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Supplementary Materials for

An orally bioavailable MrgprX1-positive allosteric modulator alleviates certain neuropathic pain–related behaviors in humanized mice

Ankit Uniyal *et al.*

Corresponding author: Yun Guan, yguan1@jhmi.edu; Takashi Tsukamoto, ttsukamoto@jhmi.edu

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

SUPPLEMENTARY METHODS

Chemical synthesis of 6-*tert*-butyl-5-(4-chlorophenyl)-4-(2-fluoro-6-(trifluoromethoxy) phenoxy)thieno[2,3-d]pyrimidine (BCFTP)

The synthesis process of BCFTP is illustrated in **Figure S1**.

6-*tert*-Butyl-5-(4-chlorophenyl)-4-methoxythieno[2,3-d]pyrimidine (2)

A suspension of **1** (0.60 g, 1.99 mmol), 4-chlorophenylboronic acid (0.62 g, 3.98 mmol), potassium carbonate (0.83 g, 5.98 mmol), and bis(triphenylphosphine)palladium(II) dichloride (0.14 g, 0.20 mmol) in DMF (15 mL) was purged with N_2 gas via an inserted long needle for 30 minutes. The mixture was then stirred at 80 °C overnight. After removing solvent in vacuo, water was added to the mixture. The mixture was extracted with dichloromethane twice, and the combined extracts were washed with brine, dried over sodium sulfate, and concentrated. A white precipitate formed after concentration was removed by filtration, and the filtrate was concentrated and purified by Biotage Isolera One using 5% EtOAc/DCM as eluent to give 0.41 g (62%) of compound **2** as a clear oil. ^{1}H NMR ($CDCl_3$) δ 1.28 (s, 9H), 3.70 (s, 3H), 7.20 (d, J = 8.3 Hz, 2H), 7.35 (d, J = 8.3 Hz, 2H), 8.57 (s, 1H).

6-*tert*-Butyl-5-(4-chlorophenyl)thieno[2,3-d]pyrimidin-4-ol (3)

To a solution of **2** (0.41 g, 1.23 mmol) in DCM (15 mL) at 0 °C was added 1 M BBr_3 solution in DCM (6.16 mL, 6.16 mmol). The mixture was stirred at 0 °C, then gradually warmed to rt and stirred until completion (7 days). The mixture was then cooled to 0 °C, and methanol was added to quench excess of BBr_3. After the removal of solvents, the crude material was partitioned between water and DCM. The organic layer was washed with brine, dried over sodium sulfate, and concentrated. The residual solid was used as is without further purification. White solid (quantitative yield). ^{1}H NMR ($CDCl_3$) δ 1.26 (s, 9H), 7.22 (d, J = 8.6 Hz, 2H), 7.36 (d, J = 8.3 Hz, 2H), 7.85 (s, 1H), 10.77 (bs, 1H).

6-*tert*-Butyl-4-chloro-5-(4-chlorophenyl)thieno[2,3-d]pyrimidine (4)

A solution of **3** (0.35 g, 1.10 mmol) in POCl3 (3 mL) was stirred at 110 °C for 1.5 h. Excess $POCl_3$ was removed in vacuo followed by co-evaporation with DCM (3 times). The residual material was passed through a short silica column using 5% EtOAc in hexanes as eluent to give 0.24 g (65%) of **4** as a white solid. ^{1}H NMR ($CDCl_3$) δ 1.31 (s, 9H), 7.25 (d, J = 8.6 Hz, 2H), 7.41 (d, J = 8.6 Hz, 2H), 8.78 (s, 1H).

6-*tert*-butyl-5-(4-chlorophenyl)-4-(2-fluoro-6-(trifluoromethoxy)phenoxy)thieno[2,3-d]pyrimidine (BCFTP)

To a solution of 2-fluoro-6-(trifluoromethoxy)phenol (0.20 g, 1.00 mmol) in DMF (3 mL) was added sodium hydride (60% dispersion in mineral oil, 0.40 g, 1.00 mmol) at rt and the mixture was stirred at rt for 5-10 min. A solution of **4** (0.17 g, 0.50 mmol) in DMF (5 mL) was then slowly added to the mixture via a syringe. The reaction mixture was heated at 65 ºC until completion. The mixture was concentrated in a vacuo. The crude material was partitioned between water and EtOAc. The organic layer was washed with brine, dried over sodium sulfate, and concentrated. The residual material was triturated in a mixture of water/MeOH to give 0.13 g of white solid after filtration. The filtrate was purified by prep-HPLC (using a Phenomenex Luna C18(2) column (5 micron, 250 x 21.2 mm) at a flow rate of 15 ml/min with an eluent gradient of 15% ACN/85% H_2O for 5 minutes followed by an increase to 30% ACN/70% H_2O over 50

minutes and then an increase to 50% ACN/50% H_2O until 60 minutes) to give an additional 0.11 g and a total of 0.24 g (97%) of BCFTP as a white solid. Mp 115 °C; ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 9H), 7.10 (m, 2H), 7.21 (m, 1H), 7.33 (s, 4H), 8.49 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 1.33 (s, 9H), 7.10 (m, 2H), 7.21 (m, 1H), 7.33 (s, 4H), 8.49 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 166.33, 161.39, 155.51 (d, J = 253.3 Hz), 152.27, 142.51, 135.53, 133.77, 132.93 (d, J = 15.1 Hz), 132.00, 131.78, 127.91 (d, J = 13.9 Hz), 127.72, 126.21 (d, J = 8.82 Hz), 120.28 (q, J = 258.8 Hz), 120.15, 117.69 (d, J = 3.78 Hz), 115.19 (d, J = 18.9 Hz), 36.65, 32.74. Anal. Calcd. for $C_{23}H_{17}ClF_4N_2O_2S$: C, 55.59; H, 3.45; N, 5.64; S, 6.45; F, 15.29; Cl, 7.13. Found: C, 55.40; H, 3.42; N, 5.65, S, 6.45; F, 15.51; Cl, 7.29. HPLC (Method A): >99% by area, retention time 2.99 min, m/z 497.1 $[M + H]^+$.

In vitro MrgprX1 assay

HEK293 cells stably transfected with human MrgprX1 were plated onto poly-D-lysine-coated, black-walled, 96-well plates at a seeding density of 12 000 cells/well and grown for 2 days. On the day of the experiment, cells were incubated with Fluo-4 AM 2 μM, pluronic acid 0.04% and Trypan Red 1% in HBSS (pH 7.4, 100 μL) for 1 h at 37 °C. BCFTP was then dissolved and serially diluted in DMSO, further diluted with Trypan Red (1%)-containing HBSS (100 μL) and added to cells (0.33% DMSO concentration by volume) to first monitor for agonist activity. After 2.5 minutes, BAM8-22 at 4-fold EC20 (50 μL) was added to cells (0.25% DMSO concentration by volume), and the cells were imaged on the FLIPR for an additional 2 minutes to monitor BCFTP for PAM activity. Finally, data were normalized to fluorescence signals obtained from 250 nM of BAM8-22 (maximum) and BAM8-22 at its EC20 concentration (minimum). EC50 of BCFTP as a PAM was defined as the concentration required to provoke a response halfway between the minimum and maximum response in the presence of EC20 concentration of BAM8-22. To investigate the effects of BCFTP on BAM8-22, the EC50 of BAM8-22 was measured in the presence of variable concentrations of BCFTP (0-960 nM).

Aqueous solubility of BCFTP

Solubility determinations were performed by Pharmaron. PBS pH 7.4, simulated gastric fluid (SGF), or simulated intestinal fluid (SIF) (485 μL) was added to a stock solution of each test compound (10 mM, 15 μL) in a vial of the cap-less Solubility Sample plate. The plate was transferred to the Eppendorf Thermomixer Comfort plate shaker and shaken at 25 °C at 1100 rpm for 2 hours. The samples were then filtered using the Vacuum Manifold. Aliquots (5 μL) of the filtrates were diluted with 5 μL of DMSO and 490 μL of H_2O-acetonitrile (1:1 mixture) containing an internal standard. Concentrations of test compounds were then measured by LC-MS/MS. Progesterone, a BCS Class II drug with limited solubility across a wide pH range, was used as a solubility control in PBS. Diclofenac, a BCS Class II drug with pH-dependent solubility, was used as a solubility control in SGF and SIF.

Metabolic stability of BCFTP in liver microsomes

The metabolic stability was evaluated using mouse, human, and rat liver microsomes as we have previously described with minor modifications. Briefly, reactions were carried out with 100 mM potassium phosphate buffer, pH 7.4, in the presence of NADPH regenerating system (1.3 mM NADPH, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, 0.4 U/mL glucose-6-phosphate dehydrogenase, 50 μM sodium citrate). Reactions in triplicate were initiated by the addition of the respective liver microsomes to the incubation mixture (1 μM compound final concentration and 0.2 mg/mL microsomes). After 60 minutes of incubation, aliquots of the mixture were

removed, and the reaction was quenched by adding three times the volume of ice-cold acetonitrile spiked with the internal standard. Compound disappearance was monitored over time using a liquid chromatography and tandem mass spectrometry (LC/MS) method on a Dionex ultra-high-performance LC system coupled with a Q-Exactive Focus orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham MA).

Bidirectional Permeability of BCFTP in MDCKII-MDR1 Cell Line

Bidirectional permeability studies were performed by Pharmaron. MDCKII-MDR1 cells ($1.56x10^6$ cells/mL), obtained from the Netherlands Cancer Institute (Amsterdam), were dispensed into the filter well of the 96-well HTS Transwell plate, and cultivated for 4-8 days at 37 °C, 5% CO_2, 95% relative humidity. The cell monolayer integrity was assessed by measuring transepithelial electrical resistance (TEER) across the monolayer using Millicell Epithelial Volt-Ohm measuring system (Millipore, USA). TEER values were confirmed to be greater than 42 ohm*cm^2, indicating a well-qualified MDCKII-MDR1 monolayer. BCFTP was added to either apical or basolateral compartments and the plates were incubated at 37 °C for 2 hours. BSA was added to the incubation system to improve the recovery rates. Concentrations of BCFTP in the acceptor wells were determined by LC-MS/MS. The apparent permeability coefficient (P_{app}), in units of centimeter per second, can be calculated using the following equation: $P_{app} = (VA \times [drug]_{acceptor}) / (Area \times Time \times [drug]_{initial,donor})$, where VA is the volume (in mL) in the acceptor well, Area is the surface area of the membrane (0.143 cm^2 for Transwell-96 Well Permeable Supports), and time is the total transport time in seconds.

The off-target activity of BCFTP

The off-target activity of BCFTP was assessed by testing the compound in CerepSafetyScreen44, a panel of 44 selected targets (Eurofins Cerep SafetyScreen 44). BCFTP showed an inhibition higher than 50% in three targets (**Table S4**) at 10 μM.

Inhibitory effects of BCFTP on hERG potassium channels

hERG inhibition assay was performed by Pharmaron. The CHO hERG-DUO cell line stably expressing hERG channel was purchased from B'SYS GmbH. The cells are cultured in a medium containing F12 (HAM) medium, 10% FBS, 100 U/mL Penicillin-Streptomycin, 100 μg/mL Hygromycin and 100 μg/mL G418. Cells are split using TrypLE™ Express about three times a week and maintain about 80% confluence. Inhibition of hERG potassium channel currents by test compounds was assessed in CHO hERG-DUO cells using the SyncroPatch 384i automated patch clamp system. The concentration-response relationship was determined for BCFTP at 5 concentrations in duplicate to calculate IC_{50} values. Cisapride was used as a positive control in the experiments to ensure a thorough quality control.

Mini-Ames assay of BCFTP in *Salmonella typhimurium*

Mini-Ames assay was performed by Pharmaron using *Salmonella typhimurium* strains TA98nd TA100 in the presence or absence of the S9 mix, along with concurrent negative/solvent control and positive controls. The dose levels tested in the Mini-Ames assay were 1.5, 4, 10, 25, 64, 160, 400, and 1000 μg/well. For BCFTP, no cytotoxicity was observed at any dose levels tested, either in the presence or absence of S9 mix in TA98 or TA100. Precipitate was observed at dose levels $\geqslant$400 μg/well.

MrgprX2 selectivity assay

Ca2+ imaging assay was employed to determine the positive allosteric effect of compound 4793 at MrgprX2 for its agonist compound 48/80 using a HEK293 cell line that stably expresses MrgprX2. Cells were incubated with Ca^{2+} sensitive dye Fluo4 solution at 37 °C for 30 minutes and at RT for 30 minutes after removing media. 5 μM of compound 4793 was added to the assay buffer with dye for 80 sec, followed by adding 0.3 μg/mL compound 48/80 (EC20 of the agonist activating MrgprX2) for 75 sec and recording the change of fluorescence by Flexstation3 imaging plate reader (**Figure S2**). 0.5 μg/mL compound 48/80 was used to activate MrgprX2 cells at the maximum level. The assay was repeated 3 times.

Pharmacokinetic studies of BCFTP in mice

All *in vivo* pharmacokinetic (PK) studies in adult mice were conducted according to protocols approved by the Animal Care and Use Committee at Johns Hopkins University. For oral bioavailability studies, adult male CD1 mice (25-30 g) were obtained from Harlan and maintained on a 12-hour light-dark cycle with ad libitum access to food and water. BCFTP was administered orally (P.O.) at 30 mg/kg (in 5% DMAC, 10% Tween80, 10% Cremophor, 40% PEG, 35% Water) or intravenously (I.V.) at 1 mg/kg (in 5% DMSO, 10% Tween80, 85% PBS). The mice were sacrificed at specific time points (0.25-8 h) post-drug administration. Blood samples were collected in heparinized microtubes by cardiac puncture, centrifuged at 2000 × g for 15 minutes, and plasma was stored at -80 °C until LC-MS/MS analysis. Dose linearity studies were conducted in a separate cohort at doses of 3-100 mg/kg. All formulations were freshly prepared before dosing. Spinal cord and plasma were collected at 2 hours post-drug administration based on the plasma PK profile. Animals were euthanized with CO2, and blood samples were collected in heparinized microtubes by cardiac puncture. For spinal cord collection, the animal was placed on its chest, the skin on the back was removed, and the muscles along the spine were excised to expose the vertebrae. The spinal column was trimmed from the base of the skull to beyond the femurs using fine-tipped bone nippers. The spinal cord was then gently removed with surgical scissors and forceps. All samples were kept on dry ice and stored at -80 °C at the end of the study.

Sample preparation and bioanalysis

Frozen samples were thawed on ice before extraction. Calibration curves were developed using plasma and/or spinal cord from naïve animals, spiked with BCFTP. Plasma samples (20 μL) were processed using a protein precipitation method by adding 100 μL of methanol containing an internal standard (IS: losartan 0.5 μM), followed by vortexing for 30 seconds and centrifugation at 14,000 rpm for 10 minutes at 4 ºC. Standards and quality controls for the spinal cord were prepared by adding methanol containing IS to the naïve tissue and homogenizing using a Geno Grinder with 2 Spex SS beads at a rate of 1500 strokes/min for 3 minutes. The homogenates were then aliquoted and spiked with BCFTP solution, followed by protein precipitation and simultaneous extraction with methanol containing IS to develop a standard curve. The treated samples were prepared by simultaneous homogenization, protein precipitation, and extraction using 100% methanol (5 μL/mg of tissue) containing IS, followed by vortexing for 30 seconds and centrifugation at 14,000 rpm for 10 minutes at 4 ºC.

The supernatants were carefully transferred to a 96-well plate, and a volume of 2 μL was injected for LC/MS-MS analysis. Chromatographic analysis was performed using a Thermo Scientific Vanquish UHPLC+ system coupled with a TSQ Altis mass spectrometer. Separation of the analyte was achieved at 35 ºC using a Zorbax Agilent Eclipse Plus column (100 mm × 2.1

mm i.d.) packed with a 1.8 μm Phenyl-Hexyl stationary phase. The mobile phase consisted of 0.1% formic acid in acetonitrile and 0.1% formic acid in water with gradient elution (35% → 98% B over 5 minutes). The $[M + H]^+$ ion transitions for BCFTP were m/z 497.07 → 236.03 and 242.04. For losartan (IS), they were m/z 423.17 → 207.125 and 377.125. Calibration curves over the range of 0.01−30 nmol/mL in plasma and 0.001–100 nmol/mg spinal cord were constructed from the peak area ratio of the analyte to the internal standard using linear regression with a weighting factor of 1/(nominal concentration).

Correlation coefficients greater than 0.99 were obtained in all analytical runs for all analytes, and good correlation was observed between brain standards and spinal cord QCs (less than 20% variation between calculated and observed values). Mean plasma concentrations of BCFTP were analyzed using a noncompartmental method as implemented in the computer software program Phoenix WinNonlin version 7.0 (Certara USA, Inc., Princeton, NJ). The maximum plasma and tissue concentration (C_{max}) and time to C_{max} (T_{max}) were the observed values. The area under the plasma and tissue concentration time curve (AUC) value was calculated up to 8 hours ($AUC_{0–t}$) using the log-linear trapezoidal rule.

Animals

All in vivo efficacy and safety experiments were conducted in adult male (6-week-old, 20 to 30 g) and female (18 to 25 g) mice. Mice were housed in standard conditions (12/12-hour light/dark cycle, *food-ad-libitum*), and all behavioral tests were carried out between 9:00 hours to 16:00 hours.

Wild-type (WT) C57BL/6 C mice (The Jackson Laboratory), transgenic MrgprX1 mice, and Mrgpr cluster knockout (aka, $Mrgpr^{-/-}$) mice were used in the present efficacy and safety studies. The details about the generation of MrgprX1 mice were published in our previous report *(26)*. Briefly, we modified a mouse BAC clone (RP23-311C15: Children's Hospital Oakland Research Institute) to generate $MrgprC11^{MrgprX1}$ mice. These mice were crossed with $Mrgpr^{–/–}$ lines to get $Mrgpr^{-/-}$, $MrgpC11^{MrgprX1}$ (aka humanized MrgprX1 mice) *(26)*.

The details on the generation of $Mrgpr^{–/–}$ have been previously published *(29)*. Briefly, chimeric $Mrgpr^{–/–}$ mice were produced by blastocyst injection of embryonic stem cells with deleted genomic DNA between the MrgprA1 and MrgprB4 loci (845 kb region). The $Mrgpr^{–/–}$ mice were generated by mating chimeric mice to C57BL/6 mice. Mice were backcrossed to C57BL/6 mice for at least 5 generations. Resulting $Mrgpr^{-/-}$ mice had 12 endogenous Mrpgrs deleted from the 845 kb region. The deleted cluster represents half of the functional Mrgprs gene repertoire, consisting of members mainly of MrgprA and MrgprC and some of the MrgprB subfamily.

Human DRG specimen

The DRGs were de-identified in compliance with the guidelines of the Institutional Review Board at Johns Hopkins Medical Institution. After dissecting and removing, the DRGs were post-fixed using a 4% paraformaldehyde (PFA) solution in phosphate-buffered saline. The samples were then prepared for RNAscope analysis.

Neuropathic pain model: chronic constriction injury (CCI) of the sciatic nerve

We performed CCI on mice as per the procedure described previously *(47, 75)*. Briefly, under aseptic conditions, mice were anesthetized using an isoflurane anesthesia unit. The left sciatic nerve was exposed at the mid-thigh level through a small incision. Three 9-0 silk ligatures were

tied around the sciatic nerve at approximately 1 mm spacing, leaving a small gap between ligatures. Care was taken to avoid any damage to the adjacent blood vessels. The muscle incision was closed with 4-0 silk sutures, followed by skin closure with a clip applier. Mice were allowed to recover in a warm and quiet environment, and a gel diet was provided for easy access to food during recovery.

Tibial spared nerve injury (SNI-t) model

The SNI-t mouse model was used for testing spontaneous pain, as this model showed more spontaneous pain-like behavior than the CCI model *(33)*. The protocol for SNI-t was reported earlier *(16, 35, 39)*. Briefly, the common peroneal and sural branches of the sciatic nerve were identified and carefully ligated and transected while keeping the tibial nerve intact. The muscle incision was closed with 4-0 silk sutures, followed by skin clipping. Mice were observed until they recovered from the anesthesia.

Inflammatory pain model induced by Complete Freund's adjuvant (CFA)

Mice received an intraplantar injection of 20 µL of CFA (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) into the left hind paw. Pain-like hypersensitivity was assessed at 24- and 72-hours post-injection.

Drugs and chemicals

Synthesis and analytical characterization of BCFTP are provided in the Supplementary methods. BAM8-22 was purchased from Tocris (#1763) and dissolved in 1X PBS for patch clamp studies. For intrathecal administration in mice, the BAM8-22 was dissolved in saline. Morphine (#M8777) and gabapentin (#60142-96-3) were purchased from Sigma and dissolved in saline.

Behavioral assessment

Thermal hyperalgesia: Hargreaves test

The Hargreaves method was used for assessing thermal hypersensitivity in mice *(76)*. The apparatus consists of a radiant heat source, an elevated glass floor, and a control unit (Plantar Test Apparatus, IITC Life Science). Mice were placed inside small acrylic enclosures kept over the glass floor, allowing heat to be applied to the plantar surface of the hind paw. The mouse was allowed to settle in the enclosures comfortably. The latency, or the time taken by the mouse to respond to the thermal stimulus by withdrawing its paw, was considered the endpoint measure. An average of 3 different readings from the same paw/mouse was taken and averaged to get the final paw withdrawal latency. The latencies were measured at pre-injury, post-injury, and post-drug administration time points.

The thermal paw withdrawal latency data were used to calculate the percent anti-hyperalgesia score using the formula: 1-(Pre-injury baseline - Post-drug effect) / (Pre-injury baseline - Pre-drug baseline) X 100. To obtain the in vivo DRC curve, the logarithmic data were normalized as top = maximum observed effect, and bottom = effect observed with the vehicle or without drug. A nonlinear fit analysis was performed to calculate the effective dose 50 (ED_{50}) and hill slope. To calculate specific EDs, the Hill Slope and ED_{50} equation, $ED_F = (F/100 - F)1/\text{hill slope} \times ED_{50,}$ was used, where F is the effect size. Isobolographic analysis was used as described by *Tallarida (77, 78)*. Experimental values below the line of additivity were considered synergistic. The combination index was calculated as: dose of drug A/dose of drug A in combination + dose of drug B/dose of drug B in combination.

Mechanical allodynia: von Frey test

The von Frey test using the frequency method assessed mechanical hypersensitivity in mice *(39)*. During the test, the mice were placed over the elevated wire mesh floor inside the acrylic enclosure. Mice were allowed to habituate to this environment for two consecutive days before conducting the trial. The calibrated filaments of 0.07 gm and 0.4 gm were applied perpendicularly for 3 seconds to the plantar surface of the hind paw of the mouse. The mechanical stimulus was applied a total of 10 times at 10-sec intervals to both hind paws. The paw withdrawal frequency was recorded as a measure of mechanical sensitivity. Data were plotted as percent paw withdrawal threshold on the y-axis and test time points on the x-axis.

Mechanical hyperalgesia: Randall Selitto test

The Randall-Selitto test assessed mechanical hyperalgesia in mice *(79)*. The device consists of a blunt probe for applying pressure and a digital display unit for recording the mechanical threshold (IITC Life Science Inc., CA). The animals were gently restrained using a cotton cloth while applying the probe to their hind paw. Gradually, the pressure was increased until the animal showed a withdrawal response, such as a paw flick, struggling, or vocalization. The force required to elicit the withdrawal response was recorded as the nociceptive mechanical threshold. A total number of three trials were performed at 10-minute intervals to calculate an average mechanical threshold for each paw of the mouse.

Spontaneous pain test

After SNI-t surgery, mice were placed in circular chambers on an elevated glass floor. A mirror positioned beneath the glass allowed high-definition video recording of mouse activity using an overhead HD camera. The test lasted for 1 hour. The total number of flinches during the test of the hind paw was observed as an endpoint measure of spontaneous pain-like behavior *(33)*.

Drug-liking behavior: conditioned place preference (CPP)

We used the CPP test to assess the drug-liking behavior in naïve mice, which is an indicator of the addictive potential of the compound. A 3-chamber unbiased test methodology was adopted for CPP *(39, 41)*. Chambers A and B consisted of distinct visual cues and floor textures, while Chamber C was a corridor allowing animals to enter any of these chambers. On day 1, mice were allowed to freely explore the apparatus for 20 minutes, and Chamber A and B preferences were measured. Exclusion criteria were set for mice showing less than 20% and more than 80% preference for any chambers. The test was counter-biased, and mice were assigned to 2 groups in such a manner that the net preference of each group for A and B remained comparable. On days 2-3, the conditioning was done in morning sessions with BCFTP (30 mg/kg, P.O.) or morphine (10 mg/kg, S.C.) with respective drug chambers for 30 minutes, followed by vehicle pairing in the afternoon sessions. Finally, on day 4, mice were allowed freely to explore the apparatus, and the test was video recorded and analyzed using a Smart 3.0 system (Panlab Harvard apparatus). The preference for drug and vehicle-paired chambers was calculated.

Tolerance study paradigm

To investigate whether tolerance develops to repeated drug treatment, we adopted a pre-established standard behavioral pharmacological approach *(38, 39)*. Mice were acclimatized to the laboratory conditions and Hargreaves apparatus before pain testing. Thermal paw withdrawal latencies were tested before and after CCI. Mice were then divided into 2 groups: one to receive repeated BCFTP treatment by oral gavage, and the other to receive vehicle treatment. On the first

day of the protocol (day 12 post-CCI), all mice orally received BCFTP (30 mg/kg, P.O.), and paw withdrawal latencies were measured at 2 hours post-administration (peak of drug effect). From the very next day, 1 group of mice received oral 30 mg/kg BCFTP twice daily (am/pm) for 3 consecutive days. Another group was administered vehicles twice daily. After this tolerance-induction paradigm, on the fifth day of the protocol, all mice received oral 30 mg/kg BCFTP, and withdrawal latencies were measured at 2 hours post-administration.

In a separate study, we examined the anti-heat hyperalgesic effect of BCFTP under morphine tolerance conditions. Mice with CCI were subcutaneously given morphine (10 mg/kg, S.C.) in the back twice a day for 3 consecutive days to induce opioid tolerance rapidly. Effects of BCFTP (30 mg/kg, P.O.) on CCI-induced PWL were tested before and after repeated morphine treatment. The % reversal of hyperalgesia to the pre-injury baseline was calculated to compare the effects.

Itch test

Mice were acclimatized to the laboratory room before testing. On the test day, MrgprX1 mice were orally administered with BCFTP (100 mg/kg, P.O.), and the behavioral response was video-recorded for 30 minutes. The scoring was done by an experimenter in a blinded manner. A scratching bout was defined as continuous scratching movements using the hind paws and wiping the face *(29)*.

Rotarod test

The rotarod test assesses motor coordination in rodents *(18, 39)*. On day 1, mice were placed on a rotating rod at 5 rpm for 60 seconds. The trial was repeated 3 times, and mice falling off the rotating rod were excluded from the study. The next day, mice were again placed on a rotating rod (5 rpm), and the speed of the rod was gradually increased up to 40 rpm over 300 seconds using an automated system (AccuRotor, Omnitech Electronics, USA). In the rotarod test, the goal is for the mouse to maintain balance and stay on the rod for as long as possible. The time the mouse stayed on the rod before falling off was recorded as a measure of motor coordination.

Open-field test

The spontaneous exploratory activity of mice was evaluated using an open arena with defined boundaries (40 x 40cm). The floor of the open field apparatus was divided into 3 zones: center, middle, and periphery. Mice were individually placed in the center of the arena, and the test was video-recorded for 10 minutes. Smart 3.0 system was used to analyze the total distance traveled as a measure of the exploratory activity of mice *(39)*.

Immunofluorescence

Mice were anesthetized and perfused intracardially with 30 mL of pre-cooled phosphate-buffered saline (PBS), followed by 30 mL of pre-cooled 4% paraformaldehyde (PFA) (vol/vol). L4 and L5 spinal cord and DRG tissue were harvested using aseptic conditions and fixed with 4% PFA. Tissues were cryoprotected in 30% sucrose (wt/vol) at 4 °C. Tissues were sectioned (spinal cord: 20 µm, DRG: 10 µm) with the cryostat (Leica), and slides were prepared. Further, tissues were hydrated, and an antigen retrieval agent was applied for 10 minutes at 95 °C using a water bath. Blocking was done with 10% goat serum, followed by the application of primary antibodies (BAM22 1:500, CGRP 1:200, β-II-Tubulin 1:200, and synaptophysin 1:200) at 4 °C overnight. Tissues were washed with 0.3% TBST. For BAM-22, we used tyramide-based signal amplification (#B40925) by incubating the horseradish peroxidase-conjugated goat anti-rabbit

IgG, followed by the application of tyramide working solution *(26)*. Fluorescent-tagged secondary antibodies were used for other cell markers. Slides were mounted with DAPI, and sections were imaged using a Leica epifluorescence microscope and Zeiss LSM 800. Image J was used to quantify the fluorescence signal intensities.

RNAscope

The RNAscope Multiplex Fluorescent Reagent Kit V2 (Cat. No. 323110) was used for *in situ* hybridization using MrgprX1 (517011-C2, ACD) and mu-opioid receptor (531151-C1, ACD) probes. The manufacturer's protocol was used to perform *in situ* hybridization. Briefly, prepared slides were dehydrated with ethanol and air-dried at room temperature, then incubated with hydrogen peroxide. Slides were then placed in pre-heated retrieval regent followed by incubation with Protease Plus. Subsequently, *MRGPRX1* and *OPRM1* probes were applied and incubated using a HybEZTM II oven. Signal amplification was done through fluorescent labeling with TSA-based technology. Samples were mounted with ProLong Gold Antifade Mountant and imaged using a confocal imaging system (Zeiss LSM 800, Germany).

Whole-cell voltage-clamp recording from spinal cord slices

A laminectomy was performed in MrgprX1 mice 12-14 days post-CCI under deep anesthesia with 2% isoflurane (Abbott Laboratories, North Chicago, IL, USA). The lumbosacral segment of the spinal cord was swiftly excised. It was immediately placed in ice-cold, low-sodium Krebs solution (in mM: 95 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4-H2O, 6 MgCl2, 1.5 CaCl2, 25 glucose, 50 sucrose, 1 kynurenic acid), which was saturated with 95% O2/5% CO2. The tissue was then trimmed and mounted on a Vibratome VT1200 tissue slicer (Leica Biosystems). Transverse slices (400 μm) with attached dorsal roots were prepared and incubated in preoxygenated low-sodium Krebs solution without kynurenic acid. The slices were allowed to recover at 34 °C for 40 minutes, followed by an additional 1-hour recovery at room temperature before experimental recordings. Whole-cell patch-clamp recordings were conducted on lamina II cells using an Olympus fixed-stage microscope system (Melville, NY, USA) under oblique illumination. The slices were transferred to a low-volume recording chamber and continuously perfused with room-temperature Krebs solution (in mM: 125 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4-H2O, 1 MgCl2, 2 CaCl2, 25 glucose) bubbled with 95% O2/5% CO2 at a flow rate of 5 mL/min. Data were acquired using pClamp10 software and a MultiClamp amplifier (Molecular Devices, Sunnyvale, CA, USA). Thin-walled glass pipettes (World Precision Instruments, Sarasota, FL, USA) were pulled using a P1000 puller (Sutter, Novato, CA, USA) to achieve resistances of 3-6 MΩ and filled with an internal saline solution (in mM: 120 K-gluconate, 20 KCl, 2 MgCl2, 0.5 EGTA, 2 Na2-ATP, 0.5 Na2-GTP, and 20 HEPES). Cells were voltage-clamped at -70 mV, with membrane current signals sampled at 10 kHz and low-pass filtered at 2 kHz. Test stimuli were delivered at a frequency of 0.05 Hz with a 500 μA intensity, followed by a 100-ms-long 5 mV depolarizing pulse to measure series resistance (R series) and input resistance (R input). Cells were discarded if R series or R input values changed by more than 20%.

SUPPLEMENTARY FIGURES:

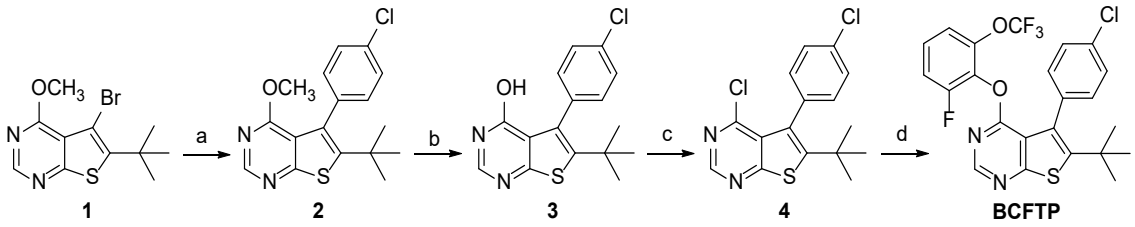

Figure S1. Synthesis of BCFTP. Reagents and conditions: (a) 4-chlorophenylboronic acid, $PdCl_2(PPh_3)_2$, K_2CO_3, DMF, 80 °C; (b) BBr_3, dichloromethane, rt; (c) $POCl_3$, 110 °C; (d) 2-fluoro-6-(trifluoromethoxy)phenol, NaH, DMF, 65 °C to rt.

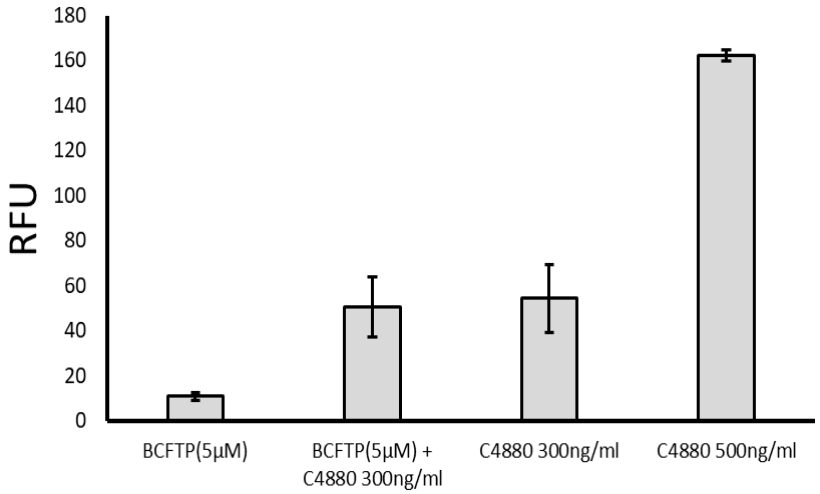

Figure S2. Calcium imaging assay to determine the selectivity of compound BCFTP over MrgprX2. EC20 of C48/80 was used to activate MrgprX2, with and without the presence of BCFTP. Data are presented as mean ± SEM (n=6/group).

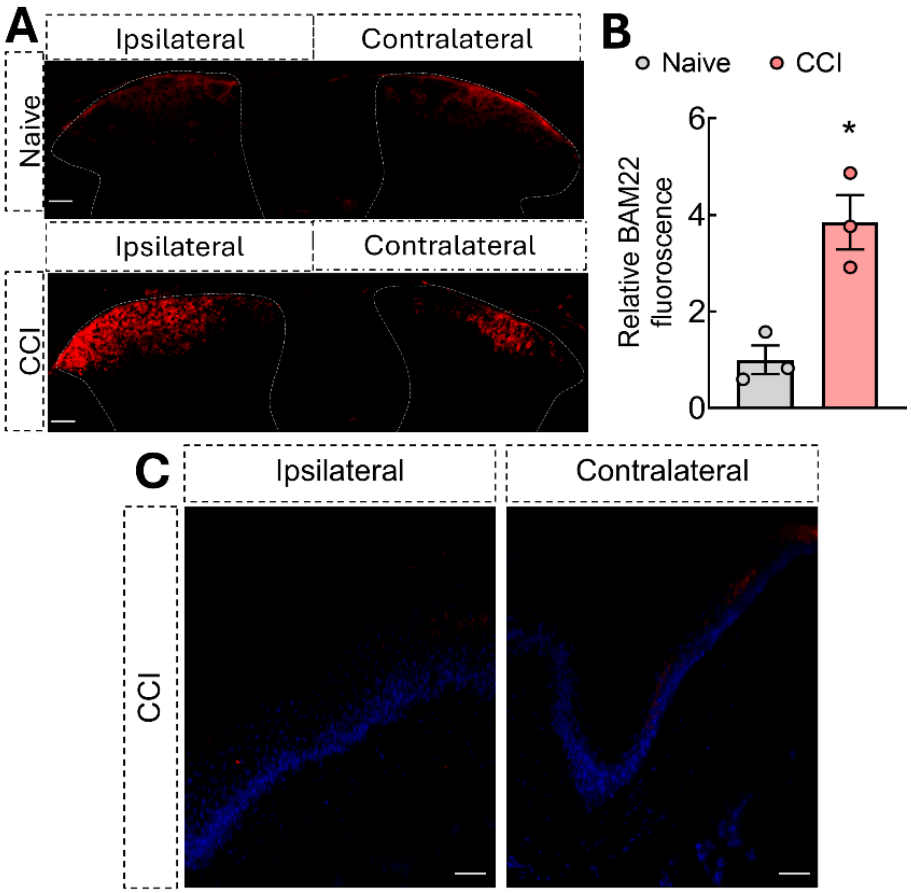

Figure S3. BAM22-immunoreactivity (IR) was increased in the ipsilateral dorsal horn of the spinal cord in humanized MrgprX1 mice after CCI. (A) Representative image of BAM22-immunoreactivity (IR, red) in the spinal cord of MrgprX1 naïve and CCI mice at day 14 post-injury. Scale bar: 100 µm. **(B)** Quantification of BAM22-IR (normalized to the mean of the contralateral side, followed by the mean of naïve mice) in the ipsilateral dorsal horn of MrgprX1 mice at 14 days post-CCI. Data are shown as mean ± SEM. *$p<0.05$ vs naive. Welch's t-test (n=3 mice/group, 2 slices/mouse). **(C)** BAM22 expression in the skin of MrgprX1 mice at day 14 post-CCI. Scale bar: 50 µm.

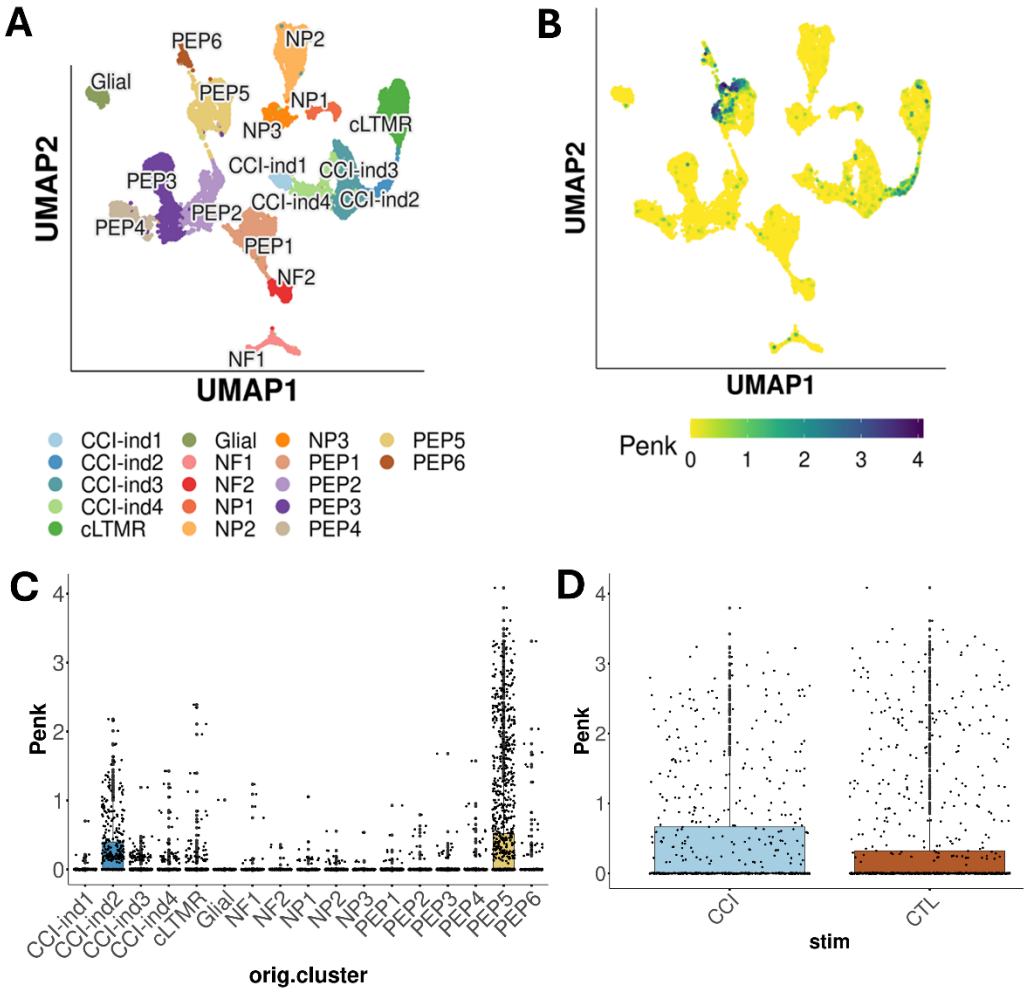

Figure S4. Single-cell RNA-sequencing (scRNA-seq) data of lumbar DRG showing *Penk* expression was upregulated in mice after chronic constriction injury (CCI) of the sciatic nerve. (A) Distinct clusters of cells in the dorsal root ganglion (DRG) of *Pirt*^{EGFPf} mice, including SGC (1), NF (2), NP (3), PEP (6), cLTMR (1), and CCI-induced clusters were identified by Seurat in scRNA-seq study. The identities of 17 cluster cells are visualized by uniform manifold approximation and projection (UMAP). **(B)** *Penk* expression in peptidergic neuron cluster (PEP5) and nerve injury-induced neuronal cluster (CCI-ind2). **(C)** *Penk* expression across different cell populations. **(D)** *Penk* expression in the PEP5 neuronal cluster of lumbar DRGS of CCI mice and uninjured control mice.

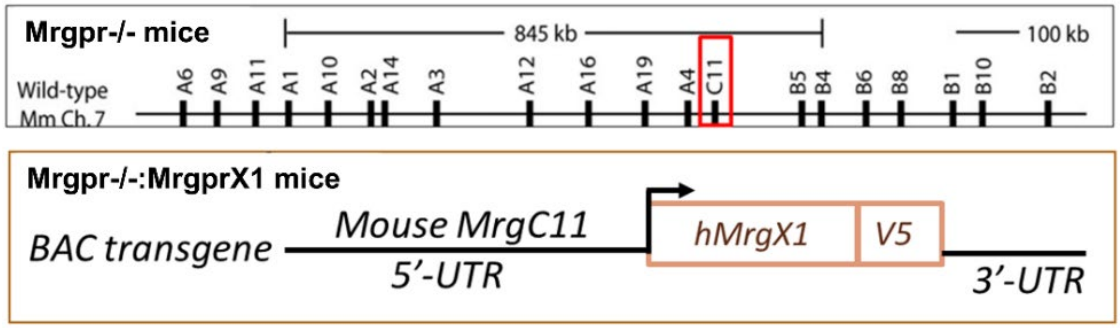

Figure S5. Scheme showing the development of Mrgpr$^{-/-}$ mice and humanized MrgprX1 mice.

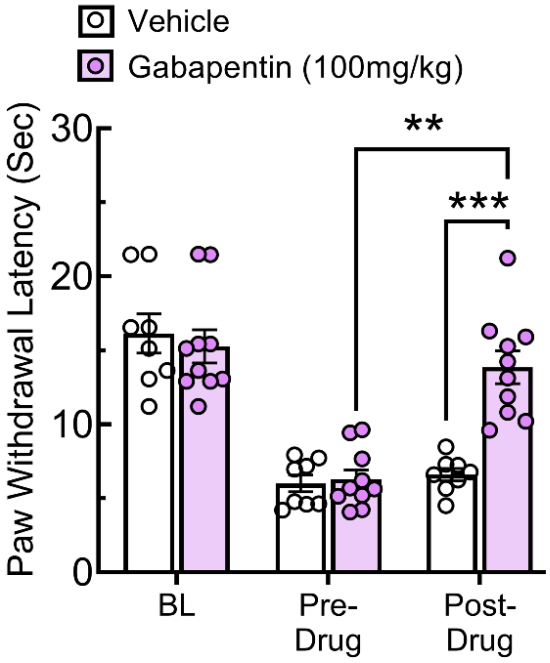

Figure S6. Effect of gabapentin on CCI-induced paw heat hyperalgesia. Effect of gabapentin (100 mg/kg, P.O.) on paw withdrawal latency (PWL) in CCI MrgprX1 mice at 2 h post-drug administration, compared to pre-drug and vehicle treatment. Data are mean ± SEM. Tukey's multiple comparison analysis (n=9-10/group). **p<0.01, ***p<0.001.

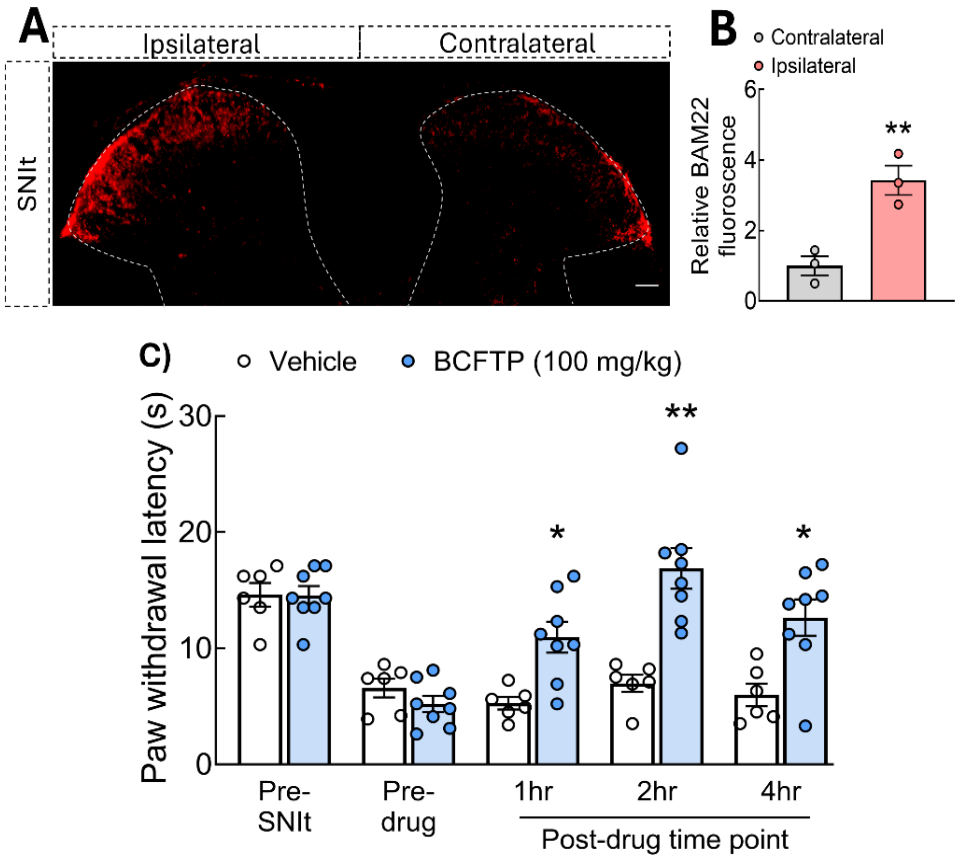

Figure S7. BCFTP attenuates heat hyperalgesia in MrgprX1 mice after SNI-t. (A) Representative image of BAM22-immunoreactivity (IR) in the spinal cord dorsal horn of MrgprX1 mice at day 40 after SNI-t. Scale bar: 100 µm. **(B)** Quantification of BAM22-immunoreactivity (IR). Data are shown as mean ± SEM. **p<0.01 vs contralateral. Welch's-t test. **(C)** Effect of oral administration of BCFTP (100 mg/kg, P.O.) on heat hyperalgesia in MrgprX1 mice at day 35 post-SNI-t (n=6-7/group). Data are shown as mean ± SEM. *p<0.05, **p<0.01 vs pre-drug. Tukey's post hoc analysis.

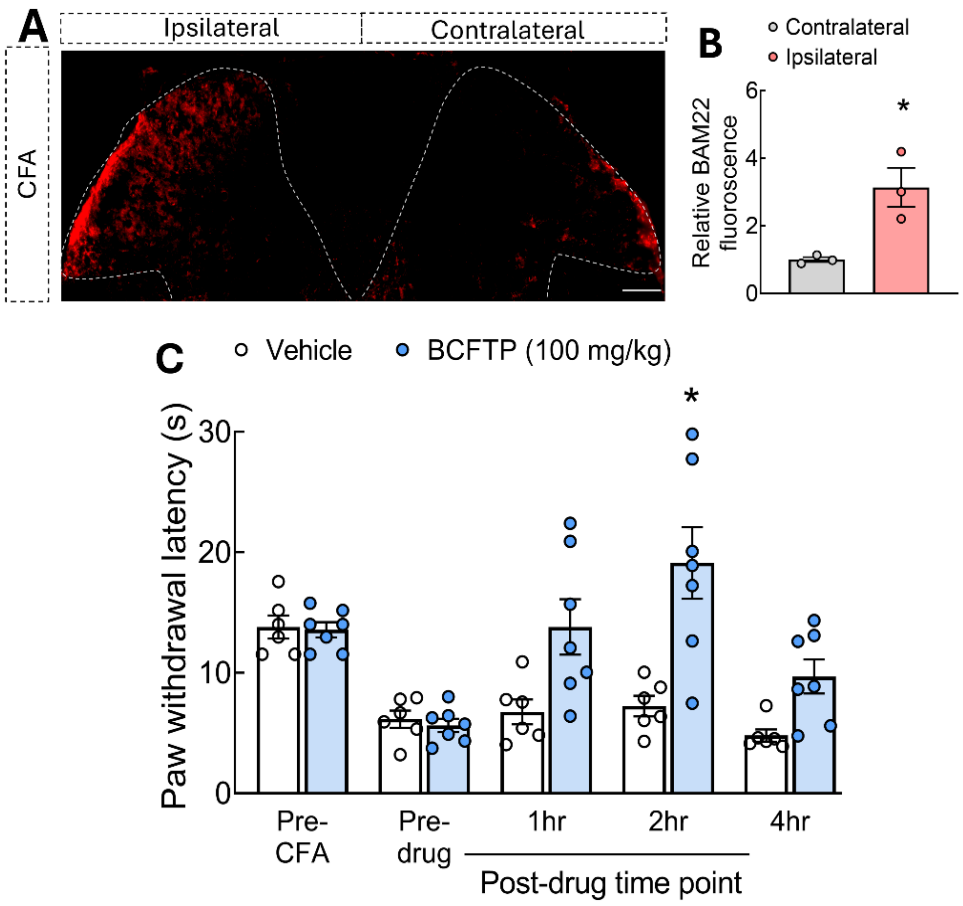

Figure S8. BCFTP attenuates CFA-induced heat hyperalgesia in MrgprX1 mice. (A) Representative image of BAM22-immunoreactivity (IR) in the spinal cord dorsal horn in MrgprX1 mice at day 5 after receiving an intraplantar injection of CFA**.** Scale bar: 100 µm. **(B)** Quantification of BAM22-IR. Data are shown as mean ± SEM. *p<0.05 vs contralateral. Welch's-t test. **(C)** Effect of oral administration of BCFTP (100 mg/kg, P.O.) on heat hyperalgesia in MrgprX1 mice injected with intraplantar CFA (n=6-8/group). Data are shown as mean ± SEM. *p<0.05 vs pre-drug. Tukey's post hoc analysis.

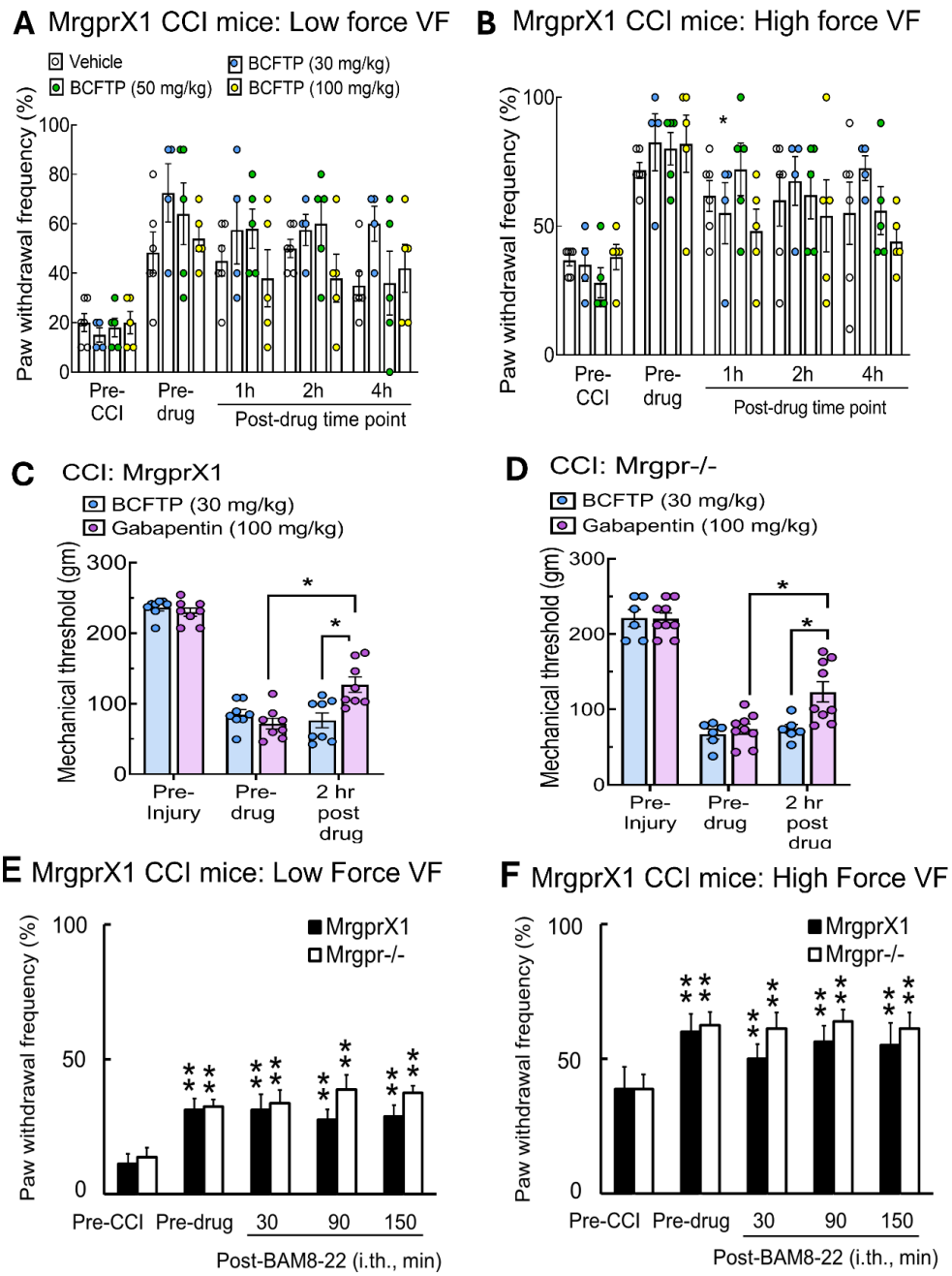

Figure S9. BCFTP did not inhibit mechanical hypersensitivity in MrgprX1 mice after nerve injury. (A-B) Ipsilateral paw withdrawal frequency (PWF) to low-force 0.07gm (A) and high-force 0.4 gm (B) von Frey filament stimulation in MrgprX1 mice after administration of different doses of BCFTP (P.O., n=8-12/group) at day 12-16 after sciatic chronic constriction injury (CCI). Data are mean ± SEM. Two-way mixed model ANOVA, followed by Bonferroni's post hoc test. **(C-D)** Effect of gabapentin (P.O.) and BCFTP (P.O.) on CCI-induced mechanical hyperalgesia in MrgprX1 (C) and Mrgpr$^{-/-}$ mice (D) in the Randall Selitto test. Data are mean ± SEM. Tukey's multiple comparison analysis (n=8-12/group). **(E-F)** Effect of intrathecal (i.th.) injection BAM8-22 (5 mM, 5 µL) on PWFs to von Frey filament stimulation in CCI MrgprX1 and Mrgpr$^{-/-}$ mice. Data are mean ± SEM. Tukey's multiple comparison analysis (n=8-12/group).

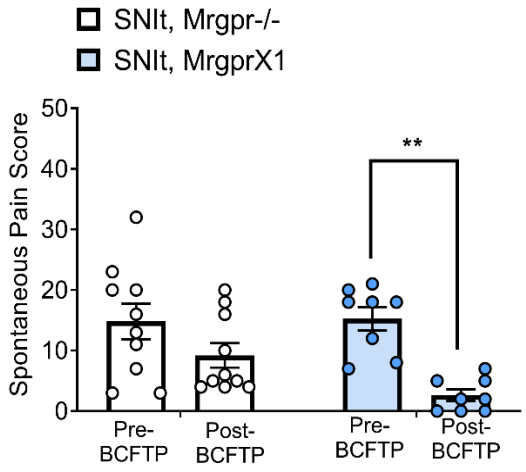

Figure S10. BCFTP attenuates the spontaneous pain in MrgprX1 mice but not in Mrgpr$^{-/-}$ mice after SNI-t. Spontaneous pain score before and after administration of BCFTP (100 mg/kg, P.O.) in Mrgpr$^{-/-}$ mice at day 14 after SNI-t (n=8-12/group). Data are mean ± SEM. **p<0.01, Tukey's post hoc analysis.

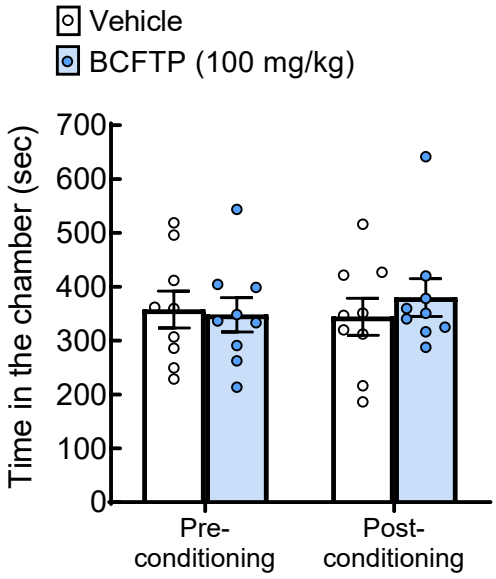

Figure S11. **MrgprX1 mice with CCI do not show a preference for the BCFTP-paired chamber.** Time spent by MrgprX1 mice (at day 14 post-CCI) in the vehicle versus drug-paired chamber at pre-conditioning and post-conditioning time points (n=9/group). Data are expressed as mean ± SEM. Tukey's post hoc analysis.

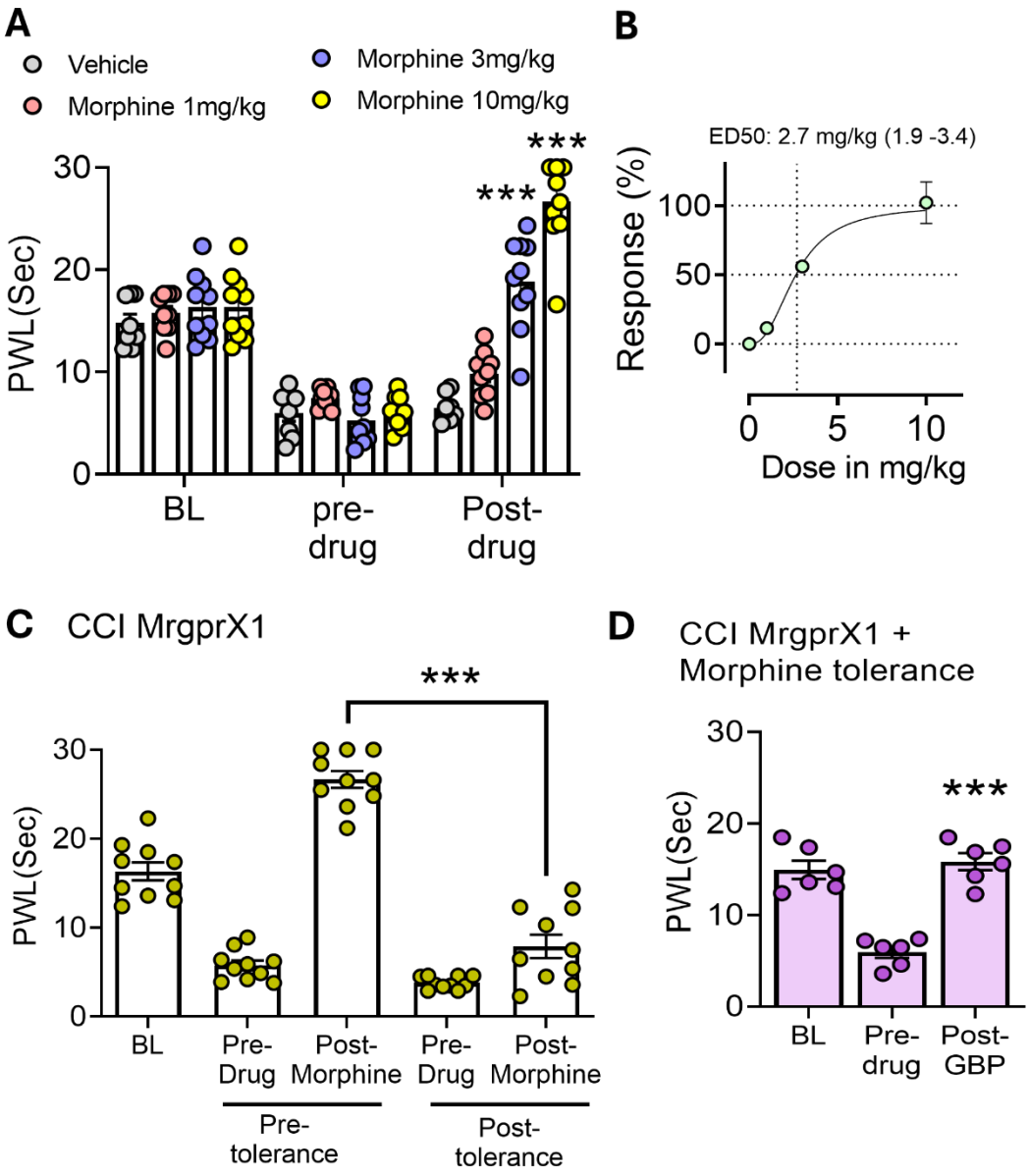

Figure S12. Gabapentin remained effective in morphine-tolerant MrgprX1 mice after nerve injury. (A) Ipsilateral paw withdrawal latency (PWL) after morphine (1, 3, 10 mg/kg, S.C.) administration in MrgprX1 mice after CCI, compared to pre-drug. Bonferroni's multiple comparison analysis (n=8-10 /group). ***p<0.001. **(B)** In vivo dose-response curve of morphine. Non-linear regression analysis, ED_{50}=2.7 mg/kg. Data are mean ± SEM. **(C)** PWLs before and after repeated administration of morphine (10 mg/kg, S.C.) twice a day for three consecutive days in MrgprX1 mice after CCI. One-way ANOVA followed by Tukey's multiple comparison analysis, n=10. ***p<0.001. **(D)** Effects of gabapentin (GBP, 100 mg/kg, P.O., n=6) on CCI-induced reduction in PWL of MrgprX1 mice with morphine tolerance. Data are mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison analysis. ***p<0.001.

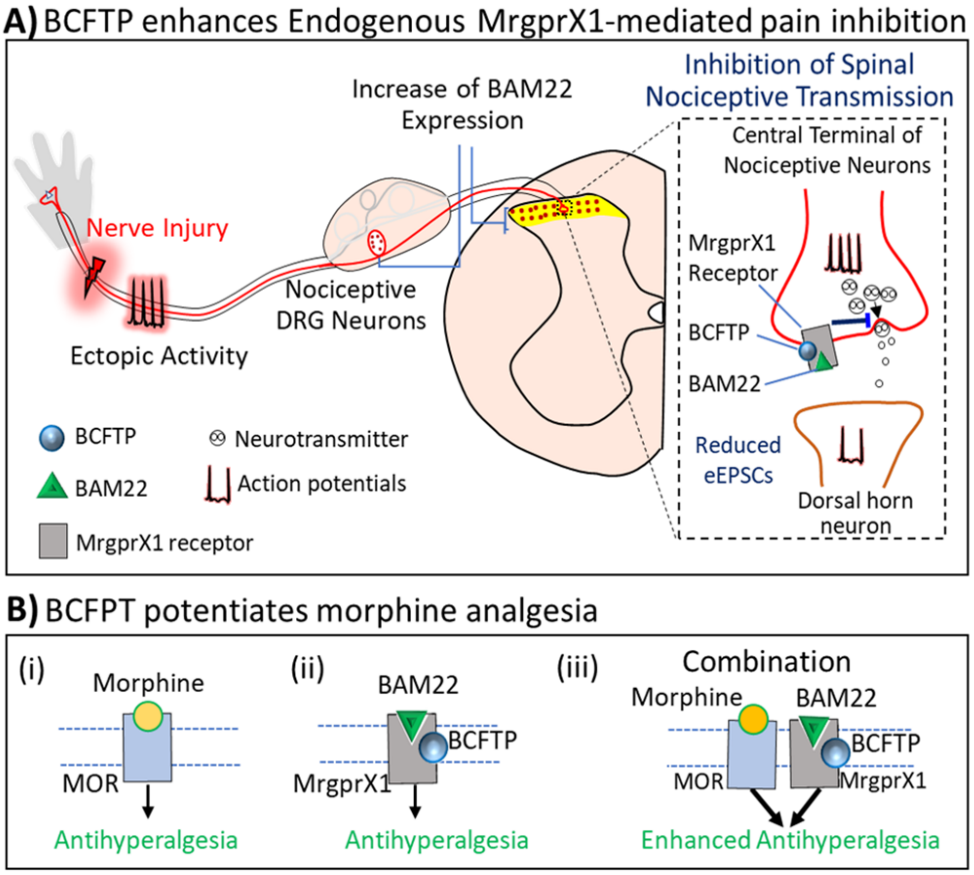

Figure S13. Summary of major findings. **(A)** BCFTP leverages the upregulated BAM22 expression in the spinal cord dorsal horn after periphery nerve injury to potentiate the endogenous MrgprX1-mediated inhibition of spinal nociceptive transmission, offering a promising non-opioid strategy for neuropathic pain management. **(B)** MOR agonist and MrgprX1 PAM combination may also provide a new strategy for opioid-sparing pain treatment.

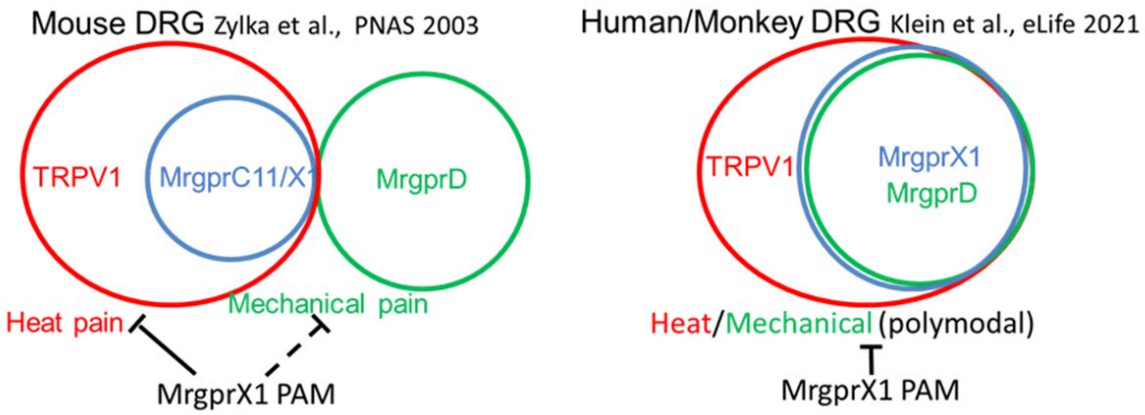

Figure S14. Differential distribution of mouse MrgprC11 and human MrgprX1 in DRG neurons. Left: In humanized MrgprX1 mice, the expression of MrgprX1 is restricted to the MrgprC11-expressing neurons, which do not overlap with a MrgprD-expressing subpopulation of neurons *(38)*. This may partly explain the lack of effect on mechanical hypersensitivity observed in MrgprX1 mice. Right: However, MrgprX1 expression in human DRG is wider and overlaps with a subpopulation of MrgprD-expressing neurons, which are important to mechanical pain hypersensitivity *(24)*.

TABLES:

Table S1. Aqueous solubility of BCFTP

Compound	Solubility in Assay Buffer (μM)		
	PBS (pH 7.4)	SGF (pH 1.2)	SIF (pH 6.8)
Control	12.14 (Progesterone)	7.68 (Diclofenac)	310.80 (Diclofenac)
BCFTP	<0.01	0.049	48.97

Table S2. Metabolic stability of BCFTP in liver microsomes

Species	% Remaining	
	30 min	60 min
Mouse	>95	>95
Human	>95	>95

Table S3. Permeability results of BCFTP and control compounds in MDCKII-MDR1 cell line (w/ 4% BSA)*

Compound ID	$P_{app\ (A-B)}$ (10^{-6}, cm/s)	$P_{app\ (B-A)}$ (10^{-6}, cm/s)	Efflux Ratio	Recovery (%) AP-BL	Recovery (%) BL-AP
Metoprolol	22.86	29.52	1.29	93.48	96.01
Digoxin	0.41	11.75	28.68	98.85	105.19
BCFTP	0.49	0.87	1.76	93.81	99.71

*BSA was added to the incubation system to improve the recovery rates

Table S4. Assays in which BCFTP displayed an inhibition higher than 50% at 10 μM

Assay	% Inhibition of Control Specific Binding
5-HT_{2B}(h) (agonist radioligand)	50.5%
Ca^{2+} channel (L, dihydropyridine site) (antagonist radioligand)	62.2%
Potassium Channel hERG (human) − [^{3}H]Dofetilide	52.1%

Table S5. hERG safety evaluation of BCFTP by SyncroPatch 384i System

Compound ID	Highest Conc. (μM)	hERG IC_{50} (μM)

Cisapride (Positive Control)	1	0.019
BCFTP	30	28.45

Table S6. Summary results of the mutagenicity assay in *Salmonella typhimurium* strain TA98

Dose Level (µg/well)	S9 Mix	Individual Revertant Colony Counts			Mean Revertant Colony Counts	SD	Ratio	Cytotoxicity Code[a]
1.5	+	5	5	3	4.33	1.15	0.87	N
4		3	7	3	4.33	2.31	0.87	N
10		2	4	11	5.67	4.73	1.13	N
25		5	2	10	5.67	4.04	1.13	N
64		6	5	3	4.67	1.53	0.93	N
160		4	4	3	3.67	0.58	0.73	N
400		4	4	4	4.00	0.00	0.80	NP
1000		8	6	7	7.00	1.00	1.40	NP
Solvent		9	5	9	5.00	3.29	1.00	N
		2	3	2				
Positive Control (2-AA)		225	274	293	264.00	35.09	52.80	N
1.5	-	4	5	4	4.33	0.58	0.87	N
4		3	4	1	2.67	1.53	0.53	N
10		3	4	2	3.00	1.00	0.60	N
25		10	4	4	6.00	3.46	1.20	N
64		4	10	3	5.67	3.79	1.13	N
160		7	6	1	4.67	3.21	0.93	N
400		6	4	6	5.33	1.15	1.07	NP
1000		3	2	7	4.00	2.65	0.80	NP
Solvent		2	8	4	5.00	2.10	1.00	N
		6	6	4				
Positive Control (2-NF)		436	421	406	421.00	15.00	84.20	N

[a]N = Normal; R = Reduced; A = absent; O = obscured by precipitate; P = Precipitate. 2-AA (2-Aminoanthracene): 0.6 µg/well. 2-NF (2-nitrofluorene): 2 µg/well.

Table S7. Pharmacokinetic characterization of BCFPT.

Treatment group	Pharmacokinetic data						
	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	V_d (mL/h/Kg)	CL (mL/h/kg)	AUC_{0-t} (ng.h/mL)	%F
IV-1 mg/kg	2575	-	2.49	1280	360	2768	
PO- 30 mg/kg	10,288	1	2.10	-	-	37,994	46%

*%F is the dose-adjusted bioavailability.