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ORIGINAL ARTICLE

Effects of TrkA inhibitory peptide on cancer-induced pain in a mouse melanoma model

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Abstract
Purpose Tropomyosin receptor kinase (Trk) A, a high-affinity receptor of nerve growth factor, is a therapeutic target for both noxious and neuropathic pain. The present study examined the effects of an inhibitory peptide of Trk activity (IPTRK) 3 that inhibits TrkA activity on cancer-induced pain in a mouse melanoma model.
Methods The hind paws of mice were inoculated with B16-F1 mouse melanoma cells on day 0. We administered IPTRK3 (20 mg/kg i.p.) repetitively on days 5, 6, 7, 8, and 9, and evaluated pain-related behaviors on days 0, 5, 10, 15, and 20 after tumor inoculation.
Results Following inoculation, mice demonstrated mechanical allodynia and thermal hyperalgesia with an increased number of flinches, and paw volume increased gradually. However, an intraperitoneal injection of IPTRK3 significantly inhibited mechanical allodynia on day 15 and suppressed the number of flinches on day 20. The increased paw volume was significantly suppressed on day 20 after tumor inoculation. IPTRK3, however, showed no significant effect on thermal hyperalgesia.
Conclusions These results suggest that TrkA inhibitory peptide likely suppress melanoma-induced pain with concomitant reduction in the increased paw volume in a mouse skin cancer pain model.

Keywords Cancer pain · Cell-penetrating peptide · Nerve growth factor · TrkA

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Introduction

Many people suffer from severe cancer pain despite recent progress in palliative care. Better management of cancer pain relief is still warranted [1], and, consequently, several novel drugs are being developed to reduce cancer pain [2]. Signal transduction systems for nerve growth factor (NGF), including tropomyosin receptor kinase (Trk) A, the high-affinity receptor of NGF at the peripheral nervous system, are potential targets for developing new analgesics [3]. After NGF binds to TrkA, activation of TrkA kinase induces peripheral sensitization for pain sensitivity. NGF signaling containing NGF, TrkA, and activated signaling proteins is transmitted retrogradely through axonal transport of TrkA from the peripheral terminals of nociceptive neurons to the cell bodies of the dorsal root ganglion, and central sensitization is then developed [4].

TrkA is a tyrosine kinase receptor, and binding of NGF to TrkA stimulates autophosphorylation of the activation loop, which further regulates downstream signaling events [5]. Previously, we designed an inhibitory peptide of Trk activity (IPTRK) 3, which includes a part of the amino acid sequence for the TrkA activation loop (i.e., 664-GMSRD IYSTDYYRVGGR-680), as well as the cell-penetrating peptide sequence, which was based on the human immunodeficiency virus type 1, transactivator of transcription

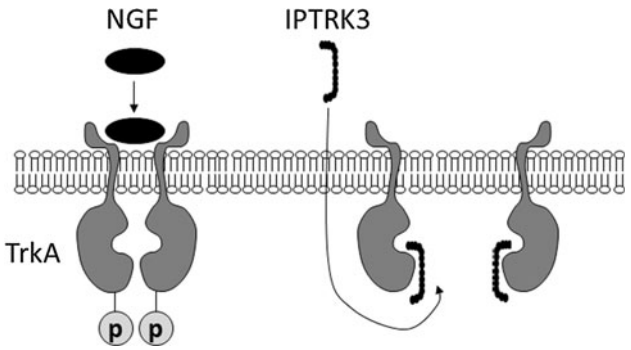

Fig. 1 A proposed mechanism of the inhibitory effects of *IPTRK3* on *TrkA* activity. *Left* Nerve growth factor (*NGF*), binding to the extracellular portion of tropomyosin receptor kinase (Trk) A, leads to autophosphorylation (P) of tyrosine residues in the intracellular portion of TrkA. NGF-stimulated autophosphorylation of TrkA activates downstream signaling and promotes either noxious or neuropathic pain. *Right* An inhibitory peptide of Trk activity (IPTRK) 3, penetrating through the nerve cell membrane, suppresses NGF-stimulated autophosphorylation of TrkA and inhibits downstream signaling

protein (TAT) (i.e., 47-YGRKKRRQRRR-57) [6]. It was found that this synthetic peptide inhibits TrkA activity and suppresses both noxious pain in a rat model of inflammation [7] and neuropathic pain in a mouse model of partial sciatic nerve ligation [8]. Figure 1 represents a proposed mechanism of the inhibitory effects of IPTRK3 on TrkA activity. In the present study, we investigated whether this peptide inhibits cancer-induced pain, using a mouse skin cancer pain model [9].

Materials and methods

Peptide synthesis

Both peptides, TAT (YGRKKRRQRRR) and IPTRK3 (YGRKKRRQRRR-acp-SRDIYSTDYYR; acp = epsilon-aminocaproic acid), were synthesized and purified by high performance liquid chromatography (Peptide Institute, Osaka, Japan).

Animals

All experimental protocols were approved by the Animal Investigation Committee at University of Fukui and adhered to the Ethical Guidelines of the International Association for the Study of Pain [10]. Male C57BL/6 mice (Charles River, Yokohama, Japan), weighing 20–25 g, were kept in a temperature-controlled room with a 12-h light:dark cycle. Food and water were provided ad libitum.

Cell culture

B16-F1 melanoma cells, which were derived from C57BL/6 mice, were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in modified Eagle's medium containing 10 % fetal bovine serum (FBS) and penicillin–streptomycin at 37 °C under a humidified atmosphere of 5 % CO_2. Cells were then subcultured or collected following an enzymatic digestion with trypsin solution.

Tumor inoculation

Following measurements of pain-related behaviors on day 0, melanoma cells (3×10^5 cells/15 μl) suspended in phosphate-buffered saline (PBS) were subcutaneously injected into the plantar region of the left hind paw under isoflurane anesthesia.

Drug administration

We prepared either vehicle (PBS) alone or IPTRK3 dissolved in PBS before the experiment. According to a protocol of mouse skin cancer pain model [9], vehicle (PBS) or IPTRK3 (20 mg/kg), with a volume of 10 μl/g body weight, was injected intraperitoneally on days 5, 6, 7, 8, and 9 after tumor inoculation. This dose was selected because half of the dose suppressed neuropathic pain almost completely after partial sciatic nerve ligation in mice [8].

Pain-related behaviors

All mice were behaviorally tested on days 0, 5, 10, 15, and 20 after a subcutaneous injection of B16-F1 cells or vehicle (PBS). Then, mice were housed in individual cages with a wire mesh floor for 30 min. Following the 30-min acclimatization period, the mice were observed for 2 min, during which spontaneous flinches were counted and mice were assessed for mechanical allodynia. Thermal hyperalgesia was evaluated in a separated study. All measurements were performed in a blinded fashion, with the investigator unaware of the injected agent, whether IPTRK3 or PBS.

Flinches were defined as the number of times the animal raised the left hind limb. Every lift with the left hind limb that was not related to walking or grooming was considered to be a flinch.

Mechanical allodynia was assessed with von Frey filaments (Stoelting, Wood Dale, IL, USA) applied to the left hind paw. Mechanical paw withdrawal threshold (PWT) was measured using a set of von Frey filaments (0.16, 0.4, 0.6, 1.0, 1.4, and 2.0 g). The filaments were poked vertically against the plantar surface with sufficient force to cause slight bending against the paw and held for 6–8 s with a

10-min interval between stimulations. Brisk withdrawal or paw flinching were considered as positive responses. Mechanical PWT was determined by sequentially increasing and decreasing the stimulus strength (i.e., the "up-and-down" method). Each mouse was tested five times per stimulus strength. The lowest von Frey filament that had three or more positive responses was regarded as the withdrawal threshold.

Thermal hyperalgesia in the hind paw was measured with a plantar test (Ugo Basile, Comerio, Italy). Mice were placed in plastic cages with a glass plate surface, and a heat stimulus was applied from beneath to the middle of the left plantar surface via a radiant heat source. When mice withdrew their paws, the sudden drop in reflected energy stopped the timer. A cutoff time of 30 s was imposed to prevent tissue damage. Thermal sensitivity was recorded as paw withdrawal latency (PWL) in seconds. Thermal PWL was measured five times in each mouse and then averaged. Stimulus interval was 5 min.

Paw volume measurement

A plethysmometer (Ugo Basile, Comerio, Italy) was used to measure the volume of the left hind paw at 0, 5, 10, 15, and 20 days after inoculation.

Hematoxylin and eosin staining and immunohistochemistry

On 20 days after experimental measurements, all mice were killed by exsanguination under deep isoflurane anesthesia, and the inoculated hind paws were amputated and immersed in 10 % buffered formalin solution. Formalin-fixed paraffin-embedded tissue sections were stained with hematoxylin and eosin.

These tissue sections (4 μm) were also processed for immunofluorescence staining. The sections received heat-induced antigen retrieval. After citrate buffer (0.01 M, pH 6.0) in a glass staining jar was preheated to 80 °C in a water bath, the slides were immersed in retrieval buffer for 30 min. The jar with buffer and slides was removed from the water bath and allowed to cool to room temperature. After being immersed in 5 % normal goat serum for 30 min, the sections were incubated overnight at 4 °C with rabbit anti-TrkA antibody(1:50; Assay Biotechnology Company, Sunnyvale, CA, USA) or rabbit anti-TrkA (Phospho-Tyr 496) antibody (1:100; Assay Biotechnology Company). This step was followed by incubation for 1 h at room temperature with Alexa Fluor 594 (goat anti-rabbit IgG, 1:200; Invitrogen, San Diego, CA, USA). The stained sections were examined with a fluorescence microscope (Olympus AX80; Olympus, Tokyo, Japan), and images were captured with a digital camera system (Olympus DP72).

Effects of synthetic peptides on proliferation of B16-F1 melanoma cells

Given that the activation of TrkA induces oncogenesis, TrkA is a potential therapeutic target against cancer [11–13]. Therefore, if TrkA inhibitory peptide, IPTRK3, suppresses melanoma proliferation, the suppression of tumor proliferation would also contribute to the analgesic effect. After we found that IPTRK3 suppresses cancer-induced pain in this mouse melanoma model, we decided to perform a cell culture study to examine the effect of IPTRK3 on cell proliferation, cell permeability, and NGF-stimulated autophosphorylation of TrkA in B16-F1 melanoma cells.

B16-F1 cell proliferation was assessed in CellTiter 96 Aqueous One Solution (Promega, Madison, WI, USA), as previously described [14], and the effects of synthetic peptides on B16-F1 cell proliferation with or without serum were examined. To evaluate the effects of synthetic peptides on serum-induced cell proliferation, B16-F1 cells were cultured in medium containing 10 % FBS with or without each synthetic peptide for 2 days. To evaluate the effects of synthetic peptides on NGF-induced cell proliferation, B16-F1 cells were cultured in serum-free medium for 5 h and then exposed to 100 ng/ml NGF (NGF 2.5S; Millipore, Billerica, MA, USA) with or without each synthetic peptide for 2 days.

Cell permeability of synthetic peptide

A fluorescein isothiocyanate (FITC)-labeled peptide (FITC-acp-YGRKKRRQRRR-acp-SRDIYSTDYYR) was synthesized. B16-F1 cells were incubated with the FITC-labeled IPTRK3 (10 μM) for 60 min, and then with Hoechst 33342 (1 μg/ml) for 10 min. Cell permeability of these peptides was evaluated via fluorescence microscopy.

Effects of synthetic peptides on NGF-stimulated autophosphorylation of TrkA in B16-F1 cells

To examine the effect of IPTRK3 on TrkA kinase activity, NGF-stimulated autophosphorylation of TrkA was evaluated. Western blot analyses were performed to examine the presence of TrkA in B16-F1 cells as well as to investigate the effects of synthetic peptides on NGF-stimulated autophosphorylation of TrkA. B16-F1 cells were detached with trypsin, and cultured to be confluent in 60-mm dishes with 10 % FBS. Cells were exposed to serum-free medium for 5 h. After the addition of synthetic peptides (100 μM) and incubation with NGF (100 ng/ml) for 10 min, the medium was replaced with 2 ml ice-cold PBS, and each sample was scraped and harvested into tubes. The supernatant was removed by centrifugation, and each sample was

suspended in lysis buffer (HEPES pH 7.5, 50 mM; NaCl, 150 mM; EDTA, 2 mM; 1 % (v/v) Nonidet P-40; 10 % (v/v) glycerol; NaF, 10 mM; sodium vanadate, 2 mM; phenylmethylsulfonyl fluoride, 1 mM; sodium pyrophosphate, 10 mM; aprotinin, 5 μg/ml; and pepstatin, 0.5 μg/ml). Insoluble material was removed via centrifugation at 15,000 rpm for 15 min. Aliquots of supernatant containing equal amounts of protein, as determined via the Bradford protein assay, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5 % (v/v) acrylamide solving gels and transferred electrophoretically to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were then blocked in 5 % (w/v) dried milk in PBS-Tween [PBS containing 0.1 % (v/v) Tween 20] for 1 h at room temperature, and then were immunoblotted with anti-TrkA antibody (1:1,000) or phospho-TrkA (Tyr 490) antibody (1:1,000) (Cell Signaling Technology, Danvers, MA, USA). Thereafter, appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2,000) were applied. The antigen–antibody complexes were visualized by chemiluminescence luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands of interest were scanned and quantified by using Light-Capture AE-6960 (ATTO, Tokyo, Japan).

Statistical analysis

A two-way repeated-measures analysis of variance (ANOVA) was adopted to compare the effect of intraperitoneal IPTRK3 on paw volume, body weight, and pain-related behaviors in a mouse melanoma model. A one-way ANOVA was used to compare the effects of IPTRK3 on cell proliferation and TrkA activity in cell culture study. The Bonferroni test was further executed as a post hoc follow-up test. $P < 0.05$ was considered statistically significant. All values were reported as the mean ± SD.

Results

IPTRK3 suppresses pain-related behaviors in a mouse melanoma model

The interaction between the two main factors (groups × days after inoculation) was significant in paw volume ($F = 2.557$, $P < 0.01$), flinches ($F = 2.606$, $P < 0.01$), and mechanical allodynia ($F = 2.557$, $P < 0.05$).

Following inoculation, there was a gradual increase in the left hind paw volume, and a significant increase on days 15 and 20 after tumor inoculation in the PBS group ($F = 4.034$, $P < 0.01$) (Fig. 2a). IPTRK3 significantly suppressed this increase in paw volume compared to PBS by day 20 ($F = 2.557$, $P < 0.001$) (Fig. 2a). Throughout

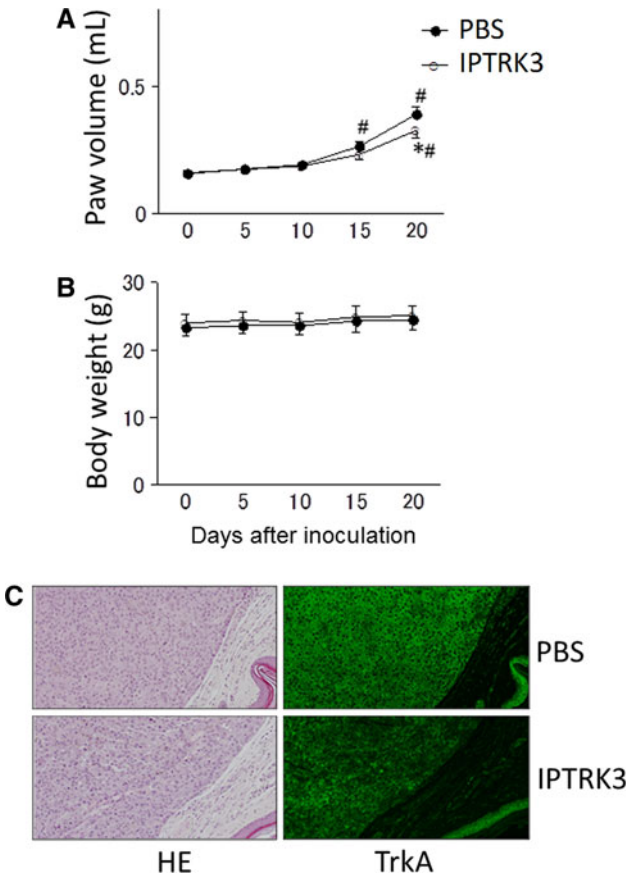

Fig. 2 Effect of IPTRK3 on paw volume and body weight in a mouse melanoma model. Inoculation of B16-F1 cells was performed on day 0. Either *IPTRK3* or phosphate-buffered saline (*PBS*) was administered intraperitoneally on days 5, 6, 7, 8, and 9 after inoculation. **a** Left hind paw volume. **b** Body weight. $^{*}P < 0.05$, compared to the PBS group at the same time point; $^{\#}P < 0.05$, compared to the baseline value on day 0 within the same group. Results represent mean ± SD of five mice in each experiment. **c** Micrographs of hematoxylin and eosin (H&E) staining (*HE*) and *TrkA* immunostaining in representative paw slices from each group on day 20. c ×100

the experiment, there were no significant differences in body weight between PBS and IPTRK3 groups (Fig. 2b). Figure 2c shows micrographs of representative paw slices, stained with hematoxylin and eosin and TrkA antibody. Proliferation of melanoma cells but not edematous tissue likely increased the left hind paw volume.

The number of flinches significantly increased on day 20 following inoculation in the PBS group ($F = 4.084$, $P < 0.05$) (Fig. 3a), and both mechanical PWT and thermal PWL gradually decreased in the PBS group (Fig. 3b,c). Mechanical PWT started to show significant decrease on day 15 ($F = 4.034$, $P < 0.05$), and thermal PWT showed significant decrease on day 10 after inoculation in the PBS group ($F = 4.034$, $P < 0.01$). In the IPTRK3 group, there were no significant changes in the number of flinches, mechanical PWT, and thermal PWL

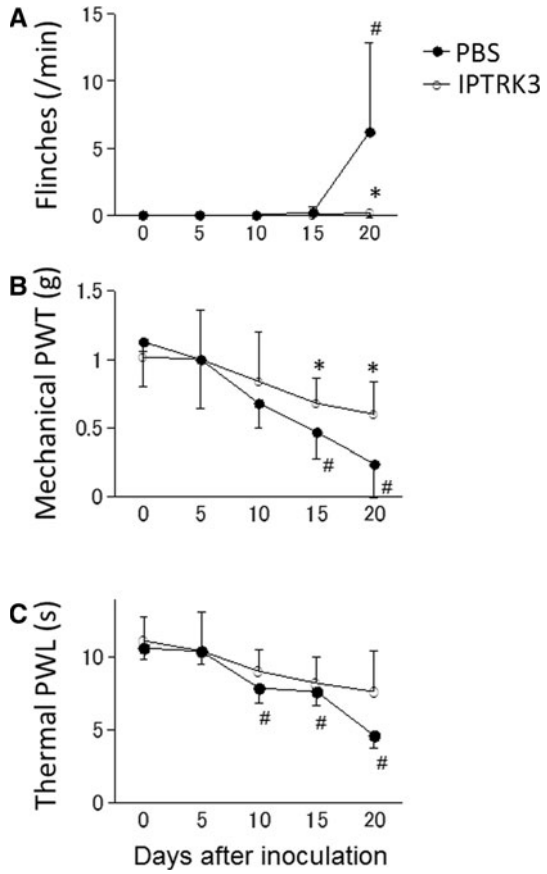

Fig. 3 Effect of IPTRK3 on pain-related behaviors in a mouse melanoma model. Inoculation of B16-F1 cells was performed on day 0. Either IPTRK3 or PBS was administered intraperitoneally on days 5, 6, 7, 8, and 9 after inoculation. **a** Number of flinches. **b** Mechanical paw withdrawal threshold (*PWT*). **c** Thermal paw withdrawal latency (*PWL*). $^{*}P < 0.05$, compared to the PBS group at the same time point; $^{\#}P < 0.05$, compared to the baseline value on day 0 within the same group. Results represent mean ± SD of five mice in each experiment

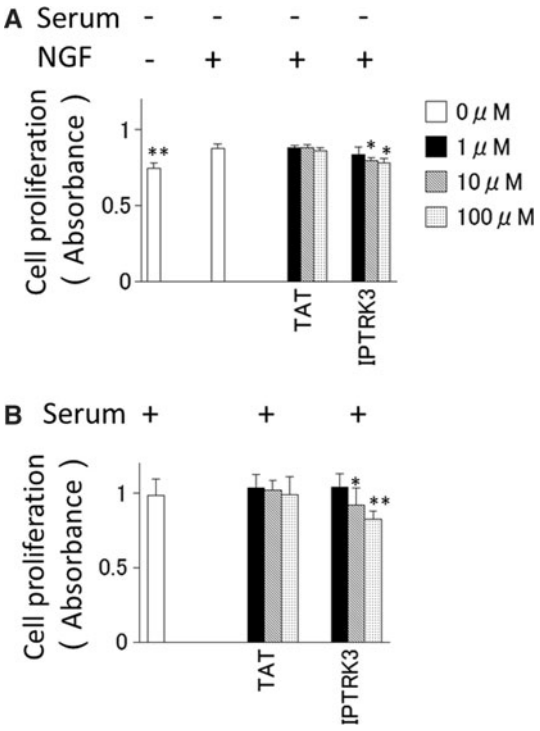

Fig. 4 Effects of synthetic peptides on B16-F1 cell proliferation with or without serum following stimulation with nerve growth factor (NGF). **a** NGF-induced cell proliferation. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to NGF-induced cell proliferation without IPTRK3. **b** Serum-induced cell proliferation. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to serum-stimulated cell proliferation without IPTRK3. Results represent mean ± SD of five separate experiments

throughout the experiment. IPTRK3 showed a significant suppression of mechanical allodynia on day 15 compared to PBS ($F = 2.557$, $P < 0.001$) (Fig. 3b). IPTRK3 also significantly suppressed the increased number of flinches compared to the PBS group at 20 days ($F = 2.606$, $P < 0.01$) (Fig. 3a). Thermal hyperalgesia, however, was not affected by IPTRK3, as the interaction effect was not significant ($F = 2.557$, $P = 0.07$) (Fig. 3c).

IPTRK3 suppresses both cell proliferation and NGF-stimulated autophosphorylation of TrkA in B16-F1 cells

Nerve growth factor increased proliferation of B16-F1 cells that were incubated without serum. IPTRK3 at a concentration of 10–100 μM significantly suppressed NGF-induced cell proliferation (Fig. 4a). IPTRK3 at a concentration of 10–100 μM significantly suppressed serum-induced cell proliferation (Fig. 4b). TAT induced no significant changes in either serum- or NGF-induced cell proliferation.

We examined the permeability of IPTRK3 into B16-F1 cells using peptides conjugated with FITC and observed that IPTRK3 penetrated into 91 % ± 4 % of cells (Fig. 5).

Immunoblots demonstrating autophosphorylation and protein content of TrkA in B16-F1 cells are presented in Fig. 6. NGF-induced tyrosine phosphorylation of TrkA was significantly suppressed via 100 μM IPTRK3. TAT at a concentration of 100 μM did not induce any significant changes in autophosphorylation.

Discussion

The present study revealed that intraperitoneal injection of IPTRK3, a cell-penetrating peptide that inhibits TrkA kinase activity, inhibits cancer-induced pain with concomitant reduction in the increase of paw volume in a mouse skin cancer pain model.

In a rat inflammatory pain model, IPTRK3 suppressed complete Freund's adjuvant (CFA)-induced pain after local administration at the site of CFA injection, together with

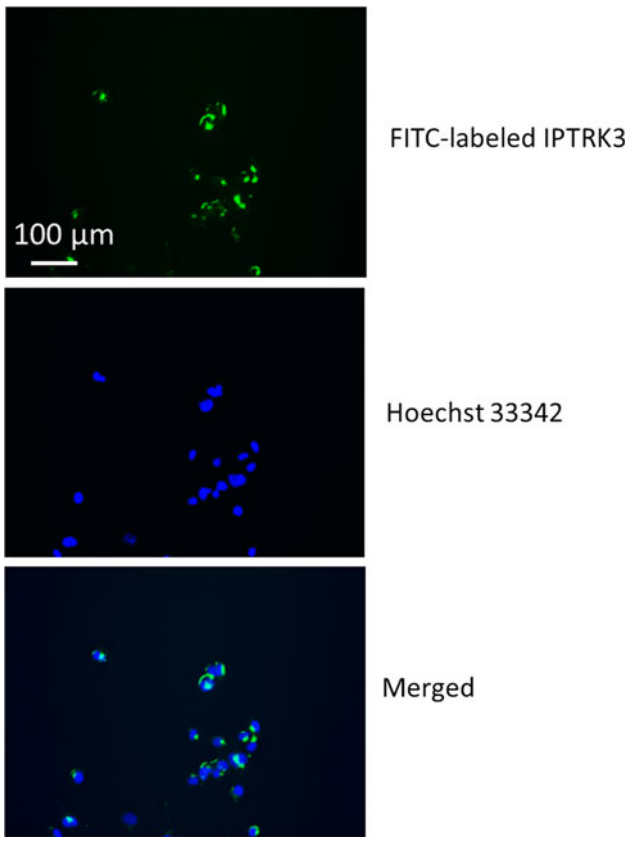

Fig. 5 Fluorescence microscopy images of B16-F1 cells treated with 10 μM fluorescein isothiocyanate (FITC)-labeled IPTRK3 (*green*) and stained with the nuclear dye Hoechst 33342 (*blue*) (color figure online)

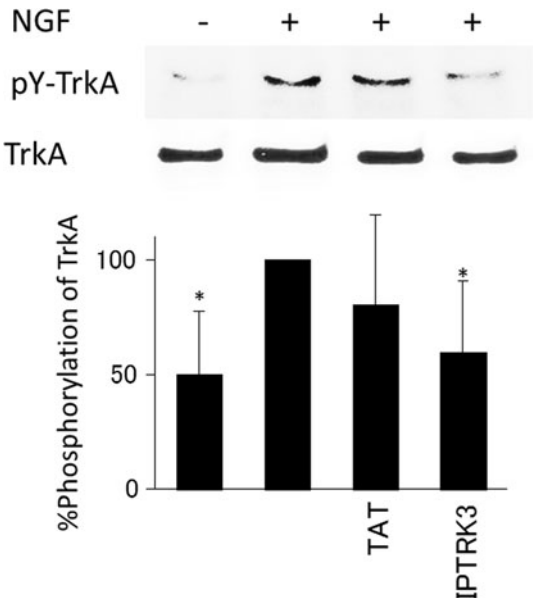

Fig. 6 Effects of synthetic peptides on nerve growth factor (NGF)-induced tyrosine phosphorylation of B16-F1 cells. Results displayed in the *upper panels* represent typical immunoblots of the autophosphorylation of TrkA and protein content. $*P < 0.05$, compared to NGF-induced autophosphorylation without IPTRK3 (100 %). Results represent mean ± SD of four separate experiments

reduction of the increased protein expression of transient receptor potential channel 1 (TRPV1) in the dorsal root ganglion [7]. Other previous studies reported that subcutaneous inoculation of sarcoma cells increases cytokines, including NGF, in tumor lysates in rats [15] and also that NGF is secreted from melanoma cells and is present in tissues adjacent to melanoma [16]. Therefore, cytokines in tumor tissues likely played a role for inducing inflammatory pain in the present study. On the other hand, intraperitoneal IPTRK3 suppressed partial sciatic nerve ligation (PSNL)-induced pain, with reduction of the increased Fos protein expression in both superficial and deep laminae of the spinal cord dorsal horn, in a mouse neuropathic pain model [8]. Nerve degeneration is reportedly observed in a mouse skin cancer pain model, and peripheral neuropathic pain is supposed to be involved in the mechanisms of pain [9]. Although we examined neither cytokines in tumors nor tumor growth in the peripheral nerve, the mechanisms of suppression of cancer-induced pain in the present study likely involve analgesic effects of IPTRK3 on both noxious and peripheral neuropathic pain.

In our previous studies, a single local injection of IPTRK3 suppressed inflammatory pain on the same day as peptide injection [7], and a single intraperitoneal injection of IPTRK3 suppressed neuropathic pain on the same day as peptide administration [8]. The analgesic effects of this peptide persisted for 2 days. However, in the present study, an intraperitoneal injection of IPTRK3 administered repeatedly from day 5 to day 9 only suppressed pain-related behaviors 15 days or more following tumor inoculation, which was concomitant with a reduction in increased paw volume. Because pain-related behaviors were not evident during the IPTRK3 administration period, the analgesic effects of IPTRK3 may not have been apparent at the time of administration. However, the analgesic effects of IPTRK3 did appear on day 15 and later. This delayed effect may be caused by the cumulative dose of this peptide following its repeated injections or suppression of tumor growth during peptide administration, which might cause a reduction in the increase of paw volume on day 20.

It is possible that the gradual increase in tumor growth, as shown in the increase of paw volume, might generate nociceptive pain. Although a small suppression of increase in paw volume by IPTRK3 was observed on day 20, this peptide inhibited mechanical allodynia on day 15. Therefore, a direct inhibitory effect of IPTRK3 on TrkA activity in nociceptive pathways, instead of the suppressive effect on paw volume increase, likely plays a predominant role in the suppression of cancer-induced pain in the present study.

The present study found that IPTRK3 suppresses proliferation of mouse melanoma cells and inhibits NGF-induced TrkA kinase activity in a cell culture study. Given that the NGF/TrkA axis likely plays a biological role in these cells, we suggest that a direct inhibitory effect of IPTRK3 on TrkA kinase activity suppresses both NGF-induced cell proliferation and NGF-stimulated autophosphorylation of TrkA in these mouse melanoma cells. In addition to IPTRK3, both antibodies for NGF and inhibitors of TrkA kinase have been previously used to inhibit the NGF/TrkA axis. Neither antibodies for NGF nor TrkA kinase inhibitors, which reportedly suppress cancer pain in rodents, showed any inhibitory effects on tumor growth in a bone metastasis model of prostate carcinoma or sarcoma [17–19]. Conversely, IPTRK3 suppressed both pain-related behaviors and the increase in paw volume following inoculation of melanoma cells in mice. Several protein kinases, with the exception of TrkA, are known to be associated with tumor growth in melanoma. The activation of c-Jun N-terminal kinase (JNK), a downstream signaling molecule of TrkA, induces cell proliferation in melanoma [20]. Protein kinase C (PKC) isoforms (i.e., α, β, γ, δ, ε, η, θ, μ) are also involved in tumor promotion in B16-F1 cells [21]. Therefore, in addition to affecting TrkA, IPTRK3 may have inhibitory effects on other protein kinases.

In summary, our findings suggest that repeated intraperitoneal administration of a TrkA inhibitory peptide, IPTRK3, suppresses cancer-induced pain in a mouse melanoma model.

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