Supplemental Online Material

Nonclinical Cardiovascular Studies of Prucalopride, a Highly Selective 5-Hydroxytryptamine Receptor 4 Agonist

Kelly Conlon, Joris H. De Maeyer, Chris Bruce, Jan A.J. Schuurkes, Lee Christie, James McRedmond, Katayoun Derakhchan, and Paul R. Wade

Journal: ***The Journal of Pharmacology and Experimental Therapeutics***

Supplemental Information 1. Study Locations

Ventricular myocyte, Purkinje fiber, and human ether-à-go-go-related gene (*hERG*)-encoded potassium channel studies were conducted at Janssen Pharmaceutica, Beerse, Belgium. Platelet aggregation studies were conducted at Java Clinical Research, Dublin, Ireland. Coronary artery constriction studies were conducted at Biopta Ltd, Glasgow, UK. Isolated atrial myocardium studies were performed by A. Kaumann, Department of Physiology, University of Cambridge, Cambridge, UK. Anesthetized rabbit, anesthetized pig, and conscious dog studies were conducted at Janssen Research Foundation, Beerse, Belgium.

Supplemental Information 2. Electrophysiological Effects On The hERG Channel In Human Embryonic Kidney Cells

Human Embryonic Kidney Cell Culture. Human embryonic kidney cells were cultured at 37ºC in a 5% CO_2 atmosphere in minimum essential medium (Gibco, Carlsbad, CA) supplemented with L-glutamate–penicillin–streptomycin (1%) (Sigma, Deisenhofen, Germany), fetal bovine serum (10%) (BioWhittaker™, Lonza, Walkersville, MD), nonessential amino acids (1%) (Gibco), 100 mM sodium pyruvate (1%) (Gibco) and geneticin (400 mg/l) (Gibco). Medium was refreshed three times a week and cells were subcultured at confluence (approximately once a week). Cells were seeded into small poly-L-lysine-coated Petri dishes (Sigma) 1–2 days before each experiment.

Experimental Procedure. Assessments were performed using a single-electrode whole-cell voltage-clamp technique (Hamill et al., 1981). Cells were continuously perfused with bath solution (150 mM NaCl, 4 mM KCl, 5 mM glucose, 10 mM HEPES, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.4 [NaOH]). Membrane currents were measured at distinct membrane potentials using an EPC-9 patch clamp amplifier (HEKA, Lambrecht [Pfalz], Germany) and patch pipettes (120 mM KCl, 5 mM EGTA, 10 mM HEPES, 4 mM MgATP, 0.5 mM $CaCl_2$, 2 mM $MgCl_2$; pH 7.2). The initial resistance of the pipettes varied between 1.1 and 2.6 MΩ.

From a holding potential of –80 mV, the hERG current (potassium-selective outward current) was defined as the maximal tail current at –40 mV following a 2-second depolarization to +60 mV (Snyders and Chaudhary, 1996; McDonald et al., 1997). Before each test, a 0.5-second pulse from the holding potential to –60 mV was given to determine the linear leak current. Cells were superfused with control or test solution using a Y-tube system (Murase et al., 1989), which enabled solutions in the vicinity of the cell to be changed in less than 0.5 seconds. Following a 5-minute equilibration period, test pulses were given every 15 seconds for 5 minutes to quantify the hERG current under control or test conditions. While the pulse protocol continued, the perfusion solution was switched to the test solution, and the effect of the drug was measured after 5 minutes. Data were acquired and analyzed using Pulse and Pulsefit (HEKA), Igor or Igor Pro (Wavemetrics, Portland, OR), and DataAccess (Bruxton, Seattle, WA) software.

Supplemental Information 3. Electrophysiological Membrane Currents in Guinea Pig Ventricular Myocytes

Cell Preparation. Single ventricular cells were isolated from the hearts of guinea pigs (350–400 g body weight) according to enzymatic dissociation methodology described previously (Bendukidze et al., 1985), using type 2 collagenase (20–22 mg; Worthington Biochemical Corporation, Lakewood, NJ) for enzymatic treatment. Isolated myocytes were

stored at room temperature before being transferred to a temperature-controlled superperfusion chamber (200 µl volume; Warner Science Products, Hofheim, Germany) mounted on the stage of an inverted microscope (Olympus IMT-2; Olympus, Tokyo, Japan). Standard patch-clamp techniques were employed to measure ion-channel activities using a HEKA EPC-9 amplifier (HEKA) with stimulation protocols and data acquisition performed using Pulse software (HEKA).

Measurement of Delayed Rectifier and Inward Rectifier Potassium Currents. Guinea pig myocytes were constantly perfused with a solution consisting of 132 mM NaCl, 1.2 mM $MgCl_2$, 4 mM KCl, 10 mM HEPES, and 10 mM glucose; pH 7.4 (NaOH) at 36°C. Pipettes contained 120 mM KCl, 6 mM $MgCl_2$, 0.154 mM $CaCl_2$, 5 mM NA_2ATP, 5 mM EGTA, and 10 mM HEPES; pH 7.2 (KOH). The glass microelectrodes used (n = 62) had a resistance range of 2.8–4.8 MΩ. The delayed rectifier potassium current (I_{Kr}) was measured during depolarizing test currents. Sodium channels were inactivated using a holding potential of –40 mV, and L-type calcium channels were blocked by adding nifedipine 1 µM to the superperfusion solution. Under these conditions, either the pulse protocol of the current–voltage relation or the envelope-of-tails test (Wang et al., 1997) was performed, followed by a slow voltage ramp. A second set of potassium current measurements was performed following 5-minute incubation in vehicle (0.1% dimethylsulfoxide) or prucalopride (10, 100, and 300 nM; and 1, 3, and 10 µM). The current–voltage relationship was determined by the application of clamp pulses (–40 to +60 mV; 500 millisecond duration; 10 mV increments; 0.1 Hz). The envelope-of-tails test was used to differentiate between the rapid and slow components of I_{Kr} (fixed step –40 to +40 mV; increasing duration 50–1922 milliseconds; 1.5 mV increments). Dofetilide (30 nM) was used to block the rapid component, revealing the slow component. I_{Kr} was defined as the difference between the initial and final currents during the depolarizing voltage step to the test potential. The amplitude of the tail current was defined as the difference between the peak tail current and the holding current level after 2 seconds. To estimate the inward rectifier potassium current (I_{K1}), slow depolarizing ramp pulses (32 mV/second; –110 to +50 mV) were applied to the myocytes.

Measurement of the Fast Sodium Current. Guinea pig myocytes were continuously perfused with superperfusion solution (30 mM NaCl, 107 mM CsCl, 0.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose; pH 7.4 [3:1 CsOH/NaOH] at room temperature). Electrophysiological recordings were taken using pipettes containing 140 mM CsCl, 4 mM $MgCl_2$, 4 mM NA_2ATP, 10 mM EGTA, and 10 mM HEPES; pH 7.2 (CsOH). The glass microelectrodes used (n = 17) had a resistance range of 1.4–2.2 MΩ. Calcium currents were blocked with nifedipine (1 µM). Clamp pulses were applied (–80 to +60 mV; duration 20 milliseconds; 0.5 Hz). The fast sodium current (I_{Na}) was measured as the difference between the peak current and the current at the end of the clamp pulse.

Measurement of the L-Type Calcium Current. Guinea pig myocytes were continuously perfused with superperfusion solution (135 mM NaCl, 3 mM CsCl, 1 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose; pH 7.4 [NaOH] at room temperature). Electrophysiological recordings were taken using pipettes containing 120 mM CsCl, 4 mM $MgCl_2$, 4 mM NA_2ATP, 10 mM EGTA, and 10 mM HEPES; pH 7.2 (CsOH). The glass microelectrodes used (n = 22) had a resistance range of 3.0–3.7 MΩ. A prepulse of 100 milliseconds (–80 to –40 mV) inactivated sodium channels; clamp pulses were then applied (–40 to +60 mV; duration 400 milliseconds; 0.5 Hz). The L-type calcium current ($I_{Ca,L}$) was measured as the difference between the peak current and the current at the end of the clamp pulse.

Supplemental Information 4. Electrophysiological Currents in Isolated Rabbit and Dog Purkinje Fibres

In rabbits and dogs, cardiac tissue was isolated within 5 minutes of sacrifice and stored in aerated (95% O_2, 5% CO_2) Tyrode's solution (136.9 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.40 mM NaH_2PO_4, 5.5 mM glucose; pH 7.4) at 35°C. Tissues were fixed to a 2.5 ml perfusion bath using cotton thread and were continuously superfused with aerated Tyrode's solution (1.5 ml/minute). Tissues were stimulated by

rectangular pulses (1.0 Hz basal rate; duration 2.0 milliseconds) at 1.5 times the threshold intensity. Intracellular potentials were recorded using 3 M KCl-filled glass microelectrodes with a tip resistance range of 15–30 MΩ. Action potentials were displayed on an oscilloscope (LeCroy, Chestnut Ridge, NY). When measured variables reached a steady state (at approximately 60 minutes), baseline values were measured, and preparations in which the action potential duration at 90% repolarization was <190 milliseconds or >400 milliseconds were discarded.

In rabbit Purkinje fibres, electrophysiological parameters were measured at 10 and 20 minutes (1 Hz stimulation rate) following continuous infusion of test solution (control [H_2O] or prucalopride 1 µM; 100 µl/minute) into a superfusion of Tyrode's solution (1 ml/minute). After 25 minutes, the stimulation rate was reduced to 0.2 Hz for 5 minutes in order to detect abnormal action potentials (early/delayed afterdepolarizations or bradycardia) (Carlsson et al., 1997; Adamantidis et al., 1998). In dog Purkinje fibres, vehicle (0.9% saline) was infused in the same volume as increasing concentrations of prucalopride (0.01–10 µM) at 30 min intervals.

Supplemental Information 5. Electrophysiological Parameters in Isolated Guinea Pig Papillary Muscle

Right ventricular papillary muscles of approximately 1 mm in diameter were dissected from guinea pig hearts and mounted horizontally in a 2.5 ml tissue chamber. Papillary muscles were continuously superfused with 10 ml/min oxygenated (5% CO_2, 95% O_2) and modified Tyrode solution (124 mmol/L NaCl, 4 mmol/L KCl, 1.1 mmol/L $MgCl_2$,1.8 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, 0.42 mmol/L NaH_2PO_4, 11 mmol/L glucose) at 35ºC. The muscles were electrically stimulated by square wave pulses of 2 ms duration at a rate of 1 Hz or 0.25 Hz (simulating bradycardia) and 50% above threshold voltage. After an equilibration period of 60 min and recording of baseline values for 30 min, prucalopride solutions prepared in dimethylsulfoxide were infused into the Tyrode solution at a rate of 10 µl/min. Contractile force was recorded isometrically by a force transducer (Gould UC 2; Gould, Oxnard, CA) and monitored on a pen recorder (Graphtec WR 7500; Graphtec, Hoofddorp, The Netherlands). Transmembrane potentials were measured using glass microelectrodes (tip resistance, 20–30 MΩ) filled with 3 mol/L KCl and recorded by an amplifier providing capacity compensation on a storage oscilloscope (Tektronix 7D20; Tektronix, Beaverton, OR). Action potentials and force of contraction data were collected by an A/D-converter.

Supplemental Information 6. Electrophysiological Effects of Prucalopride in the Isolated Rabbit Heart

Isolated rabbit hearts were perfused according to the Langendorff method, which has been described in detail previously (Hondeghem et al., 2001). Hearts were perfused with a salt solution (NaCl 118 mM, KCl 4 mM, $NaHCO_3$ 22 mM, $MgCl_2$ 1.1 mM, NaH_2PO_4 0.4 mM, $CaCl_2$ 1.8 mM, dextrose 5 mM, pyruvate 2 mM, creatine 0.038 mM). Under a dissecting microscope the atria were removed and a pair of stimulating electrodes were sutured on the His bundle. A recording electrode was placed in the subendocardium of the left ventricle and the preparation was then transferred to a SCREENIT computer system (Hondeghem 1994), where a potassium reference electrode and an epicardial recording electrode were positioned. Threshold stimulation current, automaticity and escape cycle lengths were determined and the preparation was accepted if these parameters were within the normal range.

Experimental Protocol. Prucalopride was prepared as a 10 mM stock solution in dimethyl sulfoxide, which comprised less than 0.1% of the perfusate when prucalopride was administered at the desired concentration. The following cycles were then performed: MINUTE1: a 10 beat train at 1000 ms and 300 ms was executed and the activation times and action potential duration (APD) recorded. MINUTE30: Automaticity and escape cycle lengths were determined. Following a 10 pulse train at a cycle length of 400 ms, stimulation was stopped for 15 seconds. The time to the first escape beat was saved as escape cycle

length. Conduction times and APDs for cycle lengths at 2000, 1500, 1000, 750, 500, 300 and 250 ms were determined.

The MINUTE1 protocol was executed 10 times followed by the MINUTE30 protocol. Prucalopride was then infused at each indicated concentration for 10 min; drug perfusion was terminated by a MINUTE30 protocol. Following the experiment, hearts were rinsed to remove excess drug, and frozen for chemical analysis.

Supplemental Information 7. Platelet Aggregation Studies

Platelet-rich plasma (PRP) was prepared by centrifugation at 2220 × *g* (PDQ® centrifuge; Bio/Data Corp.). For agonist assays, prucalopride succinate, tegaserod, and 5-HT were prepared in 0.002% ethanol in Hank's balanced salt solution. Test substances were incubated in whole blood at room temperature for 1 hour before the preparation of PRP, with the exception of thrombopoietin (positive control), which was incubated in PRP 5 minutes before challenge.

Supplemental Information 8. Coronary Artery Contractility

Dogs were euthanized with an intravenous overdose of pentobarbitone (1 ml/kg) before removal of the hearts, which were transferred to ambient Krebs solution (NaCl 119.0 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 24.9 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, glucose 11.1 mM) for transport on wet ice. Exsanguinated pig hearts were transferred to physiological salt solution (PSS; NaCl 119.0 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 24.9 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, glucose 11.1 mM) before transport. Human hearts were placed in transplant solution ($C_{12}H_{21}O_{12}K$ 100 mM, KH_2PO_4 25 mM, $MgSO_4$ 5 mM, raffinose 30 mM, adenosine 5 mM, glutathione 3 mM, allopurinol 1 mM, hydroxyethyl starch 50 mg/ml; UW®, Belzer, University of Wisconsin, Madison, WI) and transported on wet ice. The time in transit for human hearts (removal to experiment: approximately 21–40 hours) was mimicked for dog and pig hearts by overnight refrigerated storage (2–8ºC) in PSS before dissection and experimentation (approximately 24 hours of transit time).

Preparation of Arteries. The left anterior descending coronary artery was dissected from the heart, and distal sections of the artery were cut into 2-mm segments. Arteries were denuded of endothelium by rubbing the intimal surface with a tapered wooden stick for a maximum of 30 seconds. Arteries were mounted in organ baths (37°C, pH 7.4) containing physiological saline solution (PSS; 119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 1.2 mM KH2PO_4, 2.5 mM $CaCl_2$, 11.1 mM glucose) aerated with 95% O_2 and 5% CO_2. This solution was replaced every 15 minutes.

Standardization of Response. Changes in isometric force were measured with a force transducer (four-channel wire multimyograph models 610M and 620M; Danish Myo Technology A/S, Aarhus, Denmark) and recorded with an ADInstruments Powerlab system (ADInstruments Pty Ltd, Bella Vista, New South Wales, Australia). Following equilibration in PSS (≥30 minutes), the arteries were tensioned to ~1.5 g and allowed to stabilize over 90 minutes (washing every 15 minutes and retensioning to 1.5 g after 30 minutes if the tension dropped below 1 g). The arteries were then exposed to U46619 10 nM, a procontractile thromboxane analog. Following the plateau of the contractile response, endothelium-dependent relaxation was assessed by adding bradykinin 1 μM; arteries were rejected if a relaxation response of greater than 30% of the response to U46619 was observed. Arteries were then challenged at least three times with a priming dose of 5-HT 3 μM until stable responses were observed (the final response within 10% of the previous response). Arteries that did not demonstrate a stable response were rejected. On completion of the experiment, arterial viability was confirmed by inducing a contractile response with potassium-containing PSS.

Supplemental Information 9. Cardiovascular Parameters in Anesthetized Rabbits and Guinea Pigs

Male New Zealand White rabbits were anesthetized with an intravenous dose of methohexital sodium 5 mg/kg and α-chloralose 90 mg/kg (injected into a marginal ear

vein over 20–30 minutes). Male guinea pigs were anesthetized with sodium pentobarbital 22 mg/kg injected intraperitoneally. Tracheotomy was performed and animals were ventilated with room air (rabbits: 5–6 ml/kg body weight; stroke volume of ~22 strokes/minute; guinea pigs: stroke volume 1.1 ml/100 g; 60 strokes/min). Body temperature was maintained at 36–38ºC (rabbits) or at 34ºC (guinea pigs). Arterial blood pressure was monitored via cannulation of the right carotid artery and test solutions were administered via the left jugular vein. Needle electrodes were attached to the four limbs for the recording of surface three-lead electrocardiogram parameters.

Experimental Protocol. Following surgery and a 15–30-minute equilibration period, baseline heart rate, mean arterial blood pressure, and ECG parameters were recorded. Thereafter, methoxamine (in 0.9% NaCl) was continuously administered intravenously to rabbits at a rate of 15 µg/kg/minute. After 15 minutes, rabbits were intravenously administered water (0.125 ml/kg/minute) or prucalopride (0.04–0.31 mg/kg/minute) for 60 minutes. Guinea pigs were intravenously administered water or prucalopride over a period of 5 minutes; cardiovascular parameters were measured at 5, 10, 20 and 30 minutes after intravenous infusion. Prucalopride plasma levels in both species were then measured in blood samples drawn from the cannulated carotid artery.

QT intervals were corrected for heart-rate changes (QTc) using Bazett's formula (Bazett, 1997). QT interval dispersion (QTd) has been proposed as a marker of heterogeneous depolarization and electrical instability in the heart (Day et al., 1990; Carlsson et al., 1993; Lu et al., 1999); therefore, QTd ($QT_{max} - QT_{min}$) and QTcd ($QTc_{max} - QTc_{min}$) intervals were calculated as the difference between the longest and shortest value measured in each of the three ECG leads (I, II, III). JT intervals were rate-corrected using the formula $JT / RR^{½}$ (Berul et al., 1996; Shah et al., 1997).

Supplemental Information 10. Cardiovascular Parameters in Anesthetized Juvenile Pigs and Adult Dogs

Juvenile pigs and adult male beagle dogs were anesthetized with a mixture of scopolamine 0.015 mg/kg and lofentanil 0.070 mg/kg and relaxed with an intravenous dose of succinylcholine 1 mg/kg. Central body temperature was monitored with a thermistor positioned in the pulmonary artery and maintained at 37°C. The animals were intubated with a cuffed endotracheal tube. Intermittent positive pressure ventilation was performed with a mixture of pressurized air and oxygen (70%/30%) using a volume-controlled ventilator (Siemens-Elema, Stockholm, Sweden), with 5% v/v CO_2 at 20 breaths/minute. The CO_2 concentration in the expired air was set by adjusting the respiratory volume. Immediately after anesthesia a continuous intravenous infusion of etomidate 1.5 mg/kg/hour was initiated. Small intravenous doses of fentanyl 0.025 mg/kg were given at 60-minute intervals. If required, intravenous ketamine 0.5 mg/kg was infused to avert potential cardiovascular instability in pigs. Heparin 500 IU/kg was administered intravenously every 2 hours to prevent blood coagulation. Gentran 10%/glucose 5% was administered intravenously at 1.8 ml/kg/hour (pigs) or 2.5 mg/kg/hour (dogs) to correct for fluid loss. At regular intervals, 0.3 ml blood samples were taken from the left femoral artery to monitor blood gas and electrolyte values.

The electrocardiogram (ECG) was derived from limb leads (lead II) and used for the computerized calculation of the PQ, QRS, QT, and QTc intervals (from onset to the end of the appropriate waves). Tracings of the ECG (5, 15, and 30 minutes after each dose) were analyzed for the presence or absence of rhythm abnormalities. The carotid artery was cannulated and a high-fidelity catheter-tip micromanometer (Gaeltec Ltd, Isle of Skye, UK) was introduced and advanced to the left ventricle, allowing the measurement of left ventricular pressure. A catheter was inserted in the left femoral artery to monitor aortic blood pressure. A Swan−Ganz balloon-guided thermistor catheter (Baxter, Deerfield, IL) was placed in the pulmonary artery through a femoral vein to measure pulmonary artery blood pressure via an external pressure transducer (Gould, Oxnard, CA) positioned at mid-chest level. Zero-line calibration was achieved by simultaneously recording the pressure signal through the lumen of the catheter with an external pressure transducer (Gould) positioned at

mid-chest level and equating both zero lines on the recorder. Scale calibration of the pressure channels was performed with a DPI 601 Digital Pressure Indicator (Druck Ltd, Leicester, UK). Cardiac output (including stroke volume and systemic and pulmonary resistance) was measured using the ECG- and respiration-triggered thermodilution technique (Janssen Scientific Instruments Division; Beerse, Belgium).

Continuous registration of the analog signals was performed on a M2000-16 thermal chart recorder (Micro Movements Ltd, Hook, UK). ECG, blood pressure, temperature, and flow signals were fed via transducer amplifiers into a personal computer and the Hem 3.3 analysis system (Notocord Systems, Croissy-sur-Seine, France). Variables were acquired beat-to-beat during the experiment.

The right femoral vein was cannulated for the injection of saline (at room temperature) into the right atrium for thermodilution measurements, and for the injection of test solutions. Before and at various times after each injection of the compound, a 4-ml blood sample was taken from the femoral artery for determination of the plasma concentration of prucalopride (BioAnal Nv1-412).

Pigs were intravenously administered 5-HT–creatinine sulfate 3, 10, and 30 µg/kg (effective doses in terms of the free base, 1.3, 4.3, and 13.0 µg/kg, respectively) and isoproterenol 0.01, 0.03, and 0.10 µg/kg at 10-minute intervals. Increasing intravenous doses of prucalopride were then administered. Finally, pigs were challenged with 5-HT 30 µg/kg followed by isoproterenol 0.10 µg/kg.

Following a 20-minute stabilization period, dogs were administered increasing doses of prucalopride or control in identical volumes at 30-minute intervals by an intravenous bolus injection lasting approximately 10 seconds.

Supplemental Information 11. Cardiovascular Parameters and Behavior in Conscious Dogs

Dogs were sedated with an intravenous dose of Hypnorm® 1 ml/kg (fluanisone 10 mg/ml and fentanyl citrate 0.315 mg/ml; Janssen Pharmaceutica, Beerse, Belgium) and anesthetized with an intravenous dose of sodium pentobarbital 10 mg/kg (maintained with 75% N_2O/25% O_2). A left thoracotomy was performed through the fifth interstitial space; devices were inserted and leads were exteriorized at the dorsal side of the neck. After the animals had recovered from surgery, they were trained to accept laboratory procedures before being enrolled in pharmacological experiments.

Left ventricular pressure was measured using a catheter-tip micromanometer (JSI-400, Janssen Scientific Instruments Division, Beerse, Belgium) positioned in the left ventricle and secured on the heart. This was then connected to a transducer amplifier (JSI-1054) and calibrated in situ. Cardiac output was measured using an electromagnetic flow probe (JSI-0276, Janssen Scientific Instruments Division) mounted on the pulmonary artery and connected to an electromagnetic blood flow and velocity meter (MDL 1401, Skalar, Breda, Netherlands). An ultrasonic Doppler flow probe was placed around the left circumflex coronary artery and connected to a directional pulsed Doppler flow meter (545C-4, Bioengineering, University of Iowa, Iowa City, IA). Aortic blood pressure was recorded using a Tygon catheter inserted into the descending aorta and connected to a Gould P23 ID pressure transducer (Gould P23 ID, Oxnard, CA) calibrated with a mercury manometer. The ECG was derived from limb leads (lead II). After analog to digital conversion, the signals were fed into a digital minicomputer (PDP11/23) and the digitized signals were analyzed at 5-minute intervals using a custom-written computer program.

Baseline cardiovascular and behavioral values were recorded at 20 minutes in conscious dogs (n = 7) before intravenous administration of prucalopride dissolved in 0.9% NaCl (pH 5) or water (pH 6.1), or control (H_2O). Prucalopride plasma levels were determined in four of the seven dogs by sampling arterial blood before dosing (0 minutes) and at 5 and 30 minutes following each injection.

Supplemental Information 12. Chronotropic and Inotropic Effects of Prucalopride in Piglet and Human Atrial Myocardium

Piglet halved left atria and human right atrial trabeculae were prepared as previously described (Kaumann, 1990; Kaumann et al., 1990, 1991; De Maeyer et al., 2006) and paced at 1 Hz at 37°C.

Experimental Protocol. Experiments on piglet and human right atria were carried out in the presence of (–)-propranolol 200 nM to avoid β-adrenoreceptor-mediated effects (Kaumann et al., 1980). Experiments on piglet left atria were carried out in the presence of (–)-propranolol 200 nM, cocaine 6 μM, and/or isobutylmethylxanthine (IBMX) 20 μM to avoid β-adrenoreceptor-mediated effects, to inhibit neuronal uptake of 5-HT (Kaumann et al., 1990), and to inhibit phosphodiesterase enzyme activity that may affect 5-HT_4 receptor-mediated responses (De Maeyer et al., 2006), respectively. CCRCs were plotted for prucalopride and cisapride 1 nM to 10 μM, followed by 5-HT 200–600 μM, and finally (–)-isoprenaline 200–600 μM. Experiments were terminated by increasing the $CaCl_2$ concentration from 2.25 mM to 6.7 mM. The chronotropic and inotropic potencies of prucalopride and cisapride were expressed as a percentage of the maximum 5-HT- or (–)-isoprenaline-induced effect, as described previously (Kaumann, 1990). To measure intrinsic activity, 5-HT 200–600 μM was administered in the presence of 10 μM partial agonist, and the experiment was terminated with (–)-isoprenaline 200–600 μM. To assess the 5-HT_4 receptor nature of the agonist effects, experiments with identical protocols were carried out on separate right atria (piglet and human) preincubated for 90 minutes with the selective 5-HT_4 receptor antagonist GR113808 1 μM (Kaumann, 1993; Medhurst and Kaumann, 1993). The positive chronotropic and inotropic effects of prucalopride and cisapride were analyzed as described previously (Kaumann, 1990; Kaumann et al., 1991). The antagonism of the positive inotropic effects of 5-HT by prucalopride 10 μM was analyzed using the method of Marano and Kaumann (1976), and dissociation equilibrium constants for prucalopride were calculated.

Supplemental Information 13. Repeated Dose Studies in Rats

Rats were supplied by Biological Research Laboratories (Füllinsdorf, Basel, Switzerland) and were placed in quarantine for an acclimatisation period of approximately one week. Rats were housed individually in numbered wire mesh cages in an air conditioned room (temperature, 20–23°C; relative humidity, 40–70%; ventilation, 20–30 air replacements/hour; and illumination, 12 hours light to 12 hours dark), and were provided with water and food *ad libitum*. Rats were divided into low (5 mg/kg/day), medium (20 mg/kg/day) and high (80 mg/kg/day) prucalopride dosage groups based upon their sex and initial body weight. Each group consisted of 20 males and 20 females; an additional 10 males and 10 females were included as a control group that received demineralized water.

After a 1-month recovery period, a full necropsy was performed on all animals and all macroscopic changes were recorded. After removing adherent adipose tissue, all the organs including the heart were weighed. Heart samples from all rats were preserved in 10% buffered formalin and heart tissues were histologically examined.

Supplemental Information 14. Repeated Dose Studies in Dogs

Dogs were supplied by Janssen permanent outbred laboratory colony (Beerse, Belgium). Male and female dogs were housed in individual pens under routine test conditions (temperature, 20–23°C; relative humidity, 40–70%; ventilation, 20–30 air replacements/hour; and illumination, 12 hours light to 12 hours dark), and were provided with water *ad libitum*. The diet consisted of extruded dog feed, administered in self-raising hoppers and was fed according to the individual metabolic weight of the dogs throughout the study.

During an acclimatisation period of approximately 1 month, each dog was given a complete physical examination (including an ophthalmological examination), and baseline values for ECG, heart rate, blood pressure, hematology, serum analysis and urinalysis were determined. Dogs were divided into low (2.5 mg/kg/day), medium (10 mg/kg/day) and high (30 mg/kg/day) prucalopride dosage groups based upon their sex, initial body weight and health parameters obtained during the acclimatization period. Each group consisted of 4 males and 4 females; an additional 4 males and 4 females were included as a control group that received demineralized water.

ECG and heart rate measurements were made using Lead II of the Einthoven standard lead system, prior to the administration of the first dose and after 4, 12, 24, 40 and 52 weeks of dosing. The values of 3 channels were recorded by a standard ECG apparatus and were then assimilated with a planimeter (online records). Non-invasive, tail cuff blood pressure measurements (oscillometric principle) were carried out in parallel with ECG measurements. The cuff was wrapped around the base of the tail. For systolic and diastolic pressures, a median of three similar consecutive readings was taken to avoid false values. A full necropsy was performed on all animals and all macroscopic changes were recorded. After removing adherent adipose tissue, all the organs including the heart were weighed. Heart samples from all dogs were preserved in 10% buffered formalin. Organ and tissue samples were obtained using standardized methods, and were stained with haematoxylin-eosin and histologically examined using light microscopy.

Supplemental References

Adamantidis MM, Dumotier BM, Caron JF, and Bordet R (1998) Sparfloxacin but not levofloxacin or ofloxacin prolongs cardiac repolarization in rabbit Purkinje fibers. Fundam Clin Pharmacol 12: 70–76.

Bazett HC (1997) An analysis of the time-relations of electrocardiograms. Ann Noninvasive Electrocardiol 2: 177–194.

Bendukidze Z, Isenberg G, and Klockner U (1985) Ca-tolerant guinea-pig ventricular myocytes as isolated by pronase in the presence of 250 microM free calcium. Basic Res Cardiol 80: 13–17.

Berul CI, Aronovitz MJ, Wang PJ, and Mendelsohn ME (1996) In vivo cardiac electrophysiology studies in the mouse. Circulation 94: 2641–2648.

Day CP, McComb JM, and Campbell RW (1990) QT dispersion: an indication of arrhythmia risk in patients with long QT intervals. Br Heart J 63: 342–344.

Carlsson L, Abrahamsson C, Andersson B, Duker G, and Schiller-Linhardt G (1993) Proarrhythmic effects of the class III agent almokalant: importance of infusion rate, QT dispersion, and early afterdepolarisations. Cardiovasc Res 27: 2186–2193.

Carlsson L, Amos GJ, Andersson B, Drews L, Duker G, and Wadstedt G (1997) Electrophysiological characterization of the prokinetic agents cisapride and mosapride in vivo and in vitro: implications for proarrhythmic potential? J Pharmacol Exp Ther 282: 220–227.

De Maeyer JH, Straetemans R, Schuurkes JA, and Lefebvre RA (2006) Porcine left atrial and sinoatrial 5-HT_4 receptor-induced responses: fading of the response and influence of development. Br J Pharmacol 147: 140–157.

Hamill OP, Marty A, Neher E, Sakmann B, and Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391: 85–100.

Hondeghem LM (1994) Computer aided development of antiarrhythmic agents with class IIIa properties. J Cardiovasc Electrophysiol 5: 711–721.

Hondeghem LM, Carlsson L, and Duker G (2001) Instability and triangulation of the action potential predict serious proarrhythmia, but action potential duration prolongation is antiarrhythmic. Circulation 103: 2004–2013.

Kaumann AJ (1990) Piglet sinoatrial 5-HT receptors resemble human atrial 5-HT_4-like receptors. Naunyn Schmiedebergs Arch Pharmacol 342: 619–622.

Kaumann AJ (1993) Blockade of human atrial 5-HT_4 receptors by GR 113808. Br J Pharmacol 110: 1172–1174.

Kaumann AJ, McInerny TK, Gilmour DP, and Blinks JR (1980) Comparative assessment of beta-adrenoceptor blocking agents as simple competitive antagonists in isolated heart muscle: similarity of inotropic and chronotropic blocking potencies against isoproterenol. Naunyn Schmiedebergs Arch Pharmacol 311: 219–236.

Kaumann AJ, Sanders L, Brown AM, Murray KJ, and Brown MJ (1990) A 5-hydroxytryptamine receptor in human atrium. Br J Pharmacol 100: 879–885.

Kaumann AJ, Sanders L, Brown AM, Murray KJ, and Brown MJ (1991) A 5-HT_4-like receptor in human right atrium. Naunyn Schmiedebergs Arch Pharmacol 344: 150–159.

Lu HR, Yu F, Dai DZ, Remeysen P, and De Clerck F (1999) Reduction in QT dispersion and ventricular arrhythmias by ischaemic preconditioning in anaesthetized, normotensive and spontaneously hypertensive rats. Fundam Clin Pharmacol 13: 445–454.

Marano M and Kaumann AJ (1976) On the statistics of drug-receptor constants for partial agonists. J Pharmacol Exp Ther 198: 518–525.

McDonald TV, Yu Z, Ming Z, Palma, E, Meyers MB, Wang K-W, Goldstein SA, and Fishman GI (1997) A minK-HERG complex regulates the cardiac potassium current I(Kr). Nature 388: 289–292.

Medhurst AD and Kaumann AJ (1993) Characterization of the 5-HT_4 receptor mediating tachycardia in piglet isolated right atrium. Br J Pharmacol 110: 1023–1030.

Murase K, Ryu PD, and Randic M (1989) Excitatory and inhibitory amino acids and peptide-induced responses in acutely isolated rat spinal dorsal horn neurons. Neurosci Lett 103: 56–63.

Shah MJ, Wieand TS, Rhodes LA, Berul CI, and Vetter VL (1997) QT and JT dispersion in children with long QT syndrome. J Cardiovasc Electrophysiol 8: 642–648.

Snyders DJ and Chaudhary A (1996) High affinity open channel block by dofetilide of HERG expressed in a human cell line. Mol Pharmacol 49: 949–955.

Wang S, Liu S, Morales MJ, Strauss HC, and Rasmusson RL (1997) A quantitative analysis of the activation and inactivation kinetics of HERG expressed in *Xenopus* oocytes. J Physiol 502: 45–60.

Supplemental Table 1. Electrophysiological Effects of Prucalopride Measured in Isolated Rabbit Purkinje Fibers

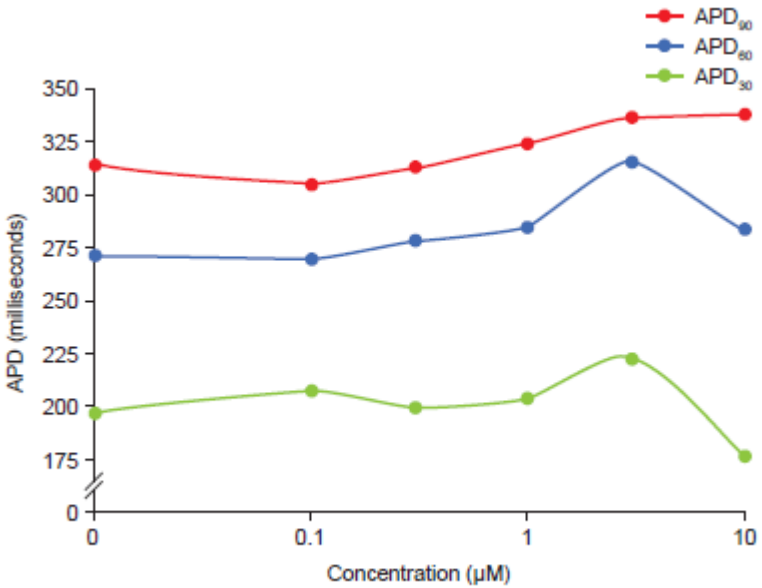
	Vehicle (*n* = 10)			Prucalopride (1 µM; *n* = 8)		
	Baseline	20 Minutes	25 Minutes	Baseline	20 Minutes	25 Minutes
Parameter	1 Hz	1 Hz	0.2 Hz	1 Hz	1 Hz	0.2 Hz
AAP (millivolts)	123 ± 2	125 ± 2	122 ± 1	126 ± 2	122 ± 4	119 ± 3
ADP_{90} (milliseconds)	275 ± 18	280 ± 18	447 ± 58	308 ± 18	326 ± 20	507 ± 42
RMP (millivolts)	85 ± 1	85 ± 2	82 ± 1	86 ± 2	86 ± 2	81 ± 1
ERP (milliseconds)	276 ± 20	279 ± 20	449 ± 59	314 ± 19	322 ± 24	522 ± 47
RT (milliseconds)	307 ± 19	309 ± 17	463 ± 58	329 ± 20	336 ± 25	526 ± 45
V_{max} (V/second)	393 ± 44	438 ± 55	446 ± 57	387 ± 35	365 ± 49	364 ± 48
Incidence of EADs (n)	-	0	0	-	0	0
Incidence of DADs (n)	-	0	0	-	0	0

Stimulations were performed under normal rhythm conditions (1 Hz) or conditions mimicking extreme bradycardia (0.2 Hz [12 pulses/minute]). Values are mean ± standard error. There were no significant differences between vehicle and prucalopride ($P > 0.05$ versus vehicle).
AAP, amplitude of resting action potential; APD_{90}, action potential duration at 90% repolarization; DAD, delayed afterdepolarization; EAD, early afterdepolarization; ERP, effective refractory period; RMP, resting membrane potential; RT, recovery time of the action potential; V_{max}, maximum rate of depolarization during the upstroke.

Supplemental Table 2. Electrophysiological Effects of Prucalopride Measured in Isolated Dog Purkinje Fibers

Parameter	Vehicle (*n* = 7)					Prucalopride (μM; *n* = 7)				
	Baseline	1st	2nd	3rd	4th	Baseline	0.01	0.1	1	10
AAP (millivolts)	117 ± 3	119 ± 2	119 ± 2	120 ± 1	119 ± 2	115 ± 1	115 ± 1	118 ± 2	114 ± 4	116 ± 2
APD_{90} (milliseconds)	382 ± 23	397 ± 26	398 ± 24	402 ± 24	404 ± 26	378 ± 24	378 ± 21	380 ± 21	392 ± 23	429 ± 27[b]
RMP (millivolts)	86 ± 1	88 ± 1	87 ± 2	88 ± 1	87 ± 1	87 ± 1	85 ± 1[a]	84 ± 1[b]	86 ± 1	87 ± 1
ERP (milliseconds)	377 ± 23	386 ± 27	396 ± 24	402 ± 22	404 ± 26	385 ±31	380 ± 25	381 ± 24	394 ± 28	416 ± 25
RT (milliseconds)	381 ± 25	384 ± 29	399 ± 25	408 ± 24	406 ± 27	391 ± 31	389 ± 25	386 ± 23	402 ± 28	444 ± 24
V_{max} (V/second)	560 ± 37	564 ± 26	568 ± 25	583 ± 26	629 ± 44	523 ± 40	499 ± 55	551± 52	516 ± 47	515 ± 49
Incidence of EADs (n)	-	-	-	-	-	-	0	0	0	0
Incidence of DADs (n)	-	-	-	-	-	-	0	0	0	0

Note: stimulations were performed under normal rhythm conditions (1 Hz) and normokalemia (KCl 4 mM). Values are mean ± standard error.
[a]*P* <0.05 vs prucalopride baseline value
[b]*P* <0.05 vs vehicle
AAP, amplitude of resting action potential; APD_{90}, action potential duration at 90% repolarization; DAD, delayed afterdepolarization; EAD, early afterdepolarization; ERP, effective refractory period; RMP, resting membrane potential; RT, recovery time of the action potential; V_{max}, maximum rate of depolarization during the upstroke.



Supplemental Fig. 1 The effect of prucalopride 0.1, 0.3, 1, 3 and 10 μM on APD_{30}, APD_{60} and APD_{90} at a cycle length of 750 milliseconds
APD, action potential duration