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RESEARCH PAPER

Comparison of the I_{Kr} blockers moxifloxacin, dofetilide and E-4031 in five screening models of pro-arrhythmia reveals lack of specificity of isolated cardiomyocytes

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BACKGROUND AND PURPOSE

Drug development requires the testing of new chemical entities for adverse effects. For cardiac safety screening, improved assays are urgently needed. Isolated adult cardiomyocytes (CM) and human embryonic stem cell-derived cardiomyocytes (hESC-CM) could be used to identify pro-arrhythmic compounds. In the present study, five assays were employed to investigate their sensitivity and specificity for evaluating the pro-arrhythmic properties of I_{Kr} blockers, using moxifloxacin (safe compound) and dofetilide or E-4031 (unsafe compounds).

EXPERIMENTAL APPROACH

Assays included the anaesthetized remodelled chronic complete AV block (CAVB) dog, the anaesthetized methoxamine-sensitized unremodelled rabbit, multi-cellular hESC-CM clusters, isolated CM obtained from CAVB dogs and isolated CM obtained from the normal rabbit. Arrhythmic outcome was defined as Torsade de Pointes (TdP) in the animal models and early afterdepolarizations (EADs) in the cell models.

KEY RESULTS

At clinically relevant concentrations (5–12 μM), moxifloxacin was free of pro-arrhythmic properties in all assays with the exception of the isolated CM, in which 10 μM induced EADs in 35% of the CAVB CM and in 23% of the rabbit CM. At supra-therapeutic concentrations (≥100 μM), moxifloxacin was pro-arrhythmic in the isolated rabbit CM (33%), in the hESC-CM clusters (18%), and in the methoxamine rabbit (17%). Dofetilide and E-4031 induced EADs or TdP in all assays (50–83%), and the induction correlated with a significant increase in beat-to-beat variability of repolarization.

CONCLUSION AND IMPLICATIONS

Isolated cardiomyocytes lack specificity to discriminate between TdP liability of the I_{Kr} blocking drugs moxifloxacin and dofetilide or E4031.

Abbreviations

AP, Action potential; APD90, action potential duration at 90% repolarization; BVR, beat-to-beat variability of repolarization; CAVB, chronic complete atrio-ventricular block; CM, cardiomyocytes; EADs, early afterdepolarizations; hESC-CM, human embryonic stem cell-derived cardiomyocytes; I_{Kr}, delayed rectifier potassium current; MAP, monophasic action potential; STV, short-term variability; TdP, Torsade de Pointes

Introduction

Healthy cardiac tissue is rather resistant to pro-arrhythmic agents. Nevertheless, the clinical incidence of class III anti-arrhythmic (dofetilide, sotalol)-induced Torsade de Pointes arrhythmias (TdP) is 1–5% (Sasse *et al.*, 1998; Pedersen *et al.*, 2007), stressing the necessity of safety pharmacological testing of new therapeutic compounds. In animal models, and in most ventricular multi-cellular preparations, the blocking of a single repolarizing current (single hit) does not cause a pro-arrhythmic outcome. Only drugs or interventions that affect multiple repolarizing currents (double hit) are pro-arrhythmic, and as a result, sensitization of the testing models is required (Biliczki *et al.*, 2002; Vos, 2008). This phenomenon is attributed to repolarization reserve (Roden, 1998). Exceptions to the rule of sensitization that are known so far include human embryonic stem cell-derived cardiomyocyte (hESC-CM) clusters (He *et al.*, 2003; Jonsson *et al.*, 2010) and Purkinje fibres (Roden and Hoffman, 1985). In these preparations an arrhythmic endpoint can be reached using only one hit, and both these models show phase 4 diastolic depolarization. However, in the course of this investigation, it was reported that a single hit is sufficient for the induction of a pro-arrhythmic outcome in isolated canine CM (Abi-Gerges *et al.*, 2010; Johnson *et al.*, 2010). These data suggest that isolated cardiomyocytes might be considered to be a potentially attractive screening model.

The most common cause of drug-induced QT prolongation is inhibition of the rapid component of the delayed rectifier potassium current (I_{Kr}) (Sanguinetti and Tristani-Firouzi, 2006). Due to its I_{Kr} blocking activity, the widely prescribed antibiotic moxifloxacin is often used as a gold standard in safety pharmacology testing: the thorough QT study (Sarapa *et al.*, 2008; Matthys *et al.*, 2010). In human volunteers (phase 1), the very stable biokinetics of moxifloxacin increases the QT interval by 7–12 ms reproducibly but without inducing any arrhythmias. The plasma concentration of moxifloxacin after a single 400 mg·day^{-1} dose amounts to 3–6 μM (1.7–3.4 μg·mL^{-1}) with a 40% protein binding (Stass *et al.*, 1998; Zeitlinger *et al.*, 2008). At this concentration, moxifloxacin has been considered to be safe (Faich *et al.*, 2004; Andriole *et al.*, 2005). However, although some recent reports have related clinical administration of moxifloxacin to induction of TdP (Sherazi *et al.*, 2008; Badshah *et al.*, 2009; Poluzzi *et al.*, 2010). It is important to stress that these tachyarrhythmias were often observed when moxifloxacin was combined with other long QT predisposing factors, such as the co-administration of diuretics.

The blocking effect of moxifloxacin has been well studied in HEK-293 cells that express K_v11.1 [the α-subunit of the hERG channel; channel nomenclature follows Alexander *et al.*, (2011)], but this is not true for native I_{Kr} [which is the result of expression of the α and β subunit (minK)] in cardiomyocytes. The IC_{50} values derived from cell culture experiments vary considerably and range from 0.75 to 129 μM (Anderson *et al.*, 2001; Kang *et al.*, 2001; Alexandrou *et al.*, 2006). Using a variety of arrhythmogenic animal models, higher concentrations (30–100 μM) of moxifloxacin induce variable results (Vos, 2008) and these variations may be attributed, in part, to the different concentrations of moxifloxacin employed in the study. However, not all variability in the results can be explained in this way. Therefore, other aspects, such as the (animal) model of choice, the type of anaesthesia or the particular test laboratory should be considered, as these factors affect the responses to moxifloxacin.

In an attempt to reduce variability, we employed five test assays in the present study. These included the anaesthetized chronic complete AV block (CAVB) dog, and the methoxamine-sensitized rabbits, both well-established *in vivo* models. The newer *in vitro* models of the multi-cellular hESC-CM and single CM, isolated from chronically remodelled dog hearts or normal, unremodelled, rabbit hearts were also used. Based on the fact that two hits are necessary for induction of arrhythmias in models with stable resting membrane potential, the CAVB dog CMs were used as a model with decreased repolarization reserve due to electrical remodelling as a first hit. In all models, the endpoint has been an arrhythmogenic outcome, ranging from triggered ectopic beats and spontaneous TdP in the first two models (Carlsson, 2008; Oros *et al.*, 2008), to induction of early afterdepolarizations (EADs) in the latter three models (Antoons *et al.*, 2010; Jonsson *et al.*, 2010). Using this comprehensive set of model systems, moxifloxacin (as an example of a safe compound) was compared dose-dependently with the 'unsafe' compounds dofetilide or E-4031, both of which are specific I_{Kr} blockers. In this way, the sensitivity (ability to detect an unsafe drug) and specificity (ability to identify a safe drug) of these drugs could be determined in our experimental models. In addition, the arrhythmogenic parameter beat-to-beat variability of repolarization (BVR), quantified as short-term variability of repolarization (STV), was taken as a surrogate predictor for TdP risk (Thomsen *et al.*, 2004). Our results indicate that assays with the isolated CM lack sufficient specificity to recognize moxifloxacin as a safe drug when tested at therapeutically relevant concentrations.

Methods

Animal care and experimental procedures was in accordance with the 'European Directive for the Protection of Vertebrate animals used for Experimental and Scientific Purpose, European Community Directive 86/609/CEE' and were approved by the Committee for Experiments on Animals of the Utrecht University, the Netherlands.

CAVB dog

AV block was induced in five dogs (Marshall, NY) by radiofrequency ablation according to methods described previously (Schoenmakers *et al.*, 2003). Experiments were performed >4 weeks after ablation thereby allowing cardiac remodelling.

Complete anaesthesia was induced by pentobarbital (25 mg·kg^{-1} i.v.) and maintained by halothane (0.5% in O_2 and N_2O, 1:2). Besides ECG, monophasic action potentials (MAP) from the endocardium of the free walls of the left and right ventricle were recorded. Perioperative care, signal processing and data recording have been described in detail previously (Van Opstal *et al.*, 2001).

Experimental protocol. Moxifloxacin was tested twice, at 2 (low) and 8 mg·kg^{-1} (intermediate dose), and administered i.v. over 5 min. This treatment regime results in moxifloxacin plasma levels of 10 μM and 50 μM respectively. The methodology for the estimation of plasma levels of drug have been published previously using the same dog model but using different anaesthetics (Thomsen *et al.*, 2006).

ECG analysis. Mean RR and QT intervals from lead LL, a left lateral pre-cordial lead placed in the sixth intercostal space near the sternum, were measured manually (ECGview, Maastricht University, the Netherlands). Durations of the MAP to 90% repolarization (MAPD) were determined semi-automatically (ECG-Auto, EMKA Technologies, France). QT intervals were corrected for heart rate (QTc) according to Van de Water's formula (Van de Water *et al.*, 1989). Measurements were performed during periods without extrasystolic activity as previously described (Thomsen *et al.*, 2004). BVR was quantified as STV using left ventricular MAPD.

Methoxamine-sensitized rabbit

The methoxamine-sensitized rabbit model of TdP as described by Carlsson *et al.* (1990) was employed but with minor modifications and a total of 16 female New Zealand White rabbits (2.7–4.0 kg) were used. Anaesthesia was induced with i.m. ketamine (35 mg·kg^{-1}) and xylazine (5 mg·kg^{-1}), and then maintained with inhaled isoflurane (1.5%) in O_2-supplemented air (1:1). After induction of anaesthesia, the analgesic rimadyl (50 mg i.v.) was administered. Subsequently, the animals were prepared as follows. The thorax was shaved, and defibrillation patches were placed on both sides and connected to an external defibrillator. The marginal ear vein and central ear artery were cannulated for infusion of drugs, measurement of arterial blood pressure and withdrawal of arterial blood samples respectively. Surface ECG (leads I-III, aVL, aVR and aVF) was recorded and stored continuously with a 12-channel ECG amplifier and the custom-made computer data acquisition system Scapsys (custom-made software, Maastricht University, the Netherlands; sampling frequency 500 Hz). To avoid large deviations in body temperature, a thermal mattress was used during all experiments.

Experimental protocol. After preparation and instrumentation, the animals were allowed to stabilize for 10 min. Baseline recordings were obtained during this entire period (Table 2). Subsequently, a continuous infusion of methoxamine (15 μg·kg^{-1}·min^{-1}, 2 mL·h^{-1}) was started. Ten minutes later, either a low-dose moxifloxacin (0.1 mg·kg^{-1}·min^{-1}, n = 4) or a high-dose moxifloxacin (3 mg·kg^{-1}·min^{-1}, n = 6) was infused for a maximum of 30 min. Low-dose infusion of moxifloxacin results in plasma concentrations of 7 ± 3 μM at 15 min and 9 ± 3 μM after 30 min. High-dose infusion results in 95 ± 17 μM after 15 min and 107 ± 15 μM after 30 min (n = 2). A separate group of six rabbits was treated with dofetilide (10 μg·kg^{-1}·min^{-1}) using the same protocol.

ECG analysis. ECG data were analysed off-line using ECGview (custom-made software, Maastricht University, the Netherlands). BVR was determined by semi-automatic measurement of 30 consecutive QT intervals (ECG-Auto, EMKA Technologies, France), whereas RR, PR, QRS duration and QT were measured manually at baseline, after 10 min methoxamine and every 5 min during drug treatment. For each time point, the displayed intervals represent the average of five beats of sinoatrial origin from the lead that provided the clearest signal (mainly lead II or III). Heart rate-corrected QT values were calculated using the formula especially developed for this particular animal model (Carlsson *et al.*, 1993). At some predetermined time points, ECG intervals could not be obtained from all animals because of arrhythmias and frequent premature ventricular contractions (PVC). After the experiments, the animals were killed.

hESC-CM

Stem cell preparation. Generation of hESC-CM clusters was preformed as described previously (Synnergren *et al.*, 2008), using the hESC line SA002 (Cellartis AB, Göteborg). Spontaneously contracting clusters were identified by visual inspection and isolated from the cultures by mechanical dissection. All experiments were performed after 42–56 days of differentiation. Clusters were transferred to collagen-coated coverslips and left to attach and recover for at least 3 days prior to experimentation. Culture medium consisted of Dulbecco's modified Eagle medium supplemented with glutamax, 1% Pen/Strep, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol and 20% heat-inactivated fetal bovine serum. Only clusters with a beating rate low enough to allow electrical pacing at 1 Hz and with the ventricular action potential duration at 90% repolarization (APD90) >300 ms were used (Jonsson *et al.*, 2010).

Action potential (AP) recordings and solutions. Measurements of field-stimulated APs were performed using a HEKA EPC-10 Double Plus amplifier (HEKA, Lambrecht/Pfalz, Germany) controlled by PatchMaster 2.20. APs were evoked by biphasic field stimulation (frequency 1 Hz, pulse length 2 ms) using two platinum electrodes and a Grass S88 stimulator (Grass Technologies, West Warwick, RI).

AP recordings were performed using sharp microelectrodes filled with 3 M KCl (pipette resistance 60–80 MΩ) in a Tyrode buffer consisting of (in mM) NaCl 130, KCl 4, $NaHCO_3$ 18, $MgCl_2$ 1.2, $CaCl_2$ 1.8, HEPES 10, glucose 10, pH 7.4/NaOH. The bath was continuously perfused and kept at 37 ± 0.5°C using a TC2BIP controller (Cell Microcontrols, Norfolk, VA).

Isolated dog and rabbit cardiomyocytes

Isolation of CAVB dog CMs. Dog CMs were enzymatically isolated from the hearts of 12 CAVB dogs. Hearts were quickly

excised and washed with cold Ca^{2+}-free standard buffer solution (in mM: NaCl 130, KCl 5.4, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 6, glucose 20, pH 7.2/NaOH). The left circumflex coronary artery was cannulated and perfused by gravity at a pressure of 80 mm H_2O with solutions warmed to 37°C in the following order: (i) Ca^{2+}-free standard buffer solution for 10 min; (ii) enzymatic solution [in 400 mL of standard buffer solution: collagenase A (Roche, Almere, the Netherlands) 420 mg, protease (Sigma-Aldrich, Zwijndrecht, the Netherlands) 32 mg, 2.5% trypsin 400 μL] for 25–35 min; and (iii) 0.2 mM Ca^{2+} standard buffer solution (Ca^{2+}-free standard buffer solution with 0.2 mM $CaCl_2$) for 10 min. Finally, mid-myocardial tissue of left ventricular free wall was minced, and the cell suspension was filtered. Isolated CMs were stored at room temperature in 0.2 mM Ca^{2+} standard buffer solution and used the same day.

Isolation of rabbit CMs. Rabbit CMs were enzymatically isolated from 10 New Zealand White rabbits. Hearts were quickly excised and washed with cold rabbit Ca^{2+}-free solution (in mM: HEPES 16.8, NaCl 146, $KHCO_3$ 3.3, $NaHCO_3$ 0.988, KH_2PO_4 1.4, $MgCl_2$ 1.9, glucose 11 and $CaCl_2$ 9.75 μM). The ascending aorta was cannulated with a glass cannula, and the heart was retrogradely perfused by gravity at a pressure of 90 mm H_2O with solutions warmed to 37°C in the following order: (i) rabbit Standard buffer solution (in mM: NaCl 128.5, KCl 4.7, $CaCl_2$ 1.45, $MgCl_2$ 0.6, $NaHCO_3$ 27, NaH_2PO_4 0.4, glucose 11) for 15 min; (ii) rabbit Ca^{2+}-free solution for 20 min; and (iii) rabbit enzymatic solution [in mg per 150 mL of Ca^{2+}-free solution: creatine hydrate (Sigma-Aldrich) 320, collagenase P (Roche) 9, collagenase B (Roche) 22, hyaluronidase (Sigma-Aldrich) 25, trypsin inhibitor (Roche) 15] for 30 min at a pressure of 60 mmH_2O. Thereafter, the left ventricle was opened, the endocardial layer removed and mid-myocardial cells of the free wall were harvested. Isolated mid-myocardial cells were shaken (2 Hz) in rabbit enzymatic solution at 37°C for 17 min. The solution was replaced by rabbit enzymatic solution with 1% bovine serum albumin and shaken (4 Hz) for an additional 10 min. Finally, the cell suspension was filtered and washed twice with washing solution (rabbit Ca^{2+}-free solution with 1% bovine serum albumin added). Isolated CMs were stored at room temperature in rabbit Ca^{2+}-free solution with addition of 0.35 mM $CaCl_2$ and 1% bovine serum albumin and were used the same day.

AP recording. The initial criteria for selecting cells for AP recordings were that they were rod-shaped, silent and did not show any spontaneous activity. In addition the myocytes showed clear and regular striation, a clean membrane and sharp edges. APs were measured with an AxoPatch 200B amplifier controlled by pClamp 9 software (Molecular Devices, Sunnyvale, CA) in the whole-cell current clamp mode. Patch pipettes were pulled with a Sutter P-2000 puller (Sutter Instrument, Novato, CA) and had resistances of 2–3 MΩ. Modified Tyrode solution with the following composition (in mM) was used: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 11.8, glucose 10, pH 7.4/NaOH. Pipette solution contained (in mm): NaCl 10, KCl 130, HEPES 10, $MgCl_2$ 0.5, MgATP 5, pH 7.2/KOH. Cells were stimulated with 2 ms current injections at a cycle length of 2000 ms. AP duration was measured at 90% repolarization (APD90). All recordings were performed at 37 ± 0.5°C in a temperature-controlled perfusion chamber (Cell Microcontrols). Recordings were made for at least 10 min after starting the drug infusion . EADs generally occurred within 4 min after the start of the infusion and if so recordings were stopped earlier.

Calculations and statistical analysis

All values are presented as mean ± SD. One or two-way repeated-measures ANOVA followed by a Tukey or Bonferroni *post hoc* test when appropriate or Student's *t*-test were used for statistical evaluation, and $P < 0.05$ was considered statistically significant. Correlations were evaluated using Pearson product moment correlation.

BVR was assessed using STV (based on 30 consecutive beats), which was calculated using the formula $STV = \Sigma|D_{n+1} - D_n| / [30\checkmark 2]$, where D represents APD90 in *in vitro* models, MAPD in CAVB dogs and QT in the methoxamine-sensitized rabbit model, respectively, and describes the mean orthogonal distance to the line-of-identity on a Poincaré plot, as described previously (Thomsen *et al.*, 2004).

The cells were considered inducible (positive arrhythmogenic outcome) when at least three EADs were observed. In dogs and rabbits, three TdP episodes was set as a minimum for animals to be considered as TdP inducible. TdP was defined as a polymorphic ventricular tachyarrhythmia with at least five consecutive undulating QRS complexes with a typical twisting around the isoelectric line of the ECG. A run of three or more consecutive PVC was classified as a short run of ventricular tachycardia (VT).

Materials

Dofetilide was initially dissolved in dimethyl sulphoxide (4.4 mg·mL^{-1}) for *in vitro* experiments and in 0.1 mL 0.1 mM HCl for *in vivo* experiments. Subsequently, it was diluted in Tyrode's solution or in 0.9% saline to the required concentration. E-4031 (Sigma-Aldrich) was dissolved in H_2O and kept as 10 mM stock solution (frozen at –20°C), which was diluted in Tyrode's solution prior to each experiment. Moxifloxacin HCl (Avelox, Bayer Healthcare, Berlin, Germany) was dissolved and diluted to the desired concentration in Tyrode's solution and used for cellular and hESC-CM cluster experiments. For *in vivo* experiments, moxifloxacin was dissolved and diluted in 0.5% lactic acid. Moxifloxacin was applied as a low, intermediate or high dose. Methoxamine HCl (Sigma-Aldrich) was dissolved and diluted in saline and used for *in vivo* rabbit experiments.

Results

The anaesthetized CAVB dog

At the lower dosage, moxifloxacin (2 mg·kg^{-1}) did not significantly change any of the electrophysiological parameters measured, except right ventricular MAPD (QTc was unchanged; 390 ± 65 vs. 405 ± 60; see Table 1 for other parameters). After 30 min of 2 mg·kg^{-1} moxifloxacin (baseline #2), most of the repolarization characterizing values were still similar as original (baseline #1) values. The intermediate dosage of moxifloxacin (8 mg·kg^{-1}) increased all ECG parameters significantly (QTc 400 ± 65 to 480 ± 75, $P < 0.01$;

Table 1), with the exception of STV, which did not change (1.9 ± 0.5 to 3.2 ± 1.3 ms). No TdP (0/5) could be induced by either dose of moxifloxacin.

Using different types of anaesthesia, we have previously shown that dofetilide caused TdP in 76% of CAVB dogs using pentobarbital (Oros *et al.*, 2008) and 100% using thiopental as anaesthetic (Thomsen *et al.*, 2006). The latter dogs are the same animals as used for the moxifloxacin experiments in the present study; however, pentobarbital was used as an anaesthetic. Dofetilide-induced TdP was associated with a significant increase in QTc and STV.

The methoxamine-sensitized anaesthetized rabbit

None of the animals experienced arrhythmias or PVCs during baseline. During infusion with methoxamine, mean blood pressure increased (62 ± 11.2 to 93 ± 22.7 mm Hg), and the RR interval was prolonged (Table 2). Other ECG parameters remained unchanged, and no arrhythmias or PVCs were observed. Subsequent infusion of moxifloxacin at a low (therapeutic, ML) or high (MH) concentration for 30 min caused significant QTc prolongation only in the high-dose group ($P < 0.05$; Table 2). STV did not increase significantly in any of the groups (from 0.8 ± 0.3 to ML 0.8 ± 0.2, $n = 4$ vs. MH 1.5 ± 0.4 ms, $n = 5$). Moxifloxacin did not induce TdP at the low dosage (0/4), whereas at the high dose, TdP was seen in one out of six rabbits (17%, Figure 1). This inducible animal experienced a total of five TdP episodes and five short runs of VT, which was associated with an STV increase to 25.7 ms just before the first TdP episode. In one other animal, PVCs were observed at the high dose of moxifloxacin. All arrhythmias ceased within 20 min after the infusion of moxifloxacin was terminated.

At 5 min, dofetilide treatment caused prolongation of the RR and QTc, while STV was increased significantly (Table 2). In five out of six animals (83%) TdP was inducible. At the same time point, only the high dose of moxifloxacin significantly prolonged QTc, but not STV (Table 2, MH-1).

The multi-cellular hESC-CM clusters

In hESC-CM clusters, control APD90 was 381 ± 47 ms ($n = 11$) and STV 2.2 ± 1.4 ($n = 10$); 10 μM moxifloxacin (low concentration, within the therapeutic range) induced a small but not significant increase in APD90 (435 ± 71 ms, $n = 11$), and also STV was unchanged (2.7 ± 2.4, $n = 9$; Figure 2A and B); 100 μM moxifloxacin (high concentration) increased APD90 to 531 ± 93 ms ($n = 11$, $P < 0.01$), comparable with the increase observed using 1 μM E-4031

Table 1

Electrophysiological parameters of CAVB dogs

	Baseline #1	Moxi-low	Baseline #2	Moxi-intermediate
RR (ms)	1264 ± 245	1360 ± 315	1330 ± 305	1575 ± 400*
QT (ms)	415 ± 85	435 ± 85	430 ± 90	530 ± 110*
QTc (ms)	390 ± 65	405 ± 60	400 ± 65	480 ± 75*
LV MAPD (ms)	345 ± 55	375 ± 90	345 ± 70	440 ± 115*
RV MAPD (ms)	300 ± 60	325 ± 70*	315 ± 65*	365 ± 85*
ΔMAPD (ms)	45 ± 10	50 ± 20	25 ± 25	75 ± 35†
STV (ms)	2.3 ± 1.2	2.5 ± 2.6	1.9 ± 0.5	3.2 ± 1.3

Values in the Table are means ± SD (n = 5). *$P < 0.01$ vs. baseline #1, baseline #2 and low dose of moxifloxacin (Moxi). †$P < 0.05$ vs. baseline #2. LV, left ventricular; RV, right ventricular.

Table 2

Rabbit electrophysiological parameters

	Baseline n = 16	Methoxamine n = 16	ML-1 n = 4	ML-2 n = 4	MH-1 n = 6	MH-2 n = 6	Dof n = 6
RR (ms)	319 ± 29	456 ± 105†	513 ± 127†	566 ± 178†	534 ± 100†	516 ± 103†	632 ± 156*†
PQ (ms)	78 ± 12	81 ± 12	88 ± 9	90 ± 8	94 ± 20	100 ± 21	93 ± 21
QRS (ms)	50 ± 4	51 ± 5	53 ± 3	52 ± 3	56 ± 6	63 ± 13*†	75 ± 18*†
QTc (ms)	170 ± 12	160 ± 13	165 ± 9	181 ± 6	223 ± 37*†	291 ± 73*†#	244 ± 36*†#c
STV (ms)	0.8 ± 0.3	0.8 ± 0.4	0.9 ± 0.1	0.8 ± 0.2	1.2 ± 0.4	1.5 ± 0.4	5.6 ± 4.5*†

Values in the Table are (mean ± SD for the number of animals shown) *$P < 0.05$ vs. methoxamine. †$P < 0.05$ vs. baseline. #$P < 0.05$ vs. ML-2. ML-1 = low-dose moxifloxacin at 5 min, ML-2 = low-dose moxifloxacin at 30 min, MH-1 = high-dose moxifloxacin at 5 min, MH-2 = high-dose moxifloxacin at 30 min, Dof = dofetilide at 5 min.

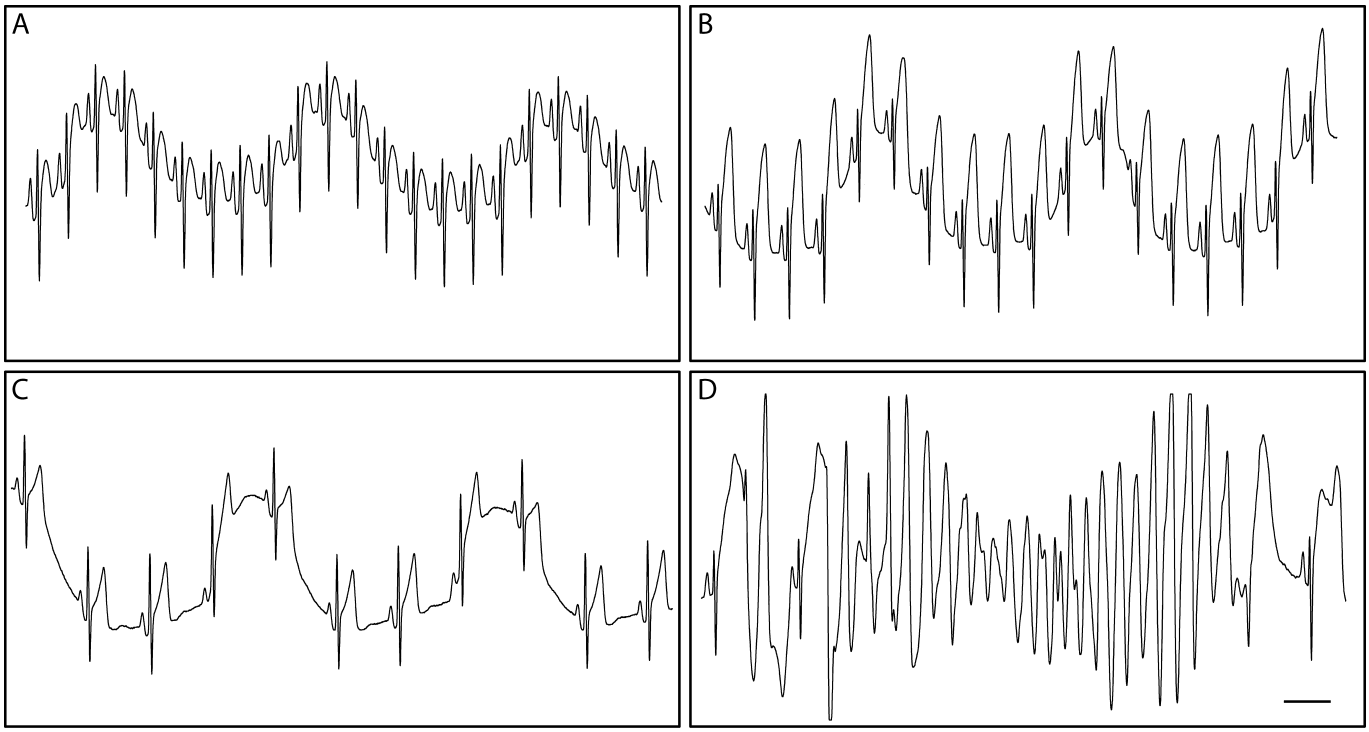

Figure 1

ECG recordings from the *in vivo* rabbit model. (A, C) Rabbit treated with low-dose moxifloxacin, A = baseline, C = 30 min moxifloxacin. (B, D) Rabbit treated with high-dose moxifloxacin showing TdP arrhythmia, B = baseline, D = 10 min moxifloxacin. Horizontal bar represents 0.5 s.

(531 ± 84 ms, n = 8, P < 0.01). The low concentration of moxifloxacin did not cause EADs, but an incidence of 18% (2 of 11) was seen with 100 μM (Figure 2C). In the same clusters, 1 μM E-4031 induced EADs in 50% (4/8). STV immediately prior to the first EAD was 5.2 ± 4.3 (n = 2, too low for statistical analysis) in the 100 μM moxifloxacin group and 9.4 ± 3.7 (n = 4, P < 0.05 compared with control) for the E-4031 group.

The isolated remodelled canine ventricular myocyte

At 10 μM, moxifloxacin prolonged APD90 (324 ± 57 to 402 ± 120 ms, P < 0.01), increased STV (15.7 ± 7 to 34.5 ± 24.5 ms, P < 0.01) and induced EADs in 7/20 (35%) cells. An example is shown in Figure 3A. Dofetilide (1 μM) prolonged APD90 (384 ± 111 to 490 ± 134, P < 0.01), increased STV (13.5 ± 10.4 to 39.8 ± 31.7 ms, P < 0.01) and caused an EAD incidence of 15 of 25 (60%).

Analysis of the differences between inducible (n = 7) and non-inducible (n = 13) cells after 10 μM moxifloxacin revealed that baseline APD90 values were higher in inducible cells (365 ± 62 vs. 302 ± 43 ms, P < 0.05), but STV (19.9 ± 9.5 vs. 13.4 ± 4 ms) was similar. In both groups, APD90 (492 ± 142, P < 0.05 vs. 354 ± 74 ms, P < 0.01) and STV (57 ± 27.7 vs. 22.5 ± 10.6 ms, P < 0.01) were significantly prolonged following moxifloxacin infusion, but increases in APD90 and STV were significantly larger in the inducible cells (127 vs. 52 ms and 37 vs. 9 ms respectively). Similarly, dofetilide (1 μM) also increased APD90 and STV more in inducible cells (534 ± 144 vs. 378 ± 130 and 56.5 ± 30.6 vs. 14.5 ± 12.7, P < 0.01; n = 15) compared with non-inducible (423 ± 98.7 vs. 394 ± 79.9 and 14.8 ± 8.2 vs. 12 ± 5.7; P > 0.05, n = 10).

The isolated unremodelled rabbit ventricular myocyte

Moxifloxacin (10 μM) significantly increased APD90 (255 ± 66 to 289 ± 71 ms, P < 0.05), STV (12 ± 6.1 to 24 ± 20.5 ms, P < 0.05) and induced EADs in 3 of 13 cells (23%, Figure 3B). An example of the corresponding time-dependent behaviour of APD90 and STV from a single rabbit CM is shown in Figure 3C. In six cells that were resistant to EADs at 10 μM, infusion of 100 μM moxifloxacin resulted in a further increase in APD90 (482 ± 163 ms, P < 0.05), in STV (44.4 ± 27.2 ms, P < 0.05) and induced EADs in two of six cells.

Administration of 1 μM dofetilide significantly increased APD90 (277 ± 45.8 to 528 ± 241 ms, P < 0.01), STV (15.4 ± 3.5 to 66 ± 38.7 ms, P < 0.01) and EADs were observed in five of eight cells (63%).

Overview of data

In Table 3, arrhythmogenic outcome for the different assays is summarized. It is clear that the isolated CM system provides acceptable sensitivity but lacks sufficient specificity to discriminate between the non-arrhythmic (moxifloxacin) and pro-arrhythmic (dofetilide) test drugs. The sensitivity (i.e. the ability to detect an unsafe drug, such as dofetilide or E4031)

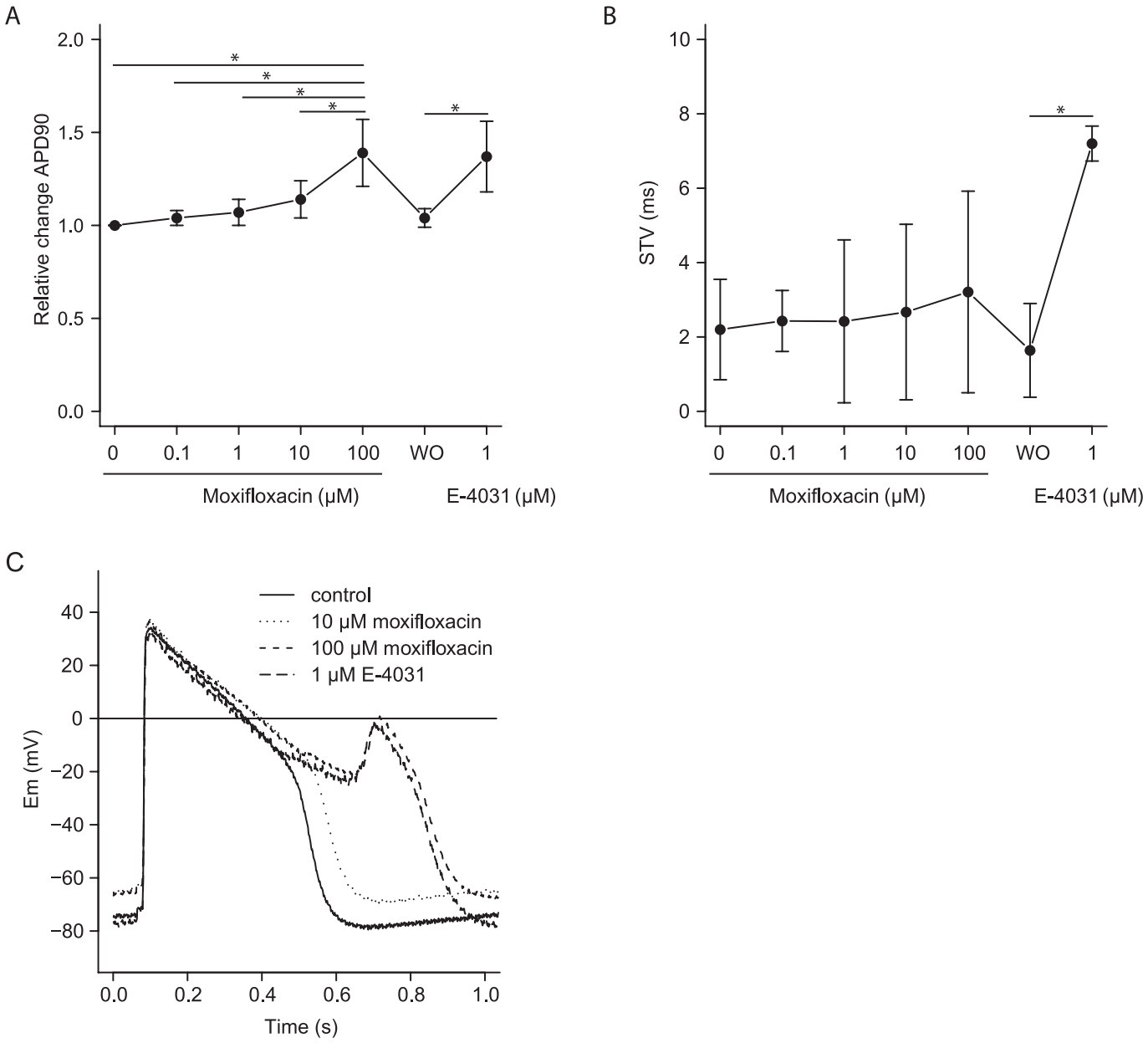

Figure 2

APD90, BVR and example of APs in hESC-CM. Alterations in APD90 (A) and BVR (B) after treatment with different concentrations of moxifloxacin (0.1, 1, 10 and 100 μM), or E-4031 (1 μM). * P<0.05 significantly different from control or wash out (WO) as indicated by horizontal bars. (C) Example of AP and induced EAD after treatment with either a low or a high concentration of moxifloxacin, or with E-4031.

is highest in animal models and lowest in hESC-CMs: 83% in methoxamine-sensitized rabbits, 76% in CAVB dogs, 63% in isolated rabbit CMs, 60% in isolated CAVB CMs and 50% in hESC-CMs. The specificity (i.e. the ability to correctly detect a safe drug – such as low-dose moxifloxacin) is 100% in both *in vivo* models and the hESC-CM clusters, 77% in isolated rabbit CMs and 65% in isolated CAVB CMs.

Importantly, STV can be regarded as a critical parameter for detecting the pro-arrhythmic potential of the test drugs used, as an increase in STV preceeds a subsequent arrhythmic outcome in all models.

Discussion

In the present work, we have demonstrated that all the multi-cellular models tested were able to identify moxifloxacin as a safe compound, despite the fact that this drug is an I_{Kr} blocker and prolongs the QT interval. On the other hand, remodelled as well as unremodelled isolated cardiac ventricular myocytes lack sufficient specificity. The behaviour of the arrhythmogenic marker STV was in line with the expectations, and only a significant increase in STV corresponded to the occurrence of EADs or TdP.

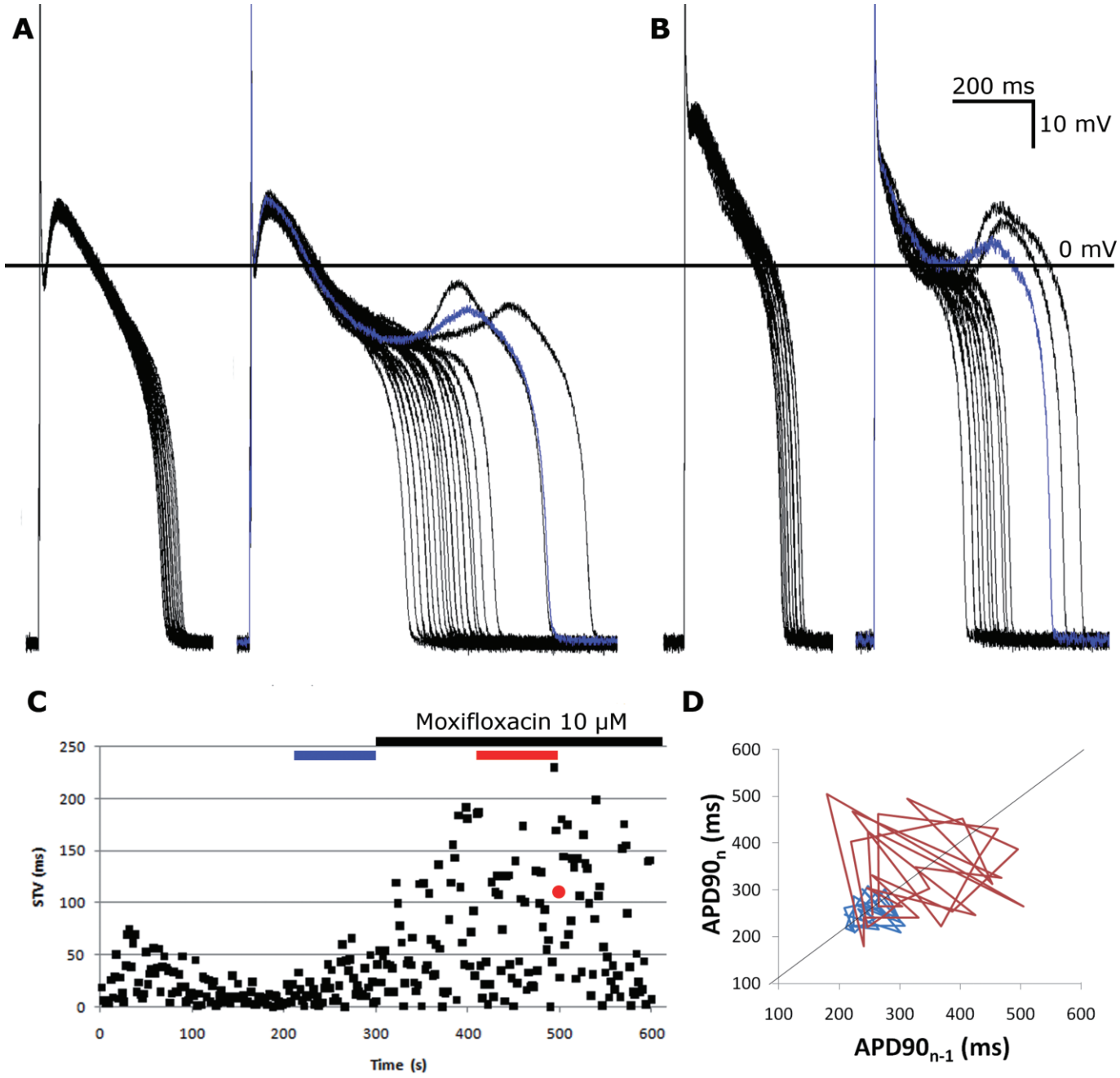

Figure 3

Examples of moxifloxacin-induced EADs in isolated cardiomyocytes. Example of control APs (left) and APs with EADs (right) after application of 10 µM moxifloxacin in CAVB dog (A) and rabbit (B) CM. (C) Example of BVR during a complete measurement in a rabbit CM. Red dot indicates first occurrence of EAD. Red and blue bars indicate which 30 beats were used to calculate STV. (D) Poincaré plot of 30 selected beats during baseline (blue) and prior to first EAD (red).

Cardiac safety assessment

The current strategy of evaluating the cardiac toxicity of new drug candidates as, for instance, applied by the FDA, is based on the thorough QT/QTC study in healthy volunteers (ICH Guideline E14, 2005). If the QTc prolongation is less than 5 ms, the drug is considered to be safe. If it is more than 20 ms, the drug is labelled as unsafe. When the QTc prolongation is between 5 and 20 ms, additional verification of cardiac safety is required (ICH Guideline S7B, 2005). At the same time, the pharmaceutical industry is trying to identify possible unsafe drugs in the early stages of drug development. For this purpose, a hERG assay is often used, in which the hERG blocking potency of the drug candidate is tested. Although informative, it must be emphasized that there is no clear correlation between hERG block, QT prolongation and the pro-arrhythmic potential of the drug (Martin *et al.*, 2004;

Table 3

Arrhythmia inducibility by moxifloxacin and dofetilide or E-4031 in all models

Model system	Arrhythmia incidence (TdP/EAD) Moxifloxacin Low dose	High dose	Dofetilide or E-4031
CAVB dog	0%		76%[a]/100%[b]
	2 mg·kg^{-1} (10 μM)		
Methoxamine rabbit	0%	17%	83%
	0.1 mg·kg^{-1}·min^{-1} (9 ± 3 μM)	3 mg·kg^{-1}·min^{-1} (107 ± 15 μM)	10 μg·kg^{-1}·min^{-1}
hESC-CM clusters	0%	18%	50%
	10 μM	100 μM	1 μM
Isolated rabbit CM	23%	33%	63%
	10 μM	100 μM	1 μM
Isolated CAVB CM	35%		60%
	10 μM		1 μM

For each model and condition (low/ high dose), the actual doses or concentrations are shown, along with the incidence (as %) of arrhthymia as TdP *in vivo* or EADs *in vitro*.
Data from earlier work is shown [a]Oros *et al.*, 2008. [b]Thomsen *et al.*, 2006.

Gintant, 2011). Thus, a new assay, not exclusively dependent on hERG block or QT prolongation, is needed, preferably in combination with a surrogate parameter of pro-arrhythmia, such as STV.

Pre-clinical model systems for cardiac safety assessment

With the exception of the isolated ventricular myocytes, the other three assays used in the present study responded as anticipated. In the hESC-CM clusters and the methoxamine-sensitized rabbit, the low concentration of moxifloxacin (10 μM) did not induce any pro-arrhythmic endpoint, whereas the high concentration (>100 μM) induced EADs (2 of 11) or TdP (1 of 6) respectively. In the anaesthetized CAVB dog, only low and intermediate dosages were tested, and neither 2 nor 8 mg·kg^{-1} moxifloxacin induced TdP. The observations were different in the isolated ventricular CMs. Already at the low concentrations (10 μM), moxifloxacin was able to induce EADs in a significant number of unremodelled rabbit CMs (3 of 13) and even more frequently (7 of 20) in the remodelled CAVB CMs. These observations clearly indicate a lower specificity of isolated ventricular CMs.

Intact animals. A previous study showed that moxifloxacin is safe (no QTc prolongation) at 10 mg·kg^{-1} in conscious telemetered dogs (Chen *et al.*, 2005). Our results support that observation in an even more sensitive model, the anaesthetized CAVB dog, which is in line with a previous study utilising a different anaesthetic (Thomsen *et al.*, 2006).

In the methoxamine-sensitized rabbits, dofetilide induced TdP with an incidence comparable to that reported by others (Carlsson *et al.*, 2009). Moxifloxacin at high dosages (>100 μM) induced TdP only in one out of six of our animals. Other authors reported no TdP arrhythmias using a similar dose, although experimental differences may account for this discrepancy [different type of anaesthesia, dose, open thorax (Chiba *et al.*, 2004); different anaesthesia regimen, lower moxifloxacin infusion rate (Anderson *et al.*, 2001)].

Multi-cellular hESC-CM. This is the first time that a negative control has been tested in multi-cellular hESC-CM clusters. We and others have shown previously a somewhat higher sensitivity of these cells to hERG block (He *et al.*, 2003; Caspi *et al.*, 2009; Jonsson *et al.*, 2010; Pekkanen-Mattila *et al.*, 2010; Peng *et al.*, 2010), with APD prolongation, increased BVR and formation of EADs (inducibility 60–69%) than our current study (50% inducibility). Here, we address specificity by showing that a therapeutic level of moxifloxacin results in slight but not significant APD prolongation and no increase in BVR or induction of EADs. Results from this model compare favourably with results from other multi-cellular preparations, such as the rabbit ventricular wedge preparation and canine Purkinje fibres (Chen *et al.*, 2005), and show that this assay merits further validation for future safety pharmacology screening.

Isolated CM assay. The ability of a single hit of dofetilide, ATXII or HMR1556 to induce EADs in isolated canine unremodelled ventricular myocytes has been reported recently (Abi-Gerges *et al.*, 2010; Johnson *et al.*, 2010). Baseline STV calculated in the isolated CM from this study (15.7 ± 7 in cells from CAVB dogs and 12.1 ± 6.1 in rabbit CM) are comparable to those reported by Abi-Gerges *et al.* (ranging from 9 ± 2 to 16 ± 4) and Johnson *et al.* (estimated 8–10 at 0.5 Hz). In remodelled CAVB left ventricular CM, we achieved moderately increased sensitivity (60%) for dofetilide-induced EAD compared with unremodelled dog left ventricular CM [55% and 42% (Abi-Gerges *et al.*, 2010; Johnson *et al.*, 2010)] at the same pacing frequency. Here, we demonstrate that even in unremodelled rabbit CMs, a single hit is enough to induce arrhythmias, whereas a second hit is mandatory in

animal models and most multi-cellular preparations like dog right ventricular papillary muscle (Biliczki *et al.*, 2002).

Unfortunately, no results concerning a negative control, like moxifloxacin, were provided in previous papers using the isolated CM test assay. Based on experience with multi-cellular preparations, we originally chose the remodelled (predisposed) cells from CAVB dogs. In the course of these experiments, the CAVB dog myocytes responded with EADs even at low concentration of moxifloxacin. As a comparison, we also included unremodelled myocytes from rabbits and evaluated the initiation of EADs. However, there was no major difference in arrhythmogenic outcome between these two types of cells with respect to moxifloxacin-induced EADs. This observation may be explained by the cell isolation procedure, which could lead to decreased repolarization reserve to such extent, that it overrides the effect of electrical remodelling of the CAVB dog cells. This hypothesis is in line with the increased STV levels in the uncoupled cells compared with the animal model from which they are derived. Although the exact mechanism of increased beat-to-beat variability of repolarization in isolated cells is not known, there are convincing data showing that beat-to-beat variability in calcium cycling results in BVR of APD through calcium-sensitive channels. In multi-cellular preparations, this variability is most likely attenuated by surrounding cells through electrotonic interaction (Pastore and Rosenbaum, 2000).

Another possible explanation could be the fact that we stimulated both cell types at 0.5 Hz. As the natural heart rate is much higher in rabbits than in dogs, this could be a confounding factor. At lower rates, the APD was increased, making the cells more susceptible to hERG block (Bányász *et al.*, 2009).

STV as an alternative arrhythmogenic marker

In patients and animal models, STV has been proposed as a better or complementary predictor of arrhythmias than QT prolongation *per se* (e.g. Thomsen *et al.*, 2004; Hinterseer *et al.*, 2008; Takahara *et al.*, 2008; Carlsson *et al.*, 2009). In all assays tested in this study, STV was increased before the occurrence of TdP or EADs. In multi-cellular assays, increased STV was present only in inducible subjects. In isolated ventricular myocytes, STV was also significantly increased in non-inducible cells treated with moxifloxacin, but not with dofetilide. There is no obvious explanation for this observation.

In our experiments, the underlying STV calculation differed in the various models (MAPD in CAVB dogs, QTc in rabbits and APD in hESC-CM clusters and isolated myocytes). In the *in vivo* models, changes in STV are comparable, independent of the parameter used for calculating STV (Thomsen *et al.*, 2007). Whether BVR in cells or clusters has a similar aetiology remains unknown.

Another way of assessing the pro-arrhythmic potential of a drug is by using triangulation, reverse use dependence, instability and dispersion of repolarization (TRIaD) (Hondeghem *et al.*, 2001). Using three or four different parameters, an overall pro-arrhythmic score is given to the drug. Using TRIaD, a previous report predicted moxifloxacin to be safe at therapeutic concentrations, but with a small safety margin (Lawrence *et al.*, 2006). In the same study dofetilide was identified as pro-torsadogenic at 20 nM (therapeutic concentration) and upwards. These results are in line with our multi-cellular and *in vivo* models, and support the hypothesis that isolated cardiomyocytes lack specificity to recognize moxifloxacin as a safe drug in clinical use.

Study limitation

The sensitivity and specificity data of the five models was based on one positive and one negative control compound only.

In summary, this study showed that a clinically relevant concentration of moxifloxacin did not cause arrhythmias in the three of the five multi-cellular assays tested, but was pro-arrhythmic in the two assays using isolated CMs. In this case, using isolated cardiomyocytes during safety pharmacology would result in a type I error by falsely rejecting a safe drug. It is concluded that the sensitive isolated CM assay, obtained from remodelled or normal hearts, lacks specificity to recognize moxifloxacin as a safe drug. Clusters of hESC-CM are an attractive *in vitro* alternative for *in vivo* models as they combine sufficient sensitivity with high specificity.

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Conflict of interest

Peter Sartipy is employed by Cellartis AB.

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