IL-6, IL-8, and MCP-1 [28]. Also, it attenuated TNF-α mediated phosphorylation of c-Jun-NH2 terminal kinase, and hence its downstream pro-inflammatory [28].

In the present study, we clearly demonstrated that resveratrol attenuated the expression of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Fig. 2a–c) at the mRNA and protein (Fig. 3a–c) level at day 14 after CCI when resveratrol was administrated by i.p. injection from day 7 to day 14. Meanwhile, it promoted the expression of anti-inflammatory cytokine IL-10. Therefore, it was concluded that resveratrol relieved neuropathic pain by attenuation of pro-inflammatory cytokines and promotion of anti-inflammatory cytokine IL-10 at the same time.

Furthermore, a cell model of microglia confirmed the effect of resveratrol on pro-inflammatory cytokines and anti-inflammatory cytokine IL-10. Microglia plays an important role as immune cells in the central nervous system. Activated microglia show a series of changes in morphology, gene expression, and function and produce and release...
various chemical mediators, including pro-inflammatory cytokines that can produce immunological actions and can also act on neurons to alter their function during neuropathic pain [17]. Diffusible factors released from activated microglia have an important role in the development of neuropathic pain, such as pro-inflammatory cytokines and anti-inflammatory cytokine IL-10 [17]. In the present study, it was confirmed that resveratrol has a beneficial immunomodulatory effect on microglia to relieve pain, such as attenuation of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and promotion of anti-inflammatory cytokine IL-10. Noteworthy, the regulation of anti-inflammatory cytokine IL-10 is a self-defensive mechanism, and it is upregulated to counteract with the effect of pro-inflammatory cytokines during nerve injury [26]. Consistently, it was found to be upregulated at day 7 after CCI injury (Figs. 2d & 3d). Resveratrol treatment (10, 20 and 40 mg/kg) after CCI further promoted its upregulation significantly (Fig. 2d & 3d), which enhanced its anti-inflammatory effect besides the direct suppression of pro-inflammatory cytokines (Fig. 2a–c, Fig. 3a–c).

It was noted that resveratrol did not relieve neuropathic pain completely, as evidenced by the partially recovered withdrawal latency and threshold (Fig. 1c–f). One possible reason is that resveratrol could not repress the upregulation of pro-inflammatory cytokines completely, as indicated by the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Figs. 2 & 3), which contributed to and correlated with neuropathic pain (Fig. 5). Also the circulation time of resveratrol is short and hence its anti-inflammatory effect would not last long after it is cleared out of the body. Since its anti-inflammatory effect is short (within several hours), therefore a significant pain relief effect was not observed when it was administrated before CCI surgery (Fig. 1a–b) in the present study since pro-inflammatory cytokines was not upregulated before CCI injury.

It was reported that the highest expression of IL-1, IL-1β and IL-6 was observed at day 7 after CCI operation and TNF at day 14 [20]. Consistently, resveratrol exhibited a more pronounced pain relief effect when it was administrated 7 days after CCI, compared to that when administrated immediately after CCI (Fig. 1e–f, compared with Fig. 1c–d), since many pro-inflammatory cytokines reached its peak concentration at day 7 which correlate with neuropathic pain (Fig. 5). It was demonstrated in the present study that neuropathic pain correlated with pro-inflammatory cytokines, such as IL-1β. It was noted that IL-10 has no correlation with withdrawal latency/threshold in the current study.

Fig. 4. Immunomodulatory effects of resveratrol on primary culture of microglia. Microglia was stimulated by lipopolysaccharides (100 ng/ml, 24 h) and treated with different doses of resveratrol (0.1, 1, 2 or 5 μM) during the entire culture. TNF-α (a), IL-1β (b), IL-6 (c) and IL-10 (d) protein content was characterized by their corresponding ELISA. Intracellular IL-10 protein expression was analyzed by Western blot (e, f) with GAPDH as control. Data were presented as mean ± SD. **p < 0.01 versus control (no LPS and resveratrol treatment), #p < 0.05 and ##p < 0.01 versus LPS group (with LPS without resveratrol treatment).
This is mainly due to the common nature of IL-10 as an anti-inflammatory cytokine. Insult would cause self-defensive responses of the body, thus leading to the release of anti-inflammatory cytokines, for instance, IL-10. And resveratrol treatment can further boost its release. So, it is not strange that IL-10 has no correlation with the pain behavior if CCI group was included in the statistical analysis, in which the body produces more IL-10 for self-defense. Another possible reason is that many different mechanisms are involved in the development of neuropathic pain besides pro-inflammation, such as the exacerbate activation of NMDA receptor and the stimulation of many other signaling pathways, as previously discussed [30].

In summary, the present study clearly showed that resveratrol exhibited a neuropathic pain relief effect as evidenced by the alleviation of thermal hyperalgesia and mechanical allodynia in neuropathic mice (CCI model) due to its anti-inflammatory property by attenuating pro-inflammatory cytokines and promotion of anti-inflammatory cytokine of IL-10 at the mRNA and protein levels at the same time. The promotion of anti-inflammatory cytokine IL-10 by resveratrol enhanced its beneficial effect against neuropathic pain. A cell model of microglia also confirmed its anti-inflammatory effect. However, possibly due to its short in vivo circulation time, its pretreatment before CCI injury did not show any pain relief effect. Taken into account the expression profiles of different pro-inflammatory cytokines after CCI injury, its best time window for administration would be 7 days after CCI, i.e. right after the peak of pain symptom, as shown in the present study (Fig. 1e & f).

In short, the present study clearly showed resveratrol displayed a significant neuropathic pain relief effect and paved a way for novel treatment of neuropathic pain. In conclusion, our data showed that resveratrol had a beneficial effect against neuropathic pain due to its inhibition of pro-inflammatory cytokines. Also, the best time window for administration of resveratrol was right after the peak of pain symptom, or 7 days after CCI injury of neuropathic mice model in the present study. The present study highlighted the role of resveratrol as a new agent for the treatment of neuropathic pain.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2016.02.033.

References


