

Inhibition of inflammatory pain and cough by a novel charged sodium channel blocker

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Abstract

Background and Purpose: Many pain-triggering nociceptor neurons express TRPV1 or TRPA1, cation-selective channels with large pores that enable permeation of QX-314, a cationic analogue of lidocaine. Co-application of QX-314 with TRPV1 or TRPA1 activators can silence nociceptors. We now describe BW-031, a novel more potent cationic sodium channel inhibitor, test whether its application alone can inhibit the pain associated with tissue inflammation, and whether this strategy can also inhibit cough. **Experimental Approach:** We characterized BW-031 inhibition of sodium channels and tested BW-031 in three models of inflammatory pain: rat paw inflammation produced by Complete Freund's Adjuvant injection or surgical incision and a mouse paw UV burn model. We also tested the ability of BW-031 to inhibit cough induced by inhalation of dilute citric acid in guinea pigs. **Key Results:** BW-031 inhibited Nav1.7 and Nav1.1 channels with ~6-fold greater potency than QX-314 when introduced inside cells and entered capsaicin-activated TRPV1 expressing sensory neurons. BW-031 inhibited inflammatory pain in all three models, producing more effective and longer-lasting inhibition of pain than QX-314 in the mouse UV burn model. BW-031 was also effective in reducing cough counts by 78-90% when applied intratracheally under isoflurane anesthesia or by aerosol inhalation in awake guinea pigs with airway inflammation produced by ovalbumin sensitization. **Conclusion and Implications:** BW-031 a novel cationic sodium channel inhibitor can be applied locally as a single agent to inhibit inflammatory pain and also effectively inhibits cough in a guinea pig model of nociceptor-activated cough, suggesting a new clinical approach to treating cough.

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Running title: Inhibition of pain and cough by BW-031

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Author Contributions

I.T., S.J., N.A., M.K., B.D.L., B.P.B. and C.J.W. designed experiments; I.T., S.J., N.A., M.K., B.D., J.S., S.T., D.R., J.L., L.H., S.M.J. carried out experiments; I.T., S.J., N.A., M.K., B.D., J.S., L.H. and S.M.J. analyzed data; I.T., S.J., N.A., M.K., B.D., J.S., B.D.L., B.P.B. and C.J.W. provided advice on the interpretation of data; I.T., B.D.L., B.P.B. and C.J.W. wrote the manuscript with input from all co-authors; B.D.L., B.P.B., and C.J.W. supervised the study. All authors approved the final manuscript.

Conflict of Interest

B.D.L., B.P.B. and C.J.W. are cofounders of and equity holders in Nocion Therapeutics which is developing charged sodium channel blockers as treatments for various disease indications, including cough, and which has licensed BW-031 from Harvard Medical School. I.T., S.J., N.A., S.T. and D.R. also have founder shares in Nocion.

Abstract

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cation-selective channels with large pores that enable permeation of QX-314, a cationic analogue of lidocaine. Co-application of QX-314 with TRPV1 or TRPA1 activators can silence nociceptors. We now describe BW-031, a novel more potent cationic sodium channel inhibitor, test whether its application alone can inhibit the pain associated with tissue inflammation, and whether this strategy can also inhibit cough.

Experimental Approach: We characterized BW-031 inhibition of sodium channels and tested BW-031 in three models of inflammatory pain: rat paw inflammation produced by Complete Freund's Adjuvant injection or surgical incision and a mouse paw UV burn model. We also tested the ability of BW-031 to inhibit cough induced by inhalation of dilute citric acid in guinea pigs.

Key Results: BW-031 inhibited $\text{Na}_v1.7$ and $\text{Na}_v1.1$ channels with ~ 6 -fold greater potency than QX-314 when introduced inside cells and entered capsaicin-activated TRPV1 expressing sensory neurons. BW-031 inhibited inflammatory pain in all three models, producing more effective and longer-lasting inhibition of pain than QX-314 in the mouse UV burn model. BW-031 was also effective in reducing cough counts by 78-90% when applied intratracheally under isoflurane anesthesia or by aerosol inhalation in awake guinea pigs with airway inflammation produced by ovalbumin sensitization.

Conclusion and Implications: BW-031 a novel cationic sodium channel inhibitor can be applied locally as a single agent to inhibit inflammatory pain and also effectively inhibits cough in a guinea pig model of nociceptor-activated cough, suggesting a new clinical approach to treating cough. (250 words)

Introduction

Most clinically-used local anaesthetics are tertiary amine molecules that exist in an equilibrium between cationic (protonated) and neutral (unprotonated) forms. The neutral molecule can gain access to the key binding site in the channel pore (Ragsdale et al., 1996) by diffusing into the lipid membrane surrounding the channel and then entering the channel protein through “fenestrations” in the wall of the pore region (Payendah et al., 2011; Martin and Corry, 2014; Gamal El-Din et al., 2018; Nguyen et al., 2019), corresponding to the “hydrophobic pathway” proposed in a classic analysis of local anesthetic action (Hille, 1977). In a second “hydrophilic pathway” proposed in this analysis, the neutral form of the drug can diffuse across the membrane into the cytoplasm, where a fraction is re-protonated. From the cytoplasm, both cationic and neutral drug molecules can enter the channel to reach the binding site in the pore, but only when the channel is open. Consistent with this hypothesis, QX-314, a permanently charged quaternary analogue of lidocaine, cannot inhibit neuronal sodium channels when applied externally, but is effective when applied intracellularly (Frazier et al., 1970; Strichartz et al., 1973).

Although QX-314 cannot readily diffuse across the cell membrane, it can enter some neurons by permeating through certain cation-selective ion channels that have unusually large pores, notably TRPV1 and TRPA1 channels (Binshtok et al., 2007; Puopolo et al., 2013). Because these channels are selectively expressed in populations of nociceptor (pain-triggering) primary sensory neurons, application of QX-314 together with activators of TRPV1 or TRPA1 channels can produce selective inhibition/silencing of nociceptors with minimal inhibition of motor neurons or non-nociceptive sensory neurons, in contrast to the non-selective nerve block produced by lidocaine (Gerner et al., 2008; Binshtok et al., 2009a; Kim et al., 2010; Roberson et al., 2011; Brenneis et al., 2013; Zhou et al., 2014).

Pain associated with tissue inflammation is mediated in part by activation of TRPV1 and TRPA1 channels in nociceptors (reviewed by Bautista et al., 2013; Julius, 2013), raising the possibility that endogenous activation of these channels might be sufficient to enable entry of QX-314 or other cationic sodium channel blockers in inflamed tissue, without requiring co-application with exogenous TRP activators. An especially intriguing possible application of the strategy is in cough, which is mediated by airway sensory neurons, including a population of nociceptors expressing TRPV1 and TRPA1 channels (Bonvini et al., 2015; Canning et al., 2014; Mazzone and Udem, 2016). Inhaled lidocaine is used to inhibit reflexive laryngospasm and cough during bronchoscopy and is highly effective for acute suppression of cough in patients with upper respiratory tract infections (Peleg and Binyamin, 2002), COPD (Chong et al., 2005; Udezue, 2001), and asthma (Slaton et al., 2013; Udezue, 2001). However, lidocaine has a short duration of action (Chong et al., 2005) and produces potential cardiac and CNS side effects (Shirk et al., 2006) as a consequence of its high lipophilicity and ready diffusion into the bloodstream. Also, because lidocaine blocks activity in motor neurons as well as sensory neurons, it inhibits swallowing and the gag reflex (Noitasaeng et al., 2016), limiting its clinical utility.

We have designed and synthesized a novel cationic compound, BW-031, with improved potency for inhibiting sodium channels compared to QX-314. We find that BW-031 when applied alone to inflamed tissue produces long-lasting inhibition of inflammatory pain in several rat and mouse models and that inhaled BW-031 can effectively inhibit cough in a guinea pig model of allergic airway inflammation. (570 words)

Methods

Chemicals. Except for BW-031, all chemicals were purchased from Sigma Aldrich or Tocris Bioscience. BW-031 (1-(1-(2, 6-dimethylphenylamino)-1-oxobutan-2-yl)-1-ethylpiperidinium) was synthesized by Acesys Pharmatech according to the pathway described in Supporting Information.

Stable cell line electrophysiology. Human embryonic kidney (HEK 293) cells stably expressing the human $\text{Na}_v1.7$ channel (Liu et al., 2012) were grown in Minimum Essential Medium (MEM, ATCC) containing 10% fetal bovine serum (FBS, Sigma), penicillin/streptomycin (Sigma), and 800 $\mu\text{g}/\text{ml}$ G418 (Sigma) under 5% CO_2 at 37°C. Human embryonic kidney (HEK 293) cells stably expressing the human $\text{Na}_v1.1$ channel (gift of Dr. Alfred L. George, Jr.) were grown in Dulbecco’s Modified Eagle Medium (DMEM, Thermo Fisher Scientific) containing 10% FBS (Sigma), penicillin/streptomycin (Sigma), and 3 $\mu\text{g}/\text{ml}$ Puromycin (Sigma) under 5% CO_2 at 37°C. Chinese Hamster Ovary (CHO-K1) cells stably expressing the human $\text{Na}_v1.8$ channel and beta 3 subunit (B’SYS GmbH) were grown in Ham’s F-12 medium (Corning) containing 10% FBS, penicillin/streptomycin (Sigma), and 3.5 $\mu\text{g}/\text{ml}$ Puromycin (Sigma) and 350 $\mu\text{g}/\text{ml}$ Hygromycin (Sigma) under 5% CO_2 at 37°C. For electrophysiological recordings, cells were re-plated on coverslips for 1 to 6 h before recording. Whole-cell recordings were obtained using patch pipettes with resistances of 2-2.5 $\text{M}\Omega$ when filled with the internal solution consisting of (in mM): 61 CsF, 61 CsCl, 9 NaCl, 1.8 MgCl_2 , 9 EGTA, 14 creatine phosphate (tris salt), 4 MgATP , and 0.3 GTP (tris salt), 9 HEPES, pH adjusted to 7.2 with CsOH. The shank of the electrode was wrapped with Parafilm in order to reduce capacitance and allow optimal series resistance compensation without oscillation. Seals were obtained and the whole-cell configuration established with cells in Tyrode’s solution consisting of (in mM): 155 NaCl, 3.5 KCl, 1.5 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH. After establishing whole-cell recording, cells were lifted off the bottom of the recording chamber and placed in front of an array of quartz flow pipes (250 μm internal diameter, 350 μm external diameter). Recordings were made using a base external solution of Tyrode’s solution with added 10 mM TEA-Cl to inhibit small endogenous potassium currents. Solution changes were made (in < 1 second) by moving the cell between adjacent pipes. Currents were recorded at room temperature (21-23°C) with an Axopatch 200B amplifier and filtered at 5 kHz with a low-pass Bessel filter. The amplifier was tuned for partial compensation of series resistance (typically 70-80% of a total series resistance of 4-10 $\text{M}\Omega$), and tuning was periodically re-adjusted during the experiment. Currents were digitized using a Digidata 1322A data acquisition interface controlled by pCLAMP 9.2 software (Axon Instruments).

Human iPSC-derived nociceptor neuron electrophysiology. Sensory neurons were differentiated from human induced pluripotent stem cells (iPSCs) as previously described (Chambers et al., 2012). Cells were cultured in 35 mm dishes (Falcon), coated with 0.1 mg/ml poly-d-lysine (Sigma) and 10 $\mu\text{g}/\text{ml}$ laminin, and grown in DMEM/F12(1:1) media (Life Technologies) containing 10% HI FBS (Life Technologies) and 35 $\mu\text{g}/\text{ml}$ Ascorbic acid (Sigma), 10 ng/ml BDNF, 10 ng/ml GDNF, 10 ng/ml NGF, 10 ng/ml NT-3 (Life technologies) for 8 weeks. Whole-cell recordings were made using patch pipettes with resistances of 1.5-2.5 $\text{M}\Omega$ when filled with the internal solution consisting of (in mM): 140 CsF, 10 NaCl, 1.1 EGTA, 10 HEPES, 20 D-glucose, pH adjusted to 7.2 with CsOH. The external solution consisted of (in mM): 130 NaCl, 20 TEA-Cl, 5 KCl, 0.1 CdCl_2 , 2 CaCl_2 , 1 MgCl_2 , 10 D-glucose, 10 HEPES, pH adjusted to 7.4 with NaOH, which was perfused during the recording using the ValveBank perfusion system (Automate Scientific). The size of neurons was measured with inverted microscope Eclipse Ti (Nikon), and neurons with a diameter of less than 25 μm were used for the experiments. Currents were recorded using a Multiclamp 700B amplifier (Molecular Devices). Data were collected and digitized at 50 kHz using a Digidata 1440 16-bit A/D converter controlled by pCLAMP 10.5 software (Molecular Devices).

Electrophysiology data analysis. Data were analyzed using programs written in IGOR Pro 4.0 (WaveMetrics, Lake Oswego, OR), using DataAccess (Buxton Software) to read pCLAMP data files into Igor Pro. Currents were corrected for linear capacitive and leak currents, which were determined using 5 mV hyperpolarizations delivered from the resting potential and then appropriately scaled and subtracted. Statistical analyses were performed using IGOR Pro. Data are given as mean \pm SEM, and statistical significance was assessed with the Mann-Whitney Test.

Mouse dorsal root ganglion (DRG) neuron culture and electrophysiology. DRG neurons were cultured as previously described (Costigan et al., 1998). DRGs from adult male C57Bl/6 mice (8-12 weeks old, Jackson Laboratories stock #000664) were dissected from into Hank's balanced salt solution (HBSS) (Life Technologies). DRGs were dissociated in 1 $\mu\text{g}/\text{ml}$ collagenase A plus 2.4 U/ml dispase II (enzymes, Roche Applied Sciences) in HEPES-buffered saline (HBSS, Sigma) for 90 min at 37 °C and then triturated down to single-cell level using glass Pasteur pipettes of decreasing size. DRGs were then centrifuged over a 10% BSA gradient and plated on laminin-coated cell culture dishes (Sigma). DRGs were cultured overnight in B27-supplemented neurobasal-A medium plus penicillin/streptomycin (Life Technologies). On the day following plating, DRG culture dishes were treated with either HBSS, 100 μM BW-031, 1 μM capsaicin or 100 μM BW-031+1 μM capsaicin in HBSS for 30 min, followed by a 5-minute perfusion of external solution to remove extracellular compounds.

Whole-cell current-clamp and voltage-clamp recordings were performed <24 hours after DRG culture using an Axopatch 200A amplifier (Molecular Devices) at 25°C. Data were sampled at 20 kHz and digitized with a Digidata 1440A A/D interface and recorded using pCLAMP 10 software (Molecular Devices). Data were low-pass filtered at 2 kHz. Patch pipettes were pulled from borosilicate glass capillaries on a Sutter Instruments P-97 puller and had resistances of 1.5–3 M Ω . Series resistance was 3–10 M Ω and compensated by at least 80% and leak currents were subtracted. Cells were classified as TRPV1⁺ or TRPV1⁻ by the presence or absence of a response to perfused 1 μM capsaicin measured in voltage clamp mode at a holding potential of -80 mV. Cells were then held at -100mV and depolarized to -10mV with a 100 ms step to activate Na_v channels. The external solution for DRG electrophysiological recordings consisted of (in mM): 30 NaCl, 90 Choline-Cl, 20 TEA-Cl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 0.1 CdCl₂, 10 HEPES, 10 Dextrose; pH 7.4, 320 mOsm. The internal pipette solution consisted of (in mM): 140 CsF, 10 NaCl, 1 EGTA, 10 HEPES, 20 Dextrose; pH 7.3.

Animals for pain studies. Male CD rats (7-8 weeks old) were purchased from Charles River and male C57BL/6J mice (8-12 weeks old) were purchased from Jackson Laboratories (stock #000664) and housed for 1 week prior to experiments. Rats were housed 3 animals per cage and mice were housed 5 animals per cage in separate rooms with constant temperature (23°C) and humidity (45-55%) with food and water available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Boston Children's Hospital.

Plantar incision surgery. Rats were placed in a chamber with 5% isoflurane and monitored until they were visibly unconscious. Once unconscious, rats were removed from the chamber and anesthesia was maintained using 2% isoflurane delivered via nose cone. A toe pinch was used to confirm that animals were fully anesthetized. The animals were then secured with surgical tape at their toes and upper leg for paw stability during surgery. The plantar surface of one hind paw was sterilized with 3 alternating wipes of betadine and ethanol. A 1.5 cm longitudinal incision was made using a scalpel along the center of the plantar surface, beginning 1 cm from the heel and extending towards the foot pad and toes. Incision was made to the minimal depth necessary to cut through skin and fascia to expose the underlying plantaris muscle, approximately 1-2 mm. Once exposed the plantaris muscle was elevated for 10 seconds with surgical forceps and gently lifted for 10 seconds. After irritation of the plantaris muscle, the wound was closed with three sutures. After surgery animals were returned to their cage and monitored until they fully recovered from anesthesia. Treatments were administered subcutaneously 24 hours after injury.

Intraplantar injection of Complete Freund's Adjuvant. Complete Freund's Adjuvant (CFA) was purchased from Sigma Aldrich (Cat. No. F5881). Rats were restrained and subcutaneously injected in the plantar region of the left paw with 50 μL of CFA (1 mg/ml). Animals receiving test compounds (2% QX-314 or 2% BW-031) were injected with these compounds dissolved in 50 μL of CFA.

Plantar ultraviolet (UV) burn. Mice were anesthetized with 3% isoflurane. UV irradiation was performed on the plantar surface of the left hind paw under maintenance anesthesia with 2% isoflurane at an intensity of 0.5 J/cm² for 1 minute at a wavelength of 305–315 nm using a fluorescent UV-B light source (XR UV LEDs 308nm, RayVio, Hayward, California, USA).

Peri-sciatic injection. Mice were anesthetized with 2.5% isoflurane. Upon achieving sufficient depth of anesthesia, mice were placed in the prone position, the fur on their left hindleg was shaved and the area was cleaned with betadine and 70% isopropanol. A 1-centimeter incision was made in the skin on the upper thigh. The sciatic nerve was identified in the intermuscular interval between the biceps femoris and gluteal muscle without the dissection of the superficial fascia layers. Then, a mixture of physiological saline with 0.5% lidocaine, 0.5% QX-314 or 0.5% BW-031 (70 μ L) was injected into the perineural space below the fascia using an insulin syringe with a 30-gauge needle. The surgical wounds were closed with stainless steel wound clips (EZ Clips, Stoelting Co.).

Behavioral measurements of sensory and motor function

Von Frey assay of mechanical sensitivity. An electronic Von Frey device (Bioseb, Model: BIO-EVF4) was used to assess mechanical sensitivity in rats before and after paw incision injury. Animals were habituated for 1 hour, one day prior to baseline testing. Animals were given 30 minutes to settle before testing. An average mechanical threshold was calculated using 5 measurements taken 5 minutes apart for each animal. For baseline measurements two testing sessions were performed on separate days prior to injury and averaged together. 50 μ L of BW-031 or saline were administered into the plantar region of the hind paw adjacent to the incision 24 hours post injury. Animals were then tested 1, 3, 5, and 24 hours post treatment. Additional timepoints were added at 7 and 9 hours for higher concentrations of treatments.

A manual Von Frey assay was used to assess mechanical sensitivity in mice before and after UV burn, as previously described (Lee et al., 2019). After mice were habituated to the testing cage (7.5 \times 7.5 \times 15 cm) with a metal grid floor for 45 min for 2 days, baseline values were measured using nine von Frey filaments with different bending forces (0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2, and 4 g). The response patterns were collected and converted into corresponding 50% withdrawal thresholds using the Up-Down Reader software and associated protocol (Gonzalez-Cano et al., 2018). Based on the baseline measurement, mice were assigned to three groups so that the baseline mechanical sensitivity among the groups was similar. Each group consisted of 10 mice, based on previous experiments showing sufficient power to detect significance with 95% confidence. Twenty-four hours after UV irradiation, mice received a 10- μ L bolus intraplantar injection of either 2% BW-031, 2% QX-314, or vehicle (normal saline) to the irradiated paw. The von Frey test was performed at 1, 3, 5, 7, and 24 hours after the drug injection.

Radiant heat assay of thermal sensitivity. Thermal hypersensitivity was measured using the plantar radiant heat test (Hargreaves et al., 1988) (Ugo Basile, Model code: 37370) in CFA injected rats. Rats were habituated to testing enclosures for 1 hour one day prior to baseline testing. Rats were given 30 minutes to settle before testing. An average paw withdrawal latency was calculated using 3 measurements taken 5 minutes apart. Animals were tested 1, 4, and 24 hours after injury from CFA injection.

Toe spread assay of motor function. Mouse toe movement was evaluated in the ipsilateral hind-paws as previously described (Ma et al., 2011) in order to assess the presence of motor block after peri-sciatic injection of lidocaine or charged sodium channel blockers. Briefly, 5 minutes after peri-sciatic injection, mice were lifted by the tail, uncovering the hind paws for clear observation. Under this condition, the digits spread, maximizing the space between them (the toe spreading reflex). This reflex was scored as previously described: 0, no spreading; 1, intermediate spreading with all toes; and 2, full spreading. Full toe spreading was defined as a complete, wide, and sustained (at least 2 seconds) spreading of the toes. Full toe spreading was observed in the contralateral paws for all mice tested.

Pinprick assay of sensory function. Mouse responses to pinprick were measured as previously described (Ma et al., 2011), with modifications. Mice were placed in wire mesh cages and habituated for 3 sessions prior to peri-sciatic injection. After peri-sciatic injection and measurement of motor function, mice were immediately placed in wire mesh cages and an Austerlitz insect pin (size 000) (FST, USA) was gently applied to the plantar surface of the paw without moving the paw or penetrating the skin. The pinprick was applied three times to the sole of the ipsilateral hind paw and three times to the sole of the contralateral hind paw. A response was considered positive (1) when the animal briskly removed its paw. If none of the

applications elicited a positive response, the overall grade was 0.

Blinding. All behavioral measurements of sensory and motor function were performed by investigators blinded to the drug treatment; the test order was randomized with multiple groups being represented in each cage.

Guinea Pig Cough Experiments

Animals and pre-screening. For cough studies, animal care and studies were conducted in alignment with applicable animal welfare regulations in an AAALAC-accredited facility. The cough studies used 5-10 week-old Dunkin Hartley guinea pigs. The initial study using intratracheal application of BW-031 under isoflurane anesthesia used female animals (range of weights of 374-505 g on the day of dosing and cough challenge), with 9 animals per each of the 4 experimental groups. The subsequent study using ovalbumin sensitization to induce lung inflammation used 6 male (416-580 g) and 6 female (456-557 g) animals for each experimental group. The number of animals per group was increased in the ovalbumin sensitization experiments because of the possibility that variable levels of sensitization might increase variability in the effectiveness with which BW-031 could enter nerve terminals. Because preliminary studies showed that some guinea pigs failed to cough in response to the citric acid challenge, each study began with 20% more animals than were planned for the protocols and animals were first pre-screened by inhalation of citric acid (400 mM for 7 minutes, with coughs counted during the 7 minute application and for 10 minutes afterward) and the lowest responders were omitted from the remaining study protocol. For the intratracheal protocol, animals with 0-1 coughs were omitted; for the ovalbumin sensitization protocol, the 6 animals of each sex with lowest cough counts (0-3 coughs) were omitted. Pre-screening was performed a minimum of 7 days before the start of the study protocol to allow animals to recover from any sensitization produced by the citric acid exposure during the pre-screening. After pre-screening, the remaining animals were allocated into each group so that each group had approximately equal group mean cough counts measured in the pre-screening protocol.

Intratracheal drug administration and citric acid challenge. Animals were dosed via the intratracheal route at a dose volume of 0.5 mL/kg of BW-031 dissolved in saline based on individual bodyweights. Animals were anaesthetized (3-5% isoflurane/oxygen mix) and secured to the intubation device by a cord around their upper incisor teeth. A rodent fiber optic laryngoscope was inserted into the animal's mouth to illuminate the posterior pharynx and epiglottis. The tongue was released and the needle of the dosing device (Penn Century intratracheal aerosol Microsprayer) guided through the vocal cords into the lumen of the trachea. Following dosing, the animals were removed from the secured position and carefully monitored until full recovery. Approximately 1 hour after intratracheal treatment, animals were placed into whole body plethysmographs connected to a Buxco Finepointe System and exposed to nebulized 400 mM citric acid for 7 minutes. Cough counts and respiratory parameters (minute volume) were recorded throughout the 7 minute exposure period and for 10 minutes following the end of nebulization period. Determinations of coughs by the Buxco Finepointe system were confirmed against manual cough counts and this was periodically re-confirmed. Recovery from the isoflurane anesthesia was complete within an hour based on the overt behavior of the animals, consistent with rapid recovery seen using measurements of physiological parameters (Schmitz et al., 2016);.

Ovalbumin sensitization/challenge. On Day 0 all animals were sensitized with intraperitoneal and subcutaneous injections of chicken egg albumin (ovalbumin). Animals were administered 1 mL of a 50 mg/mL Ovalbumin (Ova) in 0.9% w/v saline solution via the intraperitoneal route and 0.5 mL of the same solution into 2 separate subcutaneous sites (1 mL in total divided between the left and right flank). All animals were administered a single intraperitoneal dose of pyrilamine (15 mg/kg) at a dose volume of 1 mL/kg approximately 30 min prior to ovalbumin challenge on Day 14 to inhibit histamine-induced bronchospasm (Featherstone et al., 1988; Hara et al., 2005). On Day 14 animals were challenged with aerosolised ovalbumin in 0.9% w/v saline (3 mg/mL) or 0.9% w/v saline for 15 min. Animals were placed in groups in an acrylic box. 8 mL of ovalbumin in saline was placed in each of two jet nebulisers (Sidestream®). Compressed air at approximately 6 L/min was passed through each nebuliser and the output of the nebulisers passed into

the box containing the animals.

Drug administration. On Day 15 approximately 24 hours after the inflammatory challenge with ovalbumin, animals were placed in a whole-body plethysmograph (Buxco Finepointe) and dosed with vehicle or BW-031 by inhalation using an Aeroneb nebulizer (Aerogen) over 60 minutes. Upon the completion of dosing animals were returned to their home cage for approximately one hour before the cough challenge.

The inhaled dose of BW-031 was calculated according to the algorithm (Alexander et al., 2008): Inhaled dose (mg/kg) = $[C \text{ (mg/L)} \times \text{RMV (L/min)} \times D \text{ (min)}] / \text{BW (kg)}$, where C is the concentration of drug in air inhaled, RMV is respiratory minute volume, D is the of exposure in minutes, BW is bodyweight in kg, with RMV (in L/min) calculated as $0.608 \times \text{BW (kg)}^{0.852}$.

Cough/respiratory function measurement. One hour following the end of vehicle or drug administration on Day 15, the animals were placed into a whole-body plethysmograph connected to a Buxco Finepointe system. Animals were then exposed to nebulized 400 mM citric acid for 7 minutes. Cough counts were recorded throughout the 7 minute exposure period and for 10 minutes following the end of nebulization period. Animals were euthanized within approximately 60 min following the end of the cough challenge recording period by an overdose of pentobarbitone administered by the intraperitoneal route.

Tissue sampling. Upon euthanasia, 2 mL of blood were sampled from the descending vena cava from each animal. The blood was allowed to stand at room temperature for a minimum of 60 minutes but less than 120 minutes to allow the clotting process to take place. Samples were then centrifuged at 2000 g for 10 minutes at 25°C and the resulting serum was frozen at -80°C for subsequent analysis of BW-031 concentrations. Also following termination an incision was made in the neck and the muscle layers were separated by blunt dissection and the trachea isolated. A small incision was made in the trachea and a tracheal cannula inserted. The cannula was secured in place with a piece of thread. The lungs were then removed and the left lung lobe tied off and removed. The right lung was lavaged with 3 mL of phosphate buffered saline (PBS) at room temperature. The PBS was left in the airway for 10 seconds whilst the organ was gently massaged before being removed, this was repeated twice further. In total, three lots of 3 mL of PBS was used to lavage the right lung.

BAL immune cell quantification. A total and differential cell count of the BAL was performed using the XT-2000iV (Sysmex UK Ltd). The sample was vortexed for approximately 5 seconds and analyzed. A total and differential cell count (including eosinophils, neutrophils, lymphocytes and mononuclear cells (includes monocytes and macrophages)) was reported as number of cells per right lung per animal.

Liquid Chromatography/Mass Spectrometry (LC/MS). Serum samples were kept at -80degC until being assayed, at which time they were thawed at room temperature. Each serum sample was added to 100 μL of 80:20 (acetonitrile:water) solution and the mixture placed in a 1.5 mL Eppendorf Safe-Lock tubes prefilled with zirconium oxide beads (Next Advance Inc., Troy, NY). After vortexing for 30 seconds and sonicating for 10 minutes, the homogenate was centrifuged at 10,000 rpm for 10 minutes. The supernatant was then separated into a new Eppendorf tube to be stored at -80°C until the time for analysis. To 20 μL of liquid sample, 10 μL of internal standard (bupivacaine 10 ng/mL in acetonitrile:water (50:50)) were added, plus 170 μL of methanol chilled at 4°C. After vortexing for 30 seconds, the mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant solution was then transferred to a clean Eppendorf tube and evaporated to dryness under vacuum at 50°C for 40 minutes. The residue was reconstituted with 100 μL of the starting mobile phase, i.e. aqueous 0.1% formic acid:methanol (90:10), and vortexed for 30 seconds. This solution was transferred to amber screw neck vials and setup in the refrigerated autosampler tray of the chromatograph for injection. This whole procedure was also applied to spiked calibrators and quality control QC samples used in the quantification and validation methods. At least three injections were carried out from each vial. Bupivacaine, used as internal standard, methanol LC/MS grade, and formic acid LC/MS grade were purchased from Sigma-Aldrich St. Louis, MO, USA). Pure MilliQ water at 18 M Ω -cm was obtained by reserve osmosis with a Direct-Q3 UV water purifier (Millipore SAS, France).

The quantification of BW-031 in serum fluid samples was carried out with an Acquity H Class UPLC

chromatographer with a XEVO TQ MS triple quadrupole mass spectrometer detector (Water Corp., Milford, MA, USA). The assay used an Acquity UPLC BEH C18 1.7 μm 2.1x100 mm column with a VanGuard 2.1X 5 mm guard column, both kept at constant 35°C. The mobile phase was ran on a gradient of A: aqueous 0.1% formic acid and B: methanol starting at time zero with a A:B proportion of 90:10, at 3 min 10:90, and at 4.2 min 90:10 until the end of the run at 6 minutes. The flow rate was set at 0.3 mL/min, with an injection volume of 3 μL , and a post-run organic wash of 5 seconds. Multiple reaction monitoring (MRM) was used for the mass spectrometry acquisition in positive electrospray ionization (ESI) mode. The mass transitions monitored were m/z 263.22-86.02 and m/z 289.09-140.2, for BW-031 and bupivacaine respectively. The cone voltages were 36V and 30V, and the collision energies 24eV and 12eV, also respectively. The whole LC/MS system was controlled by the MassLynx v.4.2 software (Water Corp., Milford, MA, USA), including the TargetLynx Application Manager for data processing and analytes quantification. Good linearity and reproducibility were achieved in the range of 1–100 ng/mL, and the precision and accuracy of the method were 2.74% and 98.6%, respectively. The lower limit of quantification was 1.8 ng/mL.

Cardiotoxicity. Frozen human iPSC-derived cardiomyocytes (Cor.4U cardiomyocytes) were purchased from Axiogenesis AG (currently NCardia AG). Cor.4U cells were thawed and plated at a density of 10000 cells/well into 384-well plates that were pre-coated with 10 $\mu\text{g}/\text{mL}$ bovine fibronectin in sterile phosphate buffered saline, pH 7.4. Cor.4U Culture Medium (Axiogenesis) was used to maintain the cells in culture for 7 days and was changed daily. Cells exhibited synchronous beating on day 3 after plating. On day 7 after plating, the medium was changed to BMCC medium (Axiogenesis). The EarlyTox Cardiotoxicity Kit (Molecular Devices) was used to measure calcium flux as a proxy for cardiomyocyte beating activity¹¹². Cor.4U cells were incubated with the EarlyTox calcium sensitive dye in BMCC media for 1 hour, and then the plates were transferred to the FDSS700EX plate reader (Hamamatsu Photonics). The baseline calcium flux was measured for 5 minutes and then charged local anesthetics dissolved in BMCC media or media alone were added to the wells using a robot. 10 minutes after compound treatment, the calcium flux was measured again. All measurements were performed at 37°C under 95% $\text{CO}_2/5\%$ O_2 . Calcium flux parameters were measured using the Hamamatsu Analysis Software.

Statistical Analysis. Data represent mean \pm standard error of the mean (SEM). Statistical comparisons were performed using GraphPad Prism 8.0 software with the parameters described in each respective figure legend. Statistical tests were corrected for multiple comparisons where appropriate; corrections used for each data set are stated in figure legends.

Results

Among local anesthetics with an amino-amide scaffold like lidocaine, compounds like bupivacaine and mepivacaine in which the amino group is part of a piperidine ring have higher potency for sodium channel inhibition (Bräu et al., 1998; Scholz et al., 1998). Based on this observation, we designed and synthesized compounds containing a cationic charge in a piperidinium ring and identified one, BW-031 (Figure 1a), as a compound that inhibited $\text{Na}_v1.7$ sodium channels with substantially higher potency than QX-314 when applied intracellularly (Figures 1b-c). As for QX-314 (Strichartz, 1973; Schwarz et al., 1977; Yeh, 1978), inhibition by BW-031 progressively accumulates with each cycle of activation and deactivation of the sodium channel, as if the intracellular blocker can enter the channel only when it is open and is effectively trapped within the channel after the channel closes. BW-031 had minimal effect on $\text{Na}_v1.7$ currents when applied extracellularly (Figures 1d-e), suggesting that, like QX-314, it cannot effectively enter sodium channels through the narrow ion selectivity filter in the outer pore region of the channel or diffuse across the cell membrane. Intracellular BW-031 inhibited native sodium currents in nociceptors differentiated from human induced pluripotent stem cells (hiPSCs) with very similar potency as for heterologously expressed $\text{Na}_v1.7$ channels (Figures 1f-g). BW-031 inhibited $\text{Na}_v1.1$ channels with a similar potency to $\text{Na}_v1.7$ channels (Figures 2a,b). However, BW-031 was considerably less effective in inhibiting heterologously-expressed $\text{Na}_v1.8$ channels (Figures 2c,d), with intracellular 300 μM BW-031 producing only $30 \pm 8\%$ inhibition ($n=5$), similar to the effect of 30 μM BW-031 on $\text{Na}_v1.7$ channels ($37 \pm 4\%$ inhibition, $n=6$). The much weaker effect on $\text{Na}_v1.8$ channels was unexpected, because previous work showed that the uncharged piperidine-containing anesthe-

tics bupivacaine and mepivacaine have generally similar effects on native TTX-resistant sodium channels in DRG neurons and heterologously-expressed $\text{Na}_v1.8$ channels as on a variety of TTX-sensitive channels (Bräu et al., 1998; Scholz et al., 1998; Scholz et al., 2000; Leffler et al., 2010).

BW-031 applied externally to mouse DRG neurons inhibited sodium currents only when it was applied together with capsaicin to activate TRPV1 channels (Figure 3a) and the combined application of BW-031 and capsaicin had no effect on sodium currents in DRG neurons lacking expression of TRPV1 channels, as tested by the response to 1 μM capsaicin (Figure 3b). Thus, like QX-314 (Binshtok et al., 2007; Brenneis et al., 2013, 2014; Stueber et al., 2016), BW-031 can permeate through activated TRPV1 channels to block sodium channels from the inside of the cell.

We next tested the possibility that BW-031 applied alone might be able to inhibit nociceptors in several rodent models of inflammatory pain. BW-031 effectively reduced hypersensitivity in a rat model of inflammation induced by paw injection of Complete Freund's Adjuvant (CFA), in which inflammation activates TRPV1 and TRPA1 channels (Garrison and Stucky, 2014; Asgar et al., 2015; Kanai et al., 2007; Lennertz et al., 2012). In this model, latency of paw withdrawal to a thermal stimulus was decreased at 1 hour and even more at 4 hours, and BW-031 blocked this effect at both times (Figure 4a). Similarly, BW-031 also blocked mechanical hyperalgesia in a more clinically-relevant rat paw incision model of surgical pain (Brennan et al., 1996; Barabas and Stucky, 2014) in which the incision produced pronounced mechanical hyperalgesia when assayed 24 hours later. BW-031 injected near the incision greatly reduced the mechanical hyperalgesia, with strong effects at 3 hours and 5 hours after BW-031 injection that then progressively declined at later times (Figure 4b). Figure 5a shows results from a mouse model of UV-burn-induced inflammatory pain (Yin et al., 2016) where inflammatory mediators activate TRPV1 and TRPA1 channels in nociceptors (Acosta et al., 2014; Yin et al., 2016). Plantar UV-burn results in pronounced mechanical allodynia 24 hours later, at which time intra-plantar injection of 2% BW-031 produced robust mechanical analgesia lasting for at least 7 hours, with considerably longer-lasting effects than QX-314. Interestingly, in both the mouse UV burn model and the rat CFA paw-injection model, BW-031 not only reversed the tactile hypersensitivity resulting from the injury but also produced substantial long-lasting analgesia relative to the control situation, indicating a general inhibition of nociceptors at the site of administration to the inflamed tissue.

To test the selectivity of BW-031 to inhibit neurons only in conditions in which TRPV1, TRPA1 or other large-pore channels are activated, we performed perisciatic injections in naïve mice, with perisciatic injection of lidocaine as a positive control that inhibits neuronal activity without any requirement for activation of large-pore channels. We found that neither BW-031 nor QX-314 produced any block of either sensory or motor function, in contrast to the transient inhibition of both by lidocaine (Figures 5b,c), consistent with a requirement for activated TRP or other large-pore channels for neuronal inhibition by the charged blockers.

Guinea pigs are the standard pre-clinical model for studying cough (Adner et al., 2020; Bonvini et al., 2015; Lewis et al., 2007; Morice et al., 2007) as the main features of airway innervation are similar in guinea pigs and humans (West et al., 2015; Mazzone and Udem, 2016). Coughing in guinea pigs can be mediated both by a subset of bronchopulmonary C-fibers and by a distinct mechanically-sensitive and acid-sensitive subtype of myelinated airway mechanoreceptors (Canning, 2006; Canning et al., 2014; Canning et al., 2004; Chou et al., 2018b; Mazzone et al., 2009; Mazzone and Udem, 2016). The neurons mediating the C-fiber pathway have strong expression of both TRPV1 and TRPA1 channels (Bonvini et al., 2015; Canning et al., 2014; Mazzone and Udem, 2016), and coughing in both guinea pigs and humans can be evoked by both TRPV1 agonists like capsaicin (Bonvini et al., 2015; Brozmanova et al., 2012; Kanezaki et al., 2012; Laude et al., 1993) and by TRPA1 agonists (Birrell et al., 2009; Bonvini et al., 2015; Kanezaki et al., 2012; Long et al., 2019b). The importance of this population of TRPV1 and TRPA1-expressing neurons in at least some forms of cough suggested the possibility that loading charged sodium channel inhibitors into these neurons might inhibit cough.

We used two different experimental protocols to test whether BW-031 can inhibit cough in guinea pigs when applied under conditions in which TRPV1 and TRPA1 channels are likely to be activated. In the first, we delivered a small volume (0.5 mL/kg) of different doses of BW-031 intratracheally to animals under transient

isoflurane anesthesia (Figure 6), relying on the ability of isoflurane to activate TRPV1 and TRPA1 channels (Matta et al., 2008; Kichko et al., 2015). One hour after the administration of BW-031, coughing was induced by inhalation of aerosolized citric acid, which induces a low rate of coughing (typically 0.5-1 cough/minute (Tanaka and Maruyama, 2005)), and coughs were measured using whole-body plethysmography. BW-031 produced a dose-dependent reduction in the number of coughs evoked by citric acid, with administration of 7.53 mg/kg BW-031 reducing cough counts during a 17-minute period from 9.4 ± 2.4 in control to 0.9 ± 0.5 with BW-031 ($n=9$, $p=0.005$, Tukey's post-hoc test) (Figure 6b), with a complete suppression of coughing in 5 of the 9 animals tested.

With these encouraging results, we next tested BW-031 in a potentially more translationally-relevant guinea pig model of ovalbumin-induced allergic airway inflammation, which produces activation and upregulation of both TRPV1 and TRPA1 channels in the airways (Liu et al., 2015; McLeod et al., 2006; Watanabe et al., 2008). Guinea pigs were sensitized by intraperitoneal and subcutaneous injections of ovalbumin (Figure 7a). Fourteen days later, inhaled ovalbumin induced allergic airway inflammation, reflected by increased immune cell counts in the bronchoalveolar lavage (BAL) measured one day after the ovalbumin-challenge (Figure 7b). Nebulized BW-031 was administered to restrained awake guinea pigs via snout-only inhalation chambers one day after the allergen challenge, and cough was then induced by citric acid one hour after the inhalation of BW-031. BW-031 strongly inhibited the citric acid-induced cough in a dose-dependent manner (Figure 7c). At the highest dose tested (17.6 mg/kg), BW-031 reduced cough counts over 17 minutes from 10 ± 1.6 in control to 2.2 ± 0.89 with BW-031 ($n=12$, $p=0.0009$, Tukey's post-hoc test), with complete suppression of cough in 7 of the 12 animals.

The hydrophobicity of local anesthetics like lidocaine enables ready absorption from lung tissue into the blood. In principle, the absorption of cationic compounds like BW-031 might be expected to be much less. The highest dose of inhaled BW-031 (17.6 mg/kg) resulted in a serum concentration of 419 ± 46 nM ($n=12$) (Figure 8a), far lower than the average serum level of 15 μ M (3.6 μ g/mL) lidocaine measured following lidocaine spray anesthesia used for bronchoscopy (Labeledzki et al., 1983). The serum concentration of BW-031 after aerosol inhalation was many orders of magnitude below the concentration at which any effect of BW-031 was seen on contraction of human iPSC-derived cardiomyocytes (3 mM; Figure 8b). Thus, inhaled BW-031 should have a high therapeutic index with regard to *in vivo* cardiotoxicity, which is a significant concern with inhaled lidocaine (Horáček and Vymazal, 2012).

Discussion

These results show that the piperidinium-containing cationic compound BW-031 is a highly effective use-dependent sodium channel inhibitor when introduced intracellularly, acting with about 6-fold greater potency than QX-314. Like QX-314 (Binshtok et al., 2007; Puopolo et al., 2013), BW-031 had no effect on sodium currents in DRG neurons if applied in the absence of large-pore channel stimulation and in contrast to lidocaine, had no effect on nerve function when applied perisciatally in the absence of inflammation. Consistent with its greater intrinsic potency compared to QX-314, BW-031 produced more effective and longer-lasting inhibition of hypersensitivity in a mouse UV-burn model.

Both QX-314 and BW-031 applied alone to inflamed tissue were effective in inhibiting inflammatory pain, consistent with extensive evidence that tissue inflammation is associated with activation of TRPV1 and TRPA1 channels by endogenous activators (Bautista et al., 2013, Julius, 2013). Besides the models of incisional pain and UV burn we studied, the activation of TRPV1 and TRPA1 channels has been found in many other examples of inflammation, including models of colitis and inflammatory bowel disease (Zielińska et al. 2015, Utsumi et al., 2018; Csekó et al., 2019; Jain et al., 2020), dermatitis (Liu et al., 2013), cystitis (DeBerry et al., 2014) and pancreatitis (Schwartz et al., 2013). Thus, there may be many possible applications of the strategy to inhibit that inflammatory pain initiated by large pore expressing sensory neurons.

Cough may be a particularly attractive application of nerve silencing by entry of charged sodium channels blockers through large pore channels. TRPV1 and TRPA1 channels are expressed in many neurons mediating cough (Belvisi et al., 2011; Birrell et al., 2009; Bonvini et al., 2015; Brozmanova et al., 2012; Forsberg et al.,

1988; Grace and Belvisi, 2011; Jia et al., 2002; Laude et al., 1993; Undem et al., 2002) and are likely to be activated in many conditions triggering cough, including airway inflammation (Bessac and Jordt, 2008; Choi et al., 2018; Talbot et al., 2015; Talbot et al., 2020) and respiratory viral infection (Zaccone et al., 2016). Expression of TRPV1 channels is increased in airways of patients with chronic persistent cough (Groneberg et al., 2004) and after respiratory viral infection (Omar et al., 2017). In addition, increasing evidence suggests an important role for channels containing P2X3 proteins for mediating cough. P2X3 channels are expressed on sensory neurons innervating the lungs (Kollarik et al., 2019; Kwong et al., 2008; Mazzone and Undem, 2016) and P2X3 inhibitors reduce cough in guinea pig models (Bonvini et al., 2015; Garceau and Chauret, 2019; Pelleg et al., 2019) and appear promising in recent human cough trials (Morice et al., 2019; Smith et al., 2017; Smith et al., 2020a,b; Dicipinigitis et al., 2020). Interestingly, recent work has demonstrated that P2X3 receptors form large-pore channels capable of passing large cations (Harkat et al., 2017), similar to TRPV1 and TRPA1 pores. Thus, it is plausible that activated P2X3-containing channels, as well as TRPV1 and TRPA1 channels, might provide a pathway for entry of BW-031 into cough-mediating neurons.

The strategy of using activated large-pore channels to introduce charged sodium channel blockers inside sensory neurons should inhibit the activity of the neurons to all subsequent stimuli and may therefore have greater efficacy than targeting single receptors like TRPV1, TRPA1, and P2X3. Once cationic sodium channel inhibitors are loaded into a cell (concentrated by the negative intracellular potential), they will not readily diffuse out through the cell membrane and can produce effects lasting for many hours, as is the case for the analgesic effect of QX-314 (Binshatok et al., 2009a; Gerner et al., 2008; Roberson et al., 2011) and as we find here for BW-031 inhibition of pain (Figures 4,5).

Overall, the ability of BW-031 to inhibit cough adds to previous evidence that targeting peripheral afferent activity by sodium channel inhibition can be an effective strategy for inhibiting cough (Muroi et al., 2011; Sun et al., 2017; Kollarik et al., 2018; Brozmanova et al., 2019; Patil et al., 2019; Undem and Sun, 2020). Using cationic sodium channel inhibitors should have the advantage of limiting the inhibition of nerve activity only to those neurons that express large-pore channels, like TRPV1, TRPA1, and P2X3, and only under those conditions, such as inflammation or noxious irritation, where these channels are activated.

The citric acid model of guinea pig cough, although widely used, clearly has limitations for predicting drug efficacy in human disease, because while TRPV1 and TRPA1 inhibitors are reasonably effective in this model (Leung et al., 2007; Mukhopadhyay et al., 2014) this has not been replicated, so far, in patients (Khalid et al., 2014; Belvisi et al., 2017; European Medicines Agency, 2013). It would be useful in future studies to explore the efficacy of BW-031 in other modes of cough induction, such as hypo-osmotic solutions or direct mechanical stimulation, which may activate different populations of nerve fibers than citric acid (Chou et al., 2018a; Morice et al., 2007).

Figures

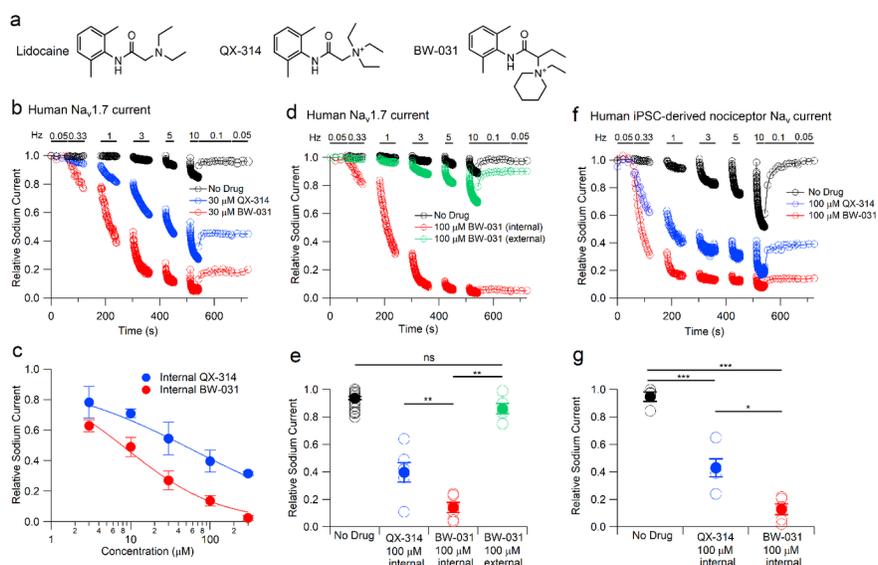


Figure 1. BW-031 inhibition of human Nav1.7 channels and sodium channels in iPSC-derived nociceptors.

a, Chemical structures of lidocaine, QX-314 and BW-031. **b**, Whole-cell patch clamp recordings illustrating use-dependent inhibition of hNav_v1.7 channels expressed in HEK 293 cells by 30 μM intracellular QX-314 (blue) or BW-031 (red) or with the use-dependent protocol run with control intracellular solution (black). hNav_v1.7 current was evoked by 20-ms depolarizations from -100 to -20 mV. After an initial stimulation at 0.05 Hz, trains of depolarizations at frequencies from 0.33 to 10 Hz were delivered, each for 1 minute (0.33 to 3 Hz) or 30 seconds (5 and 10 Hz), with a 1 minute rest between trains. **c**, Dose-dependent inhibition by various intracellular concentrations of QX-314 (blue) and BW-031 (red). Mean ± SEM (n=6 for 30, 100, and 300 μM QX-314 and for 3, 10, 30, and 100 μM BW-031, n=4 for 3 and 10 μM QX-314, n=3 for 300 μM BW-031). Solid lines, best fits to $(1/1 + [Drug]/IC_{50})$, where [Drug] is the QX-314 or BW-031 concentration and IC₅₀ is the half-blocking concentration, with IC₅₀=61 μM for QX-314 and IC₅₀=9.2 μM for BW-031. Current was quantified during the final slow (0.05 Hz) stimulation following the higher-frequency trains of stimulation. **d**, Use-dependent inhibition of hNav_v1.7 channels by 100 μM intracellular BW-031 (red) contrasted with the same voltage protocol in control (black) or after application of 100 μM extracellular BW-031 (green). **e**, Intracellular 100 μM BW-031 inhibits hNav_v1.7 more strongly (to 0.14 ± 0.03 , n=6) than intracellular 100 μM QX-314 (0.40 ± 0.07 , n=6; p=0.008, two-tailed Mann Whitney Test) or extracellular 100 μM BW-031 (0.86 ± 0.04 , n=6; p=0.008). ns p>0.05 and **p<0.01. Black symbols are mean ± SEM (0.94 ± 0.01 , n=36) for control cells in which the same sequence of stimuli was delivered using intracellular solution without compound. **f**, Use-dependent inhibition of native Na_v currents in hiPSC-derived nociceptors by 100 μM intracellular QX-314 (blue) or 100 μM intracellular BW-031 (red), and Na_v currents in untreated control neurons after the same voltage protocol (black). **g**, Collected results for hiPSC-derived nociceptors showing stronger inhibition by 100 μM intracellular BW-031 (red, 0.13 ± 0.04 , n=5) compared to 100 μM intracellular QX-314 (blue, 0.43 ± 0.07 , n=5) or control (black, 0.95 ± 0.03 , n=4). Control vs. QX-314, p=0.0004; control vs. BW-031, p<0.0001; BW-031 vs. QX-314, p=0.01, two-tailed paired t-test. *p<0.05, ***p<0.001.

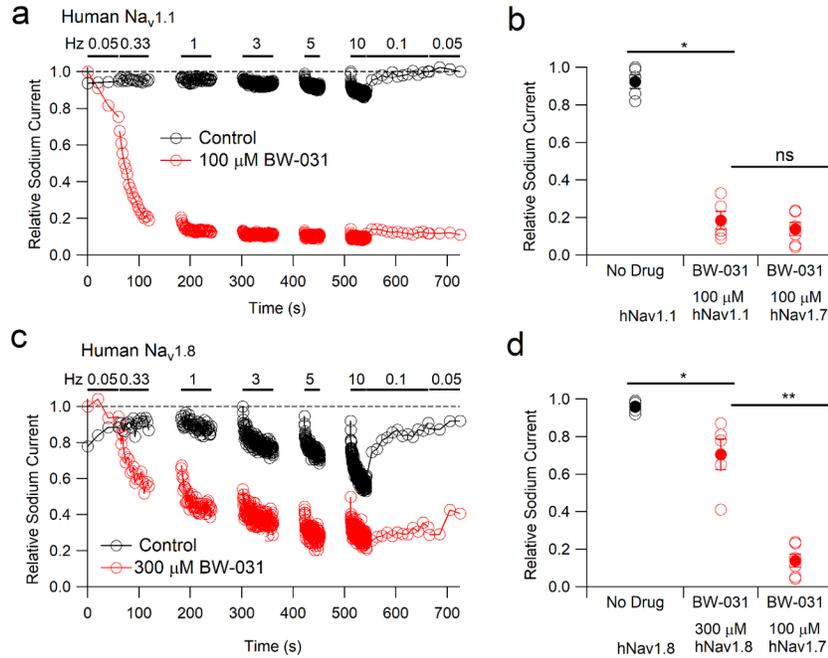


Figure 2. BW-031 inhibition of human Nav1.1 and Nav1.8 channels.

a, Use-dependent inhibition of hNav_v1.1 channels by 100 μM intracellular BW-031 (red) compared to recording with control intracellular solution (black). Na_v current was evoked by 20-ms depolarizations from -100 to 0 mV at the indicated frequencies. **b**, Collected results for hNav_v1.1 inhibition by 100 μM intracellular BW-031 (red, n=5) compared with control (black, n=5) and with inhibition of hNav_v1.7 (n=6, replotted from Figure 1e). **c**, Use-dependent inhibition of hNav_v1.8 channels by 300 μM intracellular BW-031 (red) compared to recording with control intracellular solution (black). Na_v current was evoked by 20-ms depolarizations from -70 to 0 mV at the indicated frequencies. **d**, Collected results (mean±SEM) of hNav_v1.8 inhibition by 300 μM intracellular BW-031 (red, n=5) compared with control (black, n=5) and with inhibition of hNav_v1.7 by 100 μM BW-031 (n=6, replotted from Figure 1e). Data are mean±SEM and statistics are calculated from two-tailed Mann-Whitney Test. ns p>0.05, *p<0.05, and **p<0.01.

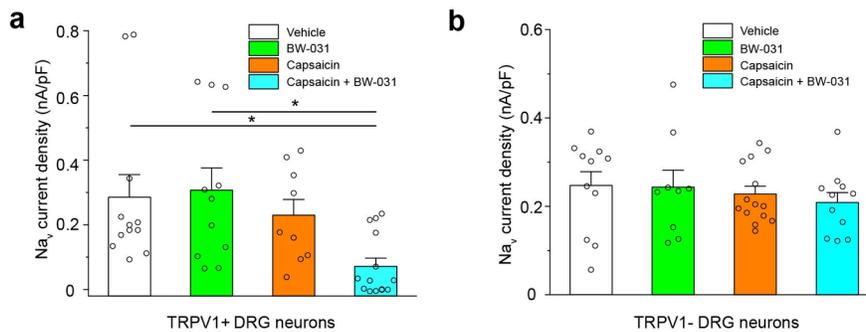


Figure 3. Extracellular application of BW-031 to DRG neurons inhibits sodium currents only with concomitant activation of TRPV1 channels.

a, Quantification of voltage-clamp recordings of sodium currents in TRPV1+ mouse DRG neurons pretreated with vehicle (white), 100 μM BW-031 (green), 1 μM capsaicin (orange) or 1 μM capsaicin + 100

μM BW-031 (cyan). Capsaicin facilitates the inhibition of Na_v currents in mouse TRPV1+ DRG neurons treated with BW-031. $N=9-14$ cells per condition, 1-way ANOVA, $[F(3, 42)=4.26]$, $p=0.01$; Tukey's post-hoc, $*p<0.05$. **b**, Quantification of the Na_v current density data from TRPV1- DRG neurons pre-treated with vehicle (white), $100 \mu\text{M}$ BW-031 (green), $1 \mu\text{M}$ capsaicin (orange) or $1 \mu\text{M}$ capsaicin + $100 \mu\text{M}$ BW-031 (cyan). Capsaicin does not facilitate the block of Na_v channels in mouse TRPV1- DRG neurons treated with BW-031. $N=9-14$ cells per group, 1-way ANOVA, $[F(3,41)=0.40]$, $p=0.75$. Bars represent mean \pm SEM for each condition, while the individual data points are displayed as open circles.

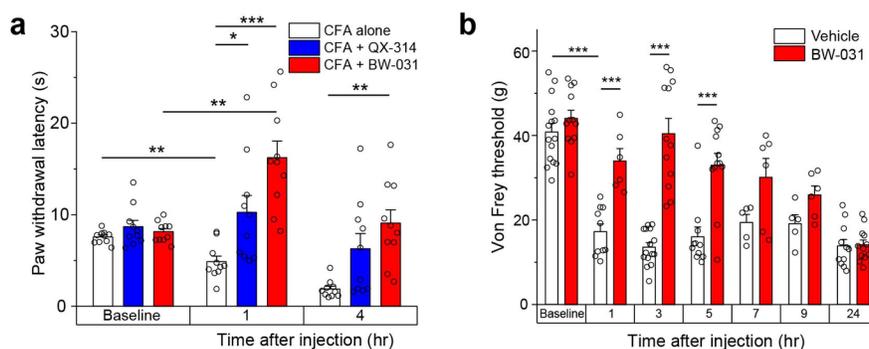


Figure 4. BW-031 produces long-lasting analgesia in rat paw CFA injection and surgical paw incision pain models.

a, Hargreaves assay of hindpaw thermal sensitivity in rats after intraplantar injection of Complete Freund's Adjuvant (CFA) alone (white), 2% QX-314 dissolved in CFA (blue) or 2% BW-031 dissolved in CFA (red). Both QX-314 and BW-031 produce robust thermal analgesia. Two-way repeated measures ANOVA with treatment as the between groups factor and time as the within groups factor. Treatment $[F(2, 27)=15.05]$, time $[F(1.980, 53.46)=14.88]$, and treatment x time interaction $[F(4, 54)=6.767]$, all $p<0.001$. Post-hoc Tukey's tests between treatment groups at each time point revealed significant increases in mechanical threshold by BW-031 at 1 and 4 hours post treatment and QX-314 at 1h post treatment. $N=10$ male rats per group, $*p<0.05$, $**p<0.01$, $***p<0.001$. **b**, Von Frey measurements of hindpaw mechanical sensitivity in rats after paw incision and intraplantar injection of vehicle or 2% BW-031. Baseline measurements were made before paw incision. Vehicle or 2% BW-031 was injected 24 hours after the incision. Two-way ANOVA (mixed-effects model) with treatment as the between groups factor and time as the within groups factor. Treatment $[F(1, 25) = 35.96]$, time $[F(6.000, 99.00) = 44.83]$ and treatment x time interaction $[F(6, 99) = 13.23]$, all $p<0.001$. Post-hoc Bonferroni tests between treatment groups at each time point revealed significant increases in mechanical threshold by BW-031 at 1, 3 and 5 hours post treatment, $p<0.001$. $N=5-15$ male rats per group, $*p<0.05$, $**p<0.01$, $***p<0.001$. Bars for panels **a-b** represent mean \pm SEM for each condition, while the individual data points are displayed as open circles.

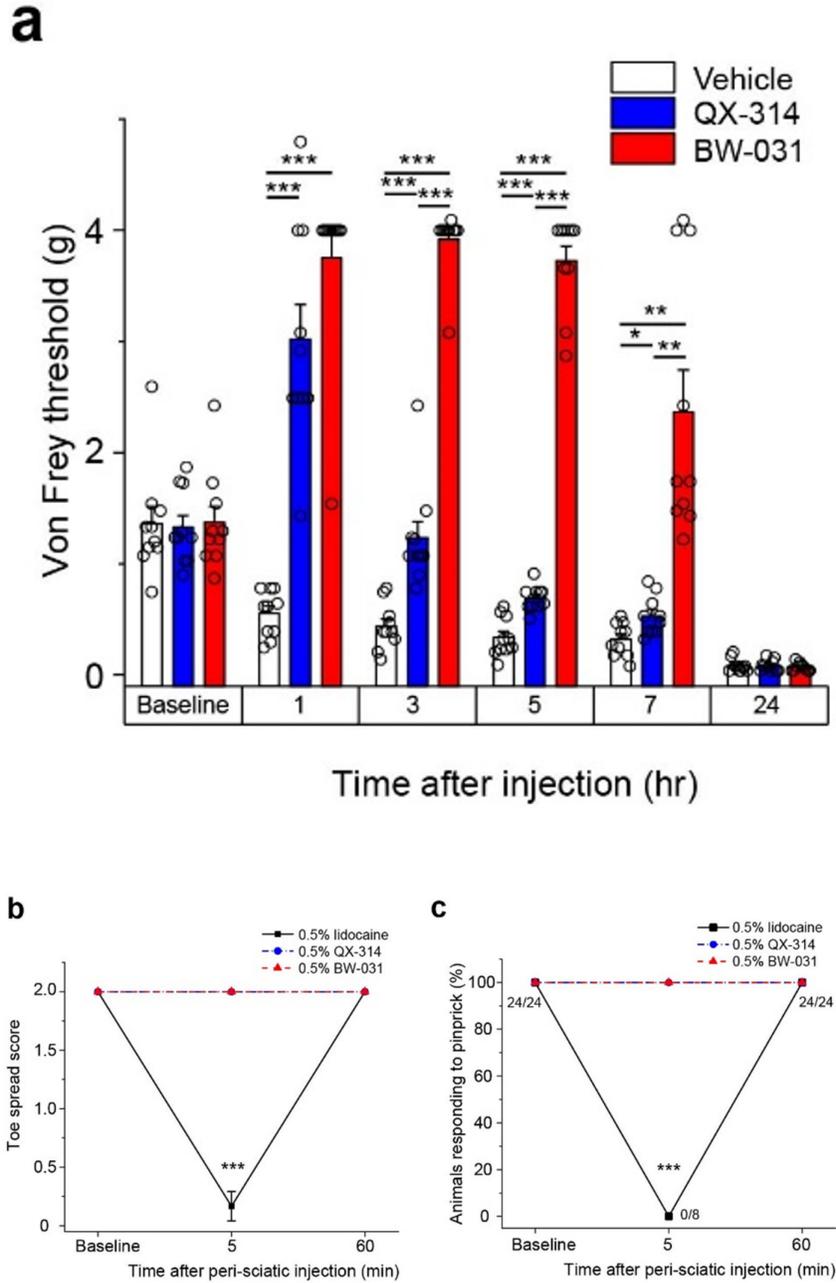


Figure 5. BW-031 produces long-lasting analgesia in a mouse paw UV-burn model and has no effect on sensory or motor function with perisciatic injection in naïve mice.

a, Von Frey measurements of hindpaw mechanical sensitivity in mice after plantar UV-burn and intraplantar injection of vehicle, 2% QX-314 or 2% BW-031. Two-way repeated measures ANOVA with treatment as the between groups factor and time as the within groups factor. Treatment [F(2, 27)=291.1], time [F(3.129, 84.49)=44.83] and treatment x time interaction [F(10, 135)=37.80], all $p < 0.001$. Post-hoc Tukey's tests between treatment groups at each time point revealed significant increases in mechanical threshold by QX-314 and BW-031 at 1, 3, 5 and 7 hours post treatment as compared with vehicle, with BW-031 treatment

producing a larger mechanical threshold than QX-314 at 3, 5 and 7 hours after treatment. N=10 male mice per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **b**, Toe spread assay of motor function in mice after perisciatic injection of 0.5% lidocaine, 0.5% QX-314 or 0.5% BW-031. Only lidocaine produces robust block of motor function in naïve mice. N=10 male mice per group, 1-way ANOVA (5 min time point), $[F(2, 21)=81]$, $p=1.3 \times 10^{-10}$; Tukey's post-hoc, *** $p < 0.001$. Data are mean \pm SEM. **c**, Plantar pinprick responses in naïve mice after perisciatic injection of 0.5% lidocaine, 0.5% QX-314 or 0.5% BW-031. Only lidocaine produces robust sensory analgesia in naïve mice. N=10 male mice per group, Fisher's exact test (5 min time point), $p=4.1 \times 10^{-6}$, *** $p < 0.001$. Data are mean \pm SEM.

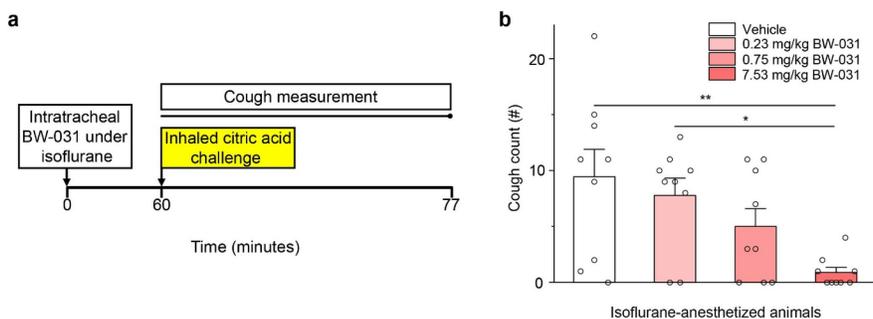


Figure 6. Intratracheal delivery of BW-031 under isoflurane anesthesia inhibits citric acid-evoked cough in guinea pigs.

a, Experimental design. BW-031 (0.23, 0.75 or 7.53 mg/kg) was delivered intratracheally to guinea pigs under isoflurane anesthesia. One hour later, the animals inhaled an aerosol with 400 mM citric acid for 7 minutes (yellow) and coughs were measured over 17 minutes during and after the citric acid challenge. **b**, Dose-dependent effect of intratracheal BW-031 on citric acid-evoked cough. N=9 female guinea pigs per group, 1-way ANOVA $[F(3, 32)=5.03]$, $p=0.0057$, Tukey's post-hoc, * $p < 0.05$, ** $p < 0.01$. Bars represent mean \pm SEM for each condition, while the individual data points are displayed as open circles.

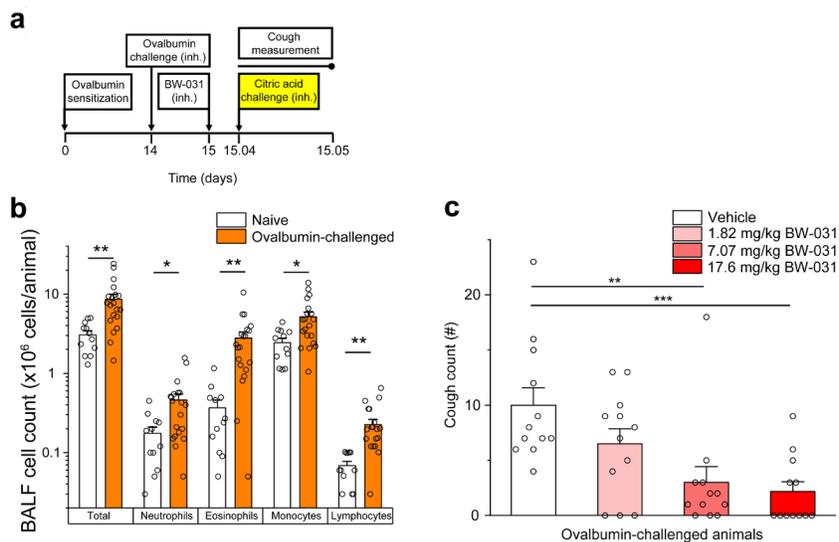


Figure 7. BW-031 inhibits cough in guinea pigs following allergic airway inflammation.

a, Experimental design. Guinea pigs were sensitized with intraperitoneal and subcutaneous ovalbumin and

challenged with inhaled (inh.) ovalbumin two weeks later. One day after ovalbumin challenge, the animals inhaled BW-031 (inhaled doses of 1.82, 7.07 or 17.6 mg/kg BW-031) followed one hour later by inhalation of 400 mM citric acid (yellow) for 7 minutes. Cough counts were measured for 17 minutes during and following the citric acid challenge. **b** , Ovalbumin sensitization and challenge causes lung inflammation, as measured by immune cell counts in bronchoalveolar lavage (BAL). N=12-20 animals per group (1:1 male:female), 1-way ANOVAs; total immune cell [F(1, 30) =10.3], p=0.003; neutrophils [F(1, 30)=5.82], p=0.02; eosinophils [F(1, 30)=11.9], p=0.002; monocytes [F(1, 30)=7.00], p=0.01; lymphocytes [F(1, 30)=10.9], p=0.003; Tukey's post-hoc, *p<0.05, **p<0.01. Bars represent mean±SEM for each condition, while the individual data points are displayed as open circles. **c** , Inhaled BW-031 produces dose-dependent inhibition of citric acid-evoked cough following allergic airway inflammation. N=12 animals per group (1:1 male:female), 1-way ANOVA, [F(3, 44)=7.113], p=0.0005; Tukey's post-hoc, **p<0.01, ***p<0.001. Bars represent mean±SEM for each condition, while the individual data points are displayed as open circles.

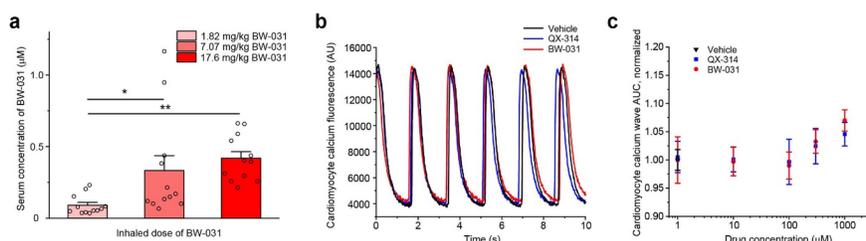


Figure 8. Inhaled BW-031 has minimal systemic distribution and is not cardiotoxic. **a** , Serum concentrations of BW-031 following inhalation. N=12 animals per group (1:1 male:female), 1-way ANOVA, [F(2, 33)=6.54], p=0.004, Tukey's post-hoc, *p<0.05, **p<0.01. Bars represent mean±SEM for each condition, while the individual data points are displayed as open circles. **b** , Representative calcium fluorescence signals from hiPSC-derived cardiomyocytes treated with vehicle, 100 µM QX-314 or 100 µM BW-031. **c** , Quantification of the effect of QX-314 and BW-031 on cardiomyocyte calcium signals as measured by area under the curve (AUC). Micromolar doses of QX-314 or BW-031 do not affect cardiomyocyte calcium signal AUC. N=5-10 wells per treatment, 1-way ANOVA (mixed-effects model), [F(2.412, 9.647)=3.27], p=0.0763; Dunnett's post hoc, *p<0.05. Data are mean±SEM.

References

- Acosta, M.C., Luna, C., Quirce, S., Belmonte, C., and Gallar, J. (2014). Corneal Sensory Nerve Activity in an Experimental Model of UV Keratitis. *Investigative Ophthalmology & Visual Science* 55, 3403-3412.
- Adner, M., Canning, B.J., Meurs, H., Ford, W., Ramos Ramírez, P., van den Berg, M.P.M., Birrell, M.A., Stoffels, E., Lundblad, L.K.A., Nilsson, G.P., et al. (2020). Back to the future: re-establishing guinea pig in vivo asthma models. *Clinical science (London, England : 1979)* 134, 1219-1242.
- Alexander, D.J., Collins, C.J., Coombs, D.W., Gilkison, I.S., Hardy, C.J., Healey, G., Karantabias, G., Johnson, N., Karlsson, A., Kilgour, J.D., et al. (2008). Association of Inhalation Toxicologists (AIT) working party recommendation for standard delivered dose calculation and expression in non-clinical aerosol inhalation toxicology studies with pharmaceuticals. *Inhalation toxicology* 20, 1179-1189.
- Asgar, J., Zhang, Y., Saloman, J.L., Wang, S., Chung, M.K., and Ro, J.Y. (2015). The role of TRPA1 in muscle pain and mechanical hypersensitivity under inflammatory conditions in rats. *Neuroscience* 310, 206-215.
- Barabas, M.E., and Stucky, C.L. (2013). TRPV1, but not TRPA1, in primary sensory neurons contributes to cutaneous incision-mediated hypersensitivity. *Mol Pain* 9: 9.
- Bautista, D.M., Pellegrino, M., and Tsunozaki, M. (2013). TRPA1: A gatekeeper for inflammation. *Annu Rev Physiol* 75: 181-200.

Belvisi, M.G., Birrell, M.A., Wortley, M.A., Maher, S.A., Satia, I., Badri, H., Holt, K., Round, P., McGarvey, L., Ford, J., et al. (2017). XEN-D0501, a Novel Transient Receptor Potential Vanilloid 1 Antagonist, Does Not Reduce Cough in Patients with Refractory Cough. *American journal of respiratory and critical care medicine* 196, 1255-1263.

Belvisi, M.G., Dubuis, E., and Birrell, M.A. (2011). Transient receptor potential A1 channels: insights into cough and airway inflammatory disease. *Chest* 140, 1040-1047.

Bessac, B.F., and Jordt, S.-E. (2008). Breathtaking TRP channels: TRPA1 and TRPV1 in airway chemosensation and reflex control. *Physiology (Bethesda)* 23, 360-370.

Binshtok, A.M., Bean, B.P., and Woolf, C.J. (2007). Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. *Nature* 449, 607-610.

Binshtok, Alexander M., Gerner, P., Oh, Seog B., Puopolo, M., Suzuki, S., Roberson, David P., Herbert, T., Wang, C.-F., Kim, D., Chung, G., et al. (2009a). Coapplication of Lidocaine and the Permanently Charged Sodium Channel Blocker QX-314 Produces a Long-lasting Nociceptive Blockade in Rodents. *Anesthesiology* 111, 127-137.

Binshtok, Alexander M.P.D., Gerner, P.M.D., Oh, Seog B.D.D.S.P.D., Puopolo, M.P.D., Suzuki, S.M.D., Roberson, David P.M.B.A., Herbert, T.B.S., Wang, C.-F.M.D., Kim, D.B.S., Chung, G.D.D.S., et al. (2009b). Coapplication of Lidocaine and the Permanently Charged Sodium Channel Blocker QX-314 Produces a Long-lasting Nociceptive Blockade in Rodents. *Anesthesiology: The Journal of the American Society of Anesthesiologists* 111, 127-137.

Birrell, M.A., Belvisi, M.G., Grace, M., Sadofsky, L., Faruqi, S., Hele, D.J., Maher, S.A., Freund-Michel, V., and Morice, A.H. (2009). TRPA1 agonists evoke coughing in guinea pig and human volunteers. *American journal of respiratory and critical care medicine* 180, 1042-1047.

Bonvini, S.J., Birrell, M.A., Smith, J.A., and Belvisi, M.G. (2015). Targeting TRP channels for chronic cough: from bench to bedside. *Naunyn-Schmiedeberg's archives of pharmacology* 388, 401-420.

Bräu, M.E., Vogel, W., and Hempelmann, G. (1998). Fundamental properties of local anesthetics: half-maximal blocking concentrations for tonic block of Na⁺ and K⁺ channels in peripheral nerve. *Anesthesia and analgesia* 87, 885-889.

Brennan, T.J., Vandermeulen, E.P., and Gebhart, G.F. (1996). Characterization of a rat model of incisional pain. *Pain* 64, 493-501.

Brenneis, C., Kistner, K., Puopolo, M., Jo, S., Roberson, D., Sisignano, M., Segal, D., Cobos, E.J., Wainger, B.J., Labocha, S., et al. (2014). Bupivacaine-induced cellular entry of QX-314 and its contribution to differential nerve block. *British journal of pharmacology* 171, 438-451.

Brenneis, C., Kistner, K., Puopolo, M., Segal, D., Roberson, D., Sisignano, M., Labocha, S., Ferreiros, N., Strominger, A., Cobos, E.J., et al. (2013). Phenotyping the function of TRPV1-expressing sensory neurons by targeted axonal silencing. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 33, 315-326.

Brozmanova, M., Mazurova, L., Ru, F., Tatar, M., and Kollarik, M. (2012). Comparison of TRPA1-versus TRPV1-mediated cough in guinea pigs. *European journal of pharmacology* 689, 211-218.

Brozmanova, M., Svajdova, S., Pavelkova, N., Muroi, Y., Undem, B.J., and Kollarik, M. (2019). The voltage-gated sodium channel Na(V)1.8 blocker A-803467 inhibits cough in the guinea pig. *Respiratory physiology & neurobiology* 270, 103267.

Camponogara, C., Brum, E.S., Pegoraro, N.S., Brusco, I., Rocha, F.G., Brandenburg, M.M., et al. (2020). Neuronal and non-neuronal transient receptor potential ankyrin 1 mediates UVB radiation-induced skin inflammation in mice. *Life Sci* 262: 118557.

- Canning, B.J. (2006). Anatomy and neurophysiology of the cough reflex: ACCP evidence-based clinical practice guidelines. *Chest* 129, 33s-47s.
- Canning, B.J., Chang, A.B., Bolser, D.C., Smith, J.A., Mazzone, S.B., and McGarvey, L. (2014). Anatomy and neurophysiology of cough: CHEST Guideline and Expert Panel report. *Chest* 146, 1633-1648.
- Canning, B.J., Mazzone, S.B., Meeker, S.N., Mori, N., Reynolds, S.M., and Undem, B.J. (2004). Identification of the tracheal and laryngeal afferent neurones mediating cough in anaesthetized guinea-pigs. *The Journal of physiology* 557, 543-558.
- Chambers, S.M., Qi, Y., Mica, Y., Lee, G., Zhang, X.J., Niu, L., Bilsland, J., Cao, L., Stevens, E., Whiting, P., et al. (2012). Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors. *Nature biotechnology* 30, 715-720.
- Choi, J.Y., Lee, H.Y., Hur, J., Kim, K.H., Kang, J.Y., Rhee, C.K., and Lee, S.Y. (2018). TRPV1 Blocking Alleviates Airway Inflammation and Remodeling in a Chronic Asthma Murine Model. *Allergy, asthma & immunology research* 10, 216-224.
- Chong, C.F., Chen, C.C., Ma, H.P., Wu, Y.C., Chen, Y.C., and Wang, T.L. (2005). Comparison of lidocaine and bronchodilator inhalation treatments for cough suppression in patients with chronic obstructive pulmonary disease. *Emergency medicine journal : EMJ* 22, 429-432.
- Chou, Y.-L., Mori, N., and Canning, B.J. (2018). Opposing effects of bronchopulmonary C-fiber subtypes on cough in guinea pigs. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 314, R489-R498.
- Costigan, M., Mannion, R.J., Kendall, G., Lewis, S.E., Campagna, J.A., Coggeshall, R.E., Meredith-Middleton, J., Tate, S., and Woolf, C.J. (1998). Heat shock protein 27: developmental regulation and expression after peripheral nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 18, 5891-5900.
- Csekő, K., Beckers, B., Keszthelyi, D., and Helyes, Z. (2019). Role of TRPV1 and TRPA1 Ion Channels in Inflammatory Bowel Diseases: Potential Therapeutic Targets? *Pharmaceuticals (Basel)* 12:.
- DeBerry, J.J., Schwartz, E.S., and Davis, B.M. (2014). TRPA1 mediates bladder hyperalgesia in a mouse model of cystitis. *Pain* 155: 1280-1287.
- Dicpinigaitis, P.V., McGarvey, L.P., and Canning, B.J. (2020). P2X3-Receptor Antagonists as Potential Antitussives: Summary of Current Clinical Trials in Chronic Cough. *Lung* 198, 609-616.
- European Medicines Agency (2013). A Phase 2a, Multi-Centre, Randomised, Double-Blind, Parallel Group, Placebo-Controlled Study to Evaluate Efficacy, Safety and Tolerability of Inhaled GRC 17536, Administered for 4 Weeks, in Patients with Refractory Chronic Cough. EU Clinical Trials Registry 2016.
- Featherstone, R.L., Hutson, P.A., Holgate, S.T., and Church, M.K. (1988). Active sensitization of guinea-pig airways in vivo enhances in vivo and in vitro responsiveness. *The European respiratory journal* 1, 839-845.
- Forsberg, K., Karlsson, J.A., Theodorsson, E., Lundberg, J.M., and Persson, C.G. (1988). Cough and bronchoconstriction mediated by capsaicin-sensitive sensory neurons in the guinea-pig. *Pulmonary pharmacology* 1, 33-39.
- Frazier, D.T., Narahashi, T., and Yamada, M. (1970). The site of action and active form of local anesthetics. II. Experiments with quaternary compounds. *J Pharmacol Exp Ther* 171: 45-51.
- Gamal El-Din, T.M., Lenaeus, M.J., Zheng, N., and Catterall, W.A. (2018). Fenestrations control resting-state block of a voltage-gated sodium channel. *Proc Natl Acad Sci U S A* 115: 13111-13116.
- Garrison, S.R., and Stucky, C.L. (2014). Contribution of transient receptor potential ankyrin 1 to chronic pain in aged mice with complete Freund's adjuvant-induced arthritis. *Arthritis Rheumatol* 66: 2380-2390.

- Garceau, D., and Chauret, N. (2019). BLU-5937: A selective P2X3 antagonist with potent anti-tussive effect and no taste alteration. *Pulm Pharmacol Ther* 56, 56-62.
- Gerner, P., Binshtok, A.M., Wang, C.F., Hevelone, N.D., Bean, B.P., Woolf, C.J., and Wang, G.K. (2008). Capsaicin combined with local anesthetics preferentially prolongs sensory/nociceptive block in rat sciatic nerve. *Anesthesiology* 109, 872-878.
- Gonzalez-Cano, R., Boivin, B., Bullock, D., Cornelissen, L., Andrews, N., and Costigan, M. (2018). Up-Down Reader: An Open Source Program for Efficiently Processing 50% von Frey Thresholds. *Frontiers in pharmacology* 9, 433.
- Grace, M.S., and Belvisi, M.G. (2011). TRPA1 receptors in cough. *Pulm Pharmacol Ther* 24, 286-288.
- Groneberg, D.A., Niimi, A., Dinh, Q.T., Cosio, B., Hew, M., Fischer, A., and Chung, K.F. (2004). Increased expression of transient receptor potential vanilloid-1 in airway nerves of chronic cough. *American journal of respiratory and critical care medicine* 170, 1276-1280.
- Hara, J., Fujimura, M., Myou, S., Oribe, Y., Furusho, S., Kita, T., Katayama, N., Abo, M., Ohkura, N., Herai, Y., et al. (2005). Comparison of cough reflex sensitivity after an inhaled antigen challenge between actively and passively sensitized guinea pigs. *Cough* 1, 6-6.
- Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32, 77-88.
- Harkat, M., Peverini, L., Cerdan, A.H., Dunning, K., Beudez, J., Martz, A., Calimet, N., Specht, A., Cecchini, M., Chataigneau, T., et al. (2017). On the permeation of large organic cations through the pore of ATP-gated P2X receptors. *Proceedings of the National Academy of Sciences of the United States of America* 114, E3786-e3795.
- Horáček, M., and Vymazal, T. (2012). Lidocaine not so innocent: Cardiotoxicity after topical anaesthesia for bronchoscopy. *Indian J Anaesth* 56, 95-96.
- Jain, P., Materazzi, S., De Logu, F., Rossi Degl'Innocenti, D., Fusi, C., Li Puma, S., et al. (2020). Transient receptor potential ankyrin 1 contributes to somatic pain hypersensitivity in experimental colitis. *Sci Rep* 10: 8632.
- Jia, Y., McLeod, R.L., Wang, X., Parra, L.E., Egan, R.W., and Hey, J.A. (2002). Anandamide induces cough in conscious guinea-pigs through VR1 receptors. *British journal of pharmacology* 137, 831-836.
- Julius, D. (2013). TRP channels and pain. *Annual review of cell and developmental biology* 29, 355-384.
- Kanai, Y., Hara, T., Imai, A., and Sakakibara, A. (2007). Differential involvement of TRPV1 receptors at the central and peripheral nerves in CFA-induced mechanical and thermal hyperalgesia. *Journal of Pharmacy and Pharmacology* 59, 733-738.
- Kanezaki, M., Ebihara, S., Gui, P., Ebihara, T., and Kohzuki, M. (2012). Effect of cigarette smoking on cough reflex induced by TRPV1 and TRPA1 stimulations. *Respiratory medicine* 106, 406-412.
- Khalid, S., Murdoch, R., Newlands, A., Smart, K., Kelsall, A., Holt, K., Dockry, R., Woodcock, A., and Smith, J.A. (2014). Transient receptor potential vanilloid 1 (TRPV1) antagonism in patients with refractory chronic cough: a double-blind randomized controlled trial. *The Journal of allergy and clinical immunology* 134, 56-62.
- Kichko, T.I., Niedermirtl, F., Leffler, A., and Reeh, P.W. (2015). Irritant volatile anesthetics induce neurogenic inflammation through TRPA1 and TRPV1 channels in the isolated mouse trachea. *Anesth Analg* 120: 467-471.
- Kim, H.Y., Kim, K., Li, H.Y., Chung, G., Park, C.-K., Kim, J.S., et al. (2010). Selectively targeting pain in the trigeminal system. *Pain* 150: 29-40.

- Kollarik, M., Ru, F., and Undem, B.J. (2007). Acid-sensitive vagal sensory pathways and cough. *Pulmonary pharmacology & therapeutics* 20, 402-411.
- Kollarik, M., Sun, H., Herbstsomer, R.A., Ru, F., Kocmalova, M., Meeker, S.N., and Undem, B.J. (2018). Different role of TTX-sensitive voltage-gated sodium channel (NaV1) subtypes in action potential initiation and conduction in vagal airway nociceptors. *The Journal of Physiology* 596, 1419-1432.
- Kwong, K., Kollarik, M., Nassenstein, C., Ru, F., and Undem, B.J. (2008). P2X2 receptors differentiate placodal vs. neural crest C-fiber phenotypes innervating guinea pig lungs and esophagus. *American journal of physiology Lung cellular and molecular physiology* 295, L858-865.
- Labeledzki, L., Ochs, H.R., Abernethy, D.R., and Greenblatt, D.J. (1983). Potentially toxic serum lidocaine concentrations following spray anesthesia for bronchoscopy. *Klin Wochenschr* 61: 379-380.
- Laude, E.A., Higgins, K.S., and Morice, A.H. (1993). A comparative study of the effects of citric acid, capsaicin and resiniferatoxin on the cough challenge in guinea-pig and man. *Pulmonary pharmacology* 6, 171-175.
- Lee, S., Jo, S., Talbot, S., Zhang, H.B., Kotoda, M., Andrews, N.A., Puopolo, M., Liu, P.W., Jacquemont, T., Pascal, M., et al. (2019). Novel charged sodium and calcium channel inhibitor active against neurogenic inflammation. *eLife* 8. e48118.
- Leffler A, Reckzeh J, Nau C. (2010) Block of sensory neuronal Na⁺ channels by the secretolytic ambroxol is associated with an interaction with local anesthetic binding sites. *Eur J Pharmacol.* 630:19-28.
- Lennertz, R.C., Kossyрева, E.A., Smith, A.K., and Stucky, C.L. (2012). TRPA1 mediates mechanical sensitization in nociceptors during inflammation. *PloS one* 7, e43597.
- Leung, S.Y., Niimi, A., Williams, A.S., Nath, P., Blanc, F.-X., Dinh, Q.T., and Chung, K.F. (2007). Inhibition of citric acid-and capsaicin-induced cough by novel TRPV-1 antagonist, V112220, in guinea-pig. *Cough* 3, 1-5.
- Lewis, C.A., Ambrose, C., Banner, K., Battram, C., Butler, K., Giddings, J., Mok, J., Nasra, J., Winny, C., and Poll, C. (2007). Animal models of cough: literature review and presentation of a novel cigarette smoke-enhanced cough model in the guinea-pig. *Pulm Pharmacol Ther* 20, 325-333.
- Liu, B., Escalera, J., Balakrishna, S., Fan, L., Caceres, A.I., Robinson, E., et al. (2013). TRPA1 controls inflammation and pruritogen responses in allergic contact dermatitis. *FASEB J* 27: 3549-3563.
- Liu, P., Jo, S., and Bean, B.P. (2012). Modulation of neuronal sodium channels by the sea anemone peptide BDS-I. *Journal of neurophysiology* 107, 3155-3167.
- Liu, Z., Hu, Y., Yu, X., Xi, J., Fan, X., Tse, C.-M., Myers, A.C., Pasricha, P.J., Li, X., and Yu, S. (2015). Allergen challenge sensitizes TRPA1 in vagal sensory neurons and afferent C-fiber subtypes in guinea pig esophagus. *Am J Physiol Gastrointest Liver Physiol* 308, G482-G488.
- Long, L., Yao, H., Tian, J., Luo, W., Yu, X., Yi, F., Chen, Q., Xie, J., Zhong, N., Chung, K.F., et al. (2019). Heterogeneity of cough hypersensitivity mediated by TRPV1 and TRPA1 in patients with chronic refractory cough. *Respiratory research* 20, 112.
- Ma, C.H., Omura, T., Cobos, E.J., Latremoliere, A., Ghasemlou, N., Brenner, G.J., van Veen, E., Barrett, L., Sawada, T., Gao, F., et al. (2011). Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice. *The Journal of clinical investigation* 121, 4332-4347.
- Martin, L.J., and Corry, B. (2014). Locating the route of entry and binding sites of benzocaine and phenytoin in a bacterial voltage gated sodium channel. *PLoS Comput Biol* 10: e1003688.
- Matta, J.A., Cornett, P.M., Miyares, R.L., Abe, K., Sahibzada, N., and Ahern, G.P. (2008). General anesthetics activate a nociceptive ion channel to enhance pain and inflammation. *Proc Natl Acad Sci U S*

A 105: 8784–8789.

Mazzone, S.B., Reynolds, S.M., Mori, N., Kollarik, M., Farmer, D.G., Myers, A.C., and Canning, B.J. (2009). Selective expression of a sodium pump isozyme by cough receptors and evidence for its essential role in regulating cough. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 29, 13662-13671.

Mazzone, S.B., and Udem, B.J. (2016). Vagal Afferent Innervation of the Airways in Health and Disease. *Physiological reviews* 96, 975-1024.

McLeod, R.L., Fernandez, X., Correll, C.C., Phelps, T.P., Jia, Y., Wang, X., and Hey, J.A. (2006). TRPV1 antagonists attenuate antigen-provoked cough in ovalbumin sensitized guinea pigs. *Cough* 2, 10-10.

Morice, A.H., Fontana, G.A., Belvisi, M.G., Birring, S.S., Chung, K.F., Dicipinigitis, P.V., Kastelik, J.A., McGarvey, L.P., Smith, J.A., Tatar, M., et al. (2007). ERS guidelines on the assessment of cough. *European Respiratory Journal* 29, 1256.

Morice, A.H., Kitt, M.M., Ford, A.P., Tershakovec, A.M., Wu, W.-C., Brindle, K., Thompson, R., Thackray-Nocera, S., and Wright, C. (2019). The Effect of Gefapixant, a P2X3 antagonist, on Cough Reflex Sensitivity: A randomised placebo-controlled study. *European Respiratory Journal*, 1900439.

Mukhopadhyay, I., Kulkarni, A., Aranake, S., Karnik, P., Shetty, M., Thorat, S., Ghosh, I., Wale, D., Bhosale, V., and Khairatkar-Joshi, N. (2014). Transient receptor potential ankyrin 1 receptor activation in vitro and in vivo by pro-tussive agents: GRC 17536 as a promising anti-tussive therapeutic. *PloS one* 9, e97005.

Muroi, Y., Ru, F., Kollarik, M., Canning, B.J., Hughes, S.A., Walsh, S., Sigg, M., Carr, M.J., and Udem, B.J. (2011). Selective silencing of NaV1. 7 decreases excitability and conduction in vagal sensory neurons. *The Journal of physiology* 589, 5663-5676.

Nguyen, P.T., DeMarco, K.R., Vorobyov, I., Clancy, C.E., and Yarov-Yarovoy, V. (2019). Structural basis for antiarrhythmic drug interactions with the human cardiac sodium channel. *Proc Natl Acad Sci U S A* 116: 2945–2954.

Noitasaeng, P., Vichitvejpaisal, P., Kaosombatwattana, U., Tassanee, J., and Suwannee, S. (2016). Comparison of Spraying and Nebulized Lidocaine in Patients Undergoing Esophago-Gastro-Duodenoscopy: A Randomized Trial. *Journal of the Medical Association of Thailand = Chotmaihet thangphaet* 99, 462-468.

Omar, S., Clarke, R., Abdullah, H., Brady, C., Corry, J., Winter, H., Touzelet, O., Power, U.F., Lundy, F., McGarvey, L.P., et al. (2017). Respiratory virus infection up-regulates TRPV1, TRPA1 and ASIC3 receptors on airway cells. *PloS one* 12, e0171681.

Ongun, S., Sarkisian, A., and McKemy, D.D. (2018). Selective cold pain inhibition by targeted block of TRPM8-expressing neurons with quaternary lidocaine derivative QX-314. *Commun Biol* 1: 53.

Patil, M.J., Sun, H., Ru, F., Meeker, S., and Udem, B.J. (2019). Targeting C-fibers for peripheral acting anti-tussive drugs. *Pulmonary Pharmacology & Therapeutics* 56, 15-19.

Payandeh, J., Scheuer, T., Zheng, N., and Catterall, W.A. (2011). The crystal structure of a voltage-gated sodium channel. *Nature* 475: 353–358.

Peleg, R., and Binyamin, L. (2002). Practice tips. Treating persistent cough. Try a nebulized mixture of lidocaine and bupivacaine. *Canadian family physician Medecin de famille canadien* 48, 275.

Pelleg, A., Xu, F., Zhuang, J., Udem, B., and Burnstock, G. (2019). DT-0111: a novel drug-candidate for the treatment of COPD and chronic cough. *Therapeutic advances in respiratory disease* 13, 1753466619877960.

- Puopolo, M., Binshtok, A.M., Yao, G.L., Oh, S.B., Woolf, C.J., and Bean, B.P. (2013). Permeation and block of TRPV1 channels by the cationic lidocaine derivative QX-314. *Journal of neurophysiology* 109, 1704-1712.
- Ragsdale, D.S., McPhee, J.C., Scheuer, T., and Catterall, W.A. (1996). Common molecular determinants of local anesthetic, antiarrhythmic, and anticonvulsant block of voltage-gated Na⁺ channels. *Proceedings of the National Academy of Sciences of the United States of America* 93: 9270–9275.
- Roberson, D.P., Binshtok, A.M., Blasl, F., Bean, B.P., and Woolf, C.J. (2011). Targeting of sodium channel blockers into nociceptors to produce long-duration analgesia: a systematic study and review. *British journal of pharmacology* 164, 48-58.
- Schmitz, S., Tacke, S., Guth, B., and Henke, J. (2016). Comparison of Physiological Parameters and Anaesthesia Specific Observations during Isoflurane, Ketamine-Xylazine or Medetomidine-Midazolam-Fentanyl Anaesthesia in Male Guinea Pigs. *PloS one* 11, e0161258.
- Scholz, A., Kuboyama, N., Hempelmann, G., and Vogel, W. (1998). Complex blockade of TTX-resistant Na⁺ currents by lidocaine and bupivacaine reduce firing frequency in DRG neurons. *Journal of neurophysiology* 79, 1746-1754.
- Scholz A, Vogel W. (2000) Tetrodotoxin-resistant action potentials in dorsal root ganglion neurons are blocked by local anesthetics. *Pain* 89:47-52..
- Schwartz, E.S., La, J.-H., Scheff, N.N., Davis, B.M., Albers, K.M., and Gebhart, G.F. (2013). TRPV1 and TRPA1 antagonists prevent the transition of acute to chronic inflammation and pain in chronic pancreatitis. *J Neurosci* 33: 5603–5611.
- Schwarz W, Palade PT, Hille B. (1977) Local anesthetics. Effect of pH on use-dependent block of sodium channels in frog muscle. *Biophys J.* 1977
- Shirk, M.B., Donahue, K.R., and Shirvani, J. (2006). Unlabeled uses of nebulized medications. *American journal of health-system pharmacy : AJHP : official journal of the American Society of Health-System Pharmacists* 63, 1704-1716.
- Slaton, R.M., Thomas, R.H., and Mbathi, J.W. (2013). Evidence for therapeutic uses of nebulized lidocaine in the treatment of intractable cough and asthma. *The Annals of pharmacotherapy* 47, 578-585.
- Smith, J.A., Kitt, M.M., Butera, P., Smith, S.A., Li, Y., Xu, Z.J., Holt, K., Sen, S., Sher, M.R., and Ford, A.P. (2020a). Gefapixant in two randomised dose-escalation studies in chronic cough. *European Respiratory Journal*, 1901615.
- Smith, J.A., Kitt, M.M., Morice, A.H., Birring, S.S., McGarvey, L.P., Sher, M.R., and Ford, A.P. (2017). MK-7264, a P2X3 Receptor Antagonist, Reduces Cough Frequency in Patients with Refractory Chronic Cough: Results from a Randomized, Controlled, Phase 2b Clinical Trial. In *B14 CLINICAL TRIALS ACROSS PULMONARY DISEASE* (American Thoracic Society), pp. A7608-A7608.
- Smith, J.A., Kitt, M.M., Morice, A.H., Birring, S.S., McGarvey, L.P., Sher, M.R., Li, Y.P., Wu, W.C., Xu, Z.J., Muccino, D.R., et al. (2020b). Gefapixant, a P2X3 receptor antagonist, for the treatment of refractory or unexplained chronic cough: a randomised, double-blind, controlled, parallel-group, phase 2b trial. *The Lancet Respiratory medicine* 8, 775-785.
- Strichartz, G.R. (1973). The inhibition of sodium currents in myelinated nerve by quaternary derivatives of lidocaine. *The Journal of general physiology* 62, 37-57.
- Stueber, T., Eberhardt, M.J., Hadamitzky, C., Jangra, A., Schenk, S., Dick, F., Stoetzer, C., Kistner, K., Reeh, P.W., Binshtok, A.M., et al. (2016). Quaternary Lidocaine Derivative QX-314 Activates and Permeates Human TRPV1 and TRPA1 to Produce Inhibition of Sodium Channels and Cytotoxicity. *Anesthesiology* 124, 1153-1165.

- Sun, H., Kollarik, M., and Udem, B.J. (2017). Blocking voltage-gated sodium channels as a strategy to suppress pathological cough. *Pulm Pharmacol Ther* 47, 38-41.
- Talbot, S., Abdunour, R.E., Burkett, P.R., Lee, S., Cronin, S.J., Pascal, M.A., Laedermann, C., Foster, S.L., Tran, J.V., Lai, N., et al. (2015). Silencing Nociceptor Neurons Reduces Allergic Airway Inflammation. *Neuron* 87, 341-354.
- Talbot, S., Doyle, B., Huang, J., Wang, J.C., Ahmadi, M., Roberson, D.P., Yekkirala, A., Foster, S.L., Browne, L.E., Bean, B.P., et al. (2020). Vagal sensory neurons drive mucous cell metaplasia. *The Journal of allergy and clinical immunology*.
- Tanaka, M., and Maruyama, K. (2005). Mechanisms of Capsaicin- and Citric-Acid-Induced Cough Reflexes in Guinea Pigs. *Journal of Pharmacological Sciences* 99, 77-82.
- Turner, R.D., and Bothamley, G.H. (2014). Cough and the Transmission of Tuberculosis. *The Journal of Infectious Diseases* 211, 1367-1372.
- Udezue, E. (2001). Lidocaine inhalation for cough suppression. *The American journal of emergency medicine* 19, 206-207.
- Udem, B.J., Carr, M.J., and Kollarik, M. (2002). Physiology and plasticity of putative cough fibres in the Guinea pig. *Pulm Pharmacol Ther* 15, 193-198.
- Udem, B.J., and Sun, H. (2020). Molecular/Ionic Basis of Vagal Bronchopulmonary C-Fiber Activation by Inflammatory Mediators. *Physiology (Bethesda)* 35, 57-68.
- Utsumi, D., Matsumoto, K., Tsukahara, T., Amagase, K., Tominaga, M., and Kato, S. (2018). Transient receptor potential vanilloid 1 and transient receptor potential ankyrin 1 contribute to the progression of colonic inflammation in dextran sulfate sodium-induced colitis in mice: Links to calcitonin gene-related peptide and substance P. *J Pharmacol Sci* 136: 121-132.
- Watanabe, N., Horie, S., Spina, D., Michael, G.J., Page, C.P., and Priestley, J.V. (2008). Immunohistochemical localization of transient receptor potential vanilloid subtype 1 in the trachea of ovalbumin-sensitized Guinea pigs. *International archives of allergy and immunology* 146 Suppl 1, 28-32.
- West, P.W., Canning, B.J., Merlo-Pich, E., Woodcock, A.A., and Smith, J.A. (2015). Morphologic Characterization of Nerves in Whole-Mount Airway Biopsies. *American journal of respiratory and critical care medicine* 192, 30-39.
- Yeh, J.Z. (1978). Sodium inactivation mechanism modulates QX-314 block of sodium channels in squid axons. *Biophysical journal* 24, 569-574.
- Yin, K., Deuis, J.R., Lewis, R.J., and Vetter, I. (2016). Transcriptomic and behavioural characterisation of a mouse model of burn pain identify the cholecystokinin 2 receptor as an analgesic target. *Mol Pain* 12, 1744806916665366.
- Zaccone, E.J., Lieu, T., Muroi, Y., Potenzieri, C., Udem, B.E., Gao, P., Han, L., Canning, B.J., and Udem, B.J. (2016). Parainfluenza 3-Induced Cough Hypersensitivity in the Guinea Pig Airways. *PloS one* 11, e0155526.
- Zhou, C., Liang, P., Liu, J., Zhang, W., Liao, D., Chen, Y., et al. (2014). Emulsified isoflurane enhances thermal transient receptor potential vanilloid-1 channel activation-mediated sensory/nociceptive blockade by QX-314. *Anesthesiology* 121: 280-289.
- Zielińska, M., Jarmuż, A., Wasilewski, A., Sałaga, M., and Fichna, J. (2015). Role of transient receptor potential channels in intestinal inflammation and visceral pain: novel targets in inflammatory bowel diseases. *Inflamm Bowel Dis* 21: 419-427.

Supporting Information: Synthesis of BW-031 (1-(1-(2, 6-dimethylphenylamino)-1-oxobutan-2-yl)-1-ethylpiperidinium)

Synthetic Scheme

Step 1: Preparation of intermediate 2

To a mixture of **1** (10.0g, 59.88mmol) was added SOCl_2 (60mL, c=1.0). The mixture was heated to reflux. After completion, the reaction mixture was concentrated under reduce pressure to give intermediate **2** (9.2g, yield=82.8%) as a yellow oil.

Step 2: Preparation of intermediate 4

To a solution of **3** (5.0g, 41.3mmol, 1.0eq) in DCM (100ml, c=0.5) was added pyridine (4.9g, 61.95mmol, 1.5eq). To the solution was added **2** (9.2g, 49.59mmol, 1.2eq) in DCM (40mL, c=1.2). The reaction mixture was stirred at room temperature overnight. Then to the solution was added water (50mL). The organic phase was washed with brine, dried over Na_2SO_4 , filtered and concentrated under reduce pressure. The residue was washed with n-hexane to give intermediate **4** (7.8g, yield=70%, HPLC: 98.6%).

Step 3: Preparation of intermediate 6

To a solution of NaH (0.35g, 14.8mmol, 2.0eq) in THF (37mL, c=0.4) was added **5** (0.75g, 8.8mmol, 1.2eq). To the solution was added **4** (2.0g, 7.4mmol, 1.0eq) in THF (20mL, c=0.37). The reaction mixture was then stirred at room temperature overnight. To the suspension was added water (20mL) and EA (50mL). The organic phase was washed with water (50mL \times 2). Then the organic phase was adjusted to pH 2, extracted with EA(40mL \times 2). The aqueous fractions were combined and adjusted to pH 9, then extracted with EA (40 \times 2). The combined organic fractions were washed with brine, dried over Na_2SO_4 , filtered and concentrated under reduce pressure. The residue was washed with n-hexane to give the intermediate **6** (0.48g, yield=24%, HPLC: 99.3%) as a solid.

Step 4: Preparation of intermediate 7

Intermediate **6** (0.48g, 1.75mmol, 1.0 eq) and MeCN (9mL, c=0.2) was added in sealed tube. To this solution, EtI (2mL, 14.0 eq) was added. After addition, the reaction mixture was stirred at 90 for 10h. After completion, the reaction mixture was concentrated under reduce pressure. The residue was purified by column chromatography to give intermediate **7** (470mg, yield=62.6%, HPLC: 99%) as a solid.

Step 4: Preparation of compound 8

To a solution of **7** (200mg, 0.465mmol, 1.0 eq) in deionized water (3ml, c=0.15) was added AgCl (133mg, 0.93mmol, 2.0 eq). After addition, the reaction mixture was stirred at room temperature overnight. The suspension was then filtered and the filtrate was lyophilized to give compound **8** (141mg, yield=89.8%) as a solid. HPLC purity: at 220nm; Mass: M+1=339.4. ^1H NMR (300 MHz, D $_2$ O): δ 7.117 (m, 3H), 4.056 (dd, J=8.1 Hz, 1H), 3.712~3.808 (m, 1H), 3.656 (m, J=13.2 Hz, 2H), 3.510~3.582 (m, 1H), 3.344 (m, 2H), 2.117 (s, 6H), 1.984~2.070 (m, 2H), 1.818 (m, 4H), 1.660 (m, 1H), 1.455 (m, 1H), 1.278 (t, J=7.2 Hz, 3H), 1.107 (t, 3H) ppm.