Pharmacology and Antitussive Efficacy of 4-\((3\text{-trifluoromethyl} \text{pyridin}-2\text{-yl})\)-piperazine-1-carboxylic Acid \((5\text{-trifluoromethyl} \text{pyridin}-2\text{-yl})\)-amide (JNJ17203212), a Transient Receptor Potential Vanilloid 1 Antagonist in Guinea Pigs

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ABSTRACT

Transient receptor potential vanilloid 1 (TRPV1) plays an integral role in modulating the cough reflex, and it is an attractive antitussive drug target. The purpose of this study was to characterize a TRPV1 antagonist, 4-\((3\text{-trifluoromethyl} \text{pyridin}-2\text{-yl})\)-piperazine-1-carboxylic acid \((5\text{-trifluoromethyl} \text{pyridin}-2\text{-yl})\)-amide (JNJ17203212), against the guinea pig TRPV1 receptor in vitro followed by a proof-of-principle study in an acid-induced model of cough. The affinity of JNJ17203212 for the recombinant guinea pig TRPV1 receptor was estimated by radioligand binding, and it was functionally characterized by antagonism of low-pH and capsaicin-induced activation of the ion channel (fluorometric imaging plate reader and electrophysiology). The nature of antagonism was further tested against the native channel in isolated guinea pig tracheal rings. Following pharmacokinetic characterization of JNJ17203212 in guinea pigs, pharmacodynamic and efficacy studies were undertaken to establish the antitussive efficacy of the TRPV1 antagonist. The pKᵢ of JNJ17203212 for recombinant guinea pig TRPV1 was 7.14 ± 0.06. JNJ17203212 inhibited both pH (pIC₅₀ of 7.23 ± 0.05) and capsaicin (pIC₅₀ of 6.32 ± 0.06)-induced channel activation. In whole-cell patch clamp, the pIC₅₀ for inhibition of guinea pig TRPV1 was 7.3 ± 0.01. JNJ17203212 demonstrated surmountable antagonism in isolated trachea, with a pKᵢ value of 6.2 ± 0.1. Intrapertoneal administration of 20 mg/kg JNJ17203212 achieved a maximal plasma exposure of 8.0 ± 4 μM, and it attenuated capsaicin evoked coughs with similar efficacy to codeine (25 mg/kg). Last, JNJ17203212 dose-dependently produced antitussive efficacy in citric acid-induced experimental cough in guinea pigs. Our data provide preclinical support for developing TRPV1 antagonists for the treatment of cough.

The transient receptor potential vanilloid 1 (TRPV1 or VR1) receptor is a ligand-gated cation channel that is activated or modulated by a variety of mediators thought to contribute to neuroinflammation (Tominaga and Tominaga, 2005; Szallis et al., 2007). This ion channel is a polymodal nociceptor that is activated by vanilloids such as capsaicin (Caterina et al., 1997), noxious heat (Cesare et al., 1999), low pH, polyamines (Ahern et al., 2006), and lipid mediators such as anandamide (Ross, 2003; De Petrocellis and Di Marzo, 2005), to mention a few. In addition, various endogenous mediators such as bradykinin, substance P, glutamate, prostaglandins, and ATP sensitize TRPV1 (Szallasi et al., 2007). As an integrator and mediator of nociceptive and/or inflammatory stimuli, TRPV1 is an attractive therapeutic target for the treatment of various neuroinflammatory disorders (Jia et al., 2006; Szallasi et al., 2007).

ABBREVIATIONS: TRPV1, transient receptor potential vanilloid 1; RTX, resiniferatoxin; JNJ17203212, 4-(3-trifluoromethyl-pyridin-2-yl)-piperazine-1-carboxylic acid (5-trifluoromethyl-pyridin-2-yl)-amide; BCTC, 4-(3-chloro-pyridin-2-yl)-piperazine-1-carboxylic acid (4-tert-butyl-phenyl)-amide; AMG517, N-[4-(6-(4-trifluoromethyl-phenyl)-pyrimidin-4-yloxy)-benzothiazol-2-yl]-acetamide; SB-705498, 1-(2-bromo-phenyl)-3-[1-(5-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl]-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-(3-trifluoromethyl-pyridin-2-yl)-pyrrolidin-3-yl)-urea; AMG9810, 3-(4-
al., 2005), and recent reviews on the discovery of TRPV1 antagonists highlight efforts to develop novel therapeutics in this area (Correll and Palani, 2006; Kyle and Tafesse, 2006; Szallasi et al., 2007).

Chronic cough is a symptomatic manifestation of airway hyperactivity. Receptors present on sensory nerve endings and in cell bodies of nonmyelinated C- and myelinated Aδ-fibers are drug targets for chronic cough, including TRPV1, acid-sensing ion channels, and G protein-coupled receptors for bradykinin, neuropeptide, and cannabinoids (Geppetti et al., 2006; Kollarik and Undem, 2006). TRPV1 plays a critical role in the sensory regulation and/or sensitization of the cough reflex in animals (Mazzone, 2004; Canning et al., 2006; Kollarik and Undem, 2006). TRPV1 is expressed on vagal afferents innervating the airway walls at the level of the guinea pig bronchi and trachea where it colocalizes with neuropeptides such as substance P and calcitonin gene-related peptide (Watanabe et al., 2006). In humans TRPV1 is up-regulated in patients with chronic cough (Groneberg et al., 2004), and capsaicin-induced cough responses are increased in patients with inflammatory lung diseases such as asthma, bronchitis, chronic obstructive pulmonary disease, and upper respiratory tract infection, probably as a result of TRPV1 sensitization. In animal models, TRPV1-mediated cough is sensitized by inflammatory pathways activated by protease-activated bradykinin receptors (Carr et al., 2003; Gatti et al., 2006). In guinea pigs, experimental cough can be induced by citric acid, capsaicin, and anandamide, all of which activate TRPV1 receptors (Ricciardolo, 2001; Jia et al., 2002). Further validation for the therapeutic rationale of TRPV1 blockade in cough has been sought from pharmacological studies. Several studies have used the TRPV1 antagonists capsazepine and iodo-resiniferatoxin (RTX). Although these studies are generally supportive (Lalloo et al., 1995; Trevisani et al., 2004), these agents are not fully efficacious, and in one study, they were not effective at all (Lewis et al., 2007). Unfortunately, capsazepine and iodo-RTX are not ideal in vivo pharmacological tools. For example, capsazepine is a relatively weak antagonist, with species and modality-specific activity and limited TRPV1 selectivity (Docherty et al., 1997; Liu and Simon, 1997; McIntyre et al., 2001; Gill et al., 2004). Iodo-RTX may be partially deiodinated in vivo and/or may possess partial agonist activity (Shimizu et al., 2005). Thus, in vivo data generated with these agents should be interpreted with a degree of caution. Perhaps the strongest pharmacological evidence for a role of TRPV1 receptors in cough comes from a recent study with the “second generation” TRPV1 antagonist BCTC. McLeod et al. (2006) reported that this compound was effective in a model of antigen-provoked cough in ovalbumin-sensitized guinea pigs. Nevertheless, additional studies, using different compounds and different cough models are required to further strengthen the therapeutic rationale of TRPV1 antagonism in cough.

In the present study we characterized the in vitro and in vivo pharmacology of JNJ17203212 (Swanson et al., 2005) at the guinea pig TRPV1 homolog, with the ultimate goal of testing the efficacy of JNJ17203212 in a guinea model of cough. Our data clearly demonstrate that JNJ17203212 is a reversible competitive antagonist at the guinea pig TRPV1 receptor, with in vivo efficacy as an antitussive agent in a citric acid-induced guinea pig cough model.

Materials and Methods

Drugs

JNJ17203212, AMG517, and SB-705498 were synthesized in the laboratories of Johnson & Johnson Pharmaceutical Research and Development, LLC. BCTC and AMG9810 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA) and Tocris Cookson Inc. (Ellisville, MO), respectively. Codeine was purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

Guinea pig TRPV1 cDNA (accession no. AJ492922.2) was generated by reverse transcription-polymerase chain reaction from guinea pig brain mRNA and stably expressed in Chinese hamster ovary (CHO) cells. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 500 μg/ml of the antibiotic Genetecin (G-418; Invitrogen, Carlsbad, CA) for selection. Cells were subcultured at approximately 90% confluence from a 150-cm² flask using 0.05% trypsin.

Radioligand Binding

Radioligand binding was carried out with membrane fractions prepared from CHO cells expressing recombinant guinea pig TRPV1 receptors. In brief, the cells were spun down in a tabletop centrifuge (1500 rpm for 5 min at 4°C), and the cell pellet was homogenized with a homogenization buffer (50 mM Tris-HCl and 5 mM EDTA, pH 7.4). The supernatant was then centrifuged at 32,000g for 30 min at 4°C to collect the membrane pellet. The membrane pellet was resuspended in assay buffer (320 mM sucrose, 2 mM MgCl₂, 5.8 mM NaCl, 5.0 mM KCl, 0.75 mM CaCl₂, and 20 mM HEPES, pH 7.4) at a final protein concentration of 100 μg/ml. ³HRTX (PerkinElmer Life and Analytical Sciences, Boston, MA) was used as the tracer for the study. Approximately 0.2 to 0.3 nM ³HRTX (KΘ of ¹³HRTX for the guinea pig TRPV1 is ~0.3 nM) was used as the tracer concentration, which was then displaced by increasing concentrations of the compounds tested. The incubation was terminated after 2 h by filtration (GF/B filters (Whatman, Maidstone, UK) presoaked with 0.3% polyethyleneimine) using a wash buffer with 50 mM Tris-HCl and 0.1% Triton-X. The filter-bound radioactivity was counted by a beta scintillation counter (PerkinElmer Life and Analytical Sciences).

Fluorescence Assays

Fluorescent assays were performed using FLIPR (MDS Analytical Technologies, Concord, ON, Canada). CHO cells expressing the guinea pig TRPV1 receptor were seeded in black-walled clear-bottomed 96-well plates at a density of 50,000 cells per well (complete media without the antibiotic), and they were cultured overnight at 37°C.

Ca²⁺ Influx Assay (FLIPR³TRA²). On the day of the experiment, cells were washed three times with HEPES-buffered saline (137 mM NaCl, 0.5 mM MgCl₂, 2 mM KCl, 5 mM dextrose, 2 mM CaCl₂, and 10 mM HEPES, pH 7.4). Fluo-3-acetoxyethyl ester (Molecular Devices, Sunnyvale, CA) was then added to the cells at a concentration of 4 μM, and then cells were incubated at room temperature in the dark for 60 min. After incubation with dye, cells were washed, and serial dilutions of test compounds were added. After a 30-min incubation at room temperature, changes in fluorescence were monitored for 3 min after the addition of agonist (1 μM capsaicin).

Low-pH Assay (FLIPR³TRA²). Cells were washed with assay buffer (130 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 10 mM HEPES, pH 7.4), and then they were loaded with 0.2 μM pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (Helliwig et al., 2004) for 30 min at 25°C in the dark, and then they were washed again with assay buffer. Test compounds were then added to the cells and incubated for an additional 30 min at room temperature. Intracellular acidification was initiated by adding the low-pH buffer (assay buffer containing 400 mM HCl, 10 mM HEPES, pH 6.0) and serial dilutions of test compounds were added. After a 30-min incubation at room temperature, changes in fluorescence were monitored for 3 min after the addition of agonist (1 μM capsaicin).
buffer with 20 mM 2-[N-morpholino]ethanesulfonic acid, pH 4.5 online, and changes of intracellular fluorescence were monitored.

**Electrophysiology**

CHO cells stably expressing the guinea pig TRPV1 receptor were plated at low density onto glass coverslips 24 to 72 h before recording. On the day of the experiment, glass coverslips containing cells were placed in a bath on the stage of an inverted microscope, and they were perfused (approximately 1 ml/min) with extracellular solution of the following composition: 137 mM NaCl, 2 mM CaCl₂, 5.4 mM KCl, 1 mM MgCl₂, 5 mM glucose, and 10 mM HEPES, pH 7.4. Pipettes were filled with an intracellular solution of the following composition: 40 mM KCl, 100 mM potassium fluoride, 2 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES, pH 7.3 to 7.4, and they had a resistance of 2 to 4 MΩ. All recordings were made at room temperature (22–24°C) in a modified, nominally calcium-free recording solution in which calcium was replaced with 2 mM Ba²⁺ using a Multiclamp 700A amplifier and pClamp 9 software (Molecular Devices). Inward TRPV1 currents were measured using the whole-cell configuration of the patch-clamp technique at a holding potential of −60 mV. The liquid junction potential was calculated to be 7.1 mV at 20°C, and voltage commands were not corrected. Current records were acquired at 5 KHz and filtered at 2 KHz. Capsaicin concentration-response curves were constructed by exposing CHO-TRPV1 cells to capsaicin (0.03–10 μM) for 10 s every 82 s using an SF-77B Fast-Step Perfusion device (Warner Instruments, Hamden, CT). Complete concentration-response curves were constructed in both ascending and descending order in each cell. Linear leak was corrected off-line, and the average response was calculated for each capsaicin concentration. To determine the IC₅₀ value for JNJ17203212, TRPV1 currents were activated using 1 μM capsaicin (approximately EC₅₀ concentration). JNJ17203212 was diluted from a 10 mM DMSO stock solution into calcium-free extracellular solution containing 1 μM capsaicin, and it was applied to cells using an SF-77B Fast-Step Perfusion device (Warner Instruments). Maximum final DMSO concentration was 0.01%. Up to five concentrations of JNJ17203212 (0.01–1 μM in half-log increments) were applied to each cell, with washout in between each concentration.

**Isolated Trachea**

Female Hartley guinea pigs were obtained from Charles River Laboratories (Kingston, NY). All procedures and experiments were carried out in accordance with the internationally accepted guidelines for the care and the use of the laboratory animals in research and approved by the local Institutional Animal Care and Use Committee. After euthanization, the trachea was quickly dissected, and rings were hung in 10-ml organ baths containing Krebs’ buffer (4.6 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 118.2 mM NaCl, 10 mM glucose, 24.8 mM NaHCO₃, and 2.5 mM CaCl₂2H₂O) at 37°C, pH 7.4, and gassed with 95% O₂, 5% CO₂. Isometric contraction was measured by a force transducer (ADInstruments, Colorado Springs, CO) connected to an electronic data acquisition system (EMKA Technologies, Falls Church, VA). Tissues were equilibrated with intermittent washes to maintain a resting tension of −1 g. Tissues were then challenged with 70 mM KCl to assess tissue viability and for normalization of tissue contraction to test substances. Tissues were then returned to baseline tone by washing and then incubated with JNJ17203212 or vehicle (0.1% DMSO) for 1 h. A concentration-response curve was then generated by cumulative addition of capsaicin (from 10 nM to 10 μM) in half-log increments. Animal numbers varied between five and eight for treatment groups.
Pharmacokinetics

Male Hartley guinea pigs were obtained from Charles River Laboratories surgically prepared with carotid artery and jugular vein catheters. Three guinea pigs (mean body weight of 411 ± 5 g) had both cannulae fitted with injection ports under isoflurane anesthesia, and, upon recovery, they were administered a single 20 mg/kg dose of JNJ17203212 in a 15% Solutol in 5% dextrose solution by i.p. injection. Blood (approximately 0.25 ml) was collected from either catheter at 15, 30, 45, 60, 75, 90, 120, 135, or 240 min after drug administration. Plasma was prepared by centrifugation of blood, and it was stored at −20°C. Plasma concentrations of JNJ17203212 were determined by liquid chromatography-tandem mass spectrometry.

Experimental Cough in Guinea Pigs

Guinea pigs were randomly assigned to various test groups, and the experimenter was blinded to the treatments. The blinding code was not revealed to the experimenter until coughs from all animals had been tallied. Guinea pigs were dosed with JNJ17203212, codeine, or vehicle (15% Solutol in 5% dextrose solution; n = 6–12 per group) via the i.p. route 1 h before the capsaicin challenge. Individual guinea pigs were placed in an exposure chamber with airflow of 3 l/min to acclimate 10 min before the capsaicin or citric acid challenge. Coughed responses were induced by exposure to capsaicin aerosol (15 μM) or citric acid aerosol (1.0 M) generated by an ultrasonic nebulizer at a nebulization rate of 0.6 ml/min for 4 and 10 min, respectively. Coughs were counted manually for a total of 15 min starting from the initiation of the irritant challenge (4-min capsaicin challenge or 10-min citric acid challenge). Animals were immediately removed from the exposure chamber and euthanized. Terminal blood samples were taken, spun down, and the plasma was aliquoted and stored at −80°C before analytical chemistry. All blood samples were taken within 1 min of the animals’ removal from the exposure chamber. Plasma concentrations of JNJ17203212 were determined by liquid chromatography-tandem mass spectrometry.

Data and Statistical Analysis

In vitro data points for concentration-response were fitted by nonlinear regression (GraphPad Prism, version 4.0; GraphPad Software Inc., San Diego, CA) using the following four-parameter general logistic equation: response = basal + (max − basal)/(1 + 10logIC50 − log agonist[Hill slope]).

Potency (pEC50 or pIC50) was estimated for the concentration that produced half-maximal effect. For Schild analysis, the shift in potency of the agonist in the presence of the antagonist was used to calculate the concentration ratio (CR) to determine pA4 or pKb estimates, according to the methods of Arunlakshana and Schild (1959). pA4 estimates were determined at two antagonist concentrations (1 and 3 μM) of JNJ17203212 from the FLIPR assay, because each concentration of the compound produced a dextral shift with no apparent suppression of the maxima. Schild estimates of pKb were obtained by plotting log (CR − 1) against log concentration of JNJ17203212 (for both the FLIPR and isolated tissue assays).

For electrophysiology, linear leak was corrected off-line, and percentage of inhibition was calculated from leak corrected records according to the following equation: % inhibition = (1 − (current, drug/[current, predrug + current, postdrug]/2)) × 100.

For the cough studies in conscious guinea pigs, a one-way analysis of variance was assessed to determine whether the treatment groups (JNJ17203212 and codeine-treated) demonstrated statistical significance from vehicle treatment, followed by the Tukey-Kramer multiple comparisons post hoc test.

Results

We determined the affinity of JNJ17203212 for recombinant guinea pig TRPV1 receptors using a tritiated-RTX binding assay. In addition, because the pharmacology of guinea pig TRPV1 has not been characterized in detail, especially for antagonists (Savidge et al., 2002; McLeod et al., 2006; Gunthorpe et al., 2007), we compared the affinity of JNJ17203212 with a number of recently described TRPV1 antagonists (Figs. 1 and 2; Table 1), namely, SB-705498 (Rami et al., 2006), AMG9810 (Gavva et al., 2005), BCTC (Valenzano et al., 2003), and AMG517 (Gavva et al., 2006). The chemical structures of the TRPV1 antagonists used in the present study are shown in Fig. 1. The equilibrium dissociation constant (Kd) of [3H]RTX for the guinea pig TRPV1 was ~0.3 nM (Fig. 2, inset). JNJ17203212 displaced tritiated-RTX from membrane preparations of CHO cells expressing the guinea pig TRPV1 ion channel (Fig. 2), with potency (pIC50) of 7.03 ± 0.07 and an affinity (pKd) of 7.14 ± 0.06. For comparison, Fig. 2 also shows displacement curves for SB-705498 and AMG9810. SB-705498 and AMG9810 exhibited similar potency to JNJ17203212, with pIC50 values of 6.95 ± 0.05 and 7.1 ± 0.10, respectively. In addition to these compounds, binding affinities for two other TRPV1 ligands, BCTC and AMG517, are shown in Table 1. The rank order of antagonist affinity for guinea pig TRPV1 was AMG517 > BCTC > AMG9810 > JNJ17203212 = SB-705498. These data show that JNJ17203212 displaces RTX binding from guinea pig TRPV1 receptor with modest affinity. Table 1 also compares the binding affinity for these five TRPV1 antagonists with their respective binding affinity at recombinant rat and human TRPV1. In general, the rank order of antagonist affinity is similar across the three species. Interestingly, the affinities measured against the guinea pig receptor are very similar to those measured against the human homolog, but they tend to be higher (up to 10-fold) than those measured against the rat receptor.

Although radioligand binding is a powerful technique for measuring receptor affinity under equilibrium conditions, it does not necessarily discriminate between agonists and antagonists, and it involves the study of TRPV1 channels under nonphysiological conditions. Therefore, we next evaluated the ability of JNJ17203212 to inhibit capsaicin-induced activation of guinea pig TRPV1 in a functional calcium mobilization assay (FLIPR). Capsaicin activated guinea pig TRPV1, with a pIC50 of 6.4 ± 0.03. As shown in Fig. 3A, JNJ17203212 inhibited 1 μM capsaicin-induced increases in calcium fluorescence, with a pIC50 of 6.32 ± 0.06. SB-705498

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Equilibrium binding affinities (pKd ± S.E.M.) of JNJ17203212 compared with TRPV1 antagonists</th>
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<tr>
<td></td>
<td>Rat TRPV1</td>
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<tr>
<td>JNJ17203212</td>
<td>6.5 ± 0.1 (n = 5)</td>
</tr>
<tr>
<td>SB-705498</td>
<td>6.1 ± 0.1 (n = 3)</td>
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<tr>
<td>BCTC</td>
<td>8.5 ± 0.08 (n &gt; 11)</td>
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<tr>
<td>AMG9810</td>
<td>6.5 ± 0.04 (n = 5)</td>
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<tr>
<td>AMG517</td>
<td>7.7 ± 0.03 (n = 5)</td>
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The number of independent replicates shown in parentheses.
To confirm TRPV1 antagonistic activity using a more direct approach, we studied the activity of JNJ17203212 against guinea pig TRPV1 using the whole-cell voltage-clamp technique (Fig. 4). In these studies, capsaicin concentration-dependently increased TRPV1 channel activity. Representative traces showing capsaicin-induced guinea pig TRPV1 inward currents are shown in Fig. 4A. As shown in this figure, capsaicin induced a slow and sustained inward current that was rapidly reversed on removal of capsaicin. On average, the activation of recombinant guinea pig TRPV1 was close to maximal at 10 μM, and the pEC50 of capsaicin was 6.1 ± 0.01. As shown in Fig. 4B, 1 μM capsaicin induced a robust inward current that exhibited only limited desensitization over a period of several minutes. JNJ17203212 produced a potent, rapid, concentration-dependent attenuation of this capsaicin response. On average, the pIC50 for inhibition of the established capsaicin response was 7.3 ± 0.01 (Fig. 4C). More importantly, this experiment demonstrated that JNJ17203212 is rapidly reversible. Together, our data demonstrate that JNJ17203212 is a competitive and reversible antagonist at the recombinant guinea pig TRPV1 receptor.

Next, we assessed the pharmacology of JNJ17203212 in a native guinea pig system to understand whether any differences exist between recombinant and native guinea pig TRPV1 before in vivo experiments. We used isolated guinea pig trachea as a native test system for JNJ17203212 (Fig. 5). Capsaicin produced a concentration-dependent contraction of isolated guinea pig tracheal rings, with a potency (pEC50) of 6.5 ± 0.07. Increasing concentrations of JNJ17203212 produced a dextral shift of the capsaicin concentration-response curve with no apparent suppression of the maximal tissue contraction, suggesting competitive antagonism, as was seen previously in the recombinant system. JNJ17203212 (1, 3, and 10 μM) shifted the potency of capsaicin to 6.2 ± 0.07, 5.9 ± 0.08, and 5.2 ± 0.07, respectively. When the agonist concentration ratios were plotted against the negative logarithm of the JNJ17203212 concentration (Schild analysis), the estimate of affinity for JNJ17203212 (pKb) as measured by the x-intercept was 6.2 ± 0.1, with a slope not significantly different from unity.

A prerequisite for in vivo studies is good exposure following drug administration via a suitable route. Therefore, we mea-
sured plasma concentrations of JNJ17203212 (20 mg/kg) in guinea pigs following administration by the i.p. route (Fig. 6). Plasma exposures of JNJ17203212 increased rapidly after a single i.p. dose, reaching a maximum concentration (plasma concentration) of 8.0 \text{M} 

\text{10 min postdose.}

Plasma concentrations were maintained above the lowest estimate of TRPV1 affinity (pK_B = 6.2 in the isolated trachea assay) for the duration of the pharmacokinetic study (4 h). Having demonstrated good systemic exposure, we tested the pharmacodynamic efficacy of JNJ17203212 in guinea pigs where the coughing reflex was initiated by aerosolized capsaicin with a local target concentration of 15 \text{M} (Fig. 7A). The mean number of capsaicin-induced cough responses recorded in vehicle-pretreated guinea pigs was 3.0 ± 0.5. This level of response was reduced significantly to 1.0 ± 0.2 coughs in JNJ17203212 (20 mg/kg i.p.)-pretreated guinea pigs (p < 0.001). For comparison, codeine (25 mg/kg i.p.), included as a positive control antitussive agent, reduced the number of coughs to 0.58 ± 0.15 (p < 0.001). These data indicate that JNJ17203212 is capable of reaching the site of capsaicin action (presumably sensory neurons innervating the upper airway) and exerting a TRPV1 antagonist effect in vivo. This model then, represents an excellent pharmacodynamic assay for JNJ17203212, but it is of questionable pathophysiological relevance. Therefore, we wanted to test the efficacy of the compound in a more disease-relevant model of experimental cough. The citric acid-induced cough model was used for that purpose (see Materials and Methods), where 1 M citric acid aerosol produced 17.7 ± 8.9 (n = 6) coughs (Fig. 7B). JNJ17203212 (n = 6 per group) dosed 1 h before the citric acid challenge reduced the number of coughs dose-dependently, and at a dose of 20 mg/kg, the number of coughs was reduced to 4.5 ± 3.6 (n = 6). The pharmacodynamic efficacy correlated well with the plasma exposure of JNJ17203212, and antitussive efficacy was seen only at plasma concentrations greater than 1 \text{M} (Fig. 7, inset). The number of coughs in the codeine-treated animals was 7.8 ± 5.5 (n = 6). Statistical analysis revealed an overall effect of JNJ17203212 (p < 0.05; analysis of variance), although post hoc analysis using the Tukey-Kramer mul-

**Fig. 4.** A, representative trace of capsaicin-induced inward current in guinea pig TRPV1-CHO cells under whole-cell voltage clamp. Horizontal bar represents the time period of capsaicin application. B and C, JNJ17203212 concentration-dependently inhibited 1 \text{M} capsaicin-induced guinea pig TRPV1 activity. Symbols represent mean data from various independent experiments. A representative trace for such an experiment is shown as an inset.

**Fig. 5.** JNJ17203212 concentration-dependently attenuated cumulative capsaicin-induced contraction of the isolated guinea pig trachea. Data are expressed as percentage of 70 mM KCl-induced contraction. Symbols represent mean contractility ± standard error of means from four to five independent experiments. The Schild plot of log (CR − 1) versus log of molar concentration of the antagonist is shown as an inset.

**Fig. 6.** Plasma concentration (Cp) against time plot of JNJ17203212 dosed intraperitoneally in guinea pigs. Symbols represent mean exposure from three animals.
multiple comparisons test indicated that no single dose of JNJ17203212 exerted a significant reduction in coughing. Given the clear trend toward dose-dependent antitussive efficacy of JNJ17203212, we postulated that the inability to demonstrate statistical significance was a reflection of the inadequate power of the study, rather than proof of the null hypothesis. To increase the power of the study, we increased the number of animals in the vehicle, high-dose JNJ17203212 (20 mg/kg), and codeine groups to \( n = 12 \) per group. As illustrated in Fig. 7C, the antitussive effects of 20 mg/kg JNJ17203212 and 25 mg/kg codeine were statistically significant \((p < 0.01)\) in the powered study. The antitussive efficacy of JNJ17203212 was comparable with codeine, where citric acid-induced coughs were reduced from 25.2 \( \pm \) 3.8 coughs in vehicle-treated guinea pigs to 9.1 \( \pm \) 2.2 and 10.1 \( \pm \) 2.4 coughs in JNJ17203212 and codeine-treated animals, respectively.

**Discussion**

In this study, we thoroughly characterized JNJ17203212 at both the recombinant and native guinea pig TRPV1 receptor and provided proof-of-concept for the efficacy of TRPV1 antagonists in experimental cough. JNJ17203212, along with four other previously described TRPV1 antagonists, competed with RTX for the binding site at the recombinant guinea pig TRPV1 receptor. Based on the binding data, it seems that the pharmacology of the guinea pig TRPV1 channel is more predictive or closely related to the human homolog, as suggested previously by Savidge et al. (2002) on the basis of capsazepine activity. Likewise, the compounds tested seem to have a lower affinity at the rat compared with human and guinea pig TRPV1. Guinea pig and human TRPV1 contain a leucine residue at position 547 (rat TRPV1 contains a methionine) that is suggested to be important for inhibition of proton activation (Gavva et al., 2004). The similarity of guinea pig and human TRPV1 with respect to antagonist affinity might suggest that this leucine residue is also involved in high-affinity antagonist binding, although the contribution of other residues common to guinea pig and human TRPV1 cannot be discounted. Regardless of the molecular basis of these findings, it seems that the guinea pig may represent a good model species for the study of TRPV1 antagonists, as suggested previously by Walker et al. (2003).

In line with the RTX competition data, JNJ17203212 was also a competitive antagonist of capsaicin-induced activation of both the recombinant (intracellular \( \text{Ca}^{2+} \) mobilization and electrophysiology) and native (tissue contraction) TRPV1 receptor. The functional estimate of affinity \( (pK_B) \) for JNJ17203212 was strikingly similar when measured at the recombinant \( (\text{Ca}^{2+} \) response of 6.12 \( \pm \) 0.05) and the native (tissue contraction of 6.2 \( \pm \) 0.1) guinea pig TRPV1 receptor. Interestingly, functional \( pK_B \) estimates were significantly different from the measured binding affinity \( (pK_i) \). It is possible that RTX (for binding) and capsaicin (for function) may present the guinea pig TRPV1 in a high- and a low-affinity state for JNJ17203212, which may then indicate that the binding site of JNJ17203212 does not completely overlap with that of capsaicin and RTX. In this context, it will be very interesting to perform a functional affinity estimate for JNJ17203212 using RTX as the stimulus. Another apparent paradox was that the potency of antagonism of JNJ17203212...
in whole-cell electrophysiology was 7.5 ± 0.01, significantly different from inhibition of capsaicin-induced Ca2+ mobilization (pIC50 of 6.32 ± 0.06). It is possible that JNJ17203212 displays a higher affinity against the "open-state" of the channel or displays some degree of voltage dependence, as was observed in a preliminary set of experiments with JNJ17203212 (A. D. Wickenden, unpublished data), and Gunthorpe et al. (2007) recently published similar data for SB-705498. Although the exact reasons for these apparent discrepancies are not clearly understood, it is likely that experimental differences in potency/affinity estimates can be accounted for by small differences in the assay conditions (e.g., method of channel activation, intact cells or membranes, extent of equilibration).

The TRPV1 receptor is endogenously gated by low pH, and protons are potentially involved in the activation of TRPV1 at the site of tissue injury, inflammation, and ischemia. Furthermore, our aim was to test the efficacy of JNJ17203212 in an acid-induced experimental cough model. Therefore, we measured the antagonist potency of JNJ17203212 using low pH as the stimulus for TRPV1 activation. Our results show that JNJ17203212 was a potent (pIC50 of 7.23 ± 0.05) inhibitor of proton-induced guinea pig TRPV1 activation, as was AMG9810. Surprisingly, SB-705498 exhibited a very different pharmacology against low pH. In fact, between the three assays (binding, capsaicin, and low pH), SB-705498 exhibited weak antagonism in only the low pH assay (pIC50 of 6.23 ± 0.05), whereas AMG9810 behaved quite similarly to JNJ17203212 in all three types of assays. Interestingly, Gunthorpe et al. (2007) recently published data suggesting SB-705498 is equipotent against both capsaicin- and low-pH-induced activation of rat and human TRPV1. The pharmacology of TRPV1 thus seems to be both species- and modality-specific, pointing to the value of studying TRPV1 antagonists against different activation modalities and also in different animal species.

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BPS, b-tubylcyclophosphorothionate; APT, aminopotentidine; cyp, cyproheptadine; NKA, neurokinin A; D-888, desmethoxyverapamil; BRL-43694, 1-methyl-N-[9-methylendo-9-azabicyclo[3.3.1]non-3-yl-1-1F]-indazole-3-carboxamide monohydrochloride; AF-DX384, 5,11-dihydro-11[2-(2-(2-dipropaminomethyl-1-piperidinyl)ethyll amino)carbonyl]-6H-pyrido[2,3-b](1,4)-benzodiazepine-6-one; SR142801, (S)-(+-)1-3-(1-benzyl-3-(3,4-dichlorophenyl)piperidin-3-yl)(pro pyl)-4-phenylepiperidin-4-yl-N-methylacetamide).
The TRPV1 antagonist JNJ17203212 demonstrated anti-
tussive efficacy in the citric acid-sensitized model of experi-
mental cough in guinea pigs. Importantly, the maximal effi-
cacy in this model was similar to that seen with the anti-
tussive agent codeine. Although it is possible that the in vivo
effects of JNJ17203212 may be mediated via a non-
TRPV1 mechanism, we think this is unlikely for the following
reasons. First, in our hands, JNJ17203212 behaves as a
highly selective TRPV1 antagonist. At 1 μM, JNJ17203212
did not significantly displace radioligands binding to a panel
of receptors and transporters (CERE, Paris, France; Table
2), nor did it inhibit related TRP channels such as TRPV2,
TRPV4, or TRPA1 (Table 3). Although 1 μM JNJ17203212
exhibited some weak TRPM8 inhibition, it is unlikely that
TRPM8 occupancy by JNJ17203212 produced antitussive ef-
cicacy, because TRPM8 is not activated by capsaicin and acid,
both of which induced cough in guinea pigs. Therefore, it is
scientifically reasonable to infer that the antitussive efficacy
of JNJ17203212 is due to TRPV1 blockade. Our data also
indicate that at the doses of JNJ17203212 used in the
present study, the compound is capable of reaching the site
capsaicin action (presumably sensory neurons innervating
the upper airway) and exerting a TRPV1 antagonist effect in
vivo. Finally, the plasma concentrations required for effect
in our study were consistent with functional affinity estimates
(μM) measured against the native guinea pig TRPV1 recep-
tor (6.2 ± 0.1). Indeed, this level of agreement between in
vitro and in vivo estimates of potency is very respectable
given the numerous confounding factors that can influence in
vivo potency, such as plasma protein binding and tissue
distribution. Thus, we think that the antitussive effects of
JNJ17203212 seen in the present study are a consequence of
TRPV1 antagonism. It is also worth mentioning that there
were no obvious behavioral abnormalities at the highest dose
(20 mg/kg) of JNJ17203212, both in the pharmacokinetic and
in the efficacy studies. We did not measure body temperature
in any of the guinea pigs in vivo studies, although JNJ17203212
causedit hyperthermia in rats (Swanson et al., 2005). Hence, our data support and extend previous findings on
the antitussive effects of TRPV1 antagonists. Capsazepine
and iodo-resiniferatoxin, both TRPV1 antagonists, have pre-
viously been shown to exhibit significant antitussive efficacy in
the guinea pig citric acid model (Laloo et al., 1995; Tre-
visani et al., 2004), although recently, Lewis et al. (2007)
reported otherwise, albeit in a modified (smoking) model of
citric acid-induced cough. In addition, the TRPV1 antagonist
BCTC was efficacious in both the capsazepin pharmacody-
namic model and in an ovalbumin-sensitized model (McLeod
et al., 2006). These previously published data, together with
our new data, provide preclinical support for developing
TRPV1 antagonists for the treatment of cough associated
with upper respiratory tract hyperactivity. Whether therapeu-
tic intervention of TRPV1 results in an improved quality
of life in patients suffering from chronic idiopathic cough
remains to be tested in the clinic. In conclusion,
JNJ17203212 is a competitive reversible antagonist of both
recombinant and native guinea pig TRPV1. JNJ17203212 is
bioavailable in guinea pigs by the intraperitoneal route, and
it demonstrates antitussive efficacy in a citric acid-induced
guinea pig cough model. To date, much emphasis has been
placed on the development of TRPV1 antagonists for the
treatment of pain. However, our data provide preclinical
support for developing TRPV1 antagonists for the treatment
of cough, and they suggest that TRPV1 antagonists may hold
promise as broad-spectrum therapeutics for the treatment of
a variety of human disorders.

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We acknowledge Stefan Masure and Marc Mercckens (Johnson &
Johnson PRD, Beerse, Belgium) for providing the guinea pig TRPV1
cell line and Ruggero Galici (Johnson and Johnson Pharmaceutical
Research & Development, LLC, San Diego, CA) for helpful comments
on statistical analyses. The guinea pig cough studies were performed
by Pneumolabs (UK) Ltd. (Harrow, Middlesex, UK).

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potent and selective TRPV1 antagonist which inhibits capsaicin, acid, and heat-

TABLE 3
Pharmacological selectivity of JNJ17203212 ascertained at 1 μM using functional assays

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<tr>
<th>TRP Channel</th>
<th>Species</th>
<th>Agonist (Conc)</th>
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<tr>
<td>TRPV4</td>
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<tr>
<td>TRPA1</td>
<td>Human</td>
<td>Allylisothiocyanate (12 μM)</td>
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<tr>
<td>TRPM8</td>
<td>Dog</td>
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