Inhibition of TRPM8 Channels Reduces Pain in the Cold Pressor Test in Humans


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ABSTRACT

The transient receptor potential (subfamily M, member 8; TRPM8) is a nonselective cation channel localized in primary sensory neurons, and is a candidate for cold thermosensing, mediation of cold pain, and bladder overactivity. Studies with TRPM8 knockout mice and selective TRPM8 channel blockers demonstrate a lack of cold sensitivity and reduced cold pain in various rodent models. Furthermore, TRPM8 blockers significantly lower body temperature. We have identified a moderately potent (IC50 = 103 nM), selective TRPM8 antagonist, PF-05105679 ([R]-3-[(1-(4-fluorophenyl)ethyl](quino-3-ylcarbonyl)amino)methylbenzoic acid). It demonstrated activity in vivo in the guinea pig bladder ice water and menthol challenge tests with an IC50 of 200 nM and reduced core body temperature in the rat (at concentrations >1219 nM). PF-05105679 was suitable for acute administration to humans and was evaluated for effects on core body temperature and experimentally induced cold pain, using the cold pressor test. Unbound plasma concentrations greater than the IC50 were achieved with 600- and 900-mg doses. The compound displayed a significant inhibition of pain in the cold pressor test, with efficacy equivalent to oxycodone (20 mg) at 1.5 hours postdose. No effect on core body temperature was observed. An unexpected adverse event (hot feeling) was reported, predominantly periorally, in 23 and 36% of volunteers (600- and 900-mg dose, respectively), which in two volunteers was non-tolerable. In conclusion, this study supports a role for TRPM8 in acute cold pain signaling at doses that do not cause hypothermia.

Introduction

Detection of environmental temperature is essential for thermoregulation and for recognition of potentially damaging (noxious) hot or cold stimuli (Premkumar and Abooj, 2013). Defects in cold sensation can lead to conditions such as cold allodynia and cold hyperalgesia, which are clinically important symptoms in patients suffering from neuropathic pain (Maijer et al., 2010), complex regional pain syndrome (Eberle et al., 2009), chemotherapy-induced allodynia (Binder et al., 2007; Attal et al., 2009), and painful bladder syndrome (Mukerji et al., 2006). Various molecular targets have been identified as candidates for signaling cold sensing, one of which is transient receptor potential (subfamily M, member 8) (TRPM8), a non-selective cation channel (McKemy et al., 2002; Peier et al., 2002). Expressed in sensory neurons, it is known to have multiple modes of activation, including cold (<28°C), cooling chemicals (such as menthol), and voltage. Preclinically, TRPM8 has been functionally linked to pain in oxaliplatin-induced (Descouer et al., 2011) and chronic nerve injury neuropathic pain models (Xing et al., 2007; Su et al., 2011), where increased receptor expression and sensitivity to menthol were observed. In oxaliplatin-treated cancer patients, an increased sensitivity to menthol suggests TRPM8 overexpression or activity (Binder et al., 2007; Kono et al., 2012). Finally, patients with idiopathic detrusor overactivity have increased TRPM8 expression in bladder sensory neurons (Mukerji et al., 2006). These patients also show involuntary sustained bladder contractions and reduced bladder threshold volumes (i.e., the volume of fluid received April 28, 2014; accepted August 13, 2014

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ABBRiEVATIONS: AE, adverse event; AUC, area under the curve; AUCcold, cumulative AUC of the cold pain time profile from 0 to 120 seconds; CI, confidence interval; CID, compound identifier; ECS, extracellular solution; FDSS, functional drug screening system; HBSS, Hanks’ balanced salt solution; HEK-293, human embryonic kidney 293; PF-05105679, ([R]-3-[(1-(4-fluorophenyl)ethyl](quino-3-ylcarbonyl)amino)methylbenzoic acid; TRPA1, transient receptor potential cation channel subfamily A, member 1; TRPM8, transient receptor potential subfamily M, member 8; TRPV1, transient receptor potential cation channel subfamily V, member 1; UGT, uridine diphosphate glucuronyltransferase; VAS, visual analog scale; WS-12, (2S,5R)-2-isopropyl-N-(4-methoxyphenyl)-5-methylcyclohexanecarboxamide.
required to elicit bladder contraction and urine expulsion) in response to installation of ice-cold water into the bladder, an effect absent in healthy adults (Al-Hayek and Abrams, 2010). In addition to a potential role in cold detection and pain, TRPM8 has been demonstrated preclinically to have a fundamental homeostatic role in the control of core body temperature, with TRPM8 antagonists causing a significant hypothermia (Almeida et al., 2012).

Although there is a significant body of work on the role of TRPM8 preclinically, confirmation of a role of TRPM8 in human physiology has yet to be determined. Therefore, we designed and characterized a novel, selective TRPM8 antagonist suitable for evaluation in humans and examined its effect on cold pain core body temperature. Our results show that TRPM8 is involved in acute cold pain detection in humans, with efficacy equivalent to the therapeutically relevant oxycodone. This effect was seen in the absence of any hypothermia.

**Materials and Methods**

The TRPM8 antagonist PF-05105679 [(R)-3-[(1-(fluorophenyl)ethyl]quinolin-3-ylcarbonylamino]methylbenzoic acid; Glossop et al., 2012] was synthesized at Pfizer Ltd. (Fig. 1). (−)-Menthol [PubChem compound identifier (CID): 16666] and WS-12 [(2S,5R)-2-isopropyl-N-(4-methoxyphenyl)-5-methylcyclohexanecarboximide; PubChem CID: 7131185] were supplied by Sigma-Aldrich (Poole, UK). Oxycodone 20 mg controlled release (4,5-dimethoxyphenyl)-5-methylcyclohexanecarboximide; PubChem CID: 19011311) was supplied by Sigma-Aldrich (Poole, UK). Oxycodeone 20 mg controlled release (4,5a-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one hydrochloride; PubChem CID: 25137874) was supplied by the Hopital Erasme pharmacy (Brussels, Belgium).

**In Vitro Characterization of the Potency of PF-05105679 against Known Activators of the TRPM8 Channel**

**WS-12 Agonist Activation.** For TRPM8 activation by WS-12 (a menthol analog; Ma et al., 2008; Sherkheli et al., 2010), intracelular calcium mobilization was measured using a functional drug screening system (FDSS 6000; Hamamatsu, Shizuoka, Japan) with the Fluoro-metric Imaging Plate Reader Calcium Assay Kit (Molecular Devices, Sunnyvale, CA). Human embryonic kidney 293 (HEK-293) cells were stably transfected with a human transient receptor potential melastatin (TRPM8) receptor construct (accession number NM_024080). Twenty-four hours before the assay, the cells were resuspended in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum supplemented with 4.5 mg/ml l-glucose, Glutamax (Life Technologies, Carlsbad, CA), 25 mM HEPES, 1 mM sodium pyruvate, and seeded 10,000 cells per well into black-walled, clear-bottom, 384-well CELL COAT poly-d-lysine–coated assay plates (Greiner Bio-One, Frickhausen, Germany) and incubated overnight at 37°C under a humidified 5% CO2 atmosphere.

Calcium influx assay was performed using an FDSS and Calcium 4 assay kit (Molecular Devices, Sunnyvale, CA). The Ca2+ indicator dye was dissolved in Hank’s balanced salt solution (HBSS) supplemented with 20 mM HEPES buffer (Invitrogen, Grand Island, NY) and 2.5 mM probenecid (Sigma-Aldrich). Prior to the start of the assay, the medium was removed and replaced with 20 μl of HBSS, and cells were loaded with 10 μl of Ca2+ dye for 1 hour at 37°C. All compounds were prepared fresh daily as 100% dimethylsulfoxide stocks and serializd in 100% dimethylsulfoxide prior to dilution in assay buffer, and 10 μl was added to the cell plates. An 11-point concentration range of PF-05105679 was tested and cells preincubated for 15 minutes in the presence of the test compound before an EC50 concentration of WS-12 was added to all test wells (determined in an initial plate with an 11-point concentration range at the beginning of the experiment; approximates to 350 nM). Changes in fluorescence were measured every 1 second for 60 seconds in the FDSS 6000. Due to the channel’s sensitivity to temperature, all solutions were warmed to 37°C prior to addition.

**Cold Activation.** The application of cold (10°C) causes a flux of Ca2+ across the cell membrane, which was measured by changes in intracelular fluorescence on a real-time polymerase chain reaction machine (ABI 7700; Applied Biosystems, Foster City, CA). The profile of PF-05105679 was assessed and concentration effect curves generated.

Cells were incubated in 225-cm2 flasks for 48 hours at 37°C in a humidified atmosphere with 5% CO2 present. After 48 hours of incubation, flasks were removed and washed with prewarmed wash buffer (HBSS without CaCl2 and MgCl2, 20 mM HEPES) and 2.5 mM probenecid, pH adjusted to 7.4). Flasks were then returned to the incubator for 30 minutes, after which they were washed twice following isolation, and cells were suspended in assay buffer (without probenecid) to achieve a concentration of 106 cells/ml. Cells were transferred to a 96-well V-bottom polypropylene optical reaction plate (Applied Biosystems) and PF-05105679 added in an 11-point concentration range. An optical seal was secured and the plate was loaded into the real-time polymerase chain reaction machine. The SDS 1.91 Alias program (Applied Biosystems) was used to run the assay, and thermal cycler conditions used were 37°C for 2 minutes, 10°C for 5 minutes, with the sample volume set as 100 μl. The camera aperture for this assay was set to 25 milliseconds. Data were collected at 37 and 10°C. Percent inhibition of cold response by PF-05105679 was calculated.

**TRPM8 Electrophysiology: Whole-Cell Manual Patch Recording**

Whole-cell patch-clamp recordings were used to obtain recordings of voltage-activated TRPM8 currents in HEK-293 cells transiently expressing guinea pig (accession number QG368453) or human TRPM8. All recordings were obtained at room temperature (20–22°C) using patch pipettes with resistances 2–6 MΩ. Coverslips containing TRPM8-transfected cells were transferred to a recording chamber and superfused with standard external solution (135 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, 5 mM glucose, pH 7.4 with TEA-0H 300 mOsM) at a rate ~2 ml/min. Cells were patched in whole-cell configuration, with a low access resistance. Voltage protocols were generated in pClamp 10 software which drove command signals to the amplifier (Axon Multiclamp 700A; Molecular Devices), and currents generated were recorded via the amplifier to a PC running pClamp (version 9 or 10). Cells were clamped at −60 mV and antagonist was applied via the bath perfusion. To determine a stable baseline for TRPM8 currents, 100-millisecond voltage step protocols from −130 to +190 mV were delivered every 1 or 2 minutes until the current amplitudes generated by the +190-mV step were stable. Following
this, single concentrations of antagonist were bath-applied until a stable plateau of TRPM8 current amplitude had been achieved once more. Multiple increasing concentrations of antagonist could be applied to cells that maintained stable recording conditions and plateaus. An additional study was performed holding the cells at 12°C and repeating the voltage step protocol to evaluate the effect of PF-05105679 under conditions of dual activation. The gigaseal seal and whole-cell configuration were obtained at room temperature (~21°C), and then the bath extracellular solution (ECS) was cooled down to 12°C with the aid of a peltier device (Harvard Apparatus, Kent, UK). Antifreeze solution maintained at 4°C from a cooled fluid circulator (DC 10 coupled with K10; Thermo Scientific, Boston, MA) was used to remove heat from the “hot” side of the peltier device to ensure greater efficiency of peltier cooling of the ECS (running over the “cold” side of the peltier) to allow the tissue bath solution to reach a target temperature of 12°C. The dead space of cooled ECS between peltier and bath was minimized by placing the peltier in the closest possible proximity to the bath, ensuring rapid and stable cooling of the bath. The bath temperature was manually controlled and adjusted with a bipolar temperature controller (model CL 100; Warner Instrument, Kent, UK), and a thermistor was placed in the bath close to the cell to allow accurate recording and monitoring of actual experimental temperature.

**In Vivo Characterization.** All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986).

**Analysis of Rodent Pharmacokinetics.** Male Wistar Han rats (Charles River, Edinburgh, UK) were dosed with either a single oral gavage of 2 mg/kg (n = 7) or 10 mg/kg (n = 3). An additional group of animals were dosed with a single intravenous bolus dose of 1 mg/kg (n = 7). For both arms of the pharmacokinetic study, the compound was administered in a standard suspension in 0.5% methylcellulose/0.1% Tween 80 in water. All samples were analyzed using a high-performance liquid chromatography–mass spectrometry system assay specific for PF-05105679. All samples analyzed were well above the quantification limit of this system (1 ng/ml).

**TRPM8 in Vivo Efficacy**

**Effect of PF-05105679 on Cold-Induced or Menthol Changes in Bladder Capacity on Discontinuous Cystometry in the Anesthetized Guinea Pig.** Details of the methodology have been published previously (Gardiner et al., 2007). In brief, halothane anesthesia was induced in female Dunkin-Hartley guinea pigs (405–435 g), followed by an intraperitoneal urethane injection (1.2 g/kg, 0.2 g/ml solution) to achieve surgical anesthesia. Core body temperature was maintained with a Harvard Apparatus homeothermic blanket control unit connected to a rectal probe. Two catheters were inserted into the bladder lumen through an incision in the dome and tied in place. The abdominal layers were then closed sutured. One catheter was connected to a pressure transducer (Spectramed, Statham, GA). The trachea was cannulated and the animals artificially ventilated with oxygen-enriched air using an Ugo-Basile rodent ventilator (Ugo-Basile, Milan, Italy). Chloralose (44 mg/kg, 10 mg/ml) was administered intravenously, and supplemental doses of urethane were given if necessary. Following a midline laparotomy, both ureters were ligated, cut rostral of the ligature, and allowed to drain into the peritoneal space. A double-lumen catheter was inserted into the bladder lumen through an incision in the dome and tied in place. The abdominal layers were then closed sutured. One catheter was connected to a pressure transducer (Spectramed) to record bladder pressure. The second catheter was used for infusion of fluid into the bladder.

For the cold-induced effects upon bladder function, the infusion of 0.9% saline was temperature controlled through a water jacket surrounding the silicone tubing, through which water circulated at 38 or 3°C. Thermosafterially controlled cooling of the infusate below room temperature was achieved by means of a Grant C1G refrigerated immersion cooler in conjunction with a Grant W6 circulating water bath (Grant Instruments, Cambridge, UK), whereas warming of the infusate was achieved by switching to water circulating at 38°C from a separate Grant W6 water bath. The infusion of the bladder with the temperature-controlled saline was controlled by a syringe infusion pump (KD Scientific, Holliston, MA). For the menthol challenge experiments, the temperature of the infusate remained at body temperature.

After the stabilization period, micturition reflexes were elicited by infusion of saline into the bladder at 6 ml/min in the cold saline model and 0.6 ml/min in the menthol model. Infusions were stopped on commencement of voiding and the residual volume drained 1 minute later via the infusion catheter, which was temporarily disconnected from the infusion system. Saline was initially infused at 15-minute intervals at 38°C until three reproducible bladder capacity (threshold volume) responses were obtained, which were averaged to provide a control value.

Following this, control micturition reflexes were obtained to infusion of the test challenge (either one for saline at 3°C at 6 ml/min or two for menthol 0.2 mM in 0.9% saline at 0.6 ml/min). Bladder infusions of the test challenge were then repeated at intervals of 20 minutes for cold saline or 15 minutes for menthol during continuous intravenous infusion of PF-05105679 or vehicle (18% glycerol formal, 17% solutol in 0.9% saline) at 0.1 ml/kg per min.

Carotid arterial blood samples were taken 1 minute prior to the start of intravesical infusion. Blood samples were centrifuged and plasma removed, frozen, and stored at −20°C for pharmacokinetic analysis. Data collected on Spike 2 software (Cambridge Electronic Devices, Cambridge, UK) included micturition interval and threshold volume. These data were used to determine the pharmacokinetic-pharmacodynamic relationship. Data for threshold volume are presented in graphical format as the arithmetic mean and S.E.M. of the raw data. Comparison between PF-05105679 and vehicle-treated groups at each time point was analyzed using analysis of covariance with the covariate value taken as the 3°C control or the second menthol control.

**Effect of PF-05105679 on Core Body Temperature in the Rat.** Male Wistar Han rats (n = 8; Charles River) were surgically implanted with telemetry devices (Data Science International, St. Paul, MN) as previously described (dePasquale et al., 2009) to enable heart rate, arterial blood pressure, and temperature to be monitored. The oral administration of PF-05105679 was used in a crossover design with all animals receiving all doses (vehicle (phosphate-buffered saline containing 0.5% methylcellulose and 0.1% polysorbate 80), 30, 100, and 300 mg/kg) via oral gavage with a minimum 48-hour washout period between treatments. Raw data were collected every minute and averaged into 15-minute mean values relative to the point of dosing. Statistical analysis compared the effects of data for PF-05105679 (30, 100, and 300 mg/kg) and control groups at each time period using analysis of variance. In a satellite group of rats (n = 4 per group), blood samples were collected predose and at 0.5, 1, 2, 3, 4, 6, and 24 hours postcompound administration. The concentration of PF-05105679 was determined in plasma from these samples.

**Clinical Methodology**

This study was conducted in compliance with the ethical principles originating in or derived from the Declaration of Helsinki, and in compliance with all International Conference on Harmonization Good Clinical Practice Guidelines. In addition, all local regulatory requirements were followed, in particular, those affording greater protection to the safety of trial participants. The study was reviewed and approved by the Ethics Committee of the Erasme Hospital in Brussels, Belgium, and was registered on ClinicalTrials.gov (identifier: #NCT01393652). All 32 subjects gave informed consent to the study, signed and dated with the option to withdraw from the study at any point. The study was carried out within the specialist Clinical Research Unit in Hopital Erasme, Brussels, Belgium.

This was a double-blind, third-party open (i.e., subject-blind, investigator-blind, and sponsor-open) crossover dose escalation study with randomized placebo and active controls. The study was split into
two parts: part 1 comprised cohorts 1 and 2, followed by part 2 comprising cohort 3, the pharmacodynamic study. Each cohort included a screening visit and up to four treatment periods, with at least a 1-week interval between doses and a follow-up visit at least 7 days after the last dosing.

Cold Pressor Test Methodology

The cold pressor test is based on the immersion of the subject’s hand into cold water for 2 minutes, and leads to a robust, transient increase in blood pressure via arterial vasoconstriction (Wolff and Hardy, 1941; Greene et al., 1965). Another component of the response is a sensation of cold pain (DePasquale et al., 2009), which may be relevant in the context of cold-sensing targets. The intensity of the pain sensation is typically measured using a visual analog scale (VAS) or similar method; in this study, the VAS scoring was continuously recorded electronically over the 120-second period of the cold pressor test. Lower AUC_{cpt0-120} [cumulative area under the curve (AUC) of the cold pain time profile from 0 to 120 seconds] values indicate greater tolerance of cold.

Baseline was the predose measurement on the day of dosing for each period. The AUC_{cpt0-120} was automatically calculated by the cold pressor test software. The AUC averaged over 120 seconds was then derived and used in the analysis, i.e., AUC/time, where time was 120 seconds for completed tests. Observed and change from baseline average AUC_{cpt0-120} values were summarized [n, mean, median, S.D., 90% confidence intervals (CIs) for the mean, minimum, maximum] by treatment and time point. Average AUC_{cpt0-120} was analyzed at each time point separately using a mixed-effects model with treatment, period, and sequence as fixed effects; subject (within sequence) as a random effect; and baseline as covariate. Two baseline covariates were used: subject-baseline (mean of baseline values across all treatment periods) and adjusted-baseline (predose value for relevant visit minus subject-baseline). Kenward-Rogers approximation was used to estimate denominator degrees of freedom. Least squares means for each treatment (S.E. and 90% CIs) were output from the model, along with point estimates (S.E. and 90% CIs) for the difference from placebo. All analyses were performed using SAS v9.2 (SAS Institute, Cary, NC).

Core Body Temperature Measurement

Core body temperature was monitored using an ingestible radio opaque device which transmitted a regular signal to an external receiver (VitalSense; Philips Respironics, Murrysville, PA; McKenzie and Ogsood, 2004). Subjects swallowed the device with up to 200 ml of room temperature water at least 2 hours prior to dosing. If the device failed electronically or was expelled from the body before 24 hours following study treatment administration, another device was ingested by the subject. Subjects were required to mark all food and drink consumption (which had to be room temperature according to the lifestyle guidelines in the protocol) on an event marker form for 24 hours postdose. Devices were allowed to pass naturally through subjects and were not recovered. In case the device was not eliminated from the body prior to discharge from the unit, an additional ambulatory visit was allowed to check that the capsule had been expelled from the body.

Following ingestion of the VitalSense device, continuous telemetric core body temperature began at least 1 hour prior to dosing and continued for at least 24 hours following study treatment administration for each period in all cohorts.

Determination of Sample Size for Clinical Study. The sample size of 8 subjects for cohorts 1 and 2 for this single-dose escalation study was chosen based on the need to minimize human subject’s first exposure to a new chemical entity and the requirement to provide adequate safety and toleration information at each dose level. The sample size of 16 male subjects in part 2 provided 80% CI for the difference between treatments of 2.4°C for cold pain threshold with 80% coverage probability. The within-subject S.D. of 4.8°C was based on data obtained from previous unpublished studies on the cold pressor test and literature standards.

Results

Compound Characterization In Vitro. PF-05105679 (molecular weight 428.463) is a lipophilic acidic molecule with a measured logD_{7.4} of 0.96 and a measured acidic pK_a of 4.09. In pH 7.4 phosphate-buffered saline, it has a limit of solubility of 84.4 μg/ml (191 μM).

PF-05105679 is a moderately potent compound when evaluated against agonist (WS-12) and cold modalities of activation of TRPM8 in plate-based assay formats, with a mean IC_{50} ± S.E.M. of 181 ± 7.21 nM (n = 25) and 480 ± 42.76 nM (n = 13) for WS-12 and cold activation, respectively. Cross-species evaluation of potency for PF-05105679 has been determined with freestyle HEK-293 (Life Technologies) transiently transfected with human, rat (accession number NM_134371), and guinea pig TRPM8 and stimulated with 350 nM WS-12 to induce calcium mobilization. PF-05105679 was roughly equipotent across all species with a mean IC_{50} ± S.E.M. of 29.9 ± 29.6, 19.3 ± 14.2, and 43.5 ± 19.6 nM (n = 3) for humans, rats, and guinea pigs, respectively.

In single-cell patch-clamp studies, voltage steps of 190 mV generated large robust TRPM8 currents in both guinea pig and human TRPM8-expressing cells at room temperature. PF-05105679 inhibited human TRPM8 currents, with a mean IC_{50} ± S.E.M. of 103 ± 29.4 nM (n = 3–4; Hill slope = 1.1 using sigmoid fitting to individual responses using ordinary least squares and no fixed parameters), and was equipotent against the guinea pig TRPM8 current with a mean IC_{50} ± S.E.M. of 109 ± 38.8 nM (n = 3, Hill slope = 0.8; Fig. 2A). When the cells are held at 12°C, PF-05105679 demonstrated increased potency compared with voltage activation alone (representative trace of TRPM8 current; Fig. 2B) with a mean IC_{50} ± S.E.M. of 13.8 ± 2.2 nM (n = 3–4, Hill slope = 1.82; Fig. 2C). Table 1 summarizes the in vitro potencies seen across the TRPM8 modalities of activation.

PF-05105679 demonstrated >100-fold selectivity against a broad panel of 90 receptors, ion channels, and enzymes in the BioPrint In Vitro Pharmacology Profile provided by CEREP (Poitiers, France). Table 2 lists hits of greater than 15% inhibition from the CEREP assay. Importantly, PF-05105679 was without effect (at 10 μM) in either agonist or antagonist format against related family members TRPV1 (transient receptor potential cation channel subfamily V, member 1; accession number NM_080704) and TRPA1 (transient receptor potential cation channel subfamily A, member 1; accession number NM_007332) at 10 μM, recorded both in Fluorometric Imaging Plate Reader Ca^{2+} mobilization assays as well as in single-cell patch-clamp studies against both voltage- and agonist-driven activation. In addition, PF-05105679 demonstrated no agonist activity against TRPM8 in any of the assays.

Summary Pharmacokinetics. Preliminary studies have shown that PF-05105679 is metabolized slowly in rat and human liver microsomes and hepatocytes. PF-05105679 also demonstrated low metabolic turnover following incubation with human liver microsomes in the presence of uridine diphosphate glucuronic acid. Preliminary data from in vitro studies carried out in uridine diphosphate glucuronotransferase (UGT) recombinant enzymes suggest that PF-05105679 is a substrate for UGT1A1, UGT1A3, UGT1A8, UGT1A9, UGT1A10, UGT2B7, and UGT2B15.

The potential for PF-05105679 to inhibit human cytochrome P450 enzymes has been investigated in human liver

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microsomes. In exploratory studies, PF-05105679 did not inhibit CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, or CYP3A4, exhibiting IC$_{50}$ values of 30 m$\mu$M.

The pharmacokinetic parameters of PF-05105679 following single intravenous and oral gavage administration to male rats were evaluated. Following single oral solution administration to male rats at a dose of 2 mg/kg ($n=7$) and 10 mg/kg ($n=3$), peak PF-05105679 plasma concentrations were achieved 1 and 0.6 hours after dosing, respectively. The mean oral bioavailability of PF-05105679 in the rat was 29% (2 mg/kg) and 62% (10 mg/kg). Following single intravenous solution administration of 1 mg/kg to male rats ($n=7$), PF-05105679 exhibited a mean plasma clearance of 19.8 ml/min per kilogram (rat liver blood flow value; 70 ml/min per kilogram) and a mean steady-state volume of distribution of 6.2 l/kg, resulting in an effective half-life of 3.6 hours.

Plasma protein binding was determined by equilibrium dialysis at a concentration of 2 m$\mu$M in rat, guinea pig, and human plasma.

**TABLE 1**

<table>
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<th>Assay</th>
<th>Species</th>
<th>IC$_{50}$ (nM)</th>
<th>S.E.M.</th>
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n/a, not applicable.
human plasma, with mean unbound fraction values determined to be 0.0316, 0.0693, and 0.0115, respectively. Initial data from a blood-partitioning study suggest that PF-05105679 is preferentially distributed in plasma relative to whole blood, with blood:plasma ratios of 0.76, 0.67, and 0.62 in rats, guinea pigs, and humans, respectively.

In Vivo Efficacy. Functional in vivo pharmacology was evaluated against cold-induced bladder contractility in the guinea pig (Gardiner et al., 2007). Cold saline (3°C) consistently reduced bladder capacity when compared with baseline responses [1.51 ± 0.15 ml compared with 2.39 ± 0.25 ml (mean ± S.E.M.) for cold saline versus 38°C saline]. In animals treated with PF-05105679 (infused intravenously at a rate of 46.67 μg/kg per minute, n = 3), full reversal of the cold-induced reduction in bladder capacity was recorded, with plasma exposures in the range of 3665–6493 nM total [with guinea pig fraction unbound measured as 0.0693, which is equivalent to 254–450 nM free (mean 341 ± 58 nM free, n = 3); Fig. 3A]. Similarly, full reversal of the menthol-induced reduction in bladder capacity was also observed, but the absolute potency was shifted compared with the effect against cold activation (n = 5; Fig. 3B). However, in single-infusion studies where either 13.06 (n = 4) or 50.63 μg/kg per minute (n = 5) doses were infused for 180 minutes, significant inhibition of the menthol-induced reduction in voiding volume was observed [2.69 ± 0.39 ml (13.06 μg/kg per minute) versus 1.71 ± 0.1 ml (vehicle) and 2.27 ± 0.26 (50.63 μg/kg per minute) versus 1.34 ± 0.08 ml (vehicle)]. Modeling of the pharmacokinetic and pharmacodynamic responses from all of

### TABLE 2

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<th>Target</th>
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<td>808-2bah</td>
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<tr>
<td>μ-Opioid (human)</td>
<td>25</td>
<td>843-h</td>
</tr>
<tr>
<td>Choline transporter 1 (human)</td>
<td>22</td>
<td>806-Uh</td>
</tr>
<tr>
<td>Adrenergic α2b (human)</td>
<td>21</td>
<td>802-1Ah</td>
</tr>
<tr>
<td>Cyclooxygenase 2 (human)</td>
<td>21</td>
<td>777-2hr</td>
</tr>
<tr>
<td>Muscarinic M3 (human)</td>
<td>21</td>
<td>806-3h</td>
</tr>
<tr>
<td>κ-Opioid (rat)</td>
<td>20</td>
<td>842-r</td>
</tr>
<tr>
<td>Neurokinin 1 (human)</td>
<td>18</td>
<td>926-1h</td>
</tr>
</tbody>
</table>

CCK2, cholecystokinin receptor 2.

![Fig. 3.](image-url) (A) Data from anesthetized guinea pigs showing the effects of vehicle (n = 3, black bars) or PF-05105679 (n = 3, gray bars) in the “ice-water” test presented as the mean ± S.E.M. Each dose infusion (16.3 and 46.7 μg/kg per minute) and bladder infusion temperature is marked as horizontal bars. Figures represent the mean plasma concentration achieved at each time point. *P < 0.05; **P < 0.01; ***P < 0.001 compared with vehicle (analysis of covariance [ANCOVA]). (B) Effect of PF-05105679 on menthol-induced reduction in bladder void volume in the anesthetized guinea pig, vehicle (n = 4, black bars), or PF-05105679 (n = 5, gray bars). Doses of 16.3, 46.47, and 153.33 μg/kg per minute or vehicle was administered sequentially intravenously. *P < 0.05; ***P < 0.001 compared with vehicle (ANCOVA). (C) Modeled normalized concentration-effect curve for effect of PF-05105679 upon menthol-induced reduction in bladder void volume. Ceff, predicted efficacious concentrations of EC90; GP, guinea pig.
the menthol challenge studies produced an EC_{50} of 200 nM and EC_{90} of 425 nM free plasma concentrations (Fig. 3C).

When tested for an effect on core body temperature, PF-05105679 (100 and 300 mg/kg but not 30 mg/kg p.o.) caused a statistically significant (P < 0.05) fall in body temperature of 0.34 and 0.46°C, respectively, during the 0–3-hour summation period (Fig. 4A). At the 300-mg/kg dose, this hypothermia was also observed in the 3–9-hour summation period. At 0.5 hour postdose, the mean plasma concentrations detected (monitored in a group of satellite animals) were approximately 10,033 and 36,910 ng/ml when administered at 100 and 300 mg/kg, respectively. The rat plasma protein binding ratio has been measured as 0.03939, thus the unbound plasma concentrations achieved during this period can be calculated to be 1219 and 4484 nM (Fig. 4B).

Human Safety, Pharmacokinetics, and Efficacy. The first human study was conducted in two arms. The first arm demonstrated the safety, tolerability, and pharmacokinetics of PF-05105679 in healthy male volunteers at single escalating oral doses of 10–900 mg. Nine hundred milligrams was set as the maximum dose due to treatment-emergent adverse events (discussed later). Exposure increased with dose with supraproportional increases in the peak plasma concentration (C_{max}) above 300 mg (Table 3). Absorption was relatively rapid with the peak concentrations occurring between 0.5 and 1.7 hours postdose. Following absorption, plasma concentrations declined rapidly with no significant exposure 4 hours postdose (Fig. 5A). The mean unbound C_{max} value for the 900-mg dose was 284 nM, although variability in total C_{max} was moderate to high (with a coefficient of variation of 41–99%). No effect was observed on core body temperature following treatment with PF-05105679 (Fig. 5B) nor on any vital signs including blood pressure and QTc intervals.

For the cold pressor test, the AUC_{0–120} for a continuous VAS was calculated at both 1.5 and 3.5 hours postdose. Negative changes from baseline imply that the subject experienced less pain from the cold water postdose. At 1.5 hours postdose, all three active treatments exhibited a reduction from baseline in mean AUC_{0–120} compared with placebo. The mean differences [S.E.] (80% CI) from placebo were estimated at −4.16 [2.62] (−7.54, −0.77), −7.08 [2.56] (−10.39, −3.78), and −5.90 [2.53] (−9.26, −2.73) for PF-05105679 600 mg, PF-05105679 900 mg, and oxycodone, respectively (Fig. 5C).

At 3.5 hours postdose, mean differences [S.E.] (80% CI) from placebo for PF-05105679 600 and 900 mg were reduced in comparison with those at 1.5 hours and were estimated at 0.16 [2.62] (−3.22, 3.55) and −0.53 [2.56] (−3.83, 2.78), respectively (Fig. 5D). The mean difference [S.E.] (80% CI) from placebo for oxycodone was also decreased at 3.5 hours postdose compared with 1.5 hours, although not by the same magnitude, −3.46 [2.53] (−6.73, −0.20), which is consistent with the clinical duration of effect of oxycodone.

The overall most frequent treatment-emergent, treatment-related adverse events (AEs) in part 1 with the compound were feeling hot (oral, face, upper body, arms, and hands) and dysgeusia (four subjects each, all of whom received PF-05105679 at a dose ≥300 mg; Table 4). For all AEs except dysgeusia, the most frequent treatment-related AEs in part 1 occurred most often in subjects receiving PF-05105679 900 mg, which in the case of two subjects was of sufficient intensity to determine this as the maximum tolerated dose. Similar effects were seen in part 2 of the study (the cold pain evaluation) where again the most frequent treatment-related AEs were feeling hot (nine subjects, all received PF-05105679), somnolence (seven subjects: four who received PF-05105679 and three who received oxycodone), and oral hypoesthesia (five subjects, all received PF-05105679). These AEs were not reported by subjects who received placebo. The mean onset of the AE feeling hot for subjects receiving PF-05105679 (600 mg) was 0.98 hours (range 0.52–1.87 hours) with a mean duration of 0.79 hours (range 0.05–1.47 hours). For subjects receiving PF-05105679 (900 mg), the mean onset was 1.04 hours (range 0.32–1.58 hours) with a mean duration of 2.19 hours (range 0.5–5.47 hours). Thus, these AEs were temporally correlated with the rise to peak (763–1227 nM) for the subjects who experienced feeling hot and then the fall of the plasma concentrations. No countermeasures were required.

Discussion

TRPM8 has been hypothesized to be a major detector of environmental cold and implicated in a number of diseases (Binder et al., 2007; Babes, 2009; Liu and Qin, 2011). To date, this evidence of a role has been shown in preclinical species (Xing et al., 2007; Descoeur et al., 2011; Su et al., 2011) and supported by work with TRPM8-null mice (Chung and Caterina, 2007; Colburn et al., 2007; Dhaka et al., 2007; Knowlton et al., 2011) and expression levels of the channel in humans (Mukerji et al., 2006; Morgan et al., 2009). The lack of availability of a TRPM8 antagonist suitable for clinical dosing
has prevented the confirmation of a functional role of TRPM8 in humans.

The compound presented here is a moderately potent but selective TRPM8 antagonist. Differences were seen in potency when it was evaluated against different modalities of activation of TRPM8 (agonist, cold, and voltage). However, this probably reflects differences in assay conditions (for example, compound incubation time, magnitude of stimulation) rather than differences in ability to block different modes of activation. Importantly, when evaluated at equilibrium in the electrophysiological studies against voltage activation of the channel, a potency value of 103 nM was achieved. When this was further studied at 12°C, a greater effect was observed suggesting cooperativity across modes of activation (Voets, 2004). The compound displayed similar potencies across species when evaluated against voltage activation for guinea pigs and humans as well as against WS-12–induced calcium flux for humans, rats, dogs, and guinea pigs.

PF-05105679 was able to completely reverse the reduction in bladder voiding volume induced by cold saline and menthol in the anesthetized guinea pig. This suggests that the primary cold detecting mechanism linked to contraction of the bladder is TRPM8. The compound, similar to other TRPM8 antagonists described in the literature (Bautista et al., 2007; Knowlton

### Table 3
Pharmacokinetic parameters for PF-05105679 in part 1 of the single escalating oral dose study in healthy human volunteers

<table>
<thead>
<tr>
<th>Parameter (Unit)</th>
<th>10 mg (n = 6)</th>
<th>30 mg (n = 6)</th>
<th>100 mg (n = 6)</th>
<th>300 mg (n = 5)</th>
<th>600 mg (n = 6)</th>
<th>900 mg (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total C\text{max} (ng/ml)</td>
<td>33.6 (80)</td>
<td>107 (45)</td>
<td>275 (41)</td>
<td>1439 (81)</td>
<td>5974 (94)</td>
<td>10,560 (99)</td>
</tr>
<tr>
<td>Unbound C\text{max} (nM)</td>
<td>0.9</td>
<td>2.9</td>
<td>7.4</td>
<td>39</td>
<td>160</td>
<td>283</td>
</tr>
<tr>
<td>T\text{max} (h)</td>
<td>0.5 (0.25–0.53)</td>
<td>0.5 (0.5–0.5)</td>
<td>0.75 (0.5–1.5)</td>
<td>1 (0.5–4)</td>
<td>1.5 (0.5–2)</td>
<td>1.5 (0.5–1.5)</td>
</tr>
<tr>
<td>Total AUC\text{24} (ng h/ml)</td>
<td>58 (46)</td>
<td>237 (42)</td>
<td>764 (32)</td>
<td>3866 (33)</td>
<td>11,630 (62)</td>
<td>20,180 (66)</td>
</tr>
</tbody>
</table>

AUC\text{24}, area under the curve between 0 and 24 hours after dosing.

### Figure 5
(A) Plasma exposure for all doses across the whole study (n = 6 for 10, 30, and 100 mg/kg; n = 5 for 300 mg; and n = 22 for 600 and 900 mg). (B) Effect of PF-05105679 (600 and 900 mg), placebo, and oxycodone on core body temperature over 24 hours measured via ingested VitalSense device. Baseline was defined as the average temperature recorded from continuous core temperature monitoring during the hour (−59 to 0 minutes) preceding dosing. (C and D) Posterior distributions for effect of PF-05105679 (600 and 900 mg) and oxycodone compared with placebo on the pain evoked by the cold pressor test at two time points post dosing: 1.5 hours (C) and 3.5 hours (D). The vertical line represents no difference from placebo, and lines within distributions show 90th and 95th percentiles.
et al., 2011; Almeida et al., 2012; Gavva et al., 2012), produced a dose-dependent reduction in core body temperature in the rat. Interestingly, the effective free concentration for the effect on core body temperature was approximately 2-fold higher than that required for the bladder effect. This may be due to a difference between the evoking stimuli of direct application of cold rather than environmental temperature, or may just be a feature of the experimental model design or species (rat versus guinea pig). From the preclinical data, primarily focused upon the in vitro potency values and the in vivo efficacy against menthol stimulation, we postulated that an unbound concentration of 450 nM would be sufficient to drive TRPM8 pharmacology in humans.

The first part of the clinical study evaluated the pharmacokinetics and safety of PF-05105679 in humans. The compound was relatively rapidly absorbed, and at the highest dose of 900 mg, a mean unbound C\text{max} plasma concentration of 284 nM was achieved. Higher doses were precluded due to the emergence of a perioral sensation of feeling hot, which in two individuals was severe enough to cause declaration of the maximal tolerated dose. Although only in the top two doses, this was not seen in all subjects, nor was it correlated with the highest exposures. TRPM8 is expressed in the trigeminal ganglia (Nealen et al., 2003; Abe et al., 2005) and oral mucosa (Abe et al., 2005), and therefore this effect could be due to the pharmacology of the molecule; however, it cannot be the only factor involved. One possible explanation is that blocking TRPM8-mediated cold sensation unmaskst constitutive activity of other temperature-sensing TRP channels expressed in the mouth, such as TRPV1 and TRPA1 (Zanotto et al., 2007; Gerhold and Bautista, 2009; Morgan et al., 2009). Furthermore, clinical and preclinical studies using agonists for the different channels have clearly demonstrated an interplay between them and an ability to modulate cold and heat sensitivity (Zanotto et al., 2007; Albin et al., 2008). However, although it was possible to agonize the channels to give rise to sensations, there was no exogenous activation in our study (such as hot drinks or food). Thus, any activity unmasked by the blockade of TRPM8 presupposes an endogenous drive via an alternative regulation of the various TRP channels in the mouth. It is known that proinflammatory mediators cause the temperature activation range of TRPV1 to be reduced from >42°C to 36–37°C (Zhang et al., 2008), suggesting that, if there is background inflammation in the mouth (e.g., gingivitis) in some volunteers, then there may be constitutive activity. This would need further careful clinical evaluation.

Despite falling short of the target 450 nM exposure, we did achieve approximately 3 times the in vitro IC\text{50} (103 nM) value at TRPM8 channels and between the EC\text{50} and EC\text{90} concentrations (200 and 425 nM, respectively) for efficacy in vivo in the ice-water bladder model. Thus, the third cohort was dosed to study the effect of PF-05105679 on the cold pressor test at 600 and 900 mg. From the pharmacokinetic analysis of the 300 mg, this maximum plasma concentration for this dose would be observed at the second cold pressor test evaluation with the 900-mg dose, and thus, was not tested in the efficacy arm of the study. PF-05105679 provided similar efficacy to the active comparator oxycodone (20 mg), the effects of which were consistent with previous studies with this opioid (Zwisler et al., 2009; Eisenberg et al., 2010). The differing time course of analgesia for both drugs is consistent with their pharmacokinetic profile. Although TRPM8 is known to be involved in the sensation of cold, a number of other ion channels have also been implicated, including TRPA1, the voltage-gated sodium channel, Na\text{v}1.8, and two-pore domain potassium channels (Viana et al., 2002; Story et al., 2003; Zimmermann et al., 2007; Babes, 2009). However, our data suggest that the main cold pain sensing channel is TRPM8 in humans. The similar efficacy observed between the TRPM8 blocker and oxycodone suggests that inhibiting TRPM8 may represent a therapeutic opportunity in cold-induced pain. It remains to be proven whether this effect in healthy volunteers can translate to analgesia in a clinically relevant patient population.
However, as the effect of PF-05105679 in the cold pressor test and the induction of the adverse events occurred at the same doses, at present we believe there is no measurable therapeutic index for this compound (and potentially this mechanism). Despite this, as the effect in the cold pressor test was observed in all subjects, whereas the perioral sensation of feeling hot and related adverse events were confined to a subset of subjects, there may be a therapeutic opportunity in patients who do not experience the emergence of adverse events upon first dosing. However, it is not clear how these patients could be identified without exposure to the compound. It may also be possible that the adverse events tolerate dose titration and/or repeated administration. PF-05105679 is not an appropriate tool to test this hypothesis due to its relatively short half-life.

Despite a clear demonstration of pharmacological activity in humans, both in terms of an effect against cold pain and the adverse events seen, the compound had no effect on core body temperature in humans. This finding contrasts with the data from rodents in the current study as well as multiple published studies in preclinical species demonstrating the role of TRPM8 in body temperature (Bautista et al., 2007; Knowlton et al., 2011). It is unlikely due to poor access to the site of action for TRPM8-induced hypothermia, as Almeida et al. (2012) eloquently demonstrated a cutaneous site of action. However, in our preclinical experiments, we required higher exposures to induce hypothermia relative to the effect on the bladder. Additionally, the clinical exposure achieved was lower than the equivalent exposure required to produce the fall in core body temperature in the rat. Thus, the most likely explanation is that a more complete blockade of the channel may be required to demonstrate an effect.

In summary, we have demonstrated that PF-05105679, a TRPM8 antagonist, is analgesic in an experimental model of cold pain in humans without a concomitant change in core body temperature. However, emergence of a tolerability issue (feeling hot) with this compound suggests that additional studies will be required to further understand the pharmacology of TRPM8 before it is possible to determine whether this mechanism is advantageous for the treatment of patients with cold pain.

Authorship Contributions

Participated in research design: Winchester, Glatt, Gardiner, Conlon, Collins, Beaumont, Reynolds.

Conducted experiments: Petit, Gardiner, Conlon, Postlethwaite, Saintot, Roberts, Gosset.

Contributed new reagents or analytic tools: Andrews, Glossop, Palmer, Clear.

Performed data analysis: Winchester, Gore, Glatt, Gardiner, Conlon, Postlethwaite, Gosset, Matsura, Collins.

Wrote or contributed to the writing of the manuscript: Winchester, Gore, Beaumont, Reynolds.

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