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RESEARCH PAPER

Investigation of mechanism of drug-induced cardiac injury and torsades de pointes in cynomolgus monkeys

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BACKGROUND AND PURPOSE

Drug candidates must be thoroughly investigated for their potential cardiac side effects. During the course of routine toxicological assessment, the compound RO5657, a CCR5 antagonist, was discovered to have the rare liability of inducing torsades de pointes (polymorphic ventricular arrhythmia) in normal, healthy animals. Studies were conducted to determine the molecular mechanism of this arrhythmia.

EXPERIMENTAL APPROACH

Toxicological effects of repeat dosing were assessed in naïve monkeys. Cardiovascular effects were determined in conscious telemetry-implanted monkeys (repeat dosing) and anaesthetized instrumented dogs (single doses). Mechanistic studies were performed in guinea-pig isolated hearts and in cells recombinantly expressing human cardiac channels.

KEY RESULTS

In cynomolgus monkeys, RO5657 caused a low incidence of myocardial degeneration and a greater incidence of ECG abnormalities including prolonged QT/QTc intervals, QRS complex widening and supraventricular tachycardia. In telemetry-implanted monkeys, RO5657 induced arrhythmias, including torsades de pointes and in one instance, degeneration to fatal ventricular fibrillation. RO5657 also depressed both heart rate (HR) and blood pressure (BP), with no histological evidence of myocardial degeneration. In the anaesthetized dog and guinea-pig isolated heart studies, RO5657 induced similar cardiovascular effects. RO5657 also inhibited Kv11.1 and sodium channel currents.

CONCLUSIONS AND IMPLICATIONS

The molecular mechanism of RO5657 is hypothesized to be due to inhibition of cardiac sodium and Kv11.1 potassium channels. These results indicate that RO5657 is arrhythymogenic due to decreased haemodynamic function (HR/BP), decreased conduction and inhibition of multiple cardiac channels, which precede and are probably the causative factors in the observed myocardial degeneration.

Abbreviations

AV, atrioventricular; BCL, basic cycle length; CCR5, C-C chemokine receptor type 5; CHL, Chinese hamster lung; CPP, coronary perfusion pressure; DAD, delayed after-depolarization; EAD, early after-depolarization; EDP, left ventricle end diastolic pressure; GLP, good laboratory practice; hERG, *human ether-a-go-go* related gene; HR, heart rate; LVDP, left ventricle developed pressure; MAPD, monophasic action potential duration at 30%, 50% and 90% ($MAPD_{30}$, $MAPD_{50}$, $MAPD_{90}$); PVC, premature ventricular contraction; PVT, severe polymorphic ventricular tachyarrhythmia; TdP, torsades de pointes; RO5657, $C_{31}H_{49}N_5O_4$

Introduction

A number of drugs have been withdrawn or had their use severely limited due to cardiovascular side effects including *torsades de pointes* (TdP), ventricular fibrillation and sudden cardiac death (for reviews, see Gintant *et al.*, 2006; Pugsley and Curtis, 2006; Farkas and Nattel, 2010; Giorgi *et al.*, 2010; Wallis, 2010). TdP is a rare polymorphic ventricular tachyarrhythmia that can degenerate into fatal ventricular arrhythmia. The majority of drugs associated with induction of TdP are also known to inhibit a human cardiac potassium channel current, Kv11.1 [encoded by KCNH2, or the *human ether-a-go-go* related gene (hERG)]. Inhibition of the Kv11.1 current prolongs action potential duration and QT intervals. Inhibition of Kv11.1 and QT interval prolongation are interconnected to such a degree that Kv11.1 inhibition is considered a surrogate biomarker for a drug's potential to induce TdP and Kv11.1 inhibition has become the centrepiece of cardiovascular safety pharmacological assessment (Gintant *et al.*, 2006; Pugsley and Curtis, 2006; Farkas and Nattel, 2010; Giorgi *et al.*, 2010; Wallis, 2010). As such, determination of a compound's inhibition of Kv11.1 channels is required as part of a specific regulatory guideline to evaluate the potential for delayed ventricular repolarization (Anon, 2005). However, despite the apparent connection between change in QT interval and risk of TdP, Kv11.1 screening alone is an imperfect predictor, as other molecular cues also contribute to the onset of drug-induced TdP (Gwathmey *et al.*, 2009).

Thus, the development of drugs requires a thorough safety assessment including cardiovascular function and electrophysiology (Anon, 2000; 2005). Assessing the potential cardiotoxicity of novel drugs extends beyond effects on ventricular repolarization; drugs may also affect the survival of cardiomyocytes, function of valves, alter blood pressure, induce ischaemia or cause arrhythmias (Hamlin, 2006; Gwathmey *et al.*, 2009; Stummann *et al.*, 2009). The tools and approaches to determine these effects range from *in silico* docking models, *in vitro* preparations (including recombinant cell lines expressing various cardiac ion channels), *ex vivo* models employing primary cardiac tissues, *in vivo* experiments in preclinical species and ultimately in humans (De Clerck *et al.*, 2002; Stummann *et al.*, 2009; Farkas and Nattel, 2010). While *in vitro* and/or *ex vivo* assays are useful to screen larger numbers of potential candidates, and can provide detailed information on specific endpoints, such assays are also usually acute and only short-term effects are captured. Early *in vivo* screening models, such as in anaesthetized or telemetry-implanted guinea-pigs, can also provide information on similar endpoints as in non-rodent telemetry and de-risk compounds moving into those non-rodent cardiovascular studies. However, metabolism in this species is generally not routinely assessed and may be different from that in other preclinical species, potentially confounding data interpretation for some compounds.

In vivo models, such as conscious dog or cynomolgus monkey telemetry, can be used to investigate drug effects in the whole animal, and are often predictive of clinical effects on the cardiovascular system, but are generally performed as single-dose studies, and therefore do not address effects of long-term drug administration or histopathological effects on the heart and cardiovascular function (De Clerck *et al.*, 2002; Stummann *et al.*, 2009; Farkas and Nattel, 2010; Pollard *et al.*, 2010). Repeated dose toxicity studies offer the advantage of morphological and structural assessment of the heart combined with longer treatment and monitoring periods, but provide limited information on cardiovascular function beyond periodic 'snapshot' ECG measurements that may not capture rare, yet problematic events, such as arrhythmias. Therefore, results of such approaches, taken together with the underlying strengths and weaknesses of each model, culminate in an abundance of evidence for understanding the cardiovascular liabilities of a molecule and must be integrated to assess the overall risks. Any findings from these studies may require follow-up experiments to discern the mechanistic effects, either direct or indirect, as they relate to potential mechanism of action and what risks identified preclinically may translate to humans.

Inhibitors of chemokine (C-C motif) receptor 5 (CCR5) are of interest as it is a co-receptor for HIV-1 entry into a target cell (for review, see Yang and Rotstein, 2010). The nature of the target receptor binding pocket drives the structural requirement of antagonists. This large receptor requires large, high molecule weight molecules to prevent activation of the receptor, and the receptor pocket is largely lipophilic. Lastly, key interaction in the receptor pocket requires a basic group in the antagonist. These receptor-driven structural requirements for high-affinity CCR5 inhibitors can also result in the inhibition of the Kv11.1 channel (Price *et al.*, 2006; 2008; Rotstein *et al.*, 2009; Barber *et al.*, 2009a,b). Therefore, assessment of effects on cardiovascular function, and particularly on ventricular repolarization, is an inherent concern when determining the safety of CCR5 inhibitors preclinically, and selecting molecules to advance into clinical testing.

Herein, the cardiovascular safety profile of RO5657, a small molecule inhibitor of CCR5, is described and the mechanism of torsadogenesis is investigated. The effects of RO5657 in a repeated dose toxicity study in cynomolgus monkeys and identification of a cardiac lesion are described, as well as effects in dedicated cardiovascular studies, using both conscious cynomolgus monkeys and anaesthetized dogs, in which BP, heart rate (HR) and ECGs were monitored. Additionally, *in vitro* ion channel profiling studies and an *ex vivo* isolated heart study are reported, as well as a proposed alternative screening pathway to determine torsadagenic risk of closely related small molecule CCR5 inhibitors.

Methods

Chemicals

RO5657 was synthesized by Roche (Rotstein *et al.*, 2009). All other chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) or as otherwise stated.

Compound formulation and administration

All animals in both the repeat dose studies were orally dosed with vehicle (0.5% hypromellose USP, 0.4% polysorbate 80, 0.9% benzyl alcohol in sterile water) or RO5657 formulated in vehicle.

In the single-dose anaesthetized dog study, compound was administered by i.v. bolus with vehicle (0.9% sodium chloride for injection, USP, pH 7.0–7.5) or RO5657 formulated in vehicle.

For all *in vitro* studies, compound was formulated in a dimethylsulfoxide (DMSO) stock solution (up to 30 mM), diluted into aqueous buffers specific to each assay, such that the final concentration of DMSO was ≤0.3%, and then bath applied.

Animal use

All animal care and experimental procedures complied with IACUC, Animal Welfare act, AAALAC, and the NIH Guide for the Care and Use of Animals and were approved by the Institute's Animal Care and Use Committee.

Two-week cynomolgus monkey toxicology study

Male and female Cynomolgus monkeys (*Macaca fascicularis*) were obtained (Biomedical Resources Foundation, Inc., Houston, TX, USA). Upon arrival at the test facility, the monkeys were quarantined in accordance with State of California regulations, and then acclimatized to laboratory conditions for at least 4 weeks before the start of the study. During the acclimatization period, the general condition of the monkeys was evaluated, and animals not considered healthy were excluded. At the start of dosing, all the monkeys weighed between 3 and 5 kg.

Two male and two female cynomolgus monkeys were each administered 0 (vehicle) and doses of 50, 250 and 750 mg kg^{-1} of RO5657 by naso-gastric tube for 14 consecutive days. Nine-lead ECGs were recorded in conscious restrained animals via surface electrodes using a Hewlett-Packard cardiograph (Palo Alto, CA, USA) at pre-dose and 3 h post-dose on Days 1 and 13, and RR, PR, QRS, QT and QT corrected by Bazzett's formula (QTcB) were measured. Samples for analysis of haematology and clinical chemistry parameters were collected, as well as tissues and organs for histopathological evaluation. Further experimental details including parameters assessed and tissues evaluated can be found in Supporting Information Appendix S1.

Repeat-dose cynomolgus monkey telemetry study

A repeat dose study for either 2 or 8 days of treatment was conducted in female telemetry-implanted monkeys where three monkeys were included in the vehicle test group and five monkeys were included in the test groups (50 and 250 mg kg^{-1} day^{-1}; 2 days and 8 days treatment, respectively). For the two test groups (50 and 250 mg kg^{-1}), animals were administered vehicle on Day 0, and cardiovascular parameters were recorded before the initiation of dosing on Day 1, so that Day 0 could serve as the vehicle control for each treated animal. At the end of the respective dosing periods, monkeys were anaesthetized and instrumented, and additional cardiovascular parameters (systemic vascular resistance, cardiac output and left ventricular pressure) were measured before they were killed using the method described previously (Cushing *et al.*, 2009). For conscious monkeys, average values taken from 10–15 cardiac cycles uninterrupted by interference of ectopic beats at 3 min intervals were used for analysis of haemodynamic parameters and ECG measurements, and then pooled into 15 min intervals. PR interval, QRS duration, QT/QTc interval and the appearance of other rhythmic disturbances (by manual inspection) were determined from the ECGs for the first 4 h of each day (Days 0–8). Systolic, diastolic and mean arterial pressures (MAPs) were determined from the pressure transducers for the first 8 h of each day (Days 0–8). Values from each individual animal were pooled to determine mean values for each variable at individual doses and time points. Limited blood samples were collected on Days 1 and Day 8 (0, 3 and 6 h) around the Tmax (previously determined from the 2 week study, between 1 and 3 h) in order to estimate the plasma concentrations and correlate free exposures to any effects observed.

Single-dose cardiovascular study in anaesthetized dogs

Dogs were obtained from Covance (Madison, WI, USA). Upon arrival at the test facility, the dogs were quarantined in accordance with appropriate regulations, and then acclimatized to laboratory conditions for at least 4 weeks before the start of the study. Female dogs were dosed with morphine (1 mg kg^{-1}, s.c.) approximately 10–20 min before administration of anaesthetic agent (α-chloralose, i.v., 120 mg kg^{-1} for induction and 35–75 mg kg^{-1} h^{-1} for maintenance) and instrumented as described previously (Cushing *et al.*, 2009).

Test compound was administered through a cephalic or sapphenous vein in-dwelling catheter. After the pre-dose equilibration period, vehicle was infused at 1.0 mL kg^{-1} for approximately 5 min, then a test compound in plasma blood sample was taken, and again after approximately 25 min. The low dose of test compound was administered for approximately 5 min, then a test compound in plasma blood sample was taken, and again after approximately 25 min. This routine was repeated for subsequent test compound concentrations (vehicle and three test compound administrations). At the end of the experiment, dogs were killed under anaesthesia via barbiturate overdose.

Values from each individual animal were pooled to determine an average for each variable at individual doses. The effect of each drug on haemodynamic (HR, systolic, diastolic, and MAP, systemic vascular resistance, cardiac output and left ventricular pressure) and electrophysiological (RR, PR, QRS, QT, and QT corrected by Van der Water's formula, or QTcV) parameters were examined for statistical significance using repeated measures ANOVA followed by a *post hoc* test for group comparisons when warranted. A value of $P < 0.05$ was considered statistically significant. All data are presented as mean ± SEM.

Guinea-pig langendorff isolated heart preparation

The detailed protocol has been described previously (Guo *et al.*, 2009) In brief, guinea-pigs were obtained from Charles River (Portage, MI, USA). Upon arrival at the test facility, the guinea-pigs were quarantined in accordance with State of New Jersey regulations, and then acclimatized to laboratory conditions for at least 1 week before the start of the study. Guinea-pigs of either sex were anaesthetized with sodium

pentobarbital (150 mg kg^{-1} i.p.) following a 5–10 min injection with heparin (1000 U kg^{-1} i.p.), and the hearts were removed rapidly via thoracotomy. Whole hearts were perfused constantly with external buffer [modified Krebs–Henseleit (KH) solution] at 36.5 ± 0.5°C and allowed to stabilize under sinus rhythm with minimal 5 min pacing at basic cycle length (BCL) of 300 ms (at approximately 1.5-fold of threshold amplitude × 2 ms duration) for a minimum period of 30 min with buffer before collection of baseline control. Modified KH solution containing ≤0.3% DMSO (vehicle) was applied to hearts, before application of the test substance, for a period of 15 min. Only hearts showing stable sinus rhythm between 130 and 190 beats min^{-1}, with left ventricle developed pressure (LVDP) ≥ 60 mmHg, and without arrhythmic activity, were accepted. Acceptable hearts were then paced at BCL 300 and 200 ms, each for approximately 100 pulses to establish baseline control measurement of haemodynamic and cardiac electrophysiological parameters.

The test substance was applied to four or more hearts at three consecutive concentrations of either 10, 30 or 100 μM while under sinus rhythm and paced at BCL of 300 and 200 ms. Each test concentration was applied for 15 min and responses were measured at the last 2 min of the 15 min application. Parameters of cardiac haemodynamics, electrophysiology and the derivatives [HR, coronary perfusion pressure, left ventricle end diastolic pressure, LVDP, maximal +dP/dt and minimal –dP/dt, RR interval, PR interval, QRS duration, QT interval and QTcF interval, monophasic action potential duration (MAPD) at 30%, 50% and 90% ($MAPD_{30}$, $MAPD_{50}$, $MAPD_{90}$)] were monitored in real time and saved to hard drive continuously using Gould ACQ-7700 acquisition system and PONEMAH P3P software (Data Sciences International, St Paul, MN, USA). QT and $MAPD_{90}$ (*cf, corrected by the sinus rate by Frederica formula*) were used for assessing the delayed repolarization. Other parameters documented as sensitive indices for pro-arrhythmogenic potential of drugs, such as triangulation, reverse-use dependence, instability, transmural dispersion of repolarization (TDR), were analysed. Triangulation was calculated as the difference between $MAPD_{90}$ and $MAPD_{30}$ ($MAPD_{90\text{-}30}$). Reverse-use dependence was determined by the slope of a liner-fitting over prolonged QT interval (% change from the pre-drug baseline level) obtained at spontaneous sinus rhythm, and when the heart was paced at 200 and 300 beats min^{-1}, respectively. TDR was measured by the duration of peak to end of the T wave (Tp-e). The beat rate instability was quantified by the coefficient of variation (CV) of MAP beat-to-beat intervals. Thirty consecutive MAP beats during the last-minute of drug exposure were selected for CV calculation using the equation: CV (%) = SD/average of beat-to-beat intervals. The amount of test substance in the perfusate was measured by HPLC and actual concentrations are reported.

Data are presented as mean ± SEM, and tabulated for each condition: control baseline, drug concentration and sinus rhythm/pacing frequency. Whenever applicable, changes in measured parameters were evaluated using one-way ANOVA followed by Dunnett's multiple comparison test with JMP (Version 5.0.1, SAS Institute, Cary, NC, USA) to determine whether the change from baseline observed after equilibration in each drug concentration was significantly different ($P < 0.05$) from that observed in the time-matched vehicle control group.

Cardiac channel patch-clamp electrophysiology

Whole-cell patch-clamp methods were used to record various channel currents from recombinant cells stably expressing human cardiac channels. An automated patch-clamp system was used to record various channel currents, except for experiments performed at physiological temperature (37 ± 1°C) where conventional manual methods were employed. Cells were voltage-clamped using either the PatchXpress (PX) 7000A – Automated Parallel Patch Clamp system (Molecular Devices, Inc., Sunnyvale, CA, USA) or a patch clamp amplifier (Axopatch 200B; Molecular Devices, Inc.), which was controlled by computer via a Digidata 1200 Interface (Molecular Devices, Inc.). Off-line analysis of data was performed using PClamp Software, Microcal Origin (OriginLab, Northampton, MA, USA) and Prism (GraphPad Software, San Diego, CA, USA). IC_{20}s and IC_{50}s were calculated using the non-linear curve-fitting function (with variable slope) of Prism software.

The following solutions were used to isolate potassium currents for both PatchXpress and conventional experiments in cultured cell lines: extracellular (in mM), NaCl (150), KCl (4), HEPES (10), $CaCl_2$ (1.2), $MgCl_2$ (1), adjusted to pH 7.4 with NaOH; intracellular (in mM), KCl (140), HEPES (10), EGTA (5), MgCl2 (6), ATP-Na_2 (5), adjusted to pH 7.2 with KOH. The following solutions were used to isolate sodium currents in cultured cell lines: extracellular (in mM), NaCl (150), DL-aspartic acid (110), KCl (1.8), $MgCl_2$ (1), $CaCl_2$ (1.8), HEPES (10), glucose (10) adjusted to pH 7.4 with NaOH; intracellular (in mM), caesium gluconate (130), CsCl (5), EGTA (0.5), $MgCl_2$ (1), ATP-Mg salt (2), GTP-Li (0.2), HEPES (10.0), NaCl (5) adjusted to pH 7.2 with KOH.

Methods for recording potassium currents from CHO-K1 cells expressing the human Kv11.1 channel have been described previously (Ly *et al.*, 2007). For automated experiments using the PX instrument, each cell was held at a negative holding potential of –80 mV and experiments were performed at approximately 30°C (the average temperature measured in the recording chamber of the instrument during each run). Cells were depolarized from –80 to –40 mV for 100 ms to measure leak current, stepped to +20 mV for 1000 ms and then repolarized to –40 mV for 500 ms to activate channels. In the physiology experiments, each cell was held at a negative holding potential of –80 mV and experiments were performed at 37°C. Cells were depolarized from –80 to –40 mV for 100 ms to measure leak current, stepped to +20 mV for 500 ms and then repolarized to –40 mV for 500 ms to activate channels. In both cases, data points were recorded at a frequency of 0.1 Hz and outward current was measured at the end of the step to –40 mV and normalized to vehicle (0.1% DMSO) controls; data are reported as % change from baseline.

Whole-cell automated patch-clamp methods were also used to record sodium currents from Chinese hamster lung cells expressing the human Nav1.5 channel. Each cell was held at a negative holding potential of –80 mV, followed by a 30 ms depolarizing pulse to –10 mV to activate channels and most closely simulate physiological conditions. Data points

were collected at frequencies of 0.1 Hz (1 stimulus 10 s^{-1}) and 3 Hz (3 stimuli s^{-1}). Peak inward current was measured during the step to –10 mV and normalized to vehicle (0.1% DMSO) controls; data are reported as % change from baseline. Flecainide or tetracaine (30 μM; Sigma Chemical Co.) was applied to each cell at the end of the experiment as a positive control.

Pharmacokinetic determination

Aliquots of monkey or dog plasma were protein precipitated with acetonitrile and analysed for RO5657 using an LC-MS/MS method. Aliquots of perfusate from guinea-pig hearts were measured by HPLC.

Results

Observation of myocardial injury and cardiovascular effects in cynomolgus monkeys

Cardiotoxicity of RO5657 was noted first in the study designed to assess the potential target organ toxicity in a 2 week repeat-dose range-finding study. Male and female cynomolgus monkeys were dosed daily with either vehicle, 50, 250 or 750 mg kg^{-1} day^{-1} RO5657. Morbidity/mortality, emesis, hypoactivity and tremors were observed in the monkeys in the two higher dose groups. One 250 mg kg^{-1} day^{-1} female animal was found dead on Day 8, with myocardial degeneration noted (see next). Animals in the high-dose group (750 mg kg^{-1} day^{-1}) were removed from the study 1 to 2 days early due to increasing severity and duration of the drug-induced clinical observations.

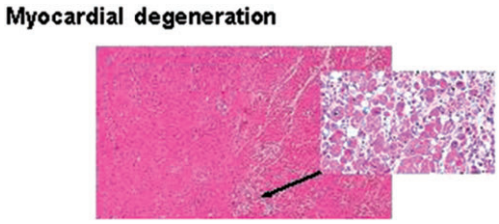

Figure 1

Photomicrograph of histopathological changes in the myocardium of an RO5657-treated monkey. Sample H&E stain from myocardium of female found dead on Day 8; evidence of extensive multifocal, moderate myocardial degeneration in the left ventricle and interventricular septum.

Treatment-related myocardial degeneration was found in the 250 mg $kg^{-1}day^{-1}$ animal (see Figure 1 for example) and probably caused its death. Myocardial degeneration was also present in another 250 mg kg^{-1} day^{-1} animal and one 750 mg kg^{-1} day^{-1} animal. No treatment-related changes in haematology or clinical chemistry parameters were observed (data not shown). No other treatment-related changes were present in the moribund or terminal death animals.

A summary of RO5657-induced findings can be found in Table 1. In the 250 and 750 mg kg^{-1} day^{-1} treated animals, abnormal ECGs including prolongation of QTc intervals, and wide complex tachycardia with prolonged QRS were noted. Specifically, both female monkeys in the 250 mg kg^{-1} day^{-1} group exhibited lengthening of the PQ interval, as well as QT/QTc intervals post-dose on both Days 1 and 13 when compared with pre-dose Day 1 values. Additionally, on Day 1, one male in the high-dose group at the post-dose measurement had either a wide QRS complex with supraventricular tachycardia (most likely) or ventricular tachycardia followed by sinus tachycardia with bundle branch block, which then resolved within approximately 30 s. On Day 13 at the post-dose measurement, the other surviving male had either wide QRS complex with supraventricular tachycardia (most likely) or ventricular tachycardia that had not resolved during the 1–2 min in which ECGs were recorded. As these findings were only seen in 250 and 750 mg kg^{-1} day^{-1} treated animals, 50 mg kg^{-1} day^{-1} was initially considered to be the "no observed adverse effect" level. The serum levels and percentage of unbound drug (i.e. free fraction) of RO5657 were determined in the treated animals. The free fraction at the peak concentration of circulating RO5657 was greater than the Kv11.1 IC_{50}. Given the ECG findings and the myocardial toxicity, the mechanistic question was whether the cardiac lesion was primary, secondary or unrelated to the effects on ECGs. These studies were undertaken to try to understand the

Table 1

Summary of findings in the 2 week RO5657-treated monkey toxicology study

	50 mg kg^{-1} day^{-1}	250 mg kg^{-1} day^{-1}	750 mg kg^{-1} day^{-1}
No. of animals	2M/2F	2M/2F	2M/2F
Morbidity/mortality	None	1F (found dead Day 8) 1M (killed Day 12)	Animals killed early on Days 13–14
Myocardial degeneration (severity)	None	1M (moderate) 1F (moderate, extensive multifocal)	1F (mild)
ECG effects	None	2F: ↓HR, ↑PQ and QTc	2M: Tachycardia with wide QRS
Free exposures at C_{max}	23 μM (12.7 μg mL^{-1})	113 μM (63.0 μg mL^{-1})	136 μM (75.8 μg mL^{-1})
C_{max}/hERG IC_{50} ratio	1.9X	9.4X	11.3X

Summary of effects in each treated dose group in 2 week repeat dose study in monkeys.

Table 2

Summary of mean haemodynamic and ECG parameters in RO5657-treated conscious telemetered monkeys

	50 mg kg^{-1} day^{-1} Day 0 (vehicle)	Day 1	Day 2	Day 8	250 mg kg^{-1} day^{-1} Day 0 (vehicle)	Day 1	Day 2	Day 8
Haemodynamics								
HR (bpm)	170 ± 4	129 ± 12	135 ± 12	124 ± 11	146 ± 16	129 ± 9	142 ± 9	106 ± 14
SAP (mmHg)	121 ± 3	113 ± 3	108 ± 2	108 ± 3	103 ± 10	90 ± 6	99 ± 7	88 ± 5
DAP (mmHg)	86 ± 3	73 ± 5	72 ± 4	67 ± 5	63 ± 8	52 ± 5	61 ± 7	48 ± 5
MAP (mmHg)	97 ± 2	86 ± 5	84 ± 3	77 ± 5	76 ± 8	65 ± 5	74 ± 7	61 ± 6
ECGs								
RR (ms)	362 ± 13	496 ± 69	466 ± 57	569 ± 85	338 ± 18	547 ± 25	441 ± 28	575 ± 70
PR (ms)	81 ± 5	81 ± 4	97 ± 12	91 ± 10	79 ± 5	90 ± 7	80 ± 6	72 ± 7
QRS (ms)	47 ± 1	52 ± 3	59 ± 6	66 ± 10	51 ± 4	67 ± 6	61 ± 5	69 ± 7
QT (ms)	200 ± 7	277 ± 18	293 ± 29	307 ± 28	192 ± 6	325 ± 33	299 ± 13	334 ± 27
QTcF (ms)	280 ± 8	354 ± 24	376 ± 26	377 ± 30	275 ± 5	407 ± 38	394 ± 15	400 ± 33
Free exposures at Cmax	BQL	4.7 μM (2.6 μg/mL)	N.D.	5.1 μM (2.8 μg/mL)	BQL	56.6 μM (31.5 μg/mL)	N.D.	72.5 μM (40.3 μg/mL)
Arrhythmias								
2° AV block (loss of 1:1 AV conduction)	2/5				4/5			
PVC	4/5 (isolated)				4/5			
PVT/TdP	4/5 (isolated incidences, generally lasting ≤3 s; 1 run lasted 10 s)				4/5 (multiple episodes, generally lasting ≤5 s; 1 degenerating into ventricular fibrillation)			

Summary of mean haemodynamic and ECG parameters in conscious non-human primates (NHPs) at 2 h post-dose administration (n = 5 animals per group). Maximal total plasma samples are presented, and corrected for protein binding (4% in NHPs). Plasma samples were not taken on Day 2, therefore exposures were not determined (N.D.). Arrhythmias for each group are noted as second degree AV block (2 AV block), premature ventricular contractions (PVC), polyventricular tachycardia (PVT) and TdP.

mechanism underlying the findings with RO5657, as well as determine the path forward for potential follow-up compounds in a screening paradigm beyond Kv11.1 and non-rodent telemetry assessment.

Extended cardiovascular assessment of RO5657 in telemetry-implanted cynomolgus monkeys

A follow-up non-good laboratory practice (non-GLP) cardiovascular function study was conducted in cynomolgus monkeys implanted with pressure catheters and s.c. electrodes to monitor ECGs. Animals were dosed daily with vehicle (Day 0) then RO5657 (50 or 250 mg kg^{-1} day^{-1}) for either 2 days (Days 1–2) or 8 days (Days 1–8), and blood pressure and ECGs were monitored. All animals were anaesthetized, at either 2 or 8 days after treatment, and additional parameters were measured before they were killed. However, data from anaesthetized monkeys were similar to data from conscious animals (except where noted), and did not provide any mechanistic insight, and data from the 2 day-treated group were also similar to those of the 8 day-treated group. Therefore, only the data from the conscious monkeys treated for 8 days with either 50 or 250 mg kg^{-1} R05657, with Day 0 serving as each animal's own vehicle control, are presented. The doses were selected based on the notable effects (250 mg kg^{-1} day^{-1}) or lack thereof (50 mg kg^{-1} day^{-1}) on the cardiovascular system noted in the aforementioned 2 week repeat dose study. The duration of dosing was chosen based on the onset of myocardial degeneration observed in the previous study.

Administration of RO5657 led to severe cardiovascular effects at both doses (for summary, see Table 2). These effects included arrhythmias as well as altered HR and BP. In both treated groups, decreases in heart rate (27% in both groups; Figure 2A,B) as well as systolic (11–15%; data not shown), diastolic (22–24%; data not shown) and mean (20% in both groups; Figure 2C,D) arterial BP were noted. The effects were maximal at approximately 90 min after dosing on each treatment day and persisted for up to 4 h; there was no correlation between the magnitude of the effects and the dose administered. While effects on HR and MAP at 250 mg kg^{-1} appeared to be greater (Figure 2B,D), when normalized to change from baseline versus the vehicle control (Day 0), effects were similar to those at 50 mg kg^{-1} (data not shown), probably due to the effects of decreased HR and MAP that occurred in those animals after vehicle administration on Day 0.

Additionally, multiple effects on ECG parameters were observed. Corresponding to the decrease in HR, an increase in RR intervals was noted, with maximal increase of approxi-

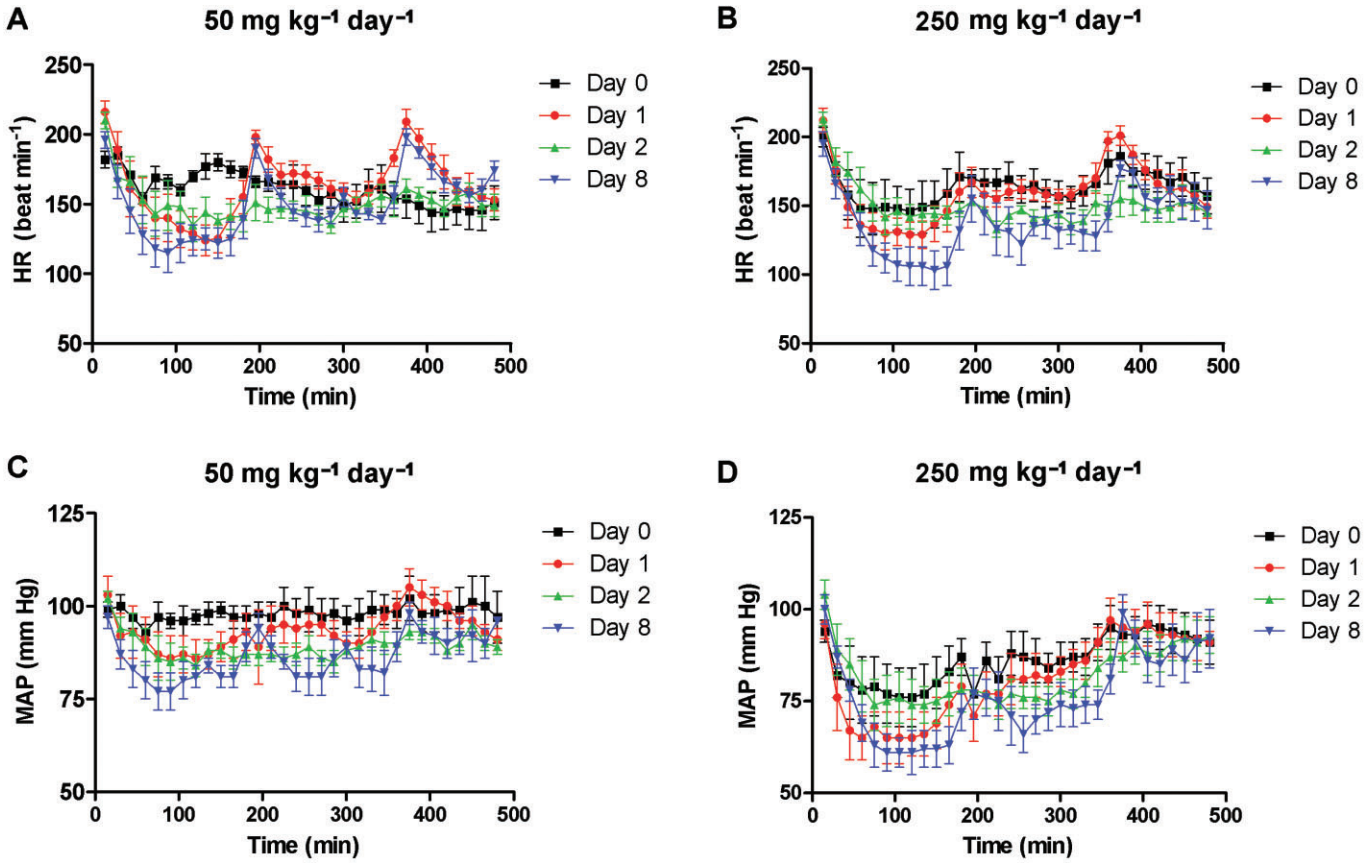

Figure 2

Mean HR and BP in RO5657-treated conscious telemetry-implanted monkeys. Summary of mean ± SEM data obtained during the first 4 h following vehicle (day 0) and RO5657 (days 1, 2 and 8) 50 mg kg^{-1} day^{-1} (HR, A; MAP, C) and 250 mg kg^{-1} day^{-1} (HR, B; MAP, D) treated groups (n = 5 animals/group).

mately 60–70% in both dose groups. Increased QRS duration was observed in both dose groups within 1 h of treatment and peaked at 50–65% above vehicle values in both treatment groups (see Figure 3A,B for the first 4 h post-dose on Days 0, 1, 2 and 8). The magnitude of effects on QRS duration was greater in the 250 mg kg^{-1} group, particularly on Days 1 and 2, and both the magnitude and duration of effects were greater on Day 8. Interestingly, the effects on QRS duration showed some dependence on days of dosing, where effects were greatest on Day 8, suggesting some cumulative effect of dosing over time. Finally, marked QT/QTc interval prolongation was observed within 1 h of dosing, and persisted for at least 4 h in both treated groups, with maximal prolongation approximately 20–65% above vehicle control values (see Figure 3C,D). In both dose groups, effects were generally similar on Days 1, 2 and 8; however, the magnitude of QTc prolongation was greater in the 250 mg kg^{-1} group. While QT intervals were well corrected by Bazzett's formula on Day 0, both groups showed slopes that were considerably far from zero as treatment progressed over time, and a large number of points where the QT intervals were much longer given similar RR intervals (Figure 3E,F). PR intervals increased approximately 30% from baseline in vehicle, 50 and 250 mg kg^{-1} groups following dosing, and remained elevated for at least 4 h every day. Because this increase occurred in the vehicle-treated group as well, this effect is thought to be produced by the vehicle and not induced by the drug. However, on the final day of the study, when monkeys were anaesthetized, PR intervals were 55–79% greater in treated animals compared with vehicle controls (data not shown).

In both 50 and 250 mg kg^{-1} day^{-1} groups, RO5657 induced 2° atrioventricular (AV) block (defined as loss of 1:1 AV conduction with variable occurrence), premature ventricular contractions, severe polymorphic ventricular tachyarrhythmias and TdP. A summary of arrhythmias can be found in Table 2. Arrhythmias were considerably more severe, both in frequency and duration, in the 250 mg kg^{-1} day^{-1} group compared with animals treated with 50 mg kg^{-1} day^{-1}. Figure 4 shows one example of a trace from a 50 mg kg^{-1} day^{-1}-treated animal, before (Figure 4A) and after (Figure 4B) compound administration. The characteristic signs of twisting along the isoelectric baseline of TdP are evident within the trace, with short-long-short beats before the tachyarrhythmia. Additionally, on Day 8, one animal in the 250 mg kg^{-1} day^{-1} group was found dead in its cage, within approximately 6 h post-dose. Retrospective analysis of this animal's ECG recordings revealed that multiple runs of TdP were evident after dosing (up to 90% of the time post-dose) that self-terminated, but

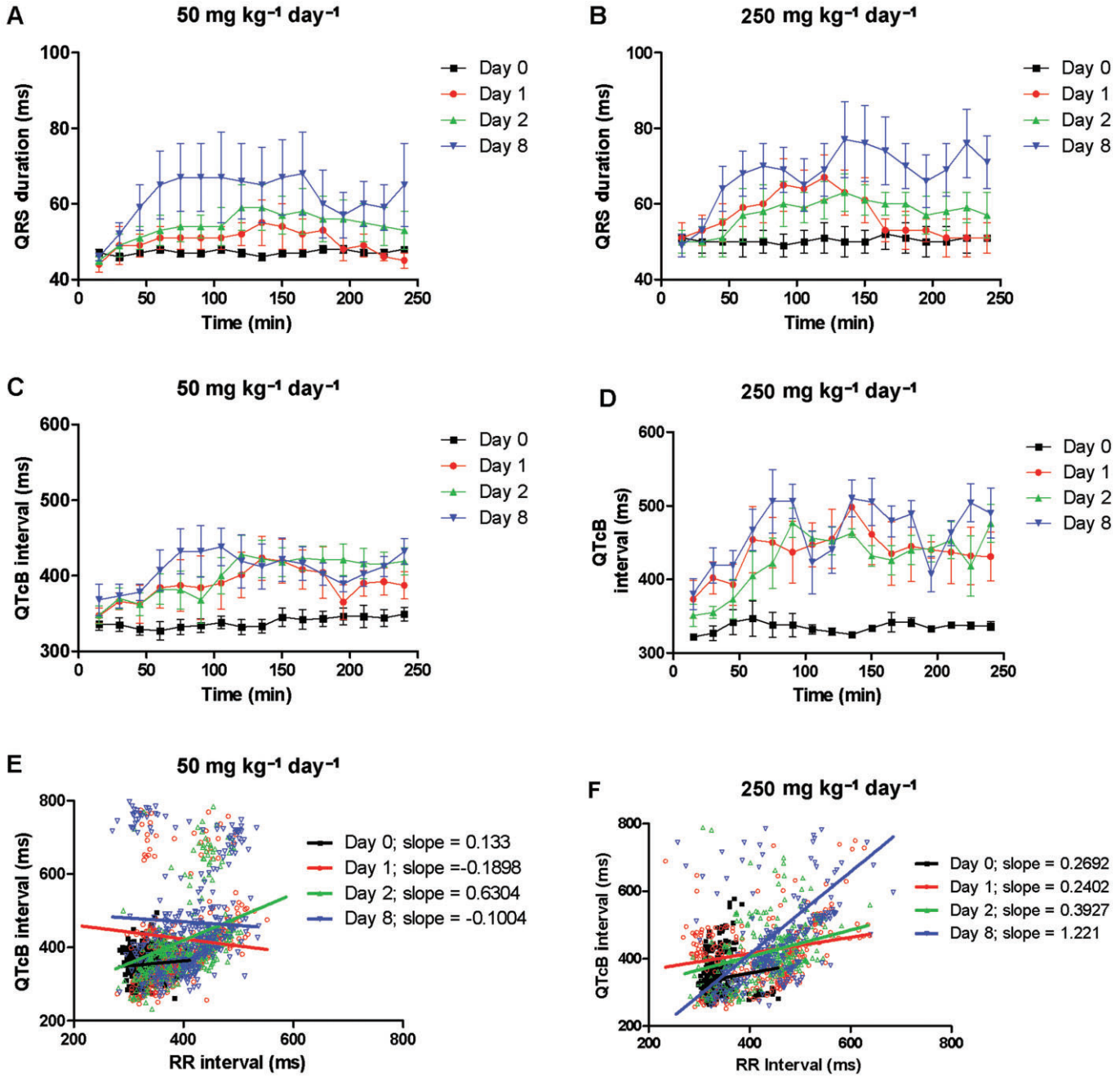

Figure 3

Mean QRS, QTcB and QT/QTcB-RR plots in RO5657-treated conscious telemetry-implanted monkeys. Summary of mean ± SEM data for the first 4 h following vehicle (day 0) and RO5657 (days 1 2, and 8) 50 mg kg^{-1} day^{-1} (QRS, A; QTcB, C) and 250 mg kg^{-1} day^{-1} (QRS, B; QTcB, D) treatment groups ($n = 5$ animals per group). Summary plots of QTcB intervals versus RR intervals for 50 mg kg^{-1} day^{-1} (E) and 250 mg kg^{-1} day^{-1} (F) treated animals (slopes of linear regression are included on plots). Additionally, when QTcB intervals were plotted against RR intervals, vehicle-treated plots (E,F; black squares) for both dose groups clearly differed from treated plots on Day 1, Day 2 and Day 8. However, between Days 1, 2 and 8, the plots were quite similar within each dose group, suggesting that maximal effects occurred within 1 day of dosing for the 50 mg kg^{-1} group. For the 250 mg kg^{-1} group, the slopes of QTcB were progressively higher on Days 2 and 8, and on Day 8 showed a slope greater than 1, suggesting that QT was not properly corrected for HR and that these effects were becoming cumulatively greater upon repeated dosing.

ultimately degenerated into fatal ventricular fibrillation (Figure 4D). ECG and BP traces from that animal pre-dose were apparently normal (Figure 4C). When the first 8 h of data for each animal on each day were reviewed qualitatively, TdP was noted in 8/10 animals (4/5 animals/group) in both treated groups; however, the incidence and the duration of arrhythmias were greater in the 250 mg kg^{-1} day^{-1} group compared with the 50 mg kg^{-1} day^{-1} group. Overall, effects on

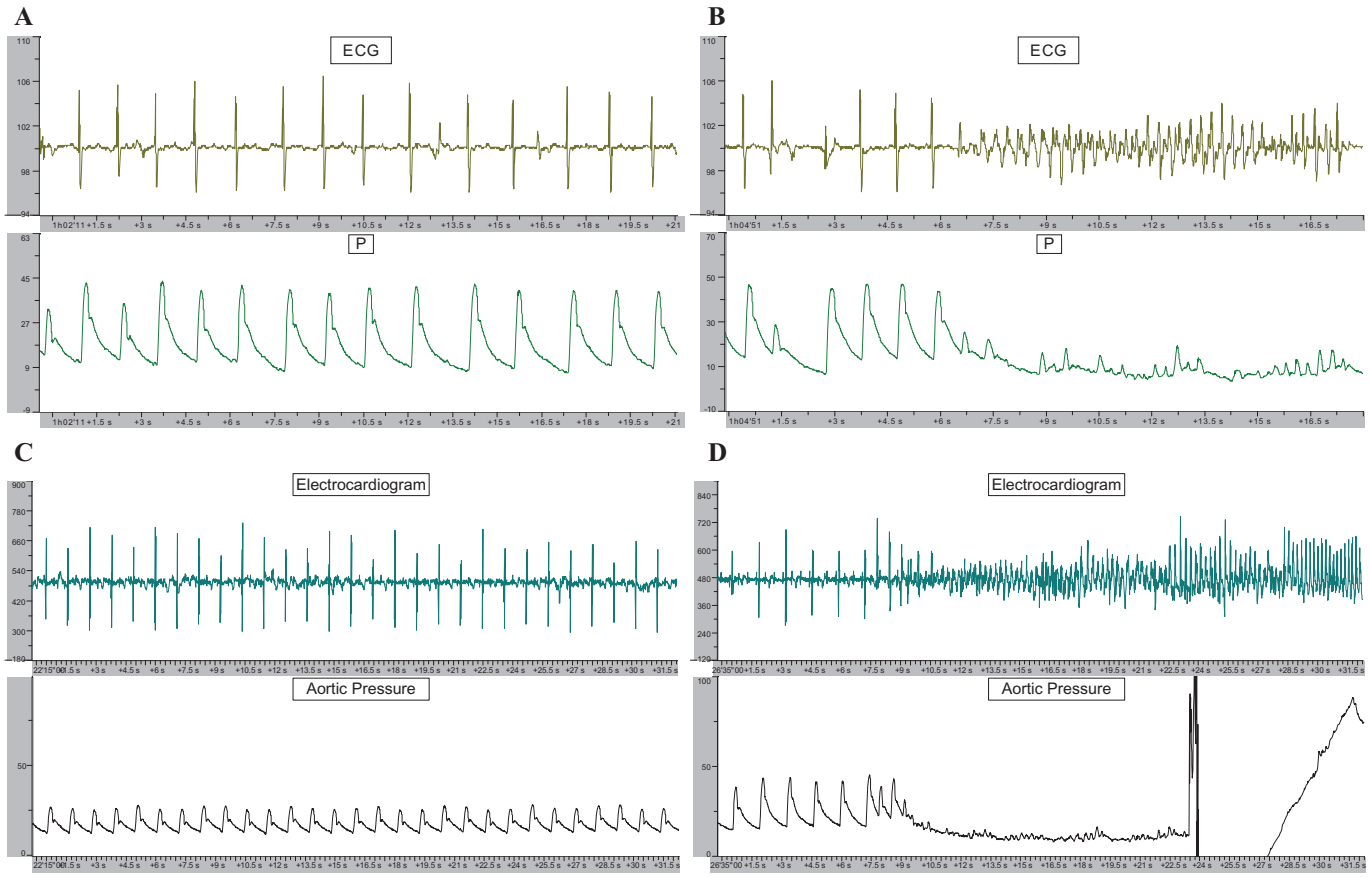

Figure 4

Sample ECG/BP traces in RO5657-treated conscious telemetry-implanted monkeys. Sample ECG and blood pressure traces from one monkey in the 50 mg kg^{-1} day^{-1} group before (A) and after treatment (B) showing ventricular tachycardia/TdP and one monkey in the 250 mg kg^{-1} day^{-1} group before (C) and after treatment (D; ~5 h post-dose on Day 8) showing ventricular fibrillation which ultimately resulted in death.

QT/QTc intervals and QRS duration were greater, and arrhythmias were more severe, both in frequency and duration, in animals treated with 250 mg kg^{-1} day^{-1}, in contrast to the apparent lack of dose-dependent effects on haemodynamic parameters. ECG data from the 250 mg kg^{-1} group also tended to be noisier, as the appearance of arrhythmias interfered with analysis of individual ECG waveforms and obtaining multiple waveforms of sufficient quality at discreet timepoints for quantification was not always possible.

Upon termination of this study, all hearts were examined for signs of morphological changes and myocardial degeneration. No cardiac histopathological findings were noted in any animals in either treatment groups (data not shown).

Cardiovascular effects of RO5657 in acute anaesthetized dog model limited to QTc prolongation and vasodilatation

RO5657 was tested in the anaesthetized dog model to establish the cross-species relationship between plasma concentrations and cardiovascular side effects. Briefly, anaesthetized dogs were treated with vehicle followed by increasing doses of RO5657 (1, 3 and 10 mg kg^{-1}), administered by i.v. bolus. Plasma samples were taken 5 and 30 min after each dose, at the same time at which haemodynamic and electrophysiological parameters were also measured (summary of results can be found in Table 3). Interestingly, dose-dependent prolongations in QT/QTc interval were observed, with maximal effects of approximately 10% at 10 mg kg^{-1}. Additionally, slight decreases in MAP were noted, with maximal effects of approximately 19% at 10 mg kg^{-1}; these effects were preceded by significant decreases in systemic vascular resistance at both 3 and 10 mg kg^{-1}, with maximal effect of 35% reduction at 10 mg kg^{-1}, suggesting that drug treatment had both a vasodilator effect as well as prolonging the QT intervals.

Similar cardiovascular effects of RO5657 noted in guinea-pig Langendorff heart

RO5657 was tested in the guinea-pig Langendorff heart preparation by bath applying ascending nominal concentrations of 10, 30 and 100 μM (Table 4 and Figure 5; actual concentrations in post-heart perfusates were 8, 26 and 94 μM). RO5657 prolonged the QT and $MAPD_{90}$ at all concentrations tested, approximately 10–14% at 8 μM up to 17–21% at 94 μM, relative to vehicle controls, when measured under spontaneous sinus rhythm. Baseline values were similar across all four hearts, and the magnitude of effects on

Table 3

Summary of mean hemodynamic and ECG parameters in RO5657-treated anesthetized dogs

	Baseline	Vehicle 5′	Vehicle 30′	1 mg kg^{-1} 5′	1 mg kg^{-1} 30′	3 mg kg^{-1} 5′	3 mg kg^{-1} 30′	10 mg kg^{-1} 5′	10 mg kg^{-1} 30′
Haemodynamics									
HR (bpm)	86 ± 7	82 ± 7	82 ± 4	79 ± 3	87 ± 4	90 ± 3	100 ± 5	97 ± 4	104 ± 6*
SAP (mmHg)	89 ± 6	84 ± 5	82 ± 3	83 ± 3	85 ± 4	84 ± 4	83 ± 4	78 ± 3	76 ± 4
DAP (mmHg)	60 ± 6	54 ± 4	54 ± 3	53 ± 3	55 ± 3	53 ± 2	53 ± 2	45 ± 1	47 ± 2
MAP (mmHg)	74 ± 6	67 ± 5	66 ± 4	67 ± 3	69 ± 4	68 ± 3	67 ± 3	60 ± 2	60 ± 3
SVR	76 ± 5	69 ± 5	70 ± 3	66 ± 3	63 ± 4	55 ± 3*	55 ± 2*	45 ± 3*	50 ± 3*
CO (L/min)	0.9 ± 0.08	0.9 ± 0.08	0.9 ± 0.05	0.9 ± 0.06	1.0 ± 0.05	1.1 ± 0.05	1.1 ± 0.05	1.2 ± 0.08*	1.1 ± 0.08*
ECGs									
RR (ms)	741 ± 75	782 ± 67	746 ± 38	770 ± 31	690 ± 28	676 ± 19	694 ± 27.7	625 ± 27	584 ± 34
PR (ms)	107 ± 3	109 ± 3	110 ± 4	113 ± 3	106 ± 3	106 ± 3	100 ± 2	93 ± 6*	96 ± 3*
QRS (ms)	58 ± 0.7	57 ± 0.6	58 ± 0.6	58 ± 0.3	59 ± 0.6	58 ± 0.6	58 ± 0.4	59 ± 0.4	59 ± 0.7
QT (ms)	278 ± 8	284 ± 7	285 ± 6	293 ± 7	278 ± 7	293 ± 5	277 ± 8	306 ± 14	298 ± 11
QTcV (ms)	302 ± 7	305 ± 8	307 ± 4	313 ± 6	304 ± 6	321 ± 5	311 ± 7	347 ± 15*	334 ± 9*
Free exposures	BQL	BQL	BQL	3.9 μM (2.2 μg mL^{-1})	0.5 μM (0.3 μg mL^{-1})	13.0 μM (7.2 μg mL^{-1})	1.8 μM (1.0 μg mL^{-1})	44 μM (24.5 μg mL^{-1})	7.7 μM (4.3 μg mL^{-1})

Baseline values were measured in dogs following a stabilization period. Vehicle, followed by ascending doses of RO5657, was administered by i.v. bolus ($n = 7$ dogs). Parameters were measured 5 min and 30 min after each bolus dose; all data are presented as mean ± SEM. The effect of each dose on haemodynamic and ECG parameters was examined for statistical significance using repeated measures ANOVA followed by a *post hoc* test (Dunnett's) for group comparisons. A value of $P < 0.05$ was considered statistically significant, and is denoted by an asterisk. No effects on left ventricular (LV) function (as measured by LV systolic pressure, LV end diastolic pressure, LV dp/dt_{max} or $LVdp/dt_{min}$) were detected (data not shown). Total plasma samples were reported, and corrected for protein binding (12% in dogs).

Table 4
Summary of findings in Langendorff isolated guinea pig hearts treated with RO5657

RO5657	8 μM (%)	26 μM (%)	94 μM (%)
HR (sinus)	–7	–18*	–16*
RR interval	6	15*	12*
QRS duration	–0.2	9	10*
QTcF	14*	21*	21*
$MAPD_{90}cF$	10*	16*	17*
Tpeak-to-end (cF)	23*	36*	28*
$MAPD_{90\text{-}30}(cF)$	17	9	47*
CPP	0.3	–4	–15*
LVDP	8	8	0.2

For each parameter assessed, the % change (an average of four hearts) relative to the time-matched vehicle control group was listed by the actual concentration measured in the perfusate. Similar measurements were obtained in hearts either under sinus rhythm or under the pacing, but only the results at sinus rate were shown in the table.
*$P < 0.05$ compared with the time-matched vehicle control group. Nominal concentrations were 10, 30 or 100 μM; however, actual measured concentrations were 8, 26 and 94 μM respectively.

QT/MAPD was also similar. At ≥26 μM, AV dissociation developed in two hearts and the prolonged (>10%) PR interval was observed when hearts were paced at 200 or 300 beats min^{-1}. A statistically significant increase in QRS duration was observed at only the highest concentration tested. Decreased sinus HR was noted at ≥26 μM, as well as a concurrent increase in RR intervals. Decreased coronary perfusion pressure, an indication of vasodilatation, was observed at ≥26 μM, when the heart was under pacing.

Although the early or delayed after-depolarization and the incidence of TdP were absent, indices of the pro-arrhythmic potential of RO5657 were also observed. Increased repolarization dispersion (as reflected by the increase in Tp-e interval; Table 4) and reverse use-dependence (greater repolarization delay at slower HRs; Figure 5A) were observed at ≥8 μM. Instability (in beat-to-beat interval MAP), AV dissociation and ventricular ectopic contractions were observed at 26 μM (Figure 5B,C). Triangulation ($MAPD_{90\text{-}30}$; Table 4), a change in the shape of the AP due to slowed repolarization, was observed at the highest concentration tested; triangulation was greater in the two hearts (#1 and #3) that developed beat-to-beat variation at 94 μM, suggesting a greater degree of responsiveness to the mixed ion channel blockade. Taken together, these results suggest that RO5657 treatment had multiple effects on parameters commonly used to assess pro-arrhythmic potential in an *ex vivo* model.

RO5657 inhibits multiple cardiac ion channels

The off-target binding affinity of RO5657 helped provide a mechanistic insight into the cardiovascular effects of RO5657. In an automated Kv11.1 potassium channel inhibitory assay, RO5657 was a moderately potent inhibitor, with an IC_{20} of ~6.0 μM and an IC_{50} of ~20.4 μM (Figure 6A). At physiologic temperature, RO5657 was slightly more potent of an inhibitor of Kv11.1, with an IC_{20} of ~2.9 μM and an IC_{50} of ~11.9 μM (Figure 6A).

Additional cardiac ion channels were also investigated. RO5657 had moderate inhibitory activity on human sodium channels (Nav1.5), as compared with the limits of the assay detection (±10%). Interestingly, a frequency-dependent inhibition was seen. The compound showed little or no ability to inhibit sodium channels when currents were recorded at 0.1 Hz, but when paced at 3 Hz, inhibition with a similar potency towards Kv11.1 was noted (IC_{20} of ~7.4 μM; Figure 6B), but was not as potent as Kv11.1 at higher concentrations tested (IC_{50} of ~118.4 μM; Figure 6B). These results correlated well with those observed in the guinea-pig isolated heart model, where prolongation of QRS intervals was observed at similar concentrations (94 μM) as the sodium channel IC_{50}. When tested against other cardiac channels at room temperature, including other potassium (hKv7.1/hminK, hKv1.5, hHCN4 and hKv4.3/hKChIP1) and L-type calcium channels (hCav1.2), the compound was found to have no significant effects (<10%) up to 30 μM (data not shown).

Additionally, the effects of RO5657 were tested on a broad panel of approximately 75 other binding and inhibition assays, including GPCRs, ion channels and transporters. At 10 μM, the compound inhibited binding by less than 30% at all the targets assessed (see Supporting Information Table S1), suggesting that its actions on the other targets tested were not involved in the observed effects.

Discussion and conclusions

Herein, we describe a small molecule drug candidate that robustly induced multiple instances of TdP in healthy, normal monkeys. Along with astemizole, RO5657 is one of the first molecules shown to induce TdP in a non-diseased, preclinical model (Ando *et al.*, 2005). The results of our analyses identified RO5657 as a potent arrhythmogenic compound *in vivo* in multiple species, with the mechanism of this arrhythmogenesis likely to be related to inhibition of Kv11.1 current and cardiac sodium channels in combination with depression of haemodynamic function. Inhibition of these cardiac channels and haemodynamic function appears to create a marked pro-arrhythmic state in healthy monkeys as nearly all animals had instances of TdP while not inducing cardiac histopathological changes in the affected animals, a traditional measure of direct cardiotoxicity. The cardiovascular effects noted in monkeys were similar to those observed in the guinea-pig Langendorff model, further validating its suitability to detect the pro-arrhythmic potential of compounds, and providing an alternative method to testing closely related molecules in the monkey (Nolte *et al.*, 1987).

The molecular mechanisms involved in the cardiovascular side effects of RO5657 were suspected to involve Kv11.1 and sodium channel inhibition, along with an effect on haemodynamics. Inhibition of Kv11.1 channels probably contributes to the effects observed, in particular involving QT

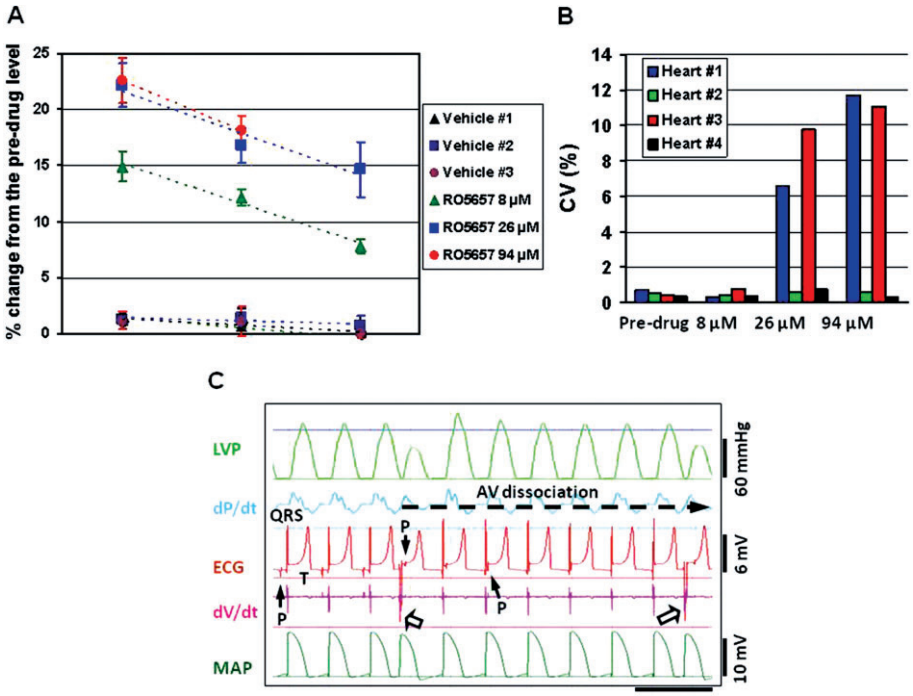

Figure 5

Effects of RO5657 on pro-arrhythmic indices in Langendorff isolated guinea-pig hearts. (A) The reverse use-dependent prolongation of QT interval induced by RO5657. The linear-fit of QT prolongation obtained at sinus rate and two paced higher rates (200 and 300 beats min^{-1}) generated a slope of −3.5, −3.8 and −4.4 for RO5657 at 8, 26 and 94 µM, respectively, compared with the slopes in the time-matched vehicle group (between −0.5 to −0.9). No data were available for effects of 94 µM RO5657 at 300 beats min^{-1} as hearts could not be paced at this rate due to an increased refractory period. (B) The increased instability of MAP beat-to-beat interval. The baseline level CV of MAP beat-to-beat interval was <1%, which was increased in two of four hearts tested to >6 at 26 µM and >11 at 94 µM, respectively. (C) The raw traces from a heart exposed to 26 µM RO5657 (Heart #1). As shown from the ECG recording, the first three beats were normal under sinus rhythm. AV block/dissociation developed following an ectopic beat (the larger, inverted QRS complex denoted by the open arrow), which probably originated from the ventricle.

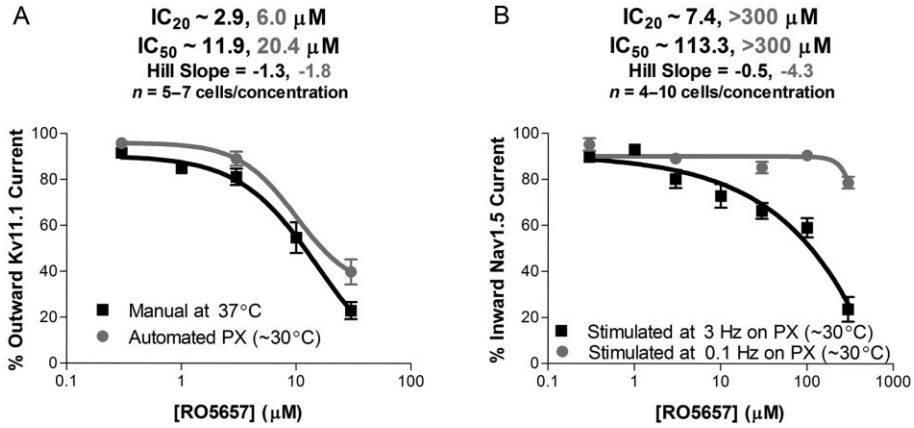

Figure 6

Effects of RO5657 on Kv11.1 potassium and Nav1.5 sodium channel currents. The effects of RO5657 on outward Kv11.1 potassium currents measured by either automated (grey) or manual methods at physiological temperature (black) are shown in (A). The effects of RO5657 on inward sodium currents as measured by automated methods are shown when stimulated at either 0.1 Hz (grey) or 3 Hz (black) in (B).

interval prolongation; however, this does not explain all the observed effects, as the decrease in HR and BP are unrelated to Kv11.1 inhibition. Multiple instances of AV block and PR interval/QRS duration prolongation were observed in monkeys as well, which are likely to be caused by antagonism of an ionic current that is not a potassium current, and are probably due to inhibition of sodium channels, given the lack of activity on other cardiac channels tested as well as a

lack of evidence of histopathological damage to the myocardium. Interestingly, minimal inhibition of sodium channels was observed at low pacing frequencies; increasing the frequency of stimulation (to 3 Hz or 180 beats min^{-1}, similar to the resting HR in monkeys) revealed a greater degree of inhibition, demonstrating that RO5657 is a use-dependent sodium channel antagonist. Both Class IC sodium channel antagonists and Class II rapid delayed-rectifier current antagonists prolong PR intervals/QRS duration and can induce arrhythmias (Sala *et al.*, 2006; Stummann *et al.*, 2009; Fabritz and Kirchhof, 2010; Farkas and Nattel, 2010), and the simultaneous inhibition of both hERG and sodium channels by RO5657 appears to have exacerbated a severe form of arrhythmia, compared with compounds that inhibit only one channel type. The combination of the Kv11.1 and sodium channel effects on cardiac conduction may contribute to the 'functional cardiotoxicity' observed, as opposed to conventionally defined 'histopathological cardiotoxicity' wherein cardiomyocytes are damaged/destroyed. Indeed, the additive risk of multiple channel blockade has been demonstrated across multiple studies, most notably with Kv11.1 and Kv7.1/minK1, whereby TdP can be more readily induced when a combination of potassium channel blockers is administered (Michael *et al.*, 2007; Shantsila *et al.*, 2007; Guerard *et al.*, 2008; Cheng and Incardona, 2009). These effects probably contributed to the electrophysiological cardiotoxicity observed, and along with the aforementioned haemodynamic depression, led to localized areas of ischaemia that induced myocardial toxicity as evidenced by morphological changes observed in the repeated-dose monkey toxicity study. Localized areas of injury within the heart may be difficult to assess by routine sectioning and histological examination, therefore monitoring physiological parameters was critical in identifying the functional cardiotoxicity induced by this compound.

The initial observation of myocardial degeneration in the monkey study confounded the understanding of the pathogenesis of the cardiovascular toxicity of RO5657. Myocardial degeneration was not observed at in mice, rats or guinea-pigs exposed to similar concentrations of RO5657 (data not shown), further indicating that direct cardiomyocyte injury as evidenced by histolopathology is not a mechanism involved in its effects. Importantly, no histological evidence of myocardial injury was noted in any of the monkeys on the telemetry-implanted study, where severe cardiovascular and electrophysiological changes were noted. Hence, the toxicological effects of RO5657 observed in the repeated-dose monkey toxicity study were probably due to secondary or ischaemic consequences of its electrophysiological effects and it does not have direct histopathological cardiotoxic effects.

Effects of RO5657 on conduction and haemodynamics may have enhanced susceptibility to arrhythmias, such that arrhythmias could be detected in healthy animals. It is rare to observe TdP in such models; in general, increased susceptibility is induced (e.g. chronic AV block, isoprenaline-stimulated rabbit heart, etc.) to facilitate induction of arrhythmias such as TdP (for review see, Sugiyama, 2008; Farkas and Nattel, 2010). Similar to the chronic AV block in monkeys, whereby HRs are reduced (to approximately 50–60 bpm) and QTc intervals and action potential duration (APD) are prolongated, RO5657 treatment induces similar effects simultaneously within the first day of dosing. Bradycardia, particularly treatment induced, can contribute to a pro-arrhythmic response by increasing the heterogeneity of repolarization at lower HRs (Roden, 2008; Fabritz and Kirchhof, 2010; Farkas and Nattel, 2010; Wallis, 2010). RO5657 decreased the HR of the monkeys. This depression of HR, as well as BP, coupled with the inhibition of sodium and Kv11.1 channel currents, may have actually magnified the cardiac channel blockade along with effects on repolarization, thereby leading to TdP. These effects on haemodynamic function and ECGs, and induction of TdP, therefore preceded, and probably induced, the myocardial degeneration observed in one monkey. In support of this hypothesis, ECG and haemodynamic effects, along with arrhythmias, were observed in the absence of myocardial degeneration and at lower plasma concentrations. Therefore, decreases in haemodynamics (HR/BP) coupled with multiple effects on conduction (PR/QRS/QT) probably contribute to the induction of arrhythmias. Focal areas of low flow/ischaemia, combined with slowing of conduction/repolarization, may have contributed to the arrhythmias and TdP observed in the monkey.

This particular target class of drugs, CCR5 inhibitors, has been associated with Kv11.1 inhibition and QT interval prolongation (De Clerck *et al.*, 2002; Dorr *et al.*, 2005; Abel *et al.*, 2008; Mansfield *et al.*, 2009; Yang and Rotstein, 2010). Maraviroc, a CCR5 inhibitor developed by Pfizer and now marketed as Celzentry, or Celsentri outside the USA, was reported to prolong QT/QTc intervals in healthy volunteers after single doses of 1200 mg, but this was not associated with arrhythmias (Abel *et al.*, 2008; Davis *et al.*, 2008). In addition, maraviroc was profiled in a number of *in vitro*/*ex vivo* assays for comparison. While maraviroc inhibited Kv11.1 channel current with an IC_{20} of approximately 0.6 μM at the same physiological temperature as our assay, it was much less potent as a sodium channel inhibitor, with an IC_{20} of approximately 12 μM (20-fold higher than for Kv11.1); an IC_{50} value could not be obtained as less than 50% inhibition was observed at the highest concentrations tested under the same conditions (100 μM; data not shown). In addition, maraviroc had no effect on any of the pro-arrhythmic indices in the guinea-pig Langendorff heart model up to 25 μM (data not shown), and prolongation of QT/QTc interval and MAPD was only observed at concentrations that significantly inhibited the Kv11.1 current (1 μM). These results further support the notion that Kv11.1 channel blockade (by a structurally similar drug) alone is not capable of inducing arrhythmias in isolation (Lawrence *et al.*, 2008). These data also illustrate the importance of an evidence-based approach to integrating cardiovascular data across assays, as Kv11.1 data alone (in this case) would have been inaccurate in predicting repolarization effects or pro-arrhythmic risks of these compounds. Importantly, neither haemodynamic effects nor arrhythmias have been reported with maraviroc treatment. This further supports the hypothesis that it is the combination of ion channel blockade and depression of haemodynamic function that form the basis for arrhythmias observed with RO5657, and demonstrates the utility of the haemodynamic and pro-arrhythmia information provided by the isolated heart model for early screening to prevent such compounds advancing into *in vivo* models.

Based on the data provided from extensive profiling of our lead molecule RO5657, along with the *in vitro/ex vivo* data generated with maraviroc, an alternative screening pathway for subsequent CCR5 inhibitor compounds beyond those required by ICS7B, mainly Kv11.1 testing and *in vivo* telemetry assessment, was devised. The Kv11.1 data (generated early in development) for RO5657 suggested that a sufficient margin between hERG effects and anticipated therapeutic concentrations (>30-fold between the IC_{20} and free exposures at Cmax) existed, and supported advancing into further characterization studies. However, this margin was dependent upon the supposition that QT/QTc prolongation could be monitored as a surrogate biomarker for a pure Kv11.1 blocker. To that end, the anaesthetized dog data were generated, and a strong correlation between concentrations of drugs inducing QT/QTc prolongation and Kv11.1 inhibition was discovered, further supporting the continued development of this compound, It was not until the compound was assessed in monkey toxicology studies (monkey selected as species, based on pharmacokinetics and metabolism) that the major cardiovascular issues were revealed.

Nearly all compounds that produce TdP in man inhibit the rapid form of the delayed rectifier potassium current Kv11.1, encoded by hERG (Gintant *et al.*, 2006; Pugsley and Curtis, 2006; Davis *et al.*, 2008; Roden, 2008; Farkas and Nattel, 2010; Giorgi *et al.*, 2010; Pollard *et al.*, 2010; Valentin, 2010; Wallis, 2010), The blockade of this channel and derived electrophysiological consequences at the cellular (prolongation of APD) and organ level (QT interval prolongation) are currently the primary parameters to predict drug-induced torsadogenesis. While associated with an increased risk of TdP, there is no reliable criterion to identify the extent of QT prolongation that is associated with a clinically meaningful increased risk of TdP. This makes it difficult for clinicians to decide what threshold of QT interval prolongation represents a risk of TdP. Other factors play an important role, for example, myocardium heterogeneity, drug–drug interactions, genetic polymorphism, electrolyte disturbances, reduced repolarization reserve and autonomic tone (Roden, 2008; Fabritz and Kirchhof, 2010; Farkas and Nattel, 2010; Wallis, 2010). An integrated approach must be considered in order to assess the risk of a drug to prolong the QT-interval and produce life-threatening arrhythmias.

There are multiple additional factors that can prolong QT coincident with drug-induced effects on repolarization. This is now known as '*multiple hit hypothesis*' (Roden, 2008; Fabritz and Kirchhof, 2010; Wallis, 2010). Electrolyte disturbances such as hypokalemia and hypomagnesemia, hypothermia, hypothyroidism and obesity are modifiable. Other factors, like advanced age, congestive heart failure and genetic polymorphism are well-known inducers of QT prolongation. More than two thirds of the cases of TdP reported occur in females, suggesting a hormonal influence. In the *in vivo* studies we describe here, female dogs and monkeys were used to increase sensitivity of the preclinical model, because females have been reported to be more susceptible to TdP in humans, but not necessarily in other species. Drug-induced TdP and QT prolongation is associated with an underlying reduced repolarization reserve in some patients (Roden, 2008; Fabritz and Kirchhof, 2010; Wallis, 2010). This indicates that prolonged repolarization could be a patient-specific response. A hypothesis supported by data demonstrating that some people can experience TdP upon exposure to different drugs, known or not known, to prolong the QTc interval, possibly due to the presence of non-symptomatic carriers of mutations involving sodium or potassium channels.

Interestingly, while QT interval prolongation and vasodilatation occurred in the anaesthetized dog, no pro-arrhythmogenic effects were produced. Systemic concentrations of RO5657 after i.v. bolus in the dog reached similar peak levels as those achieved after oral administration in the monkey. However, the pharmacokinetics in the dog revealed a higher rate of clearance of the drug compared with the monkey, resulting in sustained, elevated exposures in the latter species. It is possible that with the acute design of the anaesthetized dog model, the concentration of the drug in the target tissue was not adequate to induce arrhythmias. However, in all three species severe cardiovascular side effects of RO5657 were observed *in vivo* and subsequent work in our laboratory has revealed that RO5657 can induce the irregular or arrhythmic beats that characterize the delayed repolarization-related arrhythmia seen in human-induced pluripotent stem cell-derived cardiomyocytes (Guo *et al.*, 2011), indicating the consistent cardiovascular consequences associated with RO5657 administration.

Taken together, these data support the hypothesis that multiple factors must be introduced in order to induce arrhythmias, particularly in healthy young animals. While biomarkers of TdP, namely QT/QTc interval prolongation, are easily investigated in preclinical models, the translation to pro-arrhythmic potential is much more complex and less predictable. These studies emphasize the need to integrate data across a number of non-clinical models to accurately assess the risk of effects of novel drugs on human cardiovascular function. Additionally, we have described here, for the first time, a small molecule drug candidate capable of inducing rare arrhythmias, including TdP, in unimpaired animals. Such a molecule may serve as a valuable tool in understanding the underlying mechanisms of TdP further, and in developing new models for the pharmaceutical industry to assess pro-arrhythmic potential preclinically early, and to allow compounds with the lowest potential of risk to be prioritized through the drug development process. Additionally, this compound may help to identify assays outside the core battery that can provide such information as early as possible, as well as provide valuable information in the design of new pro-arrhythmic screening paradigms on a case-by-case basis.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplemental methods.
Table S1 List of organs and tissue collected at necropsy

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