

Original Research Article

β -Elemene alleviates bone cancer-related pain in rats by modulating N-methyl-D-aspartate receptor 2B subunit

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Abstract

Purpose: To investigate the effect of β -elemene on bone cancer-related pain (BCRP) and its underlying mechanism in a rat model.

Methods: A rat model of BCRP was established by injection of Walker 256 cells into the medullary cavity in adult male Sprague-Dawley rats. The effects of β -elemene on pain-related behaviours were assessed by paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTl). Quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting were used to analyse the transcription and translation levels of N-methyl-D-aspartate receptor 2B subunit (NR2B), respectively.

Results: This study found that PWMT and PWTl were significantly decreased in rats as evidence of successful establishment of a rat model of BCRP. Behavioral test showed that β -elemene treatment could attenuate pain-related behaviours. This analgesic effect began 1 h after drug injection, reached the highest value at 2 h, declined at 4 h, and disappeared by 24 h. qRT-PCR and western blotting indicated that intrathecal administration of β -elemene down-regulated mRNA and protein levels of NR2B.

Conclusion: The results show that β -elemene alleviates behaviours related to pain in a rat model of BCRP probably by modulating NR2B, thus indicating a new potential treatment method for cancer-related pain.

Keywords: β -Elemene, Bone cancer, Pain relief, N-methyl-D-aspartate receptor 2B subunit (NR2B), Sensitisation

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INTRODUCTION

Bone cancer-related pain (BCRP) is one of the common type of pain in malignant tumour patients [1]. Cancers with strong predilection to metastasize to bone, such as breast and prostate carcinoma, are the main causes of malignant bone disease [2,3]. The pain caused by bone

cancer significantly decreases the quality of life of patients. Despite decades of basic and clinical research on BCRP, the exact molecular mechanisms and effective therapies for BCRP are still challenging problems [4]. Current treatments for BCRP are not sufficiently effective or can cause significant adverse effects [5].

β -Elemene is a natural product found in about 50 plants used for herbal medicine that grow in tropical areas [6]. β -Elemene was reported to repress DNA synthesis and the proliferation of cancer cells in a broad range of tumours, including breast, bladder, and prostate cancers, without severe side effects [6-9]. However, the efficacy of β -elemene as a BCRP treatment is unknown, and the mechanism involved in the relief of BCRP by β -elemene remains to be determined. N-methyl-D-aspartate (NMDA) receptors are relevant to chronic neuropathic pain, injury-induced pain behaviour and persistent pain of spinal lamina II neurons [10-13]. The NMDA receptor 2B subunit (N_{R2B}) shows a relatively restricted distribution in pathways of nociceptive transmission [14]. Previous studies suggest that BCRP can be attenuated by modulation of N_{R2B} by mas-related gene C activation [15]. Furthermore, N_{R2B} in spinal cord plays a part role in inflammatory and neuropathic pain [16,17].

The aim of this research was to investigate the effects of β -elemene on pain-related behaviours and N_{R2B} expression in a rat model of BCRP, and thus a new potential therapy for BCRP were investigated.

EXPERIMENTAL

Experimental animals

Adult male Sprague-Dawley rats (body weight ranges from 230 g to 260 g) were housed in cages in groups of five and kept under a half day light/ 12 h dark cycle at 24 °C with food and water supplied *ad libitum*. These rat experiments in this study were approved by Zhejiang Cancer Hospital and in accordance with the Guidelines of the International Association for the Study of Pain [18]. Efforts were made to reduce the numbers of rats and the pain they experienced in the work.

Establishment of rat model of BCRP

Walker 256 rat mammary carcinoma cells used for implantation were grown in RPMI-1640 medium (San Diego, USA) in an incubator with 5 % CO_2 at 37 °C. The medium also comprised 100 U/mL penicillin, 0.1 mg/mL streptomycin and 15% fetal bovine serum (Biomiga, Inc.). A rat model of BCRP was established following the protocol reported by Schwei *et al.* with slight modification [19]. Anaesthetization of rats was performed by injecting pentobarbital sodium (1 % in saline) at 50 mg/kg. After shaving the rats and disinfecting with 70 % (v/v) ethanol, an incision was made on the right articulation genu.

Perforation of the tibial plateau was performed by gauge needle. The injection of 10 μ L of medium or Walker 256 cells (5×10^5 cells) into the medullary cavity of the rats in the normal control or tumour groups was performed by microsyringe (Hamilton, USA). The hole of injection was sealed by bone wax, and Gentamicin saline was used to wash the wound before closure.

Drug injection and grouping

To determine whether β -elemene alleviates pain-related behaviours, rats were injected with β -elemene followed by testing of nociceptive behaviours. β -Elemene used in this study was purchased from Yuanda Pharmaceuticals (Dalian, China). It was dissolved in dimethyl sulfoxide (DMSO) at 0.1 % and diluted by physiological saline. On day 14 after modeling, β -elemene was given intratibially at 40 mg/kg in tumor + β -elemene group, and equal amount of DMSO and physiological solution was given intratibially in tumor + vehicle group and normal control + vehicle group. Rat were divided into the following three groups (1) tumor + β -elemene group: (rats with BCRP model received intratibial injection of β -elemene); (2) tumor + vehicle group (rats with BCRP model treated by intratibial injection of equal amount of DMSO and physiological saline); (3) normal control + vehicle group (normal rats treated by intratibial injection of equal amount of DMSO and physiological saline).

Behavioral test

Before the measurements were performed, all rats acclimatized to the new environment for 30 min before tests. Mechanical allodynia was evaluated by paw withdrawal mechanical threshold (PWMT), which referred to the lowest force (in grams) causing mechanical withdrawal. Rats were kept in a glass chamber with mesh floor. The mechanical allodynia was generated by filaments in a sequentially increasing strength order, vertically against the paw to lead to paw bending for 5 s using a series of von Frey hairs (Stoelting, USA). Thermal hyperalgesia was measured by paw withdrawal thermal latency (PWTL). The animal was kept in glass chamber with a transparent bottom. Radiant heat apparatus (IITC Life Science) was placed underneath the bottom and focused onto the paw through the transparent bottom. PWTL represented the time of heat stimulation leading to the withdrawal of the paws. A cut-off period of 20 s was set to decrease empyrosis caused by radiant heat. PWMT and PWTL were examined before animal modeling, after animal modeling and after drug administration.

Western blotting analysis

Before western blotting analysis, the rats were anesthetized by isoflurane and then sacrificed by cervical dislocation. Protease and phosphatase inhibitor were used to homogenize the samples from the tissue in spinal cord L4-L6 segment. After centrifugation at 13000 rpm for 8 min at 4 °C, the resultant supernatant was analysed by BCA Protein Assay Kit (JiebeisiBiotec, Guangzhou, China) for further analysis. The samples were boiled loading buffer for 10 min. After centrifugation, protein sample in the resultant supernatant was separated by 10 % SDS-PAGE using electrophoresis. After separation by electrophoresis, the proteins on the gel were transferred to PVDF membrane (Millipore, USA). After blocked by skim milk for 2 h at 24 °C, the membrane was treated by the first antibody rabbit-N_R2B (1:1000). The membrane was washed by TBST buffer (containing 10 mM Tris-HCl, pH 8, 150 mM NaCl and 0.05 % (v/v) Tween 20). Then the membrane was treated by second goat-rabbit antibody with horseradish peroxidase (1:5000) for 120 min at 24 °C.

The bands were tested using the ECL system (Millipore, USA). The results were analysed using QuantityOne V4.40 (Bio-Rad, CA, USA), with β -actin (1:1000) as reference. All the antibodies were purchased from Abcam (UK). Western blotting analysis was performed before animal modeling, after animal modeling and after drug administration.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Before western blotting analysis, the rats were anesthetized by isoflurane and then sacrificed by cervical dislocation. The samples from the tissue of spinal cord L4–L6 segment were used for RNA extraction with Trizol Reagent (Invitrogen, USA). The concentration of RNA was analysed by Nanodrop 2000 (Thermo Fisher, USA). PrimeScrip RT reagent kit (Takara Biomedical Technology, China) was employed in reverse transcription. qRT-PCR was analysed by SYBR Fast qPCR Mix (Takara Biomedical Technology, China) on Real-time PCR System (Applied Biosystems, USA) in triplicates.

The primers of N_R2B and β -actin used for qRT-PCR were listed in Table 1 [20]. The relative expression level of N_R2B was calculated by compared with that of β -actin using CT method [21]. qRT-PCR was performed before animal modeling, after animal modeling and after drug administration.

Table 1: Primers for qRT-PCR

Gene	Forward/Reverse	Primer sequence
N _R 2B	Forward	GCATTCCTAC GACACCTTCG
	Reverse	GACCACCACT GGCTTATTGG
β -Actin	Forward	GAGACCTTCA ACACCCCAGC
	Reverse	CACAGAGTAC TTGCGCTCAG

Statistical analysis

The data in this study are presented as mean \pm standard deviation (SD). Rats were divided into normal control and tumour groups randomly. Variance analysis between two groups were analysed by Student's *t*-test. Variance analysis between more than two groups were analysed by one-way ANOVA followed by Tukey's post hoc tests. SPSS 16.0 was used for statistical analysis. $p < 0.05$ was used to define statistical significance.

RESULTS

Analysis of BCRP-related behaviour

Before model establishment (day 0), PWMT (Figure 1A) and PWTL (Figure 1B) were equivalent between rats in the normal control and tumour groups. After operation, rats in both groups showed reduced PWMT and PWTL on day 3. From day 5 to 14, PWMT and PWTL were increased in the normal control group and showed no significant differences when compared with values on day 0. The tumour group showed slightly higher PWMT and PWTL on day 5 than on day 3 ($p > 0.05$). However, from day 5 onward, PWMT and PWTL declined gradually in the tumour group. On days 7, 10, and 14, PWMT and PWTL were obviously lower in the tumour group than those in the normal control group ($p < 0.05$).

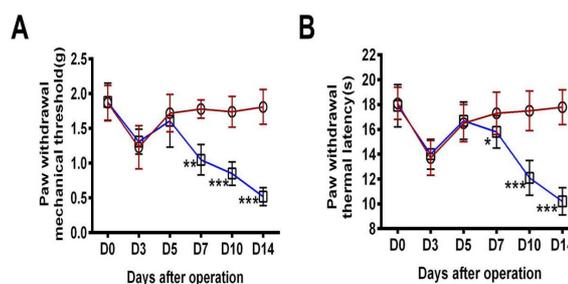


Figure 1: Behavioural testing of a rat model of BCRP. (A) PWMT in normal control and tumour groups at different time points. (B) PWTL in normal control and

tumour groups at different time points. Day 0 was before the operation, whereas days 3, 5, 7, 10, and 14 were after the operation. \circ = normal control; \square = tumour groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. normal control group

Transcription and expression of N_{R2B}

After model establishment, mRNA levels of N_{R2B} in the normal control group were markedly lower compared to those in the tumour group on days 3, 5, 7, 10, and 14 ($p < 0.05$; Figure 2A) and increased over time. Western blotting indicated that on day 3 after model establishment, the protein expression levels of N_{R2B} in the tumour group showed no obvious changes in comparison with those of normal control group (Figure 2B). Nevertheless, on days 5, 7, 10, and 14, the translation of N_{R2B} in tumour group was markedly up-regulated in a time-dependent manner.

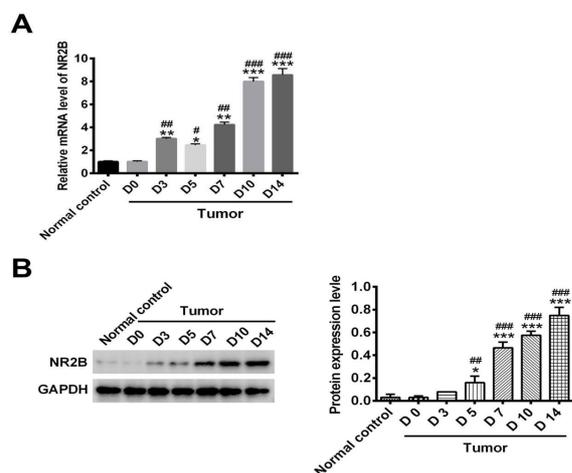


Figure 2: Relative expression levels of N_{R2B} in a rat model of BCRP. (A) Relative mRNA level of N_{R2B} . (B) Protein bands of N_{R2B} and β -actin; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs normal control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. tumour group

Effects of β -elemene on mechanical allodynia and thermal hyperalgesia

The tumour + vehicle group had a markedly lower PWMT than the normal control + vehicle group ($p < 0.05$; Figure 3A). However, the tumour + β -elemene group had a higher PWMT than the tumour + vehicle group ($p < 0.05$) and a similar PWMT as the normal control + vehicle group at 2, 4, 6, and 12 h after drug administration. At 24 h after drug administration, PWMT in the tumour + β -elemene group decreased and showed no significant difference from that in the tumour + vehicle group. PWTL showed similar variations between the two groups (Figure 3B). Thus, the analgesic effect of β -elemene showed to begin 2 h after drug

administration, reached maximal levels at 4 h, decreased at 6 h, and disappeared by 24 h.

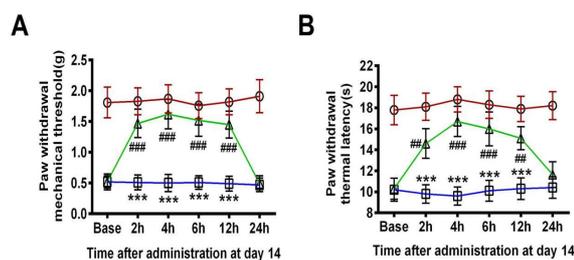


Figure 3: Changes in pain-related behaviours in response to β -elemene treatment. (A) PWMT in normal control + vehicle group, tumour + vehicle group, and tumour + β -elemene group. (B) PWTL in normal control + vehicle group, tumour + vehicle group, and tumour + β -elemene group. Baseline values were obtained before injection. ### $p > 0.05$, ## $p < 0.05$ vs. normal control + vehicle group; \circ = normal control + vehicle group; \square = tumour + vehicle group; Δ = tumour + β -elemene group; *** $p < 0.001$ vs. tumour + vehicle group

Effects of β -elemene on transcription and expression of N_{R2B}

At baseline, the mRNA level of N_{R2B} in tumour + vehicle group was greater compared to that in normal control + vehicle group (Figure 4A). However, at 1, 2, and 4 h after β -elemene injection, the mRNA level of N_{R2B} in tumour + β -elemene group was less in comparison with that in tumour + vehicle group but greater compared with that in normal control + vehicle group ($p < 0.05$). At 24 h after β -elemene injection, the difference between the tumour + β -elemene and tumour + vehicle groups disappeared ($p > 0.05$). The protein level of N_{R2B} in the tumour + β -elemene group was down-regulated in a time-dependent manner than that in tumour + vehicle group. Thus, effects of β -elemene on N_{R2B} protein expression began at 2 h after drug administration, reached maximal levels at 6 h, and decreased at 12 and 24 h. Therefore, the protein level of N_{R2B} in the tumour + β -elemene group was still higher compared with that in normal control + vehicle group, even though it was down-regulated by β -elemene.

Effect of β -elemene on histomorphometry

Compared with the normal control + vehicle group, the cancer + vehicle and cancer + β -elemene groups showed thinner and irregular bone trabeculae and destroyed bone trabecular reticulate structures (Figure 5). Thus, β -elemene treatment did not improve bone trabecular distribution.

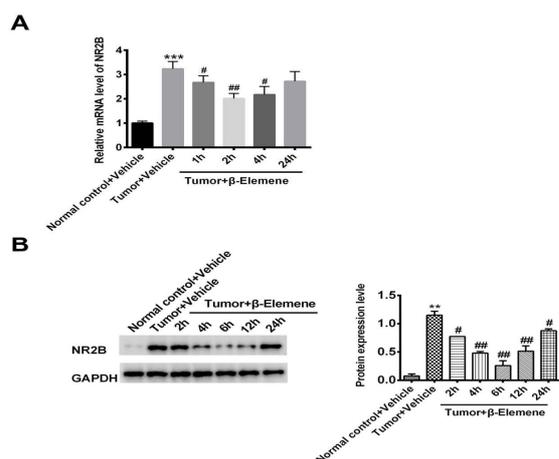


Figure 4: Relative expression level of N_{R2B} after β -elemene administration. (A) Relative mRNA level of N_{R2B} . (B) Protein bands for N_{R2B} and GAPDH; ** $p < 0.01$ vs. normal control + vehicle group; # $p < 0.05$, ## $p < 0.01$ vs. tumour + vehicle group

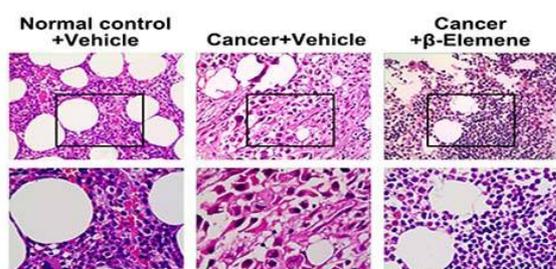


Figure 5: Histological analysis of bone trabeculae in response to β -elemene treatment

DISCUSSION

BCRP, which strongly affects the quality of life of cancer patients, can be evaluated by mechanical and thermal hyperalgesia, which involve sensitisation in the spinal cord [22]. NMDA receptor-dependent central sensitisation was accounting for pain hypersensitivity [23]. In particular, the activation of N_{R2B} was necessary for the formation of central sensitisation [24].

The present study found that PWMT and PWTL decreased in normal control and tumour groups on day 3 after operation due to effects of the operation, as both groups of rats showed recovered behaviour on day 5. On day 14, PWMT and PWTL of normal control groups recovered to basal values, whereas the tumour group showed significantly decreased PWMT and PWTL when compared with basal values before model establishment. Thus, the decreased PWMT and PWTL appeared to be caused by BCRP. These results indicate that a BCRP model was successfully established in rats and that BCRP-related behaviours tended to stabilise 14 days after operation.

The results of qRT-PCR and western blotting indicated that transcription and translation of N_{R2B} in spinal cord were markedly elevated after model establishment. The increased mRNA and protein levels of N_{R2B} , which were accompanied by BCRP-related behaviours, suggest that N_{R2B} is related to the development of BCRP. This is consistent with previous results from qRT-PCR and immunohistochemical staining [25].

In the present study, PWMT and PWTL decreased after drug administration, indicating that β -elemene can alleviate both mechanical and thermal hyperalgesia in a rat model of BCRP. Behavioural tests showed that the analgesic effect of β -elemene reached its maximal level 4 h after drug administration, decreased at 6 h, and disappeared by 24 h. Therefore, although β -elemene may quickly alleviate pain, the effect does not persist for long. Intrathecal administration of β -elemene down-regulated mRNA and protein levels of N_{R2B} , corresponding with pain-related behavioural changes. Tan *et al.* also found that gene knockdown of N_{R2B} by siRNA reduces formalin-induced nociception [26].

Qu *et al* showed that the N_{R2B} receptor antagonist ifenprodil inhibits mechanical allodynia [27]. Gu *et al* reported that N_{R2B} might play a role in BCRP in mice, as intrathecal administration of N_{R2B} receptor antagonist reduces thermal hyperalgesia and mechanical allodynia [25]. In the present study, β -elemene probably regulated pain-related behaviours by targeting N_{R2B} at the transcriptional level, both mRNA and protein levels of N_{R2B} were decreased by β -elemene in a rat model of BCRP.

CONCLUSION

These results show that β -elemene alleviates thermal hyperalgesia and mechanical hyperalgesia in a rat model of BCRP by modulating N_{R2B} , and thus presenting a new potential therapy for BCRP.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Hu Cai designed all the experiments and revised the paper. Liyan Gong, Qinfei Zhou and Xiangming Kong performed the experiments, and Liyan Gong wrote the paper.

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