

Original Article

Mas-related G-protein-coupled receptor c agonist bovine adrenal medulla 8-22 attenuates bone cancer pain in mice

Yu-E Sun*, Cui-E Lu*, Yishan Lei*, Yue Liu, Zhengliang Ma, Xiaoping Gu

Department of Anaesthesiology, Affiliated Drum-Tower Hospital of Medical College of Nanjing University, Nanjing 210008, Jiangsu Province, China. *Equal contributors.

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Abstract: Objectives: The aim of this study is to investigate the effects of Mas-related G-protein-coupled receptor C (MrgC) agonist bovine adrenal medulla 8-22 (BAM8-22) on bone cancer pain and mirror-image pain. Methods: Bone cancer pain was induced by intramedullary injection of NC2472 fibrosarcoma cells in the mice. BAM8-22 and/or anti-MrgC antibody were injected intrathecally at day 14 after bone cancer induction and their effects on pain behaviors were detected. The pain behaviours were assessed by the number of spontaneous foot lifts and paw withdrawal mechanical threshold (PWMT) tests. MrgC expression was detected using western blot analysis and immunofluorescence assay. Results: There were increased bone cancer pain and mirror-image pain in the tumor-bearing mice while not in the sham-treated mice. BAM8-22 attenuated bone cancer pain in mice dose dependently with the highest effects at 2 hr after BAM8-22 administration, and anti-MrgC antibody reversed the effects of BAM8-22. However, intrathecal administration of BAM8-22 did not affect the mirror-image pain. Furthermore, BAM8-22 stimulated the expression of MrgC in the spinal dorsal horn. Conclusions: MrgC agonist BAM8-22 could attenuate bone cancer pain in mice. This study may provide a novel strategy for the treatment of bone cancer pain.

Keywords: BAM8-22, MrgC, bone cancer pain, mirror-image pain

Introduction

Bone cancers can cause severe pain [1, 2], which lowers the quality of life in patients. Bone cancer pain is often refractory to clinical pharmacotherapies which is mainly ascribed to the limitation of the major analgesics (e.g., opioids), including the wide expression of their targeting receptors in the central nervous system [3, 4], their adverse effects and the risks of addiction and abuse [5, 6]. Nowadays, treatment of bone cancer pain is a challenging issue. Recent efforts have focused on identifying novel molecular targets on nociceptive sensory neurons in trigeminal ganglia (TG) and dorsal root ganglion (DRG), which may offer an opportunity for pain-selective pharmacologic interventions.

Mirror-image pain is characterized by mechanical hypersensitivity on the uninjured mirror-image side [7]. It accompanies many clinical pain states, including bone cancer pain.

Although mirror-image pain is usually not severe and early as the pain on the injured side, patients still suffer from pain on the contralateral side.

Mas-related G-protein-coupled receptor C (MrgC), also known as neuron-specific receptor (NSNR), plays an important role in pain sensation [8, 9]. The rodent MrgC shares substantial homogeneity with its human homologue MrgX1 [10] and expresses specifically in small diameter neurons in DRG and TG in mammals [11]. MrgC agonist bovine adrenal medulla 8-22 (BAM8-22), which is a synthesized 15-amino acid peptide, is one of the cleavage products of proenkephalin A and the precursor of Leu- and Met-enkephalin in the adrenal medulla [12]. It specifically binds to MrgC. The role of MrgC in chronic pain is controversial and activation of MrgC has a dual effect which could induce both pro-nociception and analgesia. Administration of BAM8-22 induced nociceptive pain in rodent

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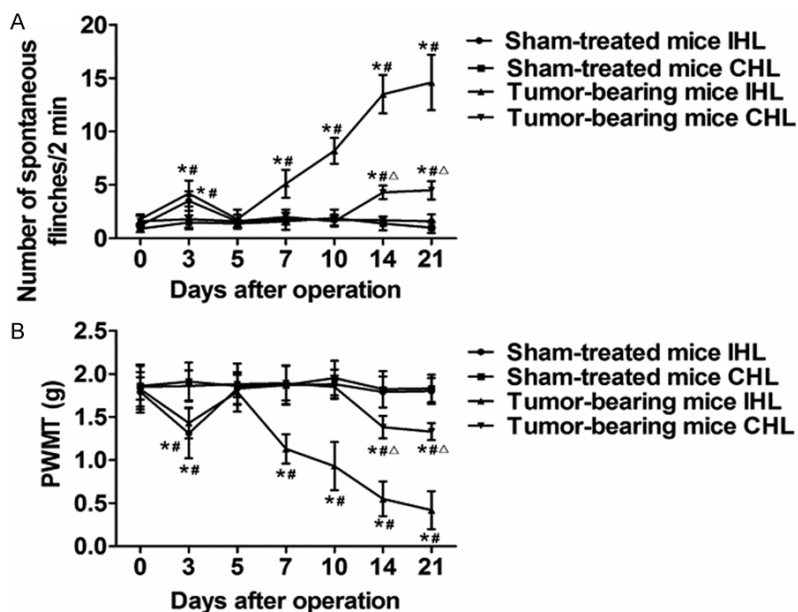


Figure 1. Increased bone cancer pain and mirror-image pain in the tumor-bearing mice. The mice receiving intramedullary injection of α -MEM containing osteosarcoma NCTC 2472 cells in the femur were designated as the tumor-bearing mice. The mice receiving intramedullary injection of α -MEM only were designated as the sham-treated mice. Spontaneous lifting (A) and PWMT (B) were examined in the tumor-bearing mice and the sham-treated mice at the indicated time points. IHL, the ipsilateral hind limb to the operation, that is, the tumor-bearing limb or the sham-treated limb (indicative of bone cancer pain); CHL, contralateral hind limb to the tumor-bearing limb or the sham-treated limb (indicative of mirror-image pain). Representative data were shown as mean \pm SD ($n = 8$). $*P < 0.05$ vs. the data detected at day 0 before intramedullary injection in the same group, $\#P < 0.05$ vs. the data from the corresponding limbs of the sham-treated mice at the corresponding time points, $\Delta P < 0.05$ vs. the data from the ipsilateral hind limb of the corresponding treated mice at the corresponding time points. PWMT, paw withdraw mechanical threshold.

models of inflammatory and neuropathic pain [13, 14]. While BAM8-22 may also be of therapeutic value to relieve pain, inhibit persistent inflammatory pain as well as chemical pain, and down-regulate spinal c-fos gene expression in an opioid-independent manner [15, 16]. Nowadays, the selectivity and mechanisms of drugs acting on the rodent MrgC receptor have not been clearly demonstrated, and the therapeutic value of its human homologue MrgX1 for pain relief is uncertain.

Ideal clinical drugs for bone cancer pain require minimal toxic side effects or non-toxic effects. Since MrgC distributed highly restrictedly in DRG, activation of MrgC could minimize the unwanted CNS adverse effects. However, very little is known about the effects of MrgC activation on bone cancer pain and mirror-image pain. We speculated that BAM8-22 might act

on bone cancer pain and mirror-image pain by activation of MrgC and thus lead to analgesia. In this study, the effects of BAM8-22 on bone cancer pain and mirror-image pain in mice were investigated.

Materials and methods

Animals

Adult (4-6 weeks old) male C3H/HeJ mice (weighing 18-22 g) were obtained from the Beijing Vital River Experimental Animal Center. The mice were habituated individually under a 12-12 hr light-dark cycle at a constant room temperature of 24°C and were given free access to food and water. All experiments were approved by the Animal Care and Use Committee of Affiliated Drum-Tower Hospital of Medical College of Nanjing University and conformed to the guidelines for the use of laboratory animals [17].

A total of 152 mice were enrolled in this study. The mice receiving the intramedullary injection of α -minimum essential medium (α -MEM) containing osteosarcoma NCTC 2472 cells were designated as the “tumor-bearing mice”, The mice receiving the injection of α -MEM only were designated as the “sham-treated mice” and the mice without treatment were designated as the “normal mice”. These mice were used in the following three independent experiments.

Spontaneous lifting behavior as well as withdrawal threshold/latency to mechanical stimulation was examined in 8 tumor-bearing mice and 8 sham-treated mice on day 0 (before the bone cancer induction), 3, 5, 7, 10, 14 and 21 after the bone cancer induction, respectively.

The mice were divided into 8 groups. In group 1 (TBO), eight tumor-bearing mice were mock

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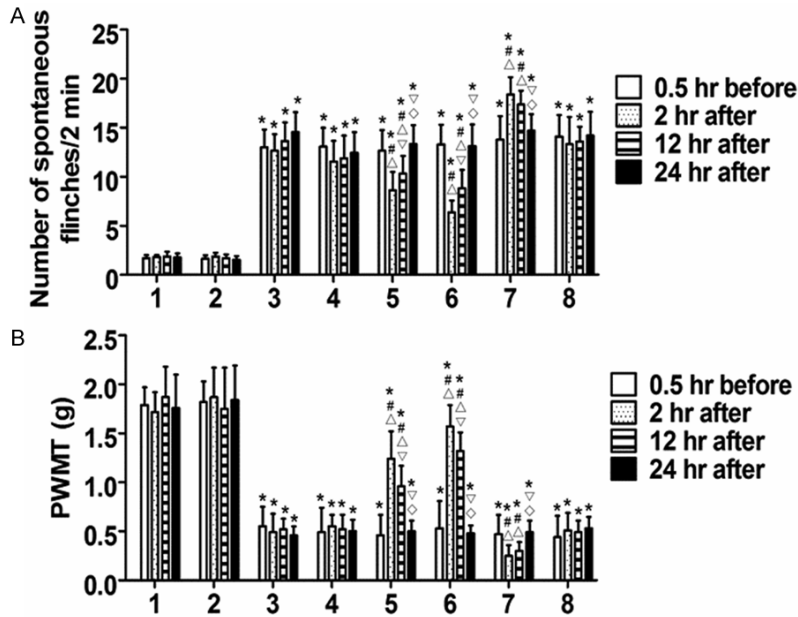


Figure 2. BAM8-22 attenuates bone cancer pain in mice. BAM8-22 and anti-MrgC antibody were injected intrathecally at day 14 after bone cancer induction. Spontaneous lifting behavior (A) and PWMT (B) of the tumor-bearing limb and the sham-treated limb were tested at 0.5 hr before and at 2 hr, 12 hr as well as 24 hr after BAM8-22 and/or anti-MrgC antibody treatment. 1, eight sham-treated mice were treated with 5 μ l of NS only (SB0); 2, eight sham-treated mice were treated with 5 μ l of NS containing 8.0 nmol BAM8-22 (SB3); 3, eight tumor-bearing mice were mocked treated with 5 μ l of NS (TB0); 4, eight tumor-bearing mice were treated with 5 μ l of NS containing 0.8 nmol BAM8-22 (TB1); 5, eight tumor-bearing mice were treated with 5 μ l of NS containing 2.4 nmol BAM8-22 (TB2); 6, eight tumor-bearing mice were treated with 5 μ l of NS containing 8.0 nmol BAM8-22 (TB3); 7, eight tumor-bearing mice were treated with 5 μ l of NS containing anti-MrgC antibody (TA); 8, eight tumor-bearing mice were treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilution) and 5 μ l of NS containing 8.0 nmol BAM8-22 (TAB). Representative data were shown as mean \pm SD ($n = 8$). * $P < 0.05$ vs. the data from group 1 at the corresponding time points, # $P < 0.05$ vs. the data from group 3 at the corresponding time points, $\Delta P < 0.05$ vs. the data detected at 0.5 hr before BAM8-22 and/or anti-MrgC antibody treatment in the same group, $\nabla P < 0.05$ vs. the data detected at 2 hr after BAM8-22 and/or anti-MrgC antibody treatment in the same group, $\diamond P < 0.05$ vs. the data detected at 12 hr after BAM8-22 and/or anti-MrgC antibody treatment in the same group, NS, normal saline, PWMT, paw withdraw mechanical threshold.

treated with 5 μ l of normal saline (NS). In group 2 (TB1), eight tumor-bearing mice were treated with 5 μ l of NS containing 0.8 nmol BAM8-22 (Sigma, St. Louis, USA). In group 3 (TB2), eight tumor-bearing mice were treated with 5 μ l of NS containing 2.4 nmol BAM8-22. In group 4 (TB3), eight tumor-bearing mice were treated with 5 μ l of NS containing 8.0 nmol BAM8-22. In group 5 (TA), eight tumor-bearing mice were treated with 5 μ l of NS containing anti-MrgC antibody (biorbyt, USA, 1:20 dilution). In group 6 (TAB), eight tumor-bearing mice were treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilu-

tion), then 5 μ l of NS containing 8.0 nmol BAM8-22. In group 7 (SB0), eight sham-treated mice were treated with 5 μ l of NS only. In group 8 (SB3), eight sham-treated mice were treated with 5 μ l of NS containing 8.0 nmol BAM8-22. BAM8-22 solution and anti-MrgC antibody solution were injected intrathecally at day 14 after bone cancer induction according to Hylden and Wilcox's report [18]. Spontaneous lifting behavior and mechanical allodynia were tested at 0.5 hr before and at 2 hr, 12 hr as well as 24 hr after BAM8-22 and/or anti-MrgC antibody treatment. In group 6, anti-MrgC antibody solution was injected 30 min before BAM8-22 administration. The dose of the drugs and the time points we adopted accorded to Jiang's report and our preliminary experiments [16].

The mice were divided into 6 groups. In group 1 (N), there were 6 normal mice without treatment. In group 2 (S), six sham-treated mice were treated with 5 μ l of NS only. In group 3 (T), six tumor-bearing mice were treated with 5 μ l of NS only. In group 4 (B), six

tumor-bearing mice were treated with 5 μ l of NS containing 8.0 nmol BAM8-22. In group 5 (A), six tumor-bearing mice were treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilution). In group 6 (AB), six tumor-bearing mice were treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilution), then 5 μ l of NS containing 8.0 nmol BAM8-22. The tissue samples were collected 2 hr after BAM8-22 and/or anti-MrgC antibody treatment. The expression of MrgC in the L₃-L₅ spinal cord was detected using western blot analysis and immunofluorescence assay.

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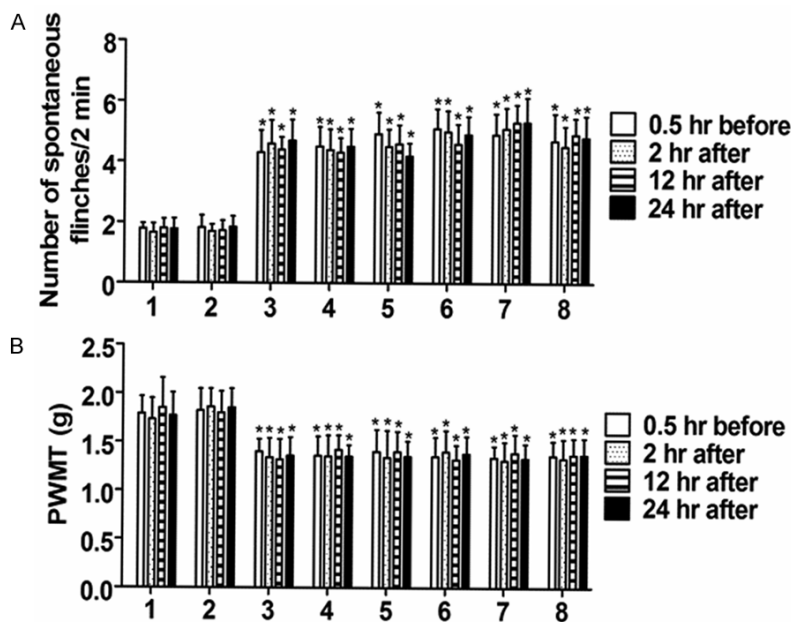


Figure 3. BAM8-22 does not affect mirror-image pain. BAM8-22 and anti-MrgC antibody were injected intrathecally at day 14 after bone cancer induction. Spontaneous lifting behavior (A) and PWMT (B) of the contralateral hind limb to the operation were tested at 0.5 hr before and at 2 hr, 12 hr as well as 24 hr after BAM8-22 and/or anti-MrgC antibody treatment. 1, eight sham-treated mice were treated with 5 μ l of NS only (SBO); 2, eight sham-treated mice were treated with 5 μ l of NS containing 8.0 nmol BAM8-22 (SB3); 3, eight tumor-bearing mice were mocked treated with 5 μ l of NS (TBO); 4, eight tumor-bearing mice were treated with 5 μ l of NS containing 0.8 nmol BAM8-22 (TB1); 5, eight tumor-bearing mice were treated with 5 μ l of NS containing 2.4 nmol BAM8-22 (TB2); 6, eight tumor-bearing mice were treated with 5 μ l of NS containing 8.0 nmol BAM8-22 (TB3); 7, eight tumor-bearing mice were treated with 5 μ l of NS containing anti-MrgC antibody (TA); 8, eight tumor-bearing mice were treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilution) and 5 μ l of NS containing 8.0 nmol BAM8-22 (TAB). Representative data were shown as mean \pm SD (n = 8). *P < 0.05 vs. the data from group 1 at the corresponding time points. NS, normal saline, PWMT, paw withdraw mechanical threshold.

Cell culture and implantation

Osteosarcoma NCTC 2472 cells (American Type Culture Collection, ATCC, 2087787) were cultured in NCTC 135 medium (Sigma-Aldrich, St. Louis, USA) with 10% horse serum (Gibco, Grand Island, USA) at 37°C in an atmosphere of 5% CO₂ and 95% air (Thermo Forma, Ohio, USA). The cells were passaged twice a week according to ATCC recommendations.

The bone cancer pain model was produced according to Schwei's report [19] and our previous studies. Briefly, the mice were anesthetized with intraperitoneal injection of 50 mg/kg pentobarbital sodium (1% in NS), and a superficial incision was made in the skin overlying the right articulation genu with eye scissors. Gonar-

throtomy was performed to expose the femur condyles. A light depression was made using a dental bur. A 30-gauge needle was used to perforate the cortex, then a volume of 20 μ l α -MEM containing 2×10^5 NCTC 2472 cells was injected into the intramedullary space of the femur using a 25 μ l microsyringe. These mice were designated as the tumor-bearing mice. In the sham-treated mice, a volume of 20 μ l α -minimum essential medium (α -MEM) only was injected into the intramedullary space of the femur. Dental amalgam was used to seal the injection hole.

Spontaneous lifting behavior assessment

All the tests were performed during the light phase. The mice were allowed to acclimatize for at least 30 min before each test. The mice were housed in individual plexiglass compartments (10 cm \times 10 cm \times 15 cm) for 30 min. The number of spontaneous

flinches of the right and left hind limb were recorded, respectively, during 2 min periods. Every lift of the hind limb not related to walking or grooming was considered to be one flinch.

Mechanical allodynia test

Von Frey filaments (Stoelting, Wood Dale, IL, USA) were used to assess mechanical allodynia according to Chaplan's report and our previous studies [20]. The mice were placed in individual transparent plexiglass compartments (10 cm \times 10 cm \times 15 cm) on a metal mesh floor (graticule: 0.5 cm \times 0.5 cm). Mechanical threshold was measured using a set of von Frey filaments (0.16 g-2.0 g bending force). The filaments were poked vertically against the plantar surface with a sufficient force until causing slight

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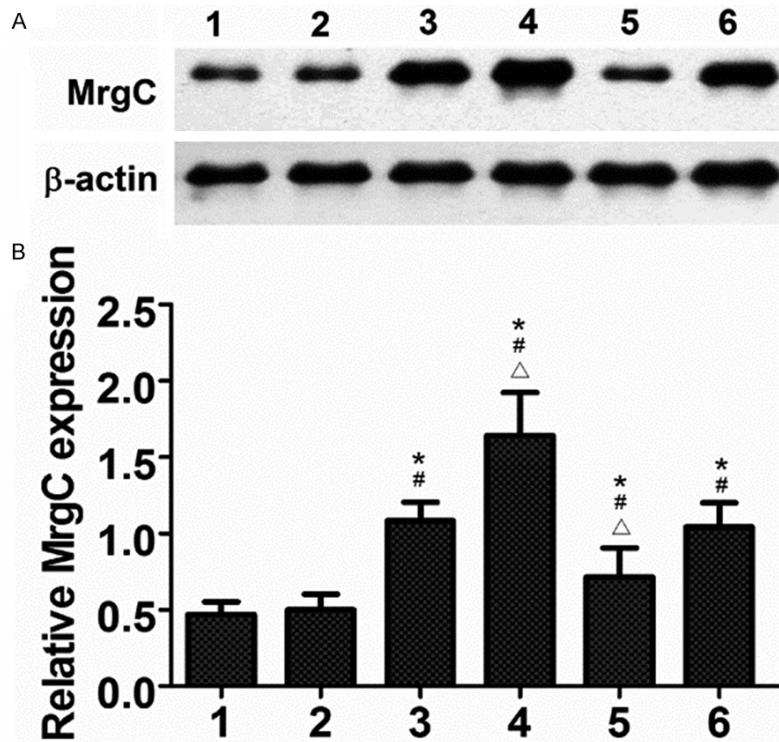


Figure 4. The expression of MrgC in the spinal dorsal horn detected using western blot analysis. BAM8-22 and anti-MrgC antibody were injected intrathecally at day 14 after bone cancer induction and MrgC expression in the spinal dorsal horn was detected at 2 hr after BAM8-22 and/or anti-MrgC antibody treatment. The representative results were shown in (A) and the quantitative comparison of the data derived from the independent experiments as in (A) were shown in (B). 1, normal mice without treatment (N); 2, sham-treated mice treated with 5 μ l of NS only (S); 3, tumor-bearing mice treated with 5 μ l of NS only (T); 4, tumor-bearing mice treated with 5 μ l of NS containing 8.0 nmol BAM8-22 (B); 5, tumor-bearing mice treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilution) (A); 6, tumor-bearing mice treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilution), then 5 μ l of NS containing 8.0 nmol BAM8-22 (AB). β -actin was used as the internal control. The data represents means \pm SD (n=6). * P < 0.05 vs. group 1, # P < 0.05 vs. group 2, ΔP < 0.05 vs. group 3.

bending against the paw. The force was held for 6-8 sec and with a 10-min interval. The positive response was defined as brisk withdrawal or paw flinching. The sequentially increasing and decreasing the stimulus strength (the "up-and-down" method) was used to determine the paw withdrawal mechanical threshold (PWMT). For each mouse, the hind paw was tested five times per stimulus strength. The cutoff force was 2.0 g. The lowest von Frey filaments which had three or more positive responses were regarded as paw withdrawal mechanical threshold (PWMT).

Immunofluorescence assay

The mice were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and perfused

transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The immunofluorescence assay was performed according to our previous studies [21]. The spinal cord L₃-L₅ segments were collected for immunofluorescence assay. Serial frozen spinal cord sections were cut on a sliding microtome to a thickness of 25 μ m, collected in PBS, and processed as free-floating sections. After washing in PBS, the sections were blocked for 60 min at room temperature with 10% (v/v) normal fetal bovine serum. The samples were incubated for 48 hours at 4°C with the primary antibody of rabbit anti-MrgC polyclonal antibody (1:250, biorbyt). After washing, the samples were incubated with the secondary antibody (Alexa Fluor 594, 1:500, Abcam) at 4°C overnight. The sections were mounted on glass slides, air-dried, and covered with coverslips using Aquamount (Fisher Scientific, Ottawa, Canada). The images were taken at 200 \times magnification using

the Leica TCS SP2 multiphoton confocal microscope (Leica Microsystems, Wetzlar, Germany) and were randomly selected for further analysis. Image-Pro Plus analysis software (Media Cybernetics, Inc., Rockville, MD) was used to analyze the fluorescence intensities of these images.

Western blot analyses

The spinal cord L₃-L₅ segments were removed rapidly and stored in liquid nitrogen. The tissue samples were homogenized in lysis buffer. The homogenate was centrifuged at 13,000 rpm for 10 minutes at 4°C, and the supernatant was removed. The BCA Protein Assay Kit (Thermo Fisher Scientific Life Science Research,

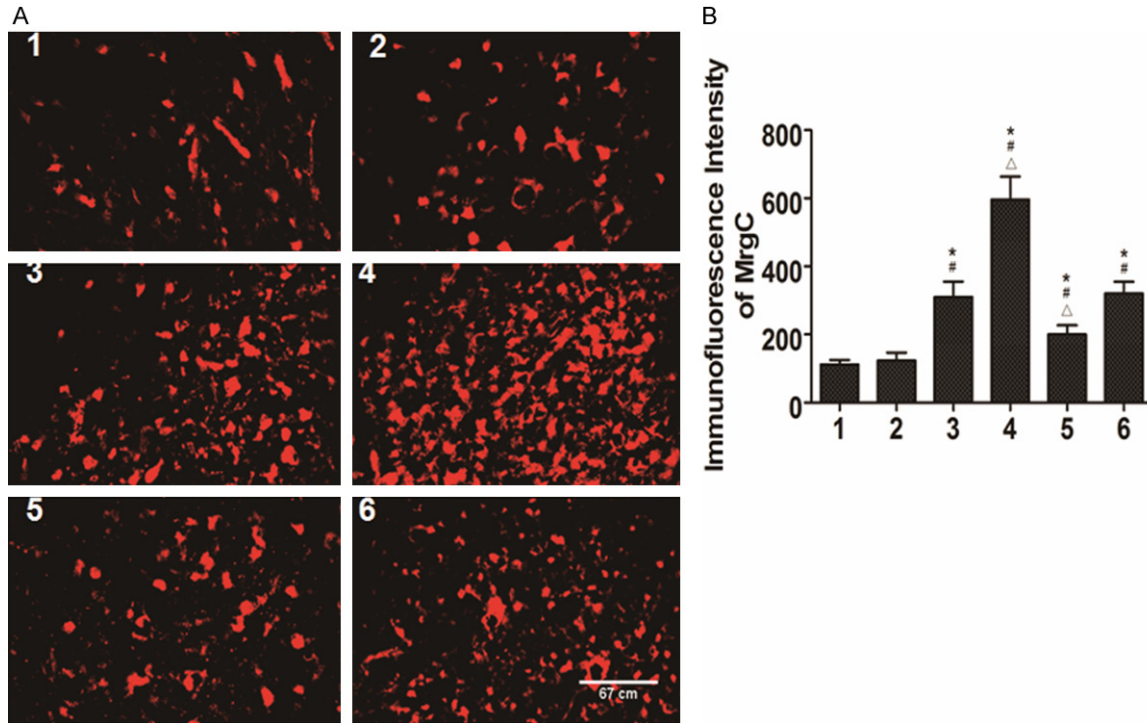


Figure 5. The expression of MrgC in the spinal dorsal horn detected using immunofluorescence assay ($\times 200$). BAM8-22 and anti-MrgC antibody were injected intrathecally at day 14 after bone cancer induction and MrgC expression in the spinal dorsal horn was detected at 2 hr after BAM8-22 and/or anti-MrgC antibody treatment. The cells with red fluorescence were MrgC-positive. The representative results were shown in (A) and the quantitative comparison of the data derived from the independent experiments as in (A) were shown in (B). 1, normal mice without treatment (N); 2, sham-treated mice treated with 5 μ l of NS only (S); 3, tumor-bearing mice treated with 5 μ l of NS only (T); 4, tumor-bearing mice treated with 5 μ l of NS containing 8.0 nmol BAM8-22 (B); 5, tumor-bearing mice treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilution) (A); 6, tumor-bearing mice treated with 5 μ l of NS containing anti-MrgC antibody (1:20 dilution), then 5 μ l of NS containing 8.0 nmol BAM8-22 (AB). The data represents means \pm SD (n = 6). * P < 0.05 vs. group 1, # P < 0.05 vs. group 2, ^ P < 0.05 vs. group 3.

Pudong, China) was used to determine the concentration of the proteins. Then the protein samples were separated on SDS-PAGE gel and transferred to polyvinylidene difluoride filters (Millipore, Billerica, MA). The filters were blocked with 5% nonfat milk and then incubated with rabbit anti-MrgC primary antibody (biorbyt, 1:500). The membrane was washed with Tris-HCl buffer saline and incubated with the secondary antibody of polyclonal goat anti-rabbit IgG (Abcam, 1:5000). Immunoblots were visualized in Electro-Chemi-Luminescence solution (Santa Cruz Biotechnology, CA, USA) for 1 minute and exposed to hyper-films (Amersham Biosciences, Piscataway, NJ) for 1 to 10 minutes. β -actin (abcam, UK, 1:1000) was used as a loading control for total protein. The gray value of each band was quantified with a. The mean normalized optical density (OD) of MrgC protein bands relative to the OD of

β -actin band from the same sample was calculated using the computer-assisted imaging analysis system (IPLab software, Scanalytics, Fairfax, VA).

Statistical analysis

All the data were expressed as mean \pm SD (standard deviation). The animals were assigned to different treatment groups in a randomized way. Repeated measures ANOVA were performed to determine the overall differences at each time point in spontaneous lifting behavior and PWMT. One-way ANOVA was used to determine the differences in the expression of MrgC among all the experimental groups. In both cases, when significant main effects were observed, LSD post hoc tests were performed to determine the source(s) of differences. P value < 0.05 was considered statistically significant.

Results

Increased bone cancer pain and mirror-image pain in the tumor-bearing mice

To assess the pain behaviors in the tumor-bearing mice and the sham-treated mice, spontaneous lifting behavior assessment and mechanical allodynia assays were performed. After bone cancer induction, the number of spontaneous flinches of the right hind limb (the ipsilateral hind limb to the operation) gradually increased with the peak at day 3, and returned to the level before operation at day 5 in both the tumor-bearing mice and the sham-treated mice (**Figure 1A**). Thus, the increase in spontaneous flinches at day 3 may be attributed to the operational injury. In the tumor-bearing mice, the number of spontaneous flinches of the tumor-bearing limb increased since day 7 and reach 13.50 ± 1.82 and 14.60 ± 2.60 at day 14 and day 21, respectively, which were significantly higher than those in the sham-treated mice (1.70 ± 0.31 , $P < 0.05$ and 1.62 ± 0.63 , $P < 0.05$, respectively). This was corresponded with the development of bone cancer. Moreover, in the tumor-bearing mice, the number of spontaneous flinches of the left hind limb (contralateral to the tumor-bearing limb) increased since day 10 and reach 4.31 ± 0.64 at day 14, which was significantly higher than that in the sham-treated mice (1.02 ± 0.50 , $P < 0.05$).

These results were further confirmed by mechanical allodynia assays. The ipsilateral hind limb to the operation of both tumor-bearing and sham-treated mice showed a significant decrease in PWMT to von Frey filaments stimulation at day 3, and recovered to the level of day 0 before operation at day 5 (**Figure 1B**). PWMT of the right hind limb decreased since day 5 and reach 0.42 ± 0.22 at day 21, which was significantly lower than that in the sham-treated mice (1.80 ± 0.15 , $P < 0.05$). Moreover, in the tumor-bearing mice, the number of spontaneous flinches of the contralateral hind limb to the operation decreased since day 10 and reached 1.33 ± 0.10 at day 21, which was significantly lower than that in the sham-treated mice (1.83 ± 0.16 , $P < 0.05$). These results indicate increased bone cancer pain and mirror-image pain in the tumor-bearing mice. Furthermore, compared to the bone cancer pain, mirror-image pain tended to occur later and at a lesser degree.

BAM8-22 attenuates bone cancer pain in mice

The effects of intrathecal administration of BAM8-22 on bone cancer pain were detected. As shown above, in the tumor-bearing mice, the bone cancer pain-related behaviors increased gradually and tended to be stable at day 14. Thus, BAM8-22 was injected intrathecally into the mice at day 14 after bone cancer induction. As shown in **Figure 2A**, there were no significant differences in the number of spontaneous flinches between the sham-treated mice and the sham-treated mice combined with BAM8-22 treatment at each time point detected, indicating that BAM8-22 itself had no effect on spontaneous lifting behavior in the sham-treated mice. As to the tumor-bearing mice, intrathecal administration of 0.8 nmol BAM 8-22 (TB1) did not affect the spontaneous pain behavior in the tumor-bearing limb at each time point detected. While at 2 hr and 12 hr after administration of 2.4 nmol BAM8-22 (TB2), the numbers of spontaneous flinches of tumor-bearing mice were 8.61 ± 1.90 and 10.35 ± 1.78 , respectively, which were significantly lower than those in the tumor-bearing mice with mock treatment (12.67 ± 1.67 , $P < 0.05$ and 13.63 ± 1.90 , $P < 0.05$, respectively). Furthermore, at 2 hr and 12 hr after administration of 8.0 nmol BAM8-22 (TB3), the numbers of spontaneous flinches of tumor-bearing mice were 6.37 ± 1.20 and 8.82 ± 1.89 , respectively, which were significantly lower than those in the tumor-bearing mice with mock treatment (12.67 ± 1.67 , $P < 0.05$ and 13.63 ± 1.90 , $P < 0.05$, respectively). It should be noticed that 24 hr after BAM8-22 administration, there were no significant differences in the number of spontaneous flinches between the tumor-bearing mice and the tumor-bearing mice combined with BAM8-22 treatment at each concentration detected.

These results were further confirmed by mechanical allodynia assays. As shown in **Figure 2B**, in the tumor-bearing mice, intrathecal administration of 0.8 nmol BAM 8-22 did not affect PWMT of the tumor-bearing limb at each time point detected. While at 2 hr and 12 hr after administration of 2.4 nmol (TB2) and 8.0 nmol BAM8-22 (TB3), PWMT was significantly higher than those in the tumor-bearing mice with mock treatment. Twenty-four hr after BAM8-22 administration, there were no significant differences in PWMT between the tumor-bearing mice and the tumor-bearing mice com-

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bined with BAM8-22 treatment at each concentration detected. These results indicate that BAM8-22 attenuates bone cancer pain in mice dose dependently with the highest effects at 2 hr after BAM8-22 administration.

The effects of intrathecal injection of the MrgC antagonist, anti-MrgC antibody, on bone cancer pain were also detected. As shown in **Figure 2A**, at 2 hr and 12 hr after administration of anti-MrgC antibody (1:20 dilution) (TA), the numbers of spontaneous flinches of tumor-bearing mice were 13.32 ± 2.80 and 13.60 ± 1.52 , respectively, which were significantly higher than those in the tumor-bearing mice with mock treatment (12.67 ± 1.67 , $P < 0.05$ and 13.63 ± 1.90 , $P < 0.05$, respectively). As mentioned above, at 2 hr and 12 hr after administration of 8.0 nmol BAM8-22 (TB3), the numbers of spontaneous flinches of tumor-bearing mice were significantly lower than those in the tumor-bearing mice with mock treatment (TBO). While there were no significant differences in the number of spontaneous flinches between the tumor-bearing mice treated with anti-MrgC antibody (1:20 dilution) as well as 8.0 nmol BAM8-22 (TAB) and the tumor-bearing mice with mock treatment (TBO) at each time point detected. These were further confirmed by mechanical allodynia assays (**Figure 2B**). These results indicate that anti-MrgC antibody aggravates bone cancer pain in mice with the highest effects at 2 hr after administration and reverses the effects of BAM8-22.

Intrathecal administration of BAM8-22 does not affect mirror-image pain

We found that in the tumor-bearing mice, the mirror-image pain-related behaviors increased significantly at day 14 after bone cancer induction. Thus, as mentioned above, BAM8-22 was injected intrathecally into the mice at day 14 and the effects of BAM8-22 on mirror-image pain were also detected. There were no significant differences in the number of spontaneous flinches between the tumor-bearing mice and the tumor-bearing mice treated with BAM8-22 at different concentrations and different time points (**Figure 3A**). These results were further confirmed by mechanical allodynia assays (**Figure 3B**). These data suggest that intrathecal administration of BAM8-22 has no signifi-

cant effect on mirror-image pain induced in the tumor-bearing mice.

BAM8-22 stimulates the expression of MrgC in the spinal dorsal horn

To investigate the effects of BAM8-22 on MrgC expression in spinal dorsal horn, western blot analysis and immunofluorescence assay were performed. BAM8-22 and anti-MrgC antibody were injected intrathecally at day 14 after bone cancer induction and MrgC expression in the spinal dorsal horn was detected at 2 hr after BAM8-22 and/or anti-MrgC antibody treatment. As shown in **Figure 4**, MrgC expression was significantly higher in the spinal dorsal horn of the tumor-bearing mice than that of the sham-treated mice (S) and the normal mice (N). Intrathecal injection of 8.0 nmol BAM8-22 (B) stimulated MrgC expression in the spinal dorsal horn, while intrathecal injection of anti-MrgC antibody (A) inhibited MrgC expression. Furthermore, pretreatment with anti-MrgC antibody reversed the stimulation effects of BAM8-22 on MrgC expression (AB). These results were further confirmed using immunofluorescence assay (**Figure 5**). These results indicate that BAM8-22 stimulates the expression of MrgC in the spinal dorsal horn.

Discussion

In this study, the effects of MrgC agonist BAM8-22 on bone cancer pain and mirror-image pain were investigated. Intrathecal treatment with BAM8-22 significantly attenuated bone cancer pain behaviors in a dose-dependent manner through upregulation of MrgC expression, and had no significant effect on the mirror-image pain. While the largest dose of BAM8-22 (TB3) we used did not reverse the bone cancer-related pain behaviors completely. This may be because MrgC is not the only factor that induces bone cancer pain. Furthermore, the dose of BAM8-22 we adopted in this study did not reach its maximum dose for treatment. We also observed that even the highest dose of BAM8-22 in our study had no effect on mirror-image pain. This may be due to different mechanisms between the bone cancer pain behaviors and mirror-image pain. It has been reported that BAM8-22 can reduce inflammatory pain and neuropathic pain, which may be associated with the inhibition of up-regulation of nNOS,

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TRPV1 and CGRP as well as c-Fos expression in the spinal dorsal horn or DRG [16, 22]. However, the specific analgesic mechanisms of BAM8-22 in bone cancer pain still need to be further elucidated.

Up to now, the treatment of various types of pain is still a real challenge as most analgesics show serious side effects owing to the wide distribution of their targeting receptors [23]. Thus, the ideal clinical analgesics should possess little or no unwanted CNS effects. It has been shown that MrgC are highly restrictly distributed in DRG. Activation of MrgC only exhibited analgesic activity instead of the adverse effects [24]. In this study, no side effects were observed with intrathecal treatment of BAM8-22 at the dose as high as 8.0 nmol. It has been reported that MrgC had no effect in pain processing under normal conditions, because activation of MrgC did not alter the basal nociceptive thresholds [25]. Therefore, there may be a promising prospect for BAM8-22 to be used in cancer pain control. In summary, MrgC agonist BAM8-22 could attenuate bone cancer pain in mice. This study may provide a novel strategy for the treatment of bone cancer pain.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhengliang Ma and Xiaoping Gu, Department of Anaesthesiology, Affiliated Drum-Tower Hospital of Medical College of Nanjing University, No. 321, Zhongshan Road, Nanjing 210008, Jiangsu Province, P. R. China. Tel: 86-025-83105502; Fax: 86-025-83105502; E-mail: mzlmz1234@126.com (ZLM); njgxp1970@163.com (XPG)

References

- [1] Falk S and Dickenson AH. Pain and nociception: mechanisms of cancer-induced bone pain. *J Clin Oncol* 2014; 32: 1647-1654.
- [2] Oliveira KG, von Zeidler SV, Podesta JR, Sena A, Souza ED, Lenzi J, Bissoli NS and Gouvea SA. Influence of pain severity on the quality of life in patients with head and neck cancer before antineoplastic therapy. *BMC Cancer* 2014; 14: 39.
- [3] Colvin L and Fallon M. Challenges in cancer pain management—bone pain. *Eur J Cancer* 2008; 44: 1083-1090.
- [4] Kwong TN, Furtado S and Gerrand C. What do we know about survivorship after treatment for extremity sarcoma? A systematic review. *Eur J Surg Oncol* 2014; 40: 1109-1124.
- [5] Boland J, Boland E and Brooks D. Importance of the correct diagnosis of opioid-induced respiratory depression in adult cancer patients and titration of naloxone. *Clin Med* 2013; 13: 149-151.
- [6] Mercadante S. Intravenous morphine for management of cancer pain. *Lancet Oncol* 2010; 11: 484-489.
- [7] Cheng CF, Cheng JK, Chen CY, Lien CC, Chu D, Wang SY and Tsaor ML. Mirror-image pain is mediated by nerve growth factor produced from tumor necrosis factor alpha-activated satellite glia after peripheral nerve injury. *Pain* 2014; 155: 906-920.
- [8] He SQ, Han L, Li Z, Xu Q, Tiwari V, Yang F, Guan X, Wang Y, Raja SN, Dong X and Guan Y. Temporal changes in MrgC expression after spinal nerve injury. *Neuroscience* 2014; 261: 43-51.
- [9] Wang D, Chen T, Zhou X, Couture R and Hong Y. Activation of Mas oncogene-related gene (Mrg) C receptors enhances morphine-induced analgesia through modulation of coupling of mu-opioid receptor to Gi-protein in rat spinal dorsal horn. *Neuroscience* 2013; 253: 455-464.
- [10] Zylka MJ, Dong X, Southwell AL and Anderson DJ. Atypical expansion in mice of the sensory neuron-specific Mrg G protein-coupled receptor family. *Proc Natl Acad Sci U S A* 2003; 100: 10043-10048.
- [11] Lembo PM, Grazzini E, Groblewski T, O'Donnell D, Roy MO, Zhang J, Hoffert C, Cao J, Schmidt R, Pelletier M, Labarre M, Gosselin M, Fortin Y, Banville D, Shen SH, Strom P, Payza K, Dray A, Walker P and Ahmad S. Proenkephalin A gene products activate a new family of sensory neuron-specific GPCRs. *Nat Neurosci* 2002; 5: 201-209.
- [12] Dores RM, McDonald LK, Steveson TC and Sei CA. The molecular evolution of neuropeptides: prospects for the '90s. *Brain Behav Evol* 1990; 36: 80-99.
- [13] Chang M, Li W, Peng YL, Gao YH, Yao J, Han RW and Wang R. Involvement of NMDA receptor in nociceptive effects elicited by intrathecal [Tyr6]

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- gamma2-MSH(6-12), and the interaction with nociceptin/orphanin FQ in pain modulation in mice. *Brain Res* 2009; 1271: 36-48.
- [14] Grazzini E, Puma C, Roy MO, Yu XH, O'Donnell D, Schmidt R, Dautrey S, Ducharme J, Perkins M, Panetta R, Laird JM, Ahmad S and Lembo PM. Sensory neuron-specific receptor activation elicits central and peripheral nociceptive effects in rats. *Proc Natl Acad Sci U S A* 2004; 101: 7175-7180.
- [15] He SQ, Li Z, Chu YX, Han L, Xu Q, Li M, Yang F, Liu Q, Tang Z, Wang Y, Hin N, Tsukamoto T, Slusher B, Tiwari V, Shechter R, Wei F, Raja SN, Dong X and Guan Y. MrgC agonism at central terminals of primary sensory neurons inhibits neuropathic pain. *Pain* 2014; 155: 534-544.
- [16] Jiang J, Wang D, Zhou X, Huo Y, Chen T, Hu F, Quirion R and Hong Y. Effect of Mas-related gene (Mrg) receptors on hyperalgesia in rats with CFA-induced inflammation via direct and indirect mechanisms. *Br J Pharmacol* 2013; 170: 1027-1040.
- [17] Brennan TJ, Vandermeulen EP and Gebhart GF. Characterization of a rat model of incisional pain. *Pain* 1996; 64: 493-501.
- [18] Hylden JL and Wilcox GL. Intrathecal morphine in mice: a new technique. *Eur J Pharmacol* 1980; 67: 313-316.
- [19] Schwei MJ, Honore P, Rogers SD, Salak-Johnson JL, Finke MP, Ramnaraine ML, Clohisy DR and Mantyh PW. Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. *J Neurosci* 1999; 19: 10886-10897.
- [20] Chaplan SR, Bach FW, Pogrel JW, Chung JM and Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53: 55-63.
- [21] Sun Y, Zhang W, Liu Y, Liu X, Ma Z and Gu X. Intrathecal injection of JWH015 attenuates remifentanyl-induced postoperative hyperalgesia by inhibiting activation of spinal glia in a rat model. *Anesth Analg* 2014; 118: 841-853.
- [22] Huang H, Li Q, Hong YG and Wang DM. [MrgC receptor activation reverses chronic morphine-evoked alterations of glutamate transporters and nNOS in rats]. *Sheng Li Xue Bao* 2014; 66: 449-456.
- [23] Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, Glaser SE and Vallejo R. Opioid complications and side effects. *Pain Physician* 2008; 11: S105-120.
- [24] Viet CT and Schmidt BL. Biologic mechanisms of oral cancer pain and implications for clinical therapy. *J Dent Res* 2012; 91: 447-453.
- [25] Cai Q, Jiang J, Chen T and Hong Y. Sensory neuron-specific receptor agonist BAM8-22 inhibits the development and expression of tolerance to morphine in rats. *Behav Brain Res* 2007; 178: 154-159.