Systemic QX-314 Reduces Bone Cancer Pain through Selective Inhibition of Transient Receptor Potential Vanilloid Subfamily 1–expressing Primary Afferents in Mice

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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. The authors examined the effects of bolus (0.01 to 3 mg/kg, n = 6 to 10) and continuous (5 mg kg\(^{-1}\) h\(^{-1}\), n = 5) administration of QX-314 on both bone cancer pain–related behaviors and phosphorylated cyclic adenosine monophosphate response element–binding protein expression in dorsal root ganglion 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 to 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 phosphorylated cyclic adenosine monophosphate response element–binding protein expression in TRPV1-positive, but not in TRPV1-negative, dorsal root ganglion 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: This 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 coadministration of TRPV1 agonists.

What We Already Know about This Topic

- In rodents, behavioral evidence of pain from bone cancer is dependent on activation of transient receptor potential vanilloid subfamily 1 ion channels in sensory nerves
- The quaternary lidocaine derivative QX-314 can enter sensory nerves through transient receptor potential vanilloid subfamily 1 channels when they are stimulated

What This Article Tells Us That Is New

- In mice with behavioral evidence of pain and dysfunction after injection of cancer cells in bone, systemic QX-314, but not lidocaine, produced a long-lasting reduction in spontaneous flinching behavior by inhibiting transient receptor potential vanilloid subfamily 1–expressing nerves, but did not reduce activity-dependent disruption of behaviors

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 increase.\textsuperscript{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.\textsuperscript{14} 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. Since TRPV1 is expressed in nociceptive neurons,\textsuperscript{15,16} QX-314 could selectively inhibit pain transmitted by TRPV1-expressing afferents with almost no impairment of motor function and proprioceptive sensation,\textsuperscript{14,17} although QX-314 administered at a dose of more than 25 mM produces both motor blockade and sensory blockade.\textsuperscript{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 coadministration of TRPV1 agonists. Previous studies have shown that systemic QX-314 inhibits thermal hyperalgesia\textsuperscript{20} and ectopic discharges from neuromas in dorsal root ganglion (DRG) neurons after nerve injury\textsuperscript{21} and suppresses increased activity of spinal dorsal horn neurons after skin incision.\textsuperscript{22} We previously reported that TRPV1 activation is involved in bone cancer pain.\textsuperscript{23,24} Thus, we hypothesized that systemic QX-314 relieves bone cancer pain through inhibition of TRPV1-expressing primary afferents without coadministration of TRPV1 agonists.

In this study, using a mouse model of bone cancer pain,\textsuperscript{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 to 25 g, Japan SLC, 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 (USA). QX-314 and lidocaine were dissolved and diluted in physiologic saline. Capsaicin was dissolved in 10% ethanol (vol/vol), 10% Tween 80 (vol/vol), and saline.\textsuperscript{26}

**Bone Cancer Model**

Murine sarcoma cells (NCTC 2472; ATCC, USA) were maintained in NCTC 135 medium containing 10% horse serum (HyClone, USA) and passaged weekly according to ATCC® recommendations. An injection of sarcoma cells was performed according to a previously described method.\textsuperscript{25} Mice were anesthetized with halothane (2% in 100% O\textsubscript{2}).

The following experiments were conducted at 14 to 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.\textsuperscript{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 × 20 × 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.\textsuperscript{23–25} 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 rotator 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., The Netherlands) for more quantitative measures of movement-evoked pain according to previously described methods.\textsuperscript{29} Briefly, mice were allowed to 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/s (model: GP-2360C, GEViCAM, USA). 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

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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 pretrained 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, Sapporo, Japan) and phosphorylated cyclic adenosine monophosphate response element–binding protein (p-CREB; 1:50, #9198, rabbit; Cell Signaling, USA). We also used biotinylated isocerin 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 to 25 g, Jackson Laboratory, USA) in addition to C3H/HeJ mice. Immunohistochemistry was performed according to a previously described method. Mice were deeply anesthetized with urethane (1.25 g/kg intraperitoneally). The left L2 DRG and spinal cord innervated by the L1 to L3 dorsal roots were removed, since a previous study has shown that L1 to L3 DRG neurons innervate the femur. Frozen samples of DRGs and spinal cords were cut at 16 and 50 μm, respectively, by using a sliding cryostat (LEICA, Japan). Photographs were taken with a confocal laser scanning microscope (ECLIPSE C1, Nikon, 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 to visualize histologic features of the bone marrow and tumor. Photographs were taken with an inverted microscope (Axio Observer Z1, Zeiss, 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, 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. 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 5,000 revolutions per minute (rpm) for 10 min at 4°C and was immediately stored at −80°C until used for analysis. Fifty microliters 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 rpm for 5 min at 20°C and then filtrated (0.44 μm, Ultrafree-MC Centrifugal Filter Units, USA). After fivefold 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, 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 length and 4.6-mm diameter (Shi-seido, Japan). The temperature was maintained 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⁻¹ h⁻¹ of QX-314 or a vehicle. Sarcoma-implanted mice received continuous subcutaneous administration of QX-314. An Alzet® Micro-Osmotic Pump (length, 1.5 cm; diameter, 0.6 cm; model: 1003D, DURECT, USA) was used for continuous subcutaneous administration of drugs. After behavioral
assessments were performed at 24 and 48 h after starting continuous subcutaneous 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\(^{-1}\) h\(^{-1}\) of QX-314, blood samples were collected 24 or 48 h after starting continuous subcutaneous 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). 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-negative or TRPV1-positive neurons was determined by counting 1,500 to 2,000 neuronal profiles from 7 to 11 DRG sections for each mouse. Because a stereologic 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 capsaicin injection, and that the effects of ablation of TRPV1-expressing afferents persisted for at least 8 weeks. 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 intrathecal capsaicin or a vehicle. During anesthesia with halothane (2% in 100% O\(_2\)), mice received intrathecal 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. Immediately after intrathecal 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 intrathecal QX-314 injection. Seven days after intrathecal 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 intrathecal 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. The percentages of intramedullary space occupied by tumors were compared using the unpaired Student’s t test between two groups. The number of flinches was compared to the basal value by using one-way ANOVA for repeated measures followed by Tukey 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 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 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 Friedman test followed by Dunn 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 test.

Results

Effects of Bolus Administration of QX-314 on Bone Cancer Pain–related Behaviors

First, we examined the effects of bolus intraperitoneal administration of QX-314 on bone cancer pain–related behaviors (figs. 1A 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 (figs. 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 at 5 to 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 0.1 mg/kg of QX-314, ‡$P < 0.01$ vs 0.1 mg/kg of QX-314, §$P < 0.01$ vs 0.01 mg/kg of QX-314, and ¶$P < 0.05$ vs vehicle). Analysis of AUCs also showed a dose-dependent effect of QX-314 (fig. 1B, $P < 0.001$). The median effective dose ($ED_{50}$) 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 the effects of continuous...
Fig. 2. Effects of bolus intraperitoneal 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, 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. *P < 0.001 versus sham, #P < 0.001 versus contralateral side.
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⁻¹ h⁻¹) 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 median scores of limb use and weight-bearing (limb use: 2 [interquartile range, 2–2], n = 5 in QX-314–treated mice and 2 [2–2], n = 5 in vehicle-treated mice at 24 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 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⁻¹ h⁻¹ 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⁻¹ h⁻¹ of QX-314. Effects of Bolus Administration of Lidocaine on Bone Cancer Pain–related Behaviors

We also examined the effects of bolus intraperitoneal administration of lidocaine on bone cancer pain–related behaviors as a potentially positive control. Three milligrams per kilogram 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, hope33,34 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 nonspecific 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. 6B, *P < 0.05 vs. sham). Continuous subcutaneous administration of QX-314 reduced p-CREB expression in TRPV1-positive, but not in TRPV1-negative, DRG neurons. QX-314 at a dose of 5 mg kg⁻¹ h⁻¹ 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. 7A, and Supplemental Digital Content, http://links.lww.com/ALN/B273). Immunohistochemistry confirmed that intrathecal capsaicin had ablated TRPV1-expressing primary afferents in the lumbar spinal cord at 21 days after intrathecal injection (fig. 7A), as shown in a previous study,26 while intrathecal capsaicin did not reduce the number of TRPV1-positive DRG neurons (Supplemental fig., Supplemental Digital Content, http://links.lww.com/ALN/B273). Ablation of the central terminals of TRPV1-expressing primary afferents was observed from the upper thoracic region to lower sacral region (data not shown). Intrathecal vehicle did not affect expression of TRPV1-expressing primary afferents in the dorsal horn (fig. 7A). Intrathecal capsaicin did not affect expression of IB4-binding primary afferents in the lumbar spinal cord at 21 days after intrathecal injection (fig. 7A), as shown in a previous study,26 while intrathecal capsaicin did not reduce the number of TRPV1-positive DRG neurons (Supplemental fig., Supplemental Digital Content, http://links.lww.com/ALN/B273). Ablation of the central terminals of TRPV1-expressing primary afferents was observed from the upper thoracic region to lower sacral region (data not shown). Intrathecal vehicle did not affect expression of TRPV1-expressing primary afferents in the dorsal horn (fig. 7A). 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afferents, which rarely express TRPV1 in mice,23 selectively ablating TRPV1-expressing primary afferents (fig. 7A).

Mice treated with intrathecal 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 intrathecal vehicle. Mice treated with intrathecal capsaicin (n = 10) exhibited a significantly smaller number of flinches (2 ± 1, \( *P < 0.001 \)) than that of mice treated with intrathecal 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...
Fig. 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 isolecitin B4 (IB4; green) in the spinal cords of sarcoma-implanted mice treated with i.t. capsaicin (upper) and of sarcoma-implanted mice treated with i.t. vehicle (lower). 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. In spontaneous flinches, *P < 0.001 versus i.t. vehicle. In max contact area and mean intensity, *P < 0.001 versus sham and #P < 0.001 versus contralateral side.
with intrathecal capsaicin were comparable to those in mice treated with intrathecal vehicle (fig. 7, C–G).

Hematoxylin and eosin 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 intrathecal capsaicin and mice treated with intrathecal 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 to 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. On the other hand, nociceptors that innervate the skin richly include both CGRP- and IB4-positive C-fibers. TRPV1 is mainly expressed in CGRP-positive C-fibers, but in very few IB4-positive C-fibers in mice. It has been shown that bone cancer proliferating in the femur causes central sensitization, resulting in mechanical allodynia of the plantar surface. 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 hind paw on the ipsilateral side. IB4-positive fibers may play an important role in transmitting bone cancer–induced referred pain and referred

Fig. 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 and n = 9 in i.t. capsaicin. Data for percentages of intramedullary space occupied by sarcoma cells are presented as means ± SDs.
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. 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 TRPV1-negative afferents each has modality specificity for bone cancer pain in mice. In the current 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. For example, Mas-related G-protein–coupled receptor d–expressing afferents and TRPV1-expressing afferents, which are nonoverlapping populations, are selectively involved in the senses of mechanical and heat pain, respectively. Our p-CREB experiment showed that the percentage of activated profiles in both TRPV1-positive and TRPV1-negative DRG neurons in sarcoma-implanted mice was significantly higher than that in sham-implanted mice. It has also been shown that unmethylated peptidergic C-fibers and myelinated fibers richly innervate bone tissues, while unmethylated nonpeptidergic C-fibers appear to be absent in bone tissues. Taken together with the results of this study, it seems that unmethylated 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 (see Study Limitations).

Selective Inhibition of TRPV1-expressing Afferents by QX-314 in Bone Cancer Pain Models

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. 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). 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. However, it has not been shown for TRPV1 yet. Another possible mechanism is enhanced permeability of TRPV1 to large cations after sustained chemical stimuli including stimulus by capsaicin, so-called pore dilation. 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. TRPV1 is present on sensory fibers in mineralized bone and bone marrow. 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. Activated osteoclasts maintain an extracellular microenvironment of low pH (4.0 to 5.0) to resorb bone. An acidic environment can activate TRPV1. Moreover, chemical mediators released from tumor cells and inflammatory cells, including endothelin-1, nerve growth factor, and prostaglandins, can sensitize TRPV1. Sensitized TRPV1 can be tonically activated at normal body temperature, 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. Intense pain associated with capsaicin injection limits the clinical use of QX-314. The current study has shown that QX-314 has analgesic effects on bone cancer pain without coadministration 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.

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 coadministration of TRPV1 agonists. It has been shown that TRPV1 activation was involved in nerve injury–induced thermal hyperalgesia. Thus, systemic administration of QX-314 may produce local anesthetic effects without coadministration 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.\textsuperscript{14,52} In the current 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.\textsuperscript{53–55} Therefore, in contrast to mice, QX-314 as well as heat pain and inflammatory pain, so-called poly-
References


