Title: Systemic QX-314 reduces bone cancer pain through selective inhibition of TRPV1-expressing primary afferents in mice.

Satoshi Fuseya, M.D.,* Katsumi Yamamoto, M.D.,* Hitoshi Minemura, M.D.,‡ Satoshi Yamaori, Ph.D.,# Tomoyuki Kawamata, M.D.,† Mikito Kawamata, M.D.§

*Staff Anesthesiologist, ‡Graduate student, †Associate Professor, §Professor and Chairman, Department of Anesthesiology and Resuscitology, Shinshu University School of Medicine, Matsumoto City, Nagano, Japan.

#Associate Professor, Department of Pharmacy, Shinshu University Hospital, Matsumoto City, Nagano, Japan

Corresponding author: Satoshi Fuseya

Address: Department of Anesthesiology and Resuscitology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto City, Nagano 390-8621, Japan.

Telephone number: +81-263372670, FAX number: +81-263352734, E-mail: sfuseya@shinshu-u.ac.jp

Disclosure of funding: This work was supported by a grant (KAKENHI) from JSPS, Chiyoda-ku, Tokyo, Japan (Grant Numbers 23390374; to TK and 23791694; to SF) and a grant from Shinshu University School of Medicine, Matsumoto City, Nagano, Japan (to SF).

Acknowledgements:

We would like to thank Masahiko Watanabe, M.D. (Hokkaido University School of Medicine, Sapporo City, Japan) for providing TRPV1 antibody, Shingo Furuse, M.D. (Nakamura Memorial Hospital, Sapporo City, Japan) for technical support in behavioral assessment and Katsunori Nakamura, Ph.D. (Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya City, Japan) and Kaori Aikawa, B.A. (Department of Pharmacy, Shinshu University Hospital, Matsumoto City, Japan) for expert

technical assistance in measurement of drug plasma concentrations.

Number of words: Abstract, 278; Introduction, 500; Discussion, 1753

Abbreviated title: QX-314 and bone cancer pain

Conflicts of interest statement: All authors declare that there was no conflict of interest.

Abstract

Background: The aim of this study was to determine whether systemic administration of QX-314 reduces bone cancer pain through selective inhibition of transient receptor potential vanilloid subfamily 1 (TRPV1)-expressing afferents.

Methods: A mouse model of bone cancer pain was used. We examined the effects of bolus (0.01-3 mg/kg, n = 6-10) and continuous (5 mg/kg/h, n = 5) administration of QX-314 on both bone cancer pain-related behaviors and phosphorylated cyclic-adenosine monophosphate response element-binding protein (p-CREB) expression in dorsal root ganglion (DRG) neurons (n = 3 or 6) and the effects of ablation of TRPV1-expressing afferents on bone cancer pain-related behaviors (n = 10).

Results: The numbers of flinches indicative of ongoing pain in QX-314-treated mice were smaller than those in vehicle-treated mice at 10 min (3 mg/kg, 4 ± 3; 1 mg/kg, 5 ± 3 vs. 12 ± 3; $P < 0.001$) (n = 8-9), 24 h (3 ± 2 vs. 13 ± 3, $P < 0.001$) and 48 h (4 ± 1 vs. 12 ± 2, $P < 0.001$) (n = 5 in each group) after QX-314 administration, but impaired limb use, weight-bearing including that examined by the CatWalk system, and rotarod performance indicative of movement-evoked pain were comparable. QX-314 selectively inhibited the increase in p-CREB expression in TRPV1-positive, but not TRPV1-negative, DRG neurons compared to that in the case of vehicle administration (32.2 ± 3.0% vs. 52.6 ± 5.9%, $P < 0.001$) (n = 6 in each group). Ablation of TRPV1-expressing afferents mimicked the effects of QX-314.

Conclusion: Our study showed that systemic administration of QX-314 in mice inhibits some behavioral aspects of bone cancer pain through selective inhibition of TRPV1-expressing afferents without co-administration of TRPV1-agonists.

Introduction

Bone cancer pain commonly includes ongoing pain and movement-evoked pain. Patients with skeletal metastases undergo surgical stabilization of bone to prevent bone fracture, which improves movement-evoked pain and weight-bearing ambulation.[1,2] However, ongoing pain is sometimes continuous even after bone fixation and is exacerbated by episodes of breakthrough pain.[3] Since ongoing cancer pain has deleterious effects on sleeping, mental health, and social activities,[4,5] relief of ongoing pain as well as movement-evoked pain is important for patients with bone cancer. Opioids are the mainstay of the structural approach to treatment for cancer pain, known as the "WHO analgesic ladder".[6,7] However, bone cancer pain is often debilitating, difficult to treat and resistant to opioids.[8-10] As a result, patients with bone cancer pain often require higher doses of morphine and suffer from opioid-induced side effects including impairment of cognition, somnolence, and constipation.[11] A novel strategy for bone cancer pain is thus needed for improvement of patients' quality of life.

QX-314, a quaternary lidocaine derivative, has a permanent positive charge that theoretically impairs its ability to cross neuronal membranes. A series of initial *in vitro* experiments in the 1970s showed that extracellular application of QX-314 to neurons caused only a slow, small decrease in the action potential rate of rise, while intracellular application of it produced a rapid decline of the action potential maximum rate of rise.[12,13] It has recently been suggested that QX-314 has clinically useful potential to produce a differential block, which inhibits pain and preserves motor function and tactile or proprioceptive sensation. Binshtok et al. reported that extracellular administration of QX-314 could produce a local anesthetic effect through entering the pore of transient receptor potential vanilloid subfamily 1 (TRPV1), when TRPV1 was activated by capsaicin.[14] Since TRPV1 is expressed in nociceptive neurons,[15,16] QX-314 thus could selectively inhibit pain transmitted by TRPV1-expressing afferents with almost no impairment of motor function and proprioceptive sensation,[14,17] although QX-314 administered at a dose of more than 25 mM produces both motor blockade and sensory

blockade.[18,19] On the other hand, a clinical problem regarding application of QX-314 combined with capsaicin for pain therapy is intense pain associated with TRPV1 activation by administration of capsaicin, which limits the clinical use of QX-314. However, if some pain conditions are caused by TRPV1 activation, systemic administration of QX-314 may produce local anesthetic effects without co-administration of TRPV1 agonists. Previous studies have shown that systemic QX-314 inhibits thermal hyperalgesia[20] and ectopic discharges from neuromas in DRG neurons after nerve injury[21] and suppresses increased activity of spinal dorsal horn neurons following skin incision.[22] We previously reported that TRPV1 activation is involved in bone cancer pain.[23,24] Thus, we hypothesized that systemic QX-314 relieves bone cancer pain through inhibition of TRPV1-expressing primary afferents without co-administration of TRPV1 agonists.

In this study, using a mouse model of bone cancer pain,[25] we examined whether systemic administration of QX-314 relieved bone cancer pain and whether the effects of QX-314 were caused by inhibition of TRPV1-expressing afferents. We also examined the effects of lidocaine compared to those of QX-314.

Materials and Methods

The protocol of this study was approved by the Animal Care and Use Committee of Shinshu University School of Medicine (reference No. 220035 and 10-018) and was in accordance with the ethical guidelines of the National Institutes of Health and of the International Association for the Study of Pain.

Animals

Experiments were conducted in adult male C3H/HeJ mice (20–25 g, Japan SLC, Hamamatsu, Japan). The mice were housed in a temperature-controlled (21 ± 1 °C) room with a 12-h light/dark cycle and given free access to food and water.

Drugs

Lidocaine N-ethyl bromide (QX-314), lidocaine hydrochloride monohydrate (lidocaine), and capsaicin were purchased from Sigma (St. Louis, MO). QX-314 and lidocaine was dissolved and diluted in physiological saline. Capsaicin was dissolved in 10% ethanol (vol/vol), 10% Tween 80 (vol/vol) and saline.[26]

Bone cancer model

Murine sarcoma cells (NCTC 2472; ATCC, Rockville, MD) were maintained in NCTC 135 medium containing 10% horse serum (HyClone, Logan, UT) and passaged weekly according to ATCC® recommendations. An injection of sarcoma cells was performed according to a previously described method.[25] Mice were anesthetized with halothane (2% in 100% oxygen).

The following experiments were conducted at 14-16 days after sarcoma implantation, because it has been shown that cancer pain-related behaviors were maximally exhibited at 14 days after implantation and maintained up to day 21.[27,28] Each animal was used in only one experiment.

Assessment of bone cancer pain-related behaviors

Mice were placed in a clear plastic box (30 x 20 x 15 cm) and allowed to habituate for 30 min. Behavioral assessments were then performed. Ongoing and movement-evoked pain behaviors were analyzed according to previously described methods.[23,24,25] Briefly, quantification of spontaneous flinches during a 2-min observation period was used for assessment of the degree of ongoing pain. Limb use during spontaneous ambulation, weight-bearing during spontaneous standing, and rotarod performance were assessed for degree of movement-evoked pain. A continuous assay for dynamic weight bearing was performed by using the CatWalk system (XT ver. 9.1, Noldus Information Technology Inc., Wageningen, Netherlands) for more quantitative measures of movement-evoked pain according to previously described methods.[29] Briefly, mice were allowed walk freely and transverse a glass plate with two dark plastic walls that created a corridor along the length of the plate, called the runway (width of 6 cm and length of 60 cm) in a dark room. Light from an enclosed fluorescent bulb was internally reflected within the glass runway and scattered only at points where a paw touched the glass, producing bright illumination of the contact area. Paw prints were recorded by a high-speed color camera mounted below the runway at 100 frames/sec (model: GP-2360C, GEViCAM, Milpitas, CA). The software analyzed the paw print information and produced many variables. We chose variables for assessment of dynamic weight-bearing including Max Contact Area (area of the paw during Maximum Contact defined as the largest part of a print of a paw that made contact with the glass) and Mean Intensity of the 15 Most Intense Pixels (Mean Intensity) (mean intensity of the 15 pixels of a paw with the highest intensity). Intensity of the signal reflects the degree of contact between the paw and the glass plate ranging from 0 to 255 arbitrary units and increases with an increase in the pressure applied. The camera detects the average intensity within a rectangular area (pixel) that includes various intensities, and aggregation of the pixels forms the paw prints. Trials in which mice stopped or changed direction were excluded from analysis. Two uninterrupted runs were analyzed and averaged to obtain the final analysis values. Mice were not pre-trained to cross the runway since they have no hesitation in crossing the runway spontaneously with sufficient speed.

Immunohistochemistry

We used polyclonal antibodies raised against the following molecules: TRPV1 (0.1 μg/mL, guinea pig; provided by Dr. Watanabe, Hokkaido University, Japan) and phosphorylated cyclic-adenosine monophosphate response element-binding protein (p-CREB) (1:50, #9198, rabbit; Cell Signaling, Danvers, MA). We also used biotinylated isolectin B4 (IB4) (1:100, L3759, Sigma). To confirm the specificity of the TRPV1 antibody used in this study, we used TRPV1-deficient mice of C57BL/6J strain (20-25 g, Jackson Laboratory, Bar Harbor, MA) in addition to C3H/HeJ mice. Immunohistochemistry was performed according to a previously described method.[23] Mice were deeply anesthetized with urethane (1.25 g/kg intraperitoneally). The left L2 dorsal root ganglion (DRG) and spinal cord innervated by the L1-3 dorsal roots were removed, since a previous study has shown that L1-3 DRG neurons innervate the femur.[30] Frozen samples of DRGs and spinal cords were cut at 16 μm and 50 μm, respectively, by using a sliding cryostat (LEICA, Tokyo, Japan). Photographs were taken with a confocal laser scanning microscope (ECLIPSE C1, Nikon, Tokyo, Japan).

Bone histology

The left femur was removed after transcardiac perfusion of 4% paraformaldehyde, decalcified in 10% EDTA (Sigma) for 2 weeks, and embedded in paraffin. The femur was cut at a thickness of 3 μm in the frontal plane and stained with hematoxylin and eosin (H&E) to visualize histological features of the bone marrow and tumor. Photographs were taken with an inverted microscope (Axio Observer Z1, Zeiss, Oberkochen, Germany) and digital imaging software (AxioVision 4.8, Zeiss). The tumor-bearing areas were analyzed by using computerized image analysis software (Win ROOF 6.1, Mitani, Fukui, Japan).

Measurement of plasma concentrations of QX-314

The plasma concentrations of QX-314 were measured by high-performance liquid chromatography (HPLC) with ultraviolet detection according to a modification of the previously described method.[22] Mice were anesthetized with urethane (1.25 g/kg intraperitoneally), and 0.5 mL of

arterial blood was collected from the left ventricle. Plasma was separated by centrifugation at 5000 r.p.m for 10 min at 4 °C and was immediately stored at −80 °C until used for analysis. Fifty μL of the serum sample was added to 10 μL of perchloric acid and mixed by a vortex mixer for 1 min. The mixture was centrifuged at 12,000 r.p.m for 5 min at 20 °C and then filtrated (0.44 μm, Ultrafree-MC Centrifugal Filter Units, Millipore, MA). After five-fold dilution by mobile phase (50-mM phosphate buffer; pH 4.0, buffer : methanol : acetonitrile = 60:30:10 vol/vol/vol, with 0.16% triethylamine), 30 μL of the solution was injected into the HPLC system. The chromatographic conditions were as follows: the HPLC system (Hitachi ELITE LaChrom; Hitachi, Tokyo, Japan) consisted of an L-2100 pump, L-2200 auto sampler, L-2300 column oven, and L-2400 UV detector. The analytical column was a SHISEIDO CAPCELL PAK MGIII C18 column of 50 mm in length and 4.6 mm in diameter (SHISEIDO, Tokyo, Japan). The temperature was kept at 40 °C for the column. Flow rate was 0.3 ml/min. The wavelength of the detector was 210 nm. Retention time for QX-314 was 5.1 min. The detectable concentration of QX314 by HPLC was 300 ng/mL.

Experimental protocols

To minimize the possibility of selection bias, mice were randomly divided by computer-generated randomization into four treatment groups: vehicle administration, lidocaine administration, QX-314 administration or TRPV1 ablation. For adequate allocation concealment, we used sequentially numbered drug containers of identical appearance. To minimize the possibility of detection bias, outcome assessors were blinded to the treatment allocation. There were no missing outcome data for mice during the experiment or in the statistical analyses.

Effects of QX-314 on bone cancer pain-related behaviors

To examine the effects of QX-314 on bone cancer pain-related behaviors, mice received bolus or continuous administration of QX-314. In the experiment with bolus administration, mice were randomly divided into five groups including four different doses of QX-314 or a vehicle. Sarcoma-implanted mice

were injected intraperitoneally at 14 days after sarcoma implantation with a volume of 5 mL/kg body weight of a vehicle or QX-314 at a dose of 0.01, 0.1, 1, or 3 mg/kg. Our preliminary study showed that a bolus injection of QX-314 at a dose of more than 5 mg/kg caused collapse-like behaviors including remaining in the same position with eyes closed in some mice, although convulsive seizure or respiratory depression was not observed. We thus decided to use a bolus injection of 3 mg/kg as the maximum dose of QX-314. Pain-related behaviors were assessed before and 5, 10, 15, 20, 30, and 60 min after drug administration. The two parameters for dynamic weight-bearing in the CatWalk system were assessed at the time when QX-314 produced a peak effect. In the experiment with continuous administration, mice were randomly divided into two groups including 5 mg/kg/h of QX-314 or a vehicle. Sarcoma-implanted mice received continuous subcutaneous (s.c.) administration of QX-314. An Alzet® micro-osmotic pump (length, 1.5 cm; diameter, 0.6 cm; Model 1003D, DURECT, Cupertino, CA) was used for continuous s.c. administration of drugs. After behavioral assessment at 14 days after sarcoma implantation, the pump was implanted. Briefly, under anesthesia with halothane (2% in 100% oxygen), a small incision was made in the skin between the scapulae. A small pocket was formed by spreading the subcutaneous connective tissues apart. The pump was inserted into the pocket with the flow moderator pointing away from the incision. The skin incision was closed with sutures. Then a vehicle or QX-314 was continuously administered at a rate of 1 μL/h for 48 h. Mice received QX-314 at a fixed infusion rate of 5 mg/kg/h by adjustment of the concentration of QX-314 to 5 mg/kg/μL. Behavioral tests were performed 24 and 48 h after starting continuous s.c. administration of QX-314 or a vehicle. Assessments of pain-related behaviors were conducted by independent investigators who were blinded to treatment received.

In another series of experiments, blood samples were collected from mice receiving QX-314 by cardiac puncture into individual heparinized containers to measure the plasma concentration of QX-314. In mice receiving 3 mg/kg of QX-314, blood samples were collected at the time when QX-314 produced a peak effect. In mice receiving 5 mg/kg/h of QX-314, blood samples were collected 24 h or 48 h after starting continuous s.c. administration of QX-314.

Effects of lidocaine on bone cancer pain-related behaviors

To examine the effects of lidocaine on bone cancer pain-related behaviors, mice received a bolus administration of lidocaine. In the experiment with bolus administration, mice were randomly divided into three groups including two different doses of lidocaine or a vehicle. Sarcoma-implanted mice were injected intraperitoneally at 14 days after sarcoma implantation with a volume of 5 mL/kg body weight of a vehicle or lidocaine at a dose of 3 or 10 mg/kg. Pain-related behaviors were assessed before and 1, 5, 10, 15, 20, 30, and 60 min after drug administration.

Effects of continuous administration of QX-314 on p-CREB expression

Some sarcoma-implanted mice receiving continuous administration of QX-314 or a vehicle for 48 h were used for immunohistochemical analysis after the behavioral tests. P-CREB expression in L2 DRG neurons was examined. The numbers of TRPV1-positive, TRPV1-negative, and p-CREB-positive neurons per DRG section were counted. The cell counts were performed using a computerized image analysis system (EZ-C1 3.90, Nikon, Tokyo, Japan). Only neurons with clearly visible nuclei were counted. The number of TRPV1-negative DRG neurons was obtained by background staining of neurons and Nomarski differential interference contrast imaging. The proportion of colocalization of p-CREB-positive profiles with TRPV1-positive or TRPV1-negative neurons was determined by counting 1,500-2,000 neuronal profiles from 7-11 DRG sections for each mouse. Because a stereological approach was not used in this study, quantification of data may have yielded biased estimates of actual numbers of cells and neurons. To prevent duplicate counting of neuronal cell bodies, sections that were 48 μm apart were counted for each DRG. An assistant who was unaware of the treatment groups of sections performed all counting.

Effects of ablation of TRPV1-expressing primary afferents on bone cancer pain-related behaviors

A previous study showed that the central terminals of TRPV1-expressing afferents were selectively ablated within 24 h after intrathecal (i.t.) capsaicin injection and that the effects of ablation of

TRPV1-expressing afferents persisted for at least 8 weeks.[31] To examine the effects of ablation of TRPV1-expressing primary afferents on bone cancer pain-related behaviors, mice were randomly divided into two groups including i.t. capsaicin or a vehicle. Under anesthesia with halothane (2% in 100% oxygen), mice received i.t. capsaicin (10 μg) or a vehicle in a volume of 5.0 μL with a 30-gauge needle attached to a Hamilton syringe at the level of the pelvic girdle according to a previously described method.[26] Immediately after i.t. capsaicin injection, mice exhibited abnormal behaviors including transient hypopnea, twisting of the trunk, and myoclonic limb movements, which were very similar to irritable behaviors caused by i.t. QX-314 injection.[32] Seven days after i.t. injection of capsaicin or a vehicle, mice were implanted with sarcoma cells into the left femur. Pain-related behaviors were assessed at 14 days after sarcoma implantation. Assessments of pain-related behaviors were conducted by independent investigators who were blinded to treatment received. In some mice, bone histology was assessed to examine the effects of ablation of TRPV1-expressing afferents on tumor growth. In addition, TRPV1 expression in the lumbar spinal cord was examined to confirm i.t. capsaicin-induced ablation of TRPV1-expressing primary afferents.

Statistical analysis

The number of flinches, area under the time-effect curve (AUC) on flinches, Max Contact Area, Mean Intensity, percentage of p-CREB-positive profiles, plasma concentrations of QX-314, and percentage of intramedullary space occupied by the tumor were expressed as means ± SDs. AUC on flinches was defined as the area between a graph line of the time-response and a line of the basal value. When the graph line was above or below the line of the basal value, the area was expressed as a negative value or a positive value, respectively. AUC was calculated by summation of both the negative value and the positive value. For continuous data, normal distribution of values was determined by the Shapiro-Wilk test. The scores of limb use, weight-bearing, and rotarod performance were expressed as medians with interquartile range (IQR). The percentages of intramedullary space occupied by tumors were compared using the unpaired *t*-test between two groups. The number of flinches was compared to the basal value by

using one-way analysis of variance (ANOVA) for repeated measures followed by Tukey's test within a single group. The numbers of flinches, Max Contact Area, and Mean Intensity were compared among the groups by using one-way ANOVA followed by Tukey's test. The numbers of flinches in each group at different time points were compared by using two-way ANOVA for repeated measures followed by Tukey's test. In order to analyze the dose-dependency of the effects of QX-314 on AUCs, the Jonckheere-Terpstra test was used. The scores of limb use, weight-bearing, and rotarod performance were compared to the basal value by using Freidman's test followed by Dunn's test and were compared between the groups by using the Mann-Whitney *U*-test. Plasma concentrations of QX-314 and the proportions of p-CREB expression were compared among the groups by using one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered to be statistically significant. IBM SPSS statistics version 21 (IBM, Armonk, NY) and GraphPad Prism software (GraphPad, San Diego, CA) were used to perform statistical analysis. Assuming that the SD of the number of flinches was 1.6 based on our preliminary study for a type 1 error of 0.05 and a power of 0.8, sample size was determined to detect a difference of three in the number of flinches. Effect size was 1.875. Power analysis showed that a minimum of 6 animals were required. In all cases, the investigator was blind to the experimental status of each animal. No adjustments were made for the interim analysis.

Results

Effects of bolus administration of QX-314 on bone cancer pain-related behaviors

First, we examined the effects of bolus i.p. administration of QX-314 on bone cancer pain-related behaviors (**figs. 1 and 2**). Before administration, the number of flinches, score of limb use, score of weight-bearing, and score of rotarod performance were comparable among the groups **(fig. 1A and 2, A-C)**. Although the vehicle did not affect the number of flinches, 0.1, 1 and 3 mg/kg of QX-314 significantly reduced the number of flinches compared to the controls **(fig. 1A, $P < 0.001$ vs. basal value within a group)**. Peak effects of QX-314 were observed 5-10 min after administration, and the reduced number of flinches returned to the basal values within 60 min after administration. We found a dose-dependent effect of QX-314 at 5, 10, and 15 min after administration **(fig. 1A, *$P < 0.001$ vs. vehicle, ¶$P < 0.05$ vs. vehicle, #$P <$ or $= 0.01$ vs. 0.01 mg/kg of QX-314, ‡$P < 0.01$ vs. 0.1 mg/kg of QX-314, †$P < 0.05$ vs. 0.1 mg/kg of QX-314)**. Analysis of AUCs also showed a dose-dependent effect of QX-314 **(fig. 1B, $P < 0.001$).** The median effective dose (ED50) of QX-314 on the number of flinches was 0.605 mg/kg ($\log_{10}$[QX-314] = -0.218) **(fig. 1B)**.

On the other hand, QX-314 at all doses used in this study did not significantly change scores of limb use, weight-bearing, and rotarod performance, Max Contact Area and Mean Intensity compared to the basal values or values with vehicle treatment (data not shown). QX-314 at a dose of 3 mg/kg, which was the highest dose used in this study did not significantly change scores of limb use, weight-bearing, and rotarod performance, Max Contact Area and Mean Intensity compared to the basal values or values with vehicle treatment **(fig. 2, A-E**).

Effects of continuous administration of QX-314 on bone cancer pain-related behaviors

Since bolus administration of QX-314 was relatively short-acting, we next examined effects of continuous administration of QX-314 on bone cancer pain-related behaviors. The number of flinches, score of limb use, and score of weight-bearing before drug administration were comparable between

vehicle-treated mice and QX-314-treated mice. The vehicle did not have any effect on number of flinches, score of limb use, or score of weight-bearing at 24 and 48 h after starting continuous administration. The number of flinches in vehicle-treated mice at 24 and 48 h after starting continuous administration were 13 ± 3, n = 5 and 12 ± 2, n = 5, respectively. QX-314 (5 mg/kg/h) significantly reduced the numbers of flinches compared to those in vehicle-treated mice at 24 and 48 h after starting continuous administration **(fig. 3, 3 ± 2 flinches, n = 5 and 4 ± 1 flinches, n = 5, respectively, *$P < 0.001$)**. On the other hand, neither the vehicle nor QX-314 had any effect on scores of limb use and weight-bearing (limb use: 2 [2-2], n = 5 in QX-314-treated mice and 2 [2-2], n = 5 in vehicle-treated mice at 24 h and 48 h after starting administration, weight-bearing: 1 [1-1], n = 5 in QX-314-treated mice and 1 [1-1], n = 5 in vehicle-treated mice at 24 h and 48 h after starting administration).

The plasma concentrations of QX-314 at 24 and 48 h after starting continuous administration of 5 mg/kg/h of QX-314 were 0.54 ± 0.18 μg/mL, n = 5 and 0.66 ± 0.41 μg/mL, n = 5, respectively. The plasma concentration of QX-314 at 10 min after bolus administration of 3 mg/kg of QX-314 was 0.73 ± 0.29 μg/mL, n= 5, which was comparable to those in the case of 5 mg/kg/h of QX-314.

Effects of bolus administration of lidocaine on bone cancer pain-related behaviors

We also examined the effects of bolus i.p. administration of lidocaine on bone cancer pain-related behaviors as a potentially positive control. Three mg/kg of lidocaine (n = 5) was not effective and 10 mg/kg of lidocaine (n = 9) exerted only a slight analgesic effect on both ongoing and movement-evoked pain-related behaviors **(fig. 4, A-C, *$P < 0.01$ vs. vehicle, #$P < 0.01$ vs. basal value within a group)**. Peak effects of lidocaine were observed 1 min after administration, and the scores of pain-related behaviors returned to the basal values within 10 min after administration.

Effects of continuous administration of QX-314 on p-CREB expression in TRPV1-positive DRG neurons

Since previous studies showed that persistent noxious stimulation induced phosphorylation of CREB in a subpopulation of DRG neurons,[33,34] we next examined the effects of continuous administration of QX-314 on p-CREB expression in TRPV1-positive or TRPV1-negative DRG neurons on the ipsilateral side to sarcoma implantation.

Before the experiments, we confirmed the specificity of the TRPV1 antibody used in this study (**fig. 5**). While the TRPV1 antibody yielded strong labeling in somata and fibers of the subpopulation of DRG neurons of naive mice, dot-like positive labeling was observed in the nuclei of most of the DRG neurons **(fig. 5, A and C).** On the other hand, no specific staining was found in the DRG of TRPV1-deficient mice, although dot-like positive reaction was found in the nuclei of most of the DRG neurons **(fig. 5, B and D)**. Therefore, we judged that labeling in somata and fibers of the neurons was a specific reaction, while dot-like positive labeling observed in the nucleus was a non-specific reaction.

Sarcoma implantation, but not sham implantation, increased the expression of p-CREB in TRPV1-positive and TRPV1-negative DRG neurons (**fig. 6**). At 14 days after sarcoma implantation, the percentage of p-CREB-positive profiles in TRPV1-positive DRG neurons in sarcoma-implanted mice was significantly higher than that in sham-implanted mice **(fig. 6B, #$P < 0.001$ vs. sham)**. The percentage of p-CREB-positive profiles in TRPV1-negative DRG neurons in sarcoma-implanted mice was also significantly higher than that in sham-implanted mice **(fig. 6C, *$P < 0.05$ vs. sham)**. Continuous s.c. administration of QX-314 reduced p-CREB expression in TRPV1-positive, but not TRPV1-negative, DRG neurons. QX-314 at a dose of 5 mg/kg/h significantly reduced the percentage of p-CREB-positive profiles in TRPV1-positive DRG neurons compared to that in the case of vehicle administration at 48 h after starting administration **(fig. 6, A and B, **$P < 0.001$ vs. vehicle)**. On the other hand, QX-314 did not reduce p-CREB expression in TRPV1-negative DRG neurons compared to that in the case of the vehicle **(fig. 6C)**.

Analgesic effects of ablation of TRPV1-expressing primary afferents on bone cancer pain-related behaviors

The results of p-CREB experiments suggested that QX-314 selectively inhibits TRPV1-positive afferents, resulting in reduction of spontaneous flinches. If QX-314 selectively inhibits TRPV1-expressing afferents, ablation of TRPV1-expressing afferents would also selectively inhibit flinching behavior. Finally, we examined analgesic effects of ablation of TRPV1-expressing afferents on bone cancer pain (**fig. 7 and S**). Immunohistochemistry confirmed that i.t. capsaicin had ablated TRPV1-expressing primary afferents in the lumbar spinal cord at 21 days after i.t. injection **(fig. 7A)**, as shown in a previous study,[26] while i.t. capsaicin did not reduce the number of TRPV1-positive DRG neurons **(fig. S**). Ablation of the central terminals of TRPV1-expressing primary afferents was observed from the upper thoracic region to lower sacral region (data not shown). I.t. vehicle did not affect expression of TRPV1-expressing primary afferents in the dorsal horn **(fig. 7A)**. I.t. capsaicin did not affect expression of IB4-binding primary afferents, which rarely express TRPV1 in mice,[23] selectively ablating TRPV1-expressing primary afferents **(fig. 7A)**.

Mice treated with i.t. vehicle (n = 5 or 10) exhibited spontaneous flinches, impaired limb use, impaired weight-bearing, impaired rotarod performance, reduced Max Contact Area, and weak Mean Intensity at 14 days after sarcoma implantation, which seemed to be comparable to those in sarcoma-implanted mice that were not administered i.t. vehicle. Mice treated with i.t. capsaicin (n = 10) exhibited a significantly smaller number of flinches (2 ± 1, $^{*}P < 0.001$) than that of mice treated with i.t. vehicle (14 ± 2 flinches) and did not show any significant difference in the number of flinches compared to that in sham-implanted mice (n = 7) **(fig. 7B)**. On the other hand, the scores of limb use, weight-bearing, and rotarod performance, Max Contact Area and Mean Intensity in mice treated with i.t. capsaicin were comparable to those in mice treated with i.t. vehicle **(fig. 7, C-G).**

H&E staining of the femur on the ipsilateral side to sarcoma implantation showed that there was no significant difference in the percentages of intramedullary space occupied by sarcoma cells between

mice treated with i.t. capsaicin and mice treated with i.t. vehicle at 14 days after sarcoma implantation **(fig. 8, A and B)**.

Discussion

The major findings of this study were that (1) systemic administration of QX-314 inhibited ongoing pain-related behavior but not movement-evoked pain-related behaviors in sarcoma-implanted mice, (2) QX-314 inhibited the increase in p-CREB expression in TRPV1-positive, but not TRPV1-negative, DRG neurons in sarcoma-implanted mice, and (3) selective ablation of TRPV1-expressing afferents mimicked analgesic effects of QX-314. Our results indicate that systemic administration of QX-314 reduces bone cancer-induced ongoing pain through selective inhibition of TRPV1-expressing afferents.

Analgesic effects of QX-314 and mechanisms of bone cancer pain

QX-314 (0.1-3 mg/kg) strongly suppressed flinches indicative of bone cancer-induced ongoing pain, while QX-314 had little effect on movement-evoked pain-related behaviors. Most of the nociceptors that innervate bone tissues including bone marrow are unmyelinated calcitonin gene-related peptide (CGRP)-labeled C-fibers, while unmyelinated nonpeptidergic IB4-labeled C-fibers appear to be absent in bone tissues.[35] On the other hand, nociceptors that innervate the skin richly include both CGRP- and IB4-positive C-fibers.[36] TRPV1 is mainly expressed in CGRP-positive C-fibers but in very few IB4-positive C-fibers in mice.[23] It has been shown that bone cancer proliferating in the femur causes central sensitization,[25,28] resulting in mechanical allodynia of the plantar surface.[37] Movement-evoked pain observed in this study might be at least in part due to bone cancer-induced referred pain and referred allodynia of the plantar skin of the hindpaw on the ipsilateral side. IB4-positive fibers may play an important role in transmitting bone cancer-induced referred pain and referred allodynia of the plantar skin without mediation by TRPV1-expressing fibers. This difference in the mechanisms of bone cancer-induced ongoing pain and movement-evoked pain might explain why QX-314 was effective for spontaneous pain assessed as flinches but not for movement-evoked pain in this study. Our previous study showed that SB366791, a selective TRPV1 antagonist, inhibits flinches but does not improve movement-evoked pain-related behaviors.[24] This may also be because movement-evoked pain might be in

part due to bone cancer-induced referred allodynia without mediation by TRPV1-expressing fibers.

Another possibility is that TRPV1-positive and -negative afferents each have modality specificity for bone cancer pain in mice. In the present study, ablation of TRPV1-expressing afferents selectively and completely abolished bone cancer-induced flinching behavior without having any effect on impaired ambulation and weight-bearing. This finding suggests that TRPV1-expressing afferents transmit ongoing pain. Recent studies have provided evidence for modality specificity of primary afferents in mice.[38] For example, Mas-related G-protein-coupled receptor d-expressing afferents and TRPV1-expressing afferents, which are non-overlapping populations, are selectively involved in the senses of mechanical pain and heat pain, respectively.[26] Our p-CREB experiment showed that the percentage of activated profiles in both TRPV1-positive and -negative DRG neurons in sarcoma-implanted mice was significantly higher than that in sham-implanted mice. It has also been shown that unmyelinated peptidergic C-fibers and myelinated fibers richly innervate bone tissues, while unmyelinated nonpeptidergic C-fibers appear to be absent in bone tissues.[35] Taken together with the results of this study, it seems that unmyelinated peptidergic C-fibers or myelinated fibers in TRPV1-negative afferents are involved in the transmission of movement-evoked pain.

Our experiments with systemic lidocaine as a potentially positive control showed analgesic effects on both bone cancer-induced ongoing pain and movement-evoked pain. However, high doses of lidocaine compared to those of QX-314 were needed for reduction of pain behaviors, and the analgesic effects of lidocaine were weak and short-lasting. While the specific reasons for these differences remain unknown, our results are consistent with the results of a previous study showing that the relative potency of QX-314 for systemic cardiac toxicity in mice is significantly higher than that of lidocaine[39] (see Study Limitations below).

Selective inhibition of TRPV1-expressing afferents by QX-314 in a bone cancer pain model

Our p-CREB experiment indicated that QX-314 selectively exerts inhibitory effects on TRPV1-expressing afferents. In addition, systemic administration of QX-314 mimicked the effects of

ablation of TRPV1-expressing afferents on bone cancer pain. Thus, our study suggests that QX-314 has analgesic effects through the inhibition of TRPV1-expressing afferents. Some mechanisms underlying selective inhibition of TRPV1-expressing neurons by QX-314 have been proposed. One is that QX-314 directly permeates through the pores of activated TRPV1 channels.[14,40] It has been suggested that the pores of TRPV1 channels are large enough to allow permeation of compounds as large as the dyes YO-PRO (molecular mass of 375 Da) and FM1-43 (molecular mass of 452 Da).[41,42] Since the molecular mass of QX-314 is 263 Da, QX-314 can enter through the pores of TRPV1 channels. Another possible mechanism is that QX-314 enters through a pathway activated secondarily by TRPV1 activation, not through the pores of TRPV1. In the case of P2X7 purinergic receptors, it has been suggested that large molecules including YO-PRO enter through large-pore pannexin channels linked to the activation of P2X7 purinergic receptors.[43] However, it has not been shown for TRPV1 yet. Another possible mechanism is enhanced permeability of TRPV1 to large cations following sustained chemical stimuli including stimulus by capsaicin, so-called "pore dilation".[44] In any case, activation of TRPV1 appears to facilitate the permeation of QX-314 into nerve fibers.

We and other researchers have shown that TRPV1 activation is involved in bone cancer pain.[23,24,45] TRPV1 is present on sensory fibers in mineralized bone and bone marrow.[45] Tumor growth and expansion induce inflammation and ischemia due to destruction of the microvasculature, resulting in an acidic environment. In addition, bone cancer generally activates osteoclasts, as previously shown in a bone cancer pain model.[46] Activated osteoclasts maintain an extracellular microenvironment of low pH (4.0-5.0) to resorb bone.[47] An acidic environment can activate TRPV1.[15] Moreover, chemical mediators released from tumor cells and inflammatory cells, including endothelin-1, nerve growth factor, and prostaglandins, can sensitize TRPV1.[16,48,49] Sensitized TRPV1 can be tonically activated at normal body temperature,[16] resulting in occurrence of spontaneous pain. Thus, QX-314 can have an inhibitory effect on TRPV1-expressing afferents, since TRPV1 is tonically activated in a bone cancer pain condition.

Clinical implication

Some previous studies have shown that activation of TRPV1 by agonists including capsaicin is needed to evoke local anesthetic effects of QX-314.[14,17] Intense pain associated with capsaicin injection limits the clinical use of QX-314. The present study has shown that QX-314 has analgesic effects on bone cancer pain without co-administration of any TRPV1 agonists. These results are consistent with results of previous studies showing that TRPV1 agonist application is not needed to evoke local anesthetic effects of QX-314.[18,19,50]

A previous study has also shown that the intravenous quaternary lidocaine derivatives QX-222 and QX-314 inhibit nerve injury-induced thermal hyperalgesia, but not mechanical hypersensitivity, without co-administration of TRPV1 agonists.[20] It has been shown that TRPV1 activation was involved in nerve injury-induced thermal hyperalgesia.[51] Thus, systemic administration of QX-314 may produce local anesthetic effects without co-administration of TRPV1 agonists in painful conditions in which TRPV1 is activated, as well as in bone cancer pain.

Previous studies have shown that when TRPV1 is activated by capsaicin or acid solution, local administration of QX-314 hardly impairs motor function.[14,52] In the present study, although we did not examine the effects of QX-314 on motor function and tactile sensation, systemic administration of QX-314 did not worsen limb use during spontaneous ambulation and weight-bearing compared to those before administration. Reduction of pain by QX-314 with almost no impairment of motor function and tactile sensation is attractive for pain management.

Finally, it is well known that in humans, capsaicin-sensitive afferents are involved in perception of mechanical pain as well as heat pain and inflammatory pain, so-called polymodal afferents.[53-55] Therefore, in contrast to mice, QX-314 may have analgesic effects on movement-evoked pain as well as ongoing pain in patients with bone cancer pain.

Study limitations

One of the weaknesses of the present study is that toxicity endpoints were not investigated. It has been hypothesized that QX-314 has less central nervous system and cardiac toxicity than that of

conventional tertiary aminoamines, since QX-314 does not rapidly penetrate a biological membrane or blood-brain barrier.[12,13,20,21] To the contrary, as mentioned in the *"Analgesic effects of QX-314 and mechanisms of bone cancer pain"* section in Discussion, it has been shown that the relative potency of QX-314 for systemic cardiac toxicity in mice is significantly higher than that of lidocaine (ED50 of QX-314, 10.6 mg/kg vs. ED50 of lidocaine, 21.2 mg/kg).[39] Our preliminary study also showed that more than 5 mg/kg of QX-314 caused collapse-like behaviors in some mice. Although we did not investigate the plasma concentrations of QX-314 at time points of collapse-like behaviors, the plasma concentrations of QX-314 for systemic toxicity may be less than those of lidocaine. Since QX-314 was detected in plasma after its i.p. or s.c. administration in our study, biological membranes were not completely impermeable to QX-314, at least in a mouse model of bone cancer pain, resulting in systemic toxicity at relatively low plasma concentrations. In addition, we observed definite abnormal behaviors after i.t. capsaicin injection, which were very similar to irritable behaviors caused by i.t. QX-314 injection.[32] Since it has been shown that more than 10 mM of QX-314 directly activates TRPV1 *in vitro*,[56] i.t. QX-314 injection at a dose of 10 mM would activate TRPV1, probably resulting in the same abnormal behavior or death. Therefore, QX-314-induced systemic toxicity may also, at least in part, be caused by activation of TRPV1. We recognize that extrapolation of effective or toxic doses of QX-314 from our mouse model to human patients requires caution. From the point of view of potential translation to human application of QX-314, further study is needed to determine not only efficacy endpoints but also toxicity endpoints.

A previous study showed that ablation of TRPV1-containing afferents including substance P- or CGRP-positive afferents by systemic s.c. capsaicin has an impact on bone remodeling in neonate rats.[57] Although we did not investigate whether ablation of TRPV1-expressing primary afferents has no impact on bone remodeling, i.t. capsaicin selectively ablated the central terminals of TRPV1-expressing primary afferents **(fig. 7A)** and did not reduce the number of TRPV1-positive DRG neurons **(fig. S**) as was observed in a previous study in mice.[26] Since i.t. capsaicin binds only to neurons existing intrathecally, peripheral terminals of TRPV1-expressing afferents in the bone are probably not ablated. I.t. capsaicin may thus have no impact on bone remodeling.

Finally, our study included male animals only. We thus cannot comment on possible sex differences as far as QX-314's actions are concerned.

Conclusion

In conclusion, our study has shown that systemic administration of QX-314 inhibits bone cancer-induced ongoing pain, which seriously affects the quality of life of patients, through selective inhibition of TRPV1-expressing afferents. Our results suggest that if generation of pain depends on TRPV1 activation in some pathophysiological conditions, QX-314 would be effective without co-administration of TRPV1 agonists.

References

1. Dijstra S, Wiggers T, van Geel BN, Boxma H: Impending and actual pathological fractures in patients with bone metastases of long bones. A retrospective study of 233 surgically treated fractures. Eur J Surg 1994; 160:535-42
2. Harrington KD: Orthopedic surgical management of skeletal complications of malignancy. Cancer 1997; 80:1614-27
3. Clohisy DR, Mantyh PW: Bone cancer pain. Cancer 2003; 97:866-73
4. Strang P: Emotional and social aspects of cancer pain. Acta Oncol 1992; 31:323-6
5. Beck SL, Dudley WN, Barsevick A: Pain, sleep disturbance, and fatigue in patients with cancer: using a mediation model to test a symptom cluster. Oncol Nurs Forum 2005; 32:542
6. Schug SA, Zech D, Dörr U: Cancer pain management according to WHO analgesic guidelines. J Pain Symptom Manage 1990; 5:27-32
7. Zech DF, Grond S, Lynch J, Hertel D, Lehmann KA: Validation of World Health Organization Guidelines for cancer pain relief: a 10-year prospective study. Pain 1995; 63:65-76
8. Mercadante S: Malignant bone pain: pathophysiology and treatment. Pain 1997; 69:1-18
9. Banning A, Sjogren P, Henriksen H: Treatment outcome in a multidisciplinary cancer pain clinic. Pain 1991; 47:129-34
10. Yamamoto J, Kawamata T, Niiyama Y, Omote K, Namiki A: Down-regulation of mu opioid receptor expression within distinct subpopulations of dorsal root ganglion neurons in a murine model of bone cancer pain. Neuroscience 2008; 151:843-53
11. Portenoy RK, Lesage P: Management of cancer pain. Lancet 1999; 353:1695-700
12. Strichartz GR: The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. J Gen Physiol 1973; 62:37-57
13. Frazier DT, Narahashi T, Yamada M: The site of action and active form of local anesthetics. II. Experiments with quaternary compounds. J Pharmacol Exp Ther 1970; 171:45-51
14. Binshtok AM, Bean BP, Woolf CJ: Inhibition of nociceptors by TRPV1-mediated entry of

impermeant sodium channel blockers. Nature 2007; 449:607-10

15. Tominaga M, Caterina MJ, Malmberg AB, Rosen TA, Gilbert H, Skinner K, Raumann BE, Basbaum AI, Julius D: The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron 1998; 21:531-43

16. Tominaga M, Tominaga T: Structure and function of TRPV1. Pflugers Arch 2005; 451:143-50

17. Kim HY, Kim K, Li HY, Chung G, Park CK, Kim JS, Jung SJ, Lee MK, Ahn DK, Hwang SJ, Kang Y, Binshtok AM, Bean BP, Woolf CJ, Oh SB: Selectively targeting pain in the trigeminal system. Pain 2010; 150:29-40

18. Sagie I, Kohane DS: Prolonged sensory-selective nerve blockade. Proc Natl Acad Sci U S A 2010; 107:3740-5

19. Lim TK, Macleod BA, Ries CR, Schwarz SK: The quaternary lidocaine derivative, QX-314, produces long-lasting local anesthesia in animal models in vivo. Anesthesiology 2007; 107:305-11

20. Chen Q, King T, Vanderah TW, Ossipov MH, Malan TP Jr, Lai J, Porreca F: Differential blockade of nerve injury-induced thermal and tactile hypersensitivity by systemically administered brain-penetrating and peripherally restricted local anesthetics. J Pain 2004; 5:281-9

21. Omana-Zepata I, Khabbaz MA, Hunter JC, Bley KR: QX-314 inhibits ectopic nerve activity associated with neuropathic pain. Brain Res 1997; 771:228-37

22. Kawamata M, Sugino S, Narimatsu E, Yamauchi M, Kiya T, Furuse S, Namiki A: Effects of systemic administration of lidocaine and QX-314 on hyperexcitability of spinal dorsal horn neurons after incision in the rat. Pain 2006; 122:68-80

23. Niiyama Y, Kawamata T, Yamamoto J, Omote K, Namiki A: Bone cancer increases transient receptor potential vanilloid subfamily 1 expression within distinct subpopulations of dorsal root ganglion neurons. Neuroscience 2007; 148:560-72

24. Niiyama Y, Kawamata T, Yamamoto J, Furuse S, Namiki A: SB366791, a TRPV1 antagonist, potentiates analgesic effects of systemic morphine in a murine model of bone cancer pain. Br J Anaesth 2009; 102:251-8

25. Honore P, Luger NM, Sabino MA, Schwei MJ, Rogers SD, Mach DB, O'keefe PF, Ramnaraine ML, Clohisy DR, Manthy PW: Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of spinal cord. Nat Med 2000; 6:521-8
26. Cavanaugh DJ, Lee H, Lo L, Shields SD, Zylka MJ, Basbaum AI, Anderson DJ: Distinct subsets of unmyelinated primary sensory fibers mediate behavioral responses to noxious thermal and mechanical stimuli. Proc Natl Acad Sci U S A 2009; 106:9075-80
27. Kawamata T, Niiyama Y, Yamamoto J, Furuse S: Reduction of bone cancer pain by CB1 activation and TRPV1 inhibition. J Anesth 2010; 24:328-32
28. Sevcik MA, Ghilardi JR, Peters CM, Lindsay TH, Halvorson KG, Jonas BM, Kubota K, Kuskowski MA, Boustany L, Shelton DL, Manthy PW: Anti-NGF therapy profoundly reduces bone cancer pain and the accompanying increase in markers of peripheral and central sensitization. Pain 2005; 115:128-41
29. Batka RJ, Brown TJ, Mcmillan KP, Meadows RM, Jones KJ, Haulcomb MM: The need for speed in rodent locomotion analyses. Anat Rec (Hoboken) 2014; 297:1839-64
30. Peters CM, Ghilardi JR, Keyser CP, Kubota K, Lindsay TH, Luger NM, Mach DB, Schwei MJ, Sevcik MA, Manthy PW: Tumor-induced injury of primary afferent sensory nerve fibers in bone cancer pain. Exp Neurol 2005; 193:85-100
31. Shields SD, Cavanaugh DJ, Lee H, Anderson DJ, Basbaum AI: Pain behavior in the formalin test persists after ablation of the great majority of C-fiber nociceptors. Pain 2010; 151:422-9
32. Schwarz SK, Cheung HM, Ries CR, Lee SM, Wang JT, MacLeod BA: Lumbar intrathecal administration of the quaternary lidocaine derivative, QX-314, produces irritation and death in mice. Anesthesiology 2010; 113:438-44
33. Tamura S, Morikawa Y, Senba E: Up-regulated phosphorylation of signal transducer and activator of transcription 3 and cyclic AMP-responsive element binding protein by peripheral inflammation in primary afferent neurons possibly through oncostain M receptor. Neuroscience 2005; 133:797-806
34. Nakanishi M, Hata K, Nagayama T, Sakurai T, Nishisho T, Wakabayashi H, Hiraga T, Ebisu S,

Yoneda T: Acid activation of Trpv1 leads to an up-regulation of calcitonin gene-rerated peptide expression in dorsal root ganglion neurons via the CaMK-CREB cascade: A potential mechanism of inflammatory pain. Mol Biol Cell 2010; 21:2568-77

35. Mach DB, Rogers SD, Sabino MC, Luger NM, Schwei MJ, Pomonis JD, Keyser CP, Clohisy DR, Adams DJ, O'Leary P, Mantyh PW: Origins of skeletal pain: sensory and sympathetic innervation of the mouse femur. Neuroscience 2002; 113:155-66

36. Ambalavanar R, Moritani M, Haines A, Hilton T, Dessem D: Chemical phenotypes of muscle and cutaneous afferent neurons in the rat trigeminal ganglion. J Comp Neurol 2003; 460:167-79

37. Honore P, Rogers SD, Schwei MJ, Salak-Johnson JL, Luger NM, Sabino MC, Clohisy DR, Mantyh PW: Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. Neuroscience 2000; 98:585-98

38. Ma Q: Population cording of somatic sensation. Neurosci Bull 2012; 28:91-9

39. Cheung HM, Lee SM, MacLeod BA, Ries CR, Schwarz SK: A comparison of the systemic toxicity of lidocaine versus its quaternary derivative QX-314 in mice. Can J Anaesth 2011; 58:443-50

40. Puopolo M, Binshtok AM, Yao GL, Oh SB, Woolf CJ, Bean BP: Permeation and block of TRPV1 channels by the cationic lidocaine derivative QX-314. J Neurophysiol 2013; 109:1704-12

41. Banke TG, Chaplan SR, Wickenden AD: Dynamic changes in the TRPA1 selectivity filter lead to progressive but reversible pore dilation. Am J Physiol Cell Physiol 2010; 298:C1457-68

42. Meyers JR, MacDonald RB, Duggan A, Lenzi D, Standaert DG, Corwin JT, Corey DP: Lighting up the senses: FM1-43 loading of sensory cells through nonselective ion channels. J Neurosci 2003; 23:4054-65

43. Pelegrin P, Surprenant A: Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1β release through a dye uptake-independent pathway. J Biol Chem 2007; 282:2386-94

44. Chung MK, Güler AD, Caterina MJ: TRPV1 shows dynamic ionic selectivity during agonist stimulation. Nat Neurosci 2008; 11:555-64

45. Ghilardi JR, Röhrich H, Lindsay TH, Sevcik MA, Schwei MJ, Kubota K, Halvorson KG, Poblete J,

Chaplan SR, Dubin AE, Carruthers NI, Swanson D, Kuskowski M, Flores CM, Julius D, Mantyh PW: Selective blockade of the capsaicin receptor TRPV1 attenuates bone cancer pain. J Neurosci 2005; 25:3126-31

46. Sevcik MA, Luger NM, Mach DB, Sabino MA, Peters CM, Ghilardi JR, Schwei MJ, Röhrich H, De Felipe C, Kuskowski MA, Mantyh PW: Bone cancer pain: the effects of the bisphosphonate alendronate on pain, skeletal remodeling, tumor growth and tumor necrosis. Pain 2004; 111:169-80
47. Delaisse JM, Vaes G: Biology and Physiology of the Osteoclast. Ann Arbor, CPC-Press, 1992, pp 289-314
48. Jimenez-Andrade JM, Mantyh WG, Bloom AP, Ferng AS, Geffre CP, Mantyh PW: Bone cancer pain. Ann N Y Acad Sci 2010; 1198:173-81
49. Zhang X, Huang J, McNaughton PA: NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. EMBO J 2005; 24:4211-23
50. Ries CR, Pillai R, Chung CC, Wang JT, MacLeod BA, Schwarz SK: QX-314 produces long-lasting local anesthesia modulated by transient receptor potential vanilloid receptors in mice. Anesthesiology 2009; 111:122-6
51. Vilceanu D, Honore P, Hogan QH, Stucky CL: Spinal nerve ligation in mouse upregulates TRPV1 heat function in injured IB4 positive nociceptors. J Pain 2010; 11:588-99
52. Liu H, Zhang HX, Hou HY, Lu XF, Wei JQ, Wang CG, Zhang LC, Zeng YM, Wu YP, Cao JL: Acid solution is a suitable medium for introducing QX-314 into nociceptors through TRPV1 channels to produce sensory-specific analgesic effects. PLoS One 2011; 6:e29395
53. Schmelz M, Schmid R, Handwerker HO, Torebjörk HE: Encording of burning pain from capsaicin-treated human skin in two categories of unmyelinated nerve fibers. Brain 2000; 123:560-71
54. Nolano M, Simone DA, Wendelschafer-Crabb G, Johnson T, Hazen E, Kennedy WR: Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation. Pain 1999; 81:135-45
55. Chizh BA, O'Donnell MB, Napolitano A, Wang J, Brooke AC, Aylott MC, Bullman JN, Gray EJ, Lai RY, Williams PM, Appleby JM: The effects of the TRPV1 antagonist SB-705498 on TRPV1

receptor-mediated activity and inflammatory hyperalgesia in humans. Pain 2007; 132:132-41

56. Rivera-Acevedo RE, Pless SA, Ahern CA, Schwarz SK: The quaternary lidocaine derivative, QX-314, exerts biphasic effects on transient receptor potential vanilloid subtype 1 channels in vitro. Anesthesiology 2011; 114:1425-34

57. Hill EL, Turner R, Elde R: Effects of neonatal sympathectomy and capsaicin treatment on bone remodeling in rats. Neuroscience 1991; 44:747-55

Figure legends

Figure 1. Effects of bolus intraperitoneal (i.p.) administration of QX-314 on bone cancer pain-related behaviors. (A), time courses of effects of bolus i.p. administration of QX-314 (0.01, 0.1, 1 and 3 mg/kg) or a vehicle on number of flinches. (B), Dose-response relationship of QX-314 with area under the curve (AUC) of flinches. QX-314 significantly reduced the number of flinches in a dose-dependent manner (A and B). In spontaneous flinches; n = 8 for vehicle; n = 6 for 0.01 mg/kg of QX-314; n = 9 for 0.1, 1 and 3 mg/kg of QX-314. Data for number of flinches and AUC of flinches are presented as means ± SDs. **P* < 0.001 vs. vehicle, ¶*P* < 0.05 vs. vehicle, #*P* < or = 0.01 vs. 0.01 mg/kg of QX-314, ‡*P* < 0.01 vs. 0.1 mg/kg of QX-314, †*P* < 0.05 vs. 0.1 mg/kg of QX-314 at each observation point.

Figure 2. Effects of bolus intraperitoneal (i.p.) administration of QX-314 on bone cancer pain-related behaviors. (A), time course of effects of 3 mg/kg of QX-314 on scores of limb use. (B), time course of effects of 3 mg/kg of QX-314 on scores of weight-bearing. (C), time course of effects of 3 mg/kg of QX-314 on scores of rotarod performance. (D), effects of 3 mg/kg of QX-314 on Max Contact Area. (E), effects of 3 mg/kg of QX-314 on Mean Intensity of the 15 Most Intense Pixels (Mean Intensity). QX-314 at the highest dose used in this study did not significantly change scores of limb use, weight-bearing, and rotarod performance, Max Contact Area and Mean Intensity compared to the basal values or values with vehicle treatment (A-E). There is no variability in the measures of scores of limb use and weight-bearing (A and B). In limb use and weight-bearing; n = 9 for 3 mg/kg of QX-314. In rotarod performance; n = 10 for 3 mg/kg of QX-314. In Max contact area and Mean Intensity; n = 10 in each group. Data for Max contact area and Mean Intensity are presented as means ± SDs. Data for scores of limb use, weight-bearing, and rotarod performance are presented as medians with interquartile range (IQR). **P* < 0.001 vs. sham, #*P* < 0.001 vs. contralateral side.

Figure 3. Time courses of effects of continuous subcutaneous administration of QX-314 (5 mg/kg/h) or

a vehicle on number of flinches. QX-314 significantly reduced the numbers of flinches compared to that in the case of the vehicle at 24 and 48 h after starting continuous administration. n = 5 in each group. Data for number of flinches are presented as means ± SDs. *$P < 0.001$ vs. vehicle at each observation point.

Figure 4. Effects of bolus intraperitoneal (i.p.) administration of lidocaine on bone cancer pain-related behaviors. (A), time courses of effects of bolus i.p. administration of lidocaine (3 and 10 mg/kg) or a vehicle on number of flinches. (B), time courses of effects of lidocaine (3 and 10 mg/kg) or a vehicle on scores of limb use. (C), time courses of effects of lidocaine (3 and 10 mg/kg) or a vehicle on scores of weight-bearing. Three mg/kg of lidocaine was not effective and 10 mg/kg of lidocaine significantly reduced the number of flinches (A) and improved scores of limb use and weight-bearing (B and C). n = 8 for vehicle; n = 5 for 3 mg/kg of lidocaine; n = 9 for 10 mg/kg of lidocaine. Data for number of flinches are presented as means ± SDs. Data for scores of limb use and weight-bearing are presented as medians with interquartile range (IQR). *$P < 0.01$ vs. vehicle at the observation point, #$P < 0.01$ vs. basal value within a group.

Figure 5. Immunohistochemical staining of transient receptor potential vanilloid subfamily 1 (TRPV1) in the dorsal root ganglion (DRG). (A) and (C) show the DRG of a naive mouse. (B) and (D) show the DRG of a TRPV1 knockout (KO) mouse. Strong labeling in somata and fibers of the DRG neurons was a specific reaction of the anti-TRPV1 antibody, while dot-like positive labeling observed in the nucleus was a non-specific reaction. Scale bar = 50 μm. (C) and (D) are higher powered magnification of the areas in (A) and (B) as indicated by the boxes, respectively.

Figure 6. Effects of continuous subcutaneous (s.c.) administration of QX-314 on phosphorylated cyclic-adenosine monophosphate response element-binding protein (p-CREB) expression in L2 dorsal root ganglion (DRG) neurons of sarcoma-implanted mice. (A), immunohistochemical staining for transient receptor potential vanilloid subfamily 1 (TRPV1) (*green*) with p-CREB (*red*) in L2 DRG

neurons of sarcoma-implanted mice after continuous s.c. administration of a vehicle or QX-314 (5 mg/kg/h) for 48 h. White arrows indicate p-CREB-immunoreactive neurons expressed in TRPV1-immunoreactive neurons. Scale bar = 50 μm. (B), percentages of p-CREB-positive profiles in TRPV1-positive DRG neurons. n = 3 for naive mice, n = 6 in the other groups. (C), percentages of p-CREB-positive profiles in TRPV1-negative DRG neurons. n = 3 for naive mice, n = 6 in the other groups. Data for percentages of p-CREB-positive profiles in TRPV1-positive DRG neurons or in TRPV1-negative DRG neurons are presented as means ± SDs. #$P < 0.001$ vs. sham. *$P < 0.05$ vs. sham. **$P < 0.001$ vs. vehicle.

In naive mice, 33.8 ± 2.1% of TRPV1-positive DRG neurons were p-CREB-positive (p-CREB-positive count/total TRPV1-positive count = 658/1948) and 31.1 ± 7.5% of TRPV1-negative DRG neurons were p-CREB-positive (p-CREB-positive count/total TRPV1-negative count = 867/2787) (B and C). These results represent averaged proportions obtained from 27 DRG sections.

Sarcoma implantation, but not sham implantation, significantly increased the expression of p-CREB in TRPV1-positive and TRPV1-negative DRG neurons (B and C). QX-314 reduced p-CREB expression in TRPV1-positive, but not TRPV1-negative, DRG neurons (B and C). In QX-314-treated mice, 32.2 ± 3.0% of TRPV1-positive DRG neurons were p-CREB-positive (p-CREB-positive count/total TRPV1-positive count = 926/2887), and in vehicle-treated mice, 52.6 ± 5.9% of TRPV1-positive DRG neurons were p-CREB-positive (p-CREB-positive count/total TRPV1-positive count = 1703/3266). In QX-314-treated mice, 41.7 ± 4.5% of TRPV1-negative DRG neurons were p-CREB-positive (p-CREB-positive count/total TRPV1-negative count = 1586/3777), and in vehicle-treated mice, 42.1 ± 4.7% of TRPV1-negative DRG neurons were p-CREB-positive (p-CREB-positive count/total TRPV1-negative count = 2146/5107). These results represent averaged proportions obtained from 54 DRG sections in QX-314-treated mice and 49 DRG sections in vehicle-treated mice.

Figure 7. Effects of intrathecal (i.t.) capsaicin on expression of transient receptor potential vanilloid subfamily 1 (TRPV1)-expressing primary afferents and analgesic effects of ablation of

TRPV1-expressing primary afferents. (A), immunohistochemical staining for TRPV1 (*red*) or biotinylated isolectin B4 (IB4) (*green*) in the spinal cords of sarcoma-implanted mice treated with i.t. capsaicin (upper panels) and of sarcoma-implanted mice treated with i.t. vehicle (lower panel). I.t. capsaicin ablated TRPV1-expressing, but not IB4-binding, primary afferents in the dorsal horn of the L2 lumbar spinal cord. Scale bar = 50 μm. (B), effects of ablation of TRPV1-expressing primary afferents on number of flinches. (C), effects of ablation of TRPV1-expressing primary afferents on scores of limb use. (D), effects of ablation of TRPV1-expressing primary afferents on scores of weight-bearing. (E), effects of ablation of TRPV1-expressing primary afferents on scores of rotarod performance. (F), effects of ablation of TRPV1-expressing primary afferents on Max Contact Area. (G), effects of ablation of TRPV1-expressing primary afferents on Mean Intensity of the 15 Most Intense Pixels (Mean Intensity). I.t. capsaicin significantly reduced the number of flinches compared to i.t. vehicle treatment and did not show any significant difference in the number of flinches compared to sham implantation (B). On the other hand, the scores of limb use, weight-bearing, and rotarod performance, Max Contact Area and Mean Intensity in mice treated with i.t. capsaicin were comparable to those in mice treated with i.t. vehicle (C-G). In spontaneous flinches, limb use, and weight bearing; n = 5 in i.t. vehicle; n = 10 in i.t. capsaicin; n = 7 in sham. In rotarod performance, Max Contact Area, and Mean Intensity; n = 10 in each group. Data for number of flinches, Max Contact Area, and Mean Intensity are presented as means ± SDs. Data for scores of limb use, weight-bearing, and rotarod performance are presented as medians with interquartile range (IQR). In spontaneous flinches; $*P < 0.001$ vs. i.t. vehicle. In Max contact area and Mean Intensity; $*P < 0.001$ vs. sham, $\#P < 0.001$ vs. contralateral side.

Figure 8. Hematoxylin and eosin **(**H&E) staining of a long-axis cross-sectional sarcoma-bearing femur 14 days after sarcoma implantation. (A), sarcoma-bearing femur of mice treated with intrathecal (i.t.) vehicle or i.t. capsaicin. Area surrounded by dashed line shows intramedullary space occupied by sarcoma cells. Scale bar = 1 mm. (B), percentages of intramedullary space occupied by sarcoma cells in mice treated with i.t. vehicle or i.t. capsaicin at 14 days after sarcoma implantation. There was no significant

difference in the percentages of intramedullary space occupied by sarcoma cells between mice treated with i.t. capsaicin and mice treated with i.t. vehicle. n = 7 in i.t. vehicle, n = 9 in i.t. capsaicin. Data for percentages of intramedullary space occupied by sarcoma cells are presented as means ± SDs.

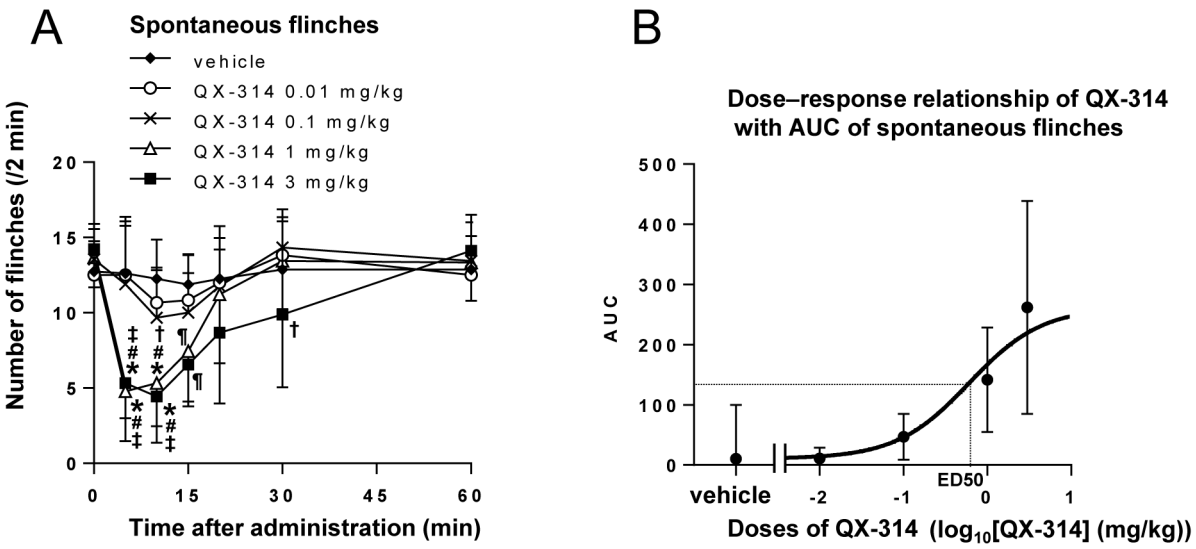

A
Spontaneous flinches
vehicle
QX-314 0.01 mg/kg
QX-314 0.1 mg/kg
QX-314 1 mg/kg
QX-314 3 mg/kg
Number of flinches (/2 min)
Time after administration (min)
B
Dose–response relationship of QX-314
with AUC of spontaneous flinches
AUC
vehicle
ED50
Doses of QX-314 (log10[QX-314] (mg/kg))

Fig. 1

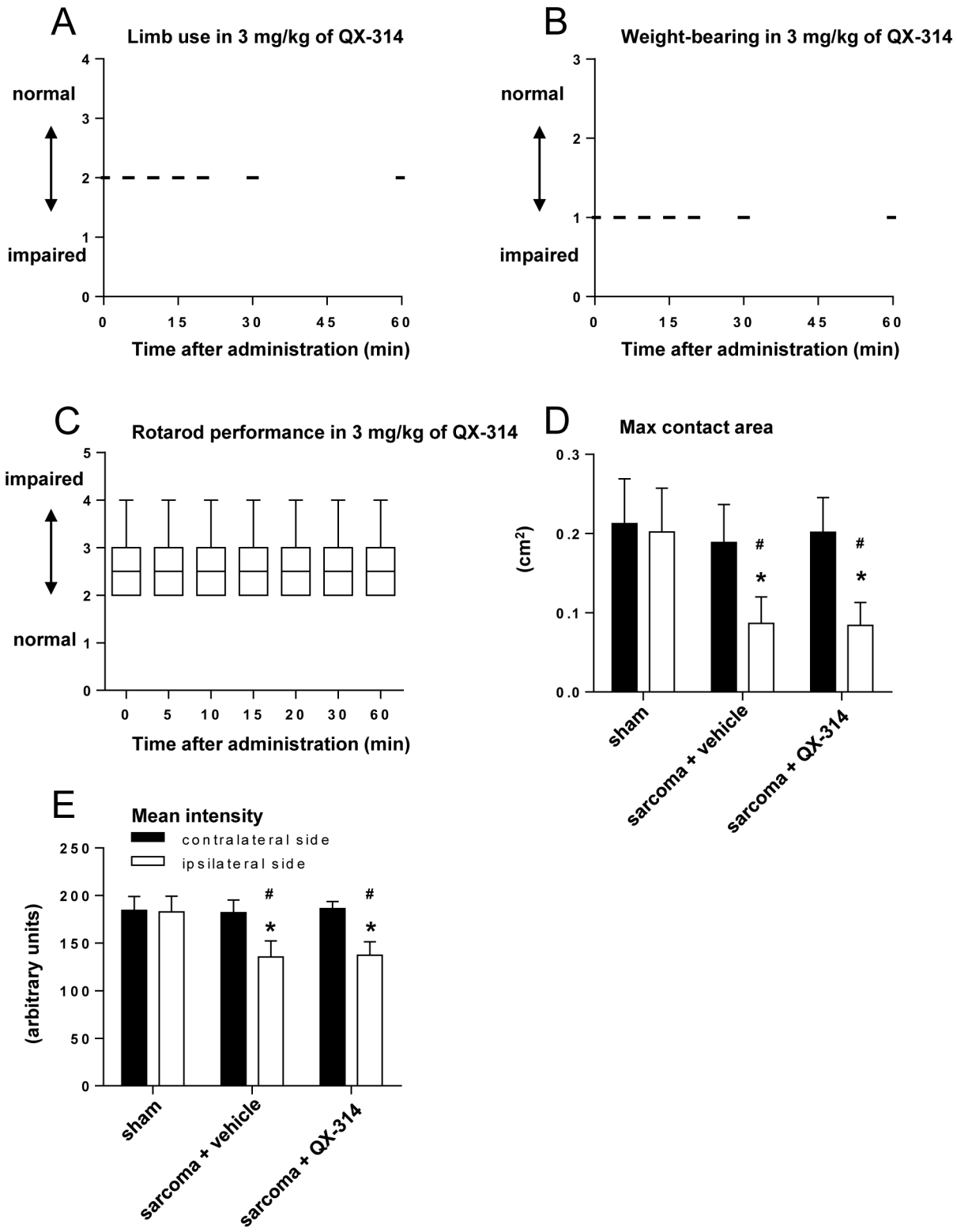

A
Limb use in 3 mg/kg of QX-314
normal
impaired
Time after administration (min)
B
Weight-bearing in 3 mg/kg of QX-314
normal
impaired
Time after administration (min)
C
Rotarod performance in 3 mg/kg of QX-314
impaired
normal
Time after administration (min)
D
Max contact area
(cm²)
sham
sarcoma + vehicle
sarcoma + QX-314
E
Mean intensity
contralateral side
ipsilateral side
(arbitrary units)
sham
sarcoma + vehicle
sarcoma + QX-314

Fig. 2

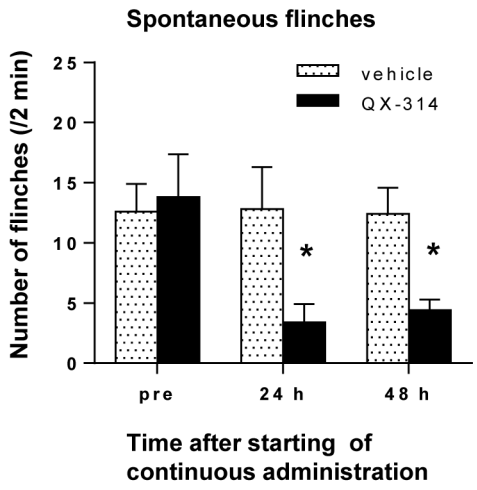

Spontaneous flinches
Number of flinches (/2 min)
25
20
15
10
5
0
vehicle
QX-314
*
*
pre
24 h
48 h
Time after starting of
continuous administration

Fig. 3

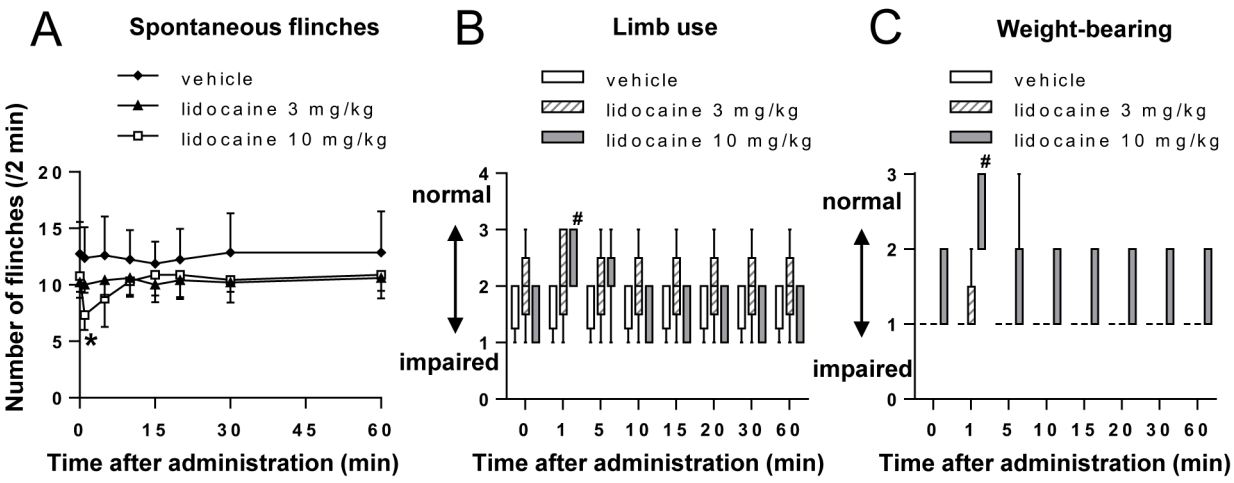

A
Spontaneous flinches
vehicle
lidocaine 3 mg/kg
lidocaine 10 mg/kg
Number of flinches (/2 min)
20
15
10
5
0
*
0
15
30
45
60
Time after administration (min)
B
Limb use
vehicle
lidocaine 3 mg/kg
lidocaine 10 mg/kg
normal
impaired
4
3
2
1
0
#
0
1
5
10
15
20
30
60
Time after administration (min)
C
Weight-bearing
vehicle
lidocaine 3 mg/kg
lidocaine 10 mg/kg
normal
impaired
3
2
1
0
#
0
1
5
10
15
20
30
60
Time after administration (min)

Fig. 4

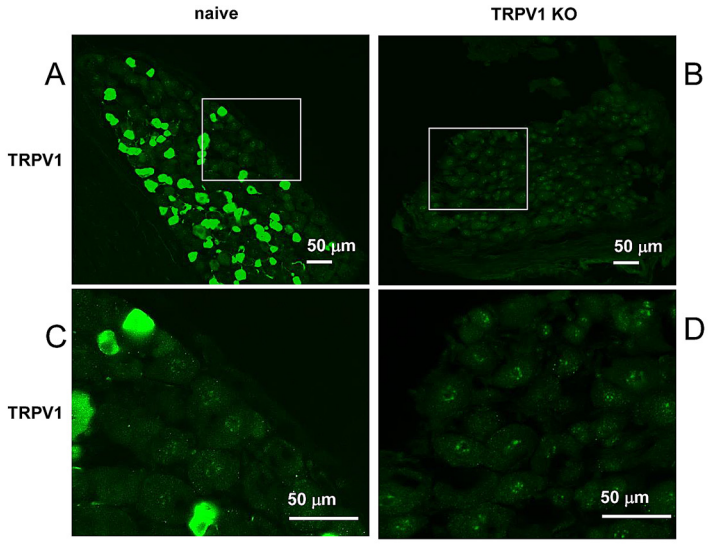


Fig. 5

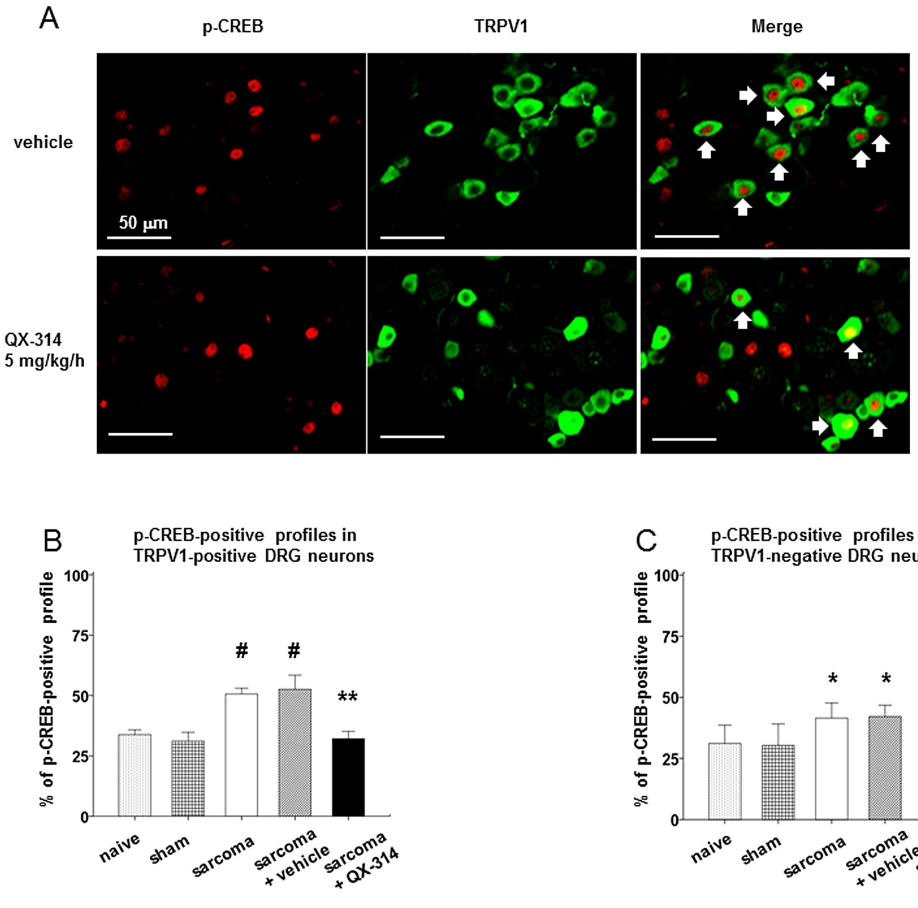

A
p-CREB
TRPV1
Merge
vehicle
QX-314
5 mg/kg/h
50 µm
B
p-CREB-positive profiles in
TRPV1-positive DRG neurons
% of p-CREB-positive profile
100
75
50
25
0
#
#
**
naive
sham
sarcoma
sarcoma + vehicle
sarcoma + QX-314
C
p-CREB-positive profiles in
TRPV1-negative DRG neurons
% of p-CREB-positive profile
100
75
50
25
0
*
*
*
naive
sham
sarcoma
sarcoma + vehicle
sarcoma + QX-314

Fig. 6

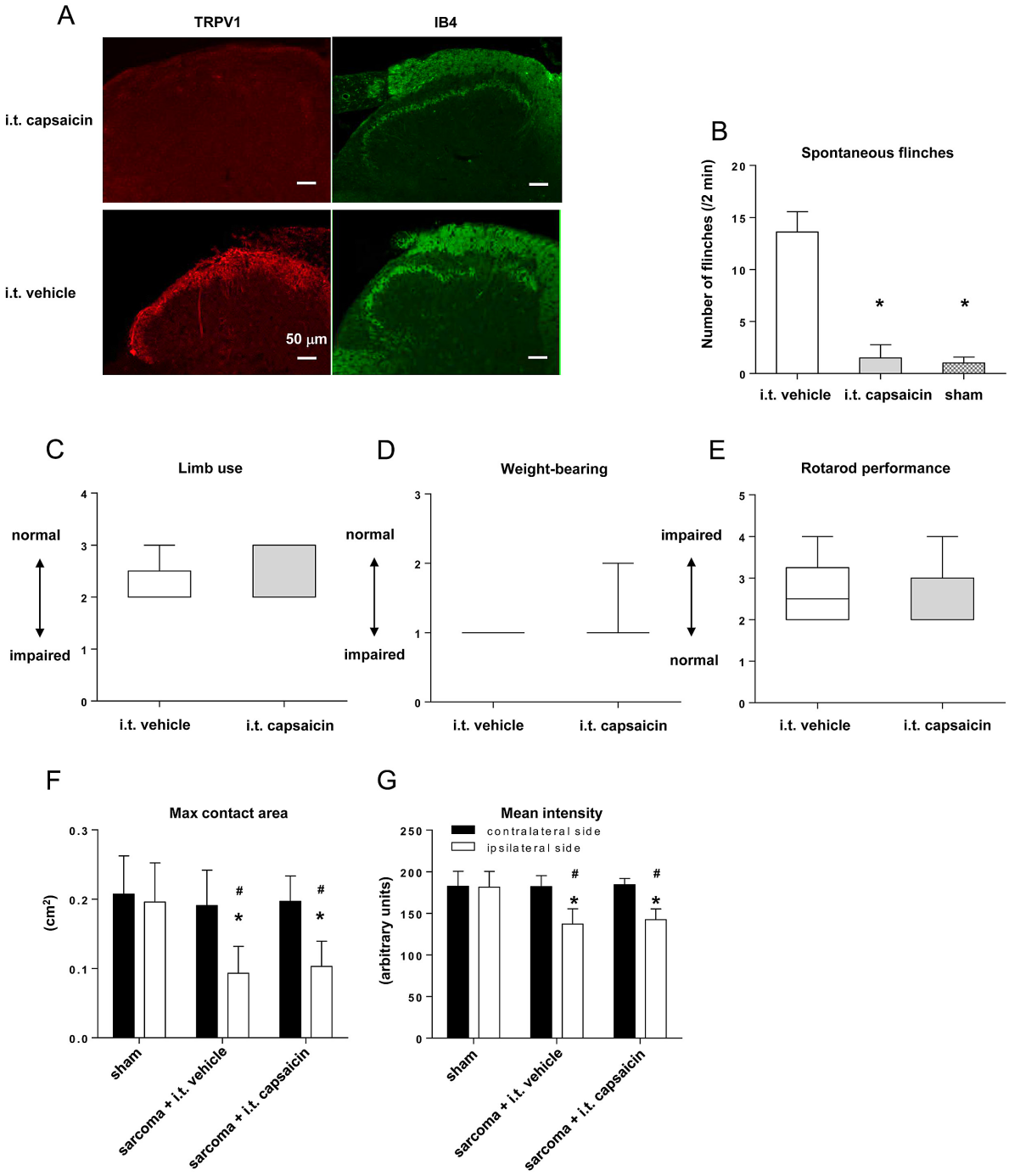
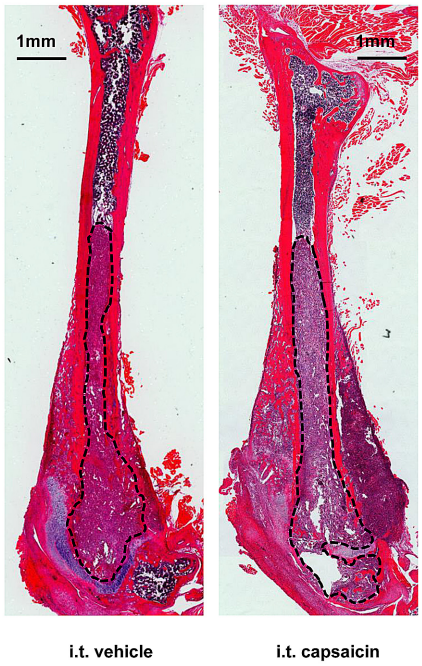

A
TRPV1
IB4
i.t. capsaicin
i.t. vehicle
50 μm
B
Spontaneous flinches
Number of flinches (/2 min)
i.t. vehicle
i.t. capsaicin
sham
C
Limb use
normal
impaired
i.t. vehicle
i.t. capsaicin
D
Weight-bearing
normal
impaired
i.t. vehicle
i.t. capsaicin
E
Rotarod performance
impaired
normal
i.t. vehicle
i.t. capsaicin
F
Max contact area
(cm²)
sham
sarcoma + i.t. vehicle
sarcoma + i.t. capsaicin
G
Mean intensity
contralateral side
ipsilateral side
(arbitrary units)
sham
sarcoma + i.t. vehicle
sarcoma + i.t. capsaicin

Fig. 7

A


H&E staining of the tumor-bearing femur
1mm
1mm
i.t. vehicle
i.t. capsaicin

B

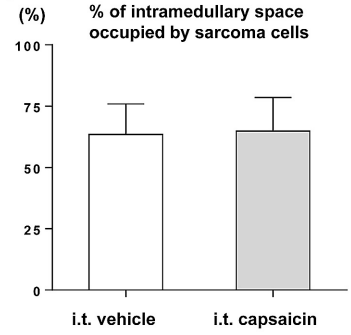

(%)
% of intramedullary space occupied by sarcoma cells
100
75
50
25
0
i.t. vehicle
i.t. capsaicin

Fig. 8

Fig. S

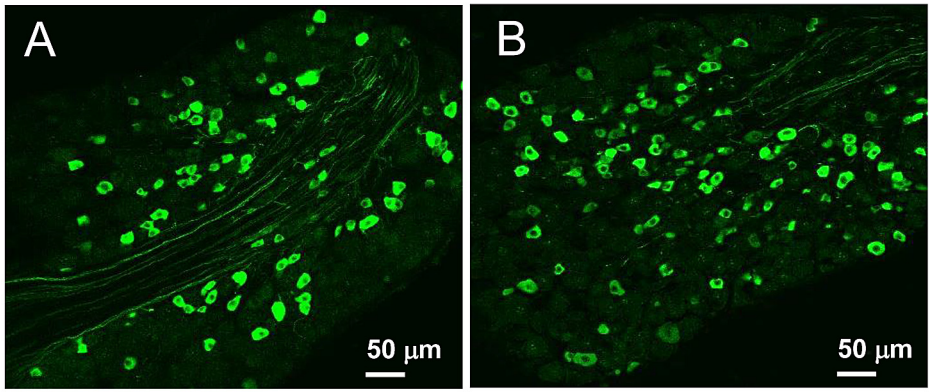


Figure S. Immunohistochemical staining of transient receptor potential vanilloid subfamily 1 (TRPV1) (*green*) in the L2 dorsal root ganglion (DRG). (A) shows the DRG of a sarcoma-implanted mouse treated with intrathecal (i.t.) vehicle. (B) shows the DRG of a sarcoma-implanted mouse treated with i.t. capsaicin. I.t. capsaicin did not reduced the number of TRPV1-positive DRG neurons in the L2 DRG. Scale bar = 50 μm.