Original Article

Accurate detection of drug-induced delayed ventricular repolarization with a suitable correction formula in Langendorff guinea pig heart

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ABSTRACT — The aims of this study were to determine a suitable method to correct the ventricular repolarization period against the RR interval in isolated perfused Langendorff guinea pig heart and to clarify the reliability of this model using several drugs. QT and RR intervals from an electrocardiogram and the epicardial monophasic action potential duration (MAP_{90}) were measured. Two drugs clinically known to be QT-prolonging (E-4031, moxifloxacin) and two known to be non-QT-prolonging (verapamil, zatebradine) were used for the study. To determine a method of correcting the ventricular repolarization period against RR interval, heart rates were slowed with 0.3 μ M zatebradine, a specific bradycardiac agent, and then accelerated with atrial pacing to obtain a wide range of MAP_{90} /RR relationships. An exponential rate-correction model elicited the most appropriate algorithm for the relationship among the four models tested. Based on linear regression analysis, the exponential showed superior dissociation of corrected MAP_{90} s against RR intervals than generic Bazett's and Fridericia's formulae. E-4031 and moxifloxacin prolonged the corrected QT (QTc) intervals and MAP_{90} under atrial pacing at a cycle length of 0.25 sec ($MAP_{90(pacing)}$) dose-dependently; verapamil and zatebradine failed to prolong them, indicating that the reliability of this model was excellent. $MAP_{90(pacing)}$ prolongation by moxifloxacin, the positive compound in the clinical "Thorough QT/QTc Study", was seen at around QTc-prolonging concentrations in clinic, suggesting that the sensitivity would be appropriate for QT evaluation. We therefore concluded that the isolated guinea pig heart model is sufficiently sensitive and useful for assessing the potential QT prolongation of drugs.

Key words: QT interval prolongation, Exponential rate-correction, Isolated perfused guinea pig heart, Langendorff, Moxifloxacin

INTRODUCTION

Recent clinical observations have raised concerns regarding the QT interval prolonging potential of non-cardiac drugs (De Ponti *et al.*, 2001) and the pharmaceutical industry needs a reliable preclinical assay to screen for potential risk. Most drugs that increase the QT interval prolong the cardiac action potential duration by inhibiting rapidly activating delayed rectifier K^+ currents (I_{Kr}) encoded by the human ether-a-go-go-related gene (hERG) (Redfern *et al.*, 2003). Hence, a hERG assay using the patch-clamp method (Guth *et al.*, 2004; Kirsch *et al.*, 2004) has been widely performed in early-stage develop-

ment when only small amounts of test drugs are available, but the assay is known to sometimes show false positive signals (Redfern *et al.*, 2003). Recently, an isolated perfused guinea pig heart model using the Langendorff method was proposed for early screening of QT risk because of its expected high reliability (Hamlin *et al.*, 2004; Guo *et al.*, 2009) and small amount of test drug required.

When examining the effect of a drug on QT interval, it is necessary to correct the QT intervals against RR intervals using an appropriate QT/RR correction method because the QT interval adapts to changes in the RR interval (or heart rate). Concerning guinea pigs, the general correction formula proposed by Bazett (1920) or

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Fridericia (1920) has been used for correcting QT intervals against RR intervals in isolated hearts (Hamlin *et al.*, 2004; Guo *et al.*, 2009) and in anesthetized (Hamlin *et al.*, 2003a; Testai *et al.*, 2004; Hauser *et al.*, 2005; Tabo *et al.*, 2007) or conscious animals (Hamlin *et al.*, 2003a). However, there is inadequate information about which equation corrects the QT interval appropriately for the RR interval in isolated guinea pig hearts. Cardio-acceleration by electrical pacing and/or treatment with drugs which change the heart rate were performed to construct a relationship between QT and RR intervals over a wide range to develop suitable QT/RR correction methods (Hamlin *et al.*, 2003b; Tabo *et al.*, 2006). Zatebradine is a specific bradycardiac agent that blocks the hyperpolarization-activated pacemaker current (I_h) and was reported to reduce the heart rate without changing the actual ventricular repolarization period in isolated guinea pig cardiac preparations (Kobinger *et al.*, 1984; Hamlin *et al.*, 2004), indicating that zatebradine would be appropriate for establishing plots of the ventricular repolarization period against the RR interval in isolated guinea pig hearts.

A recent guidance of the International Conference on Harmonization (ICH) for clinical studies on delayed ventricular repolarization (ICH E14) requires the reliable detection of changes in QT intervals using a positive control. Moxifloxacin, a fluoroquinolone antibiotic known to prolong QT interval (Balfour and Wiseman, 1999), has been used as the standard positive compound to evaluate the sensitivity of clinical studies to detect small but significant increases in the QT interval (Shah, 2005), and data on the effect of moxifloxacin on QT interval in clinic is abundant at present. Thus, when demonstrating the appropriateness of preclinical assays for predicting QT risk in human, it is necessary to confirm the reactivity of moxifloxacin and compare it with clinical outcomes (Chen *et al.*, 2005; Chaves *et al.*, 2006; Holzgrefe *et al.*, 2007; Komatsu *et al.*, 2010). To our knowledge, however, there are no such reports at present regarding the guinea pig Langendorff model.

The purpose of the present study was twofold. (1) To clarify the relationship between ventricular repolarization periods and RR intervals under the combination of zatebradine treatment and atrial pacing to validate the equations for correcting QT interval against RR interval in isolated guinea pig heart model. (2) To clarify the reliability and sensitivity of the present model by evaluating the reactivity of moxifloxacin (the standard positive compound in the clinical "Thorough QT/QTc Study") and E-4031 (a selective I_{K_r} channel blocker), both of which are known to prolong QT interval, and verapamil (a multi-

channel blocker) and zatebradine (a specific bradycardiac agent), both known to not prolong QT interval.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs obtained from Japan SLC, Inc. (Shizuoka, Japan), weighing from 507.6 to 814.9 g, were used in this study. The animals received solid food and water *ad libitum* and were individually housed in stainless steel cages (W: 350 mm, D: 420 mm, H: 200 mm) in environmentally controlled, HEPA-filtered rooms with 12 hr of light (7:00-19:00), a temperature range of $23 \pm 3^\circ\text{C}$, and a relative humidity range of $55 \pm 20\%$. The care of the animals and the present protocols complied with the "General Consideration for Animal Experiments" promulgated in Chugai Pharmaceutical Co., Ltd. and was approved by the Institutional Animal Care and Use Committee.

Drugs

Zatebradine hydrochloride and verapamil hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA), E-4031 hydrochloride from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and moxifloxacin hydrochloride from CHEMOS GmbH (Regensburg, Germany). Stock solutions of test substances were prepared by dissolving the substances in dimethyl sulfoxide (DMSO, Junsei Chemical Co., Ltd., Tokyo, Japan) and then each stock solution was sequentially diluted to the proper concentrations with DMSO. The DMSO-diluted solutions were then diluted 1000-fold with Krebs-Henseleit solution immediately before use to achieve the desired final concentration, so that the final concentration of DMSO in the perfusate was 0.1% (v/v). The composition of Krebs-Henseleit solution included (in mM) NaCl, (120.0), KCl (4.7), Na-pyruvate (2.0), CaCl_2 (1.8), MgSO_4 (1.2), KH_2PO_4 (1.2), NaHCO_3 (25.0), and glucose (11.1).

Electrophysiological measurement in Langendorff guinea pig heart

Guinea pigs were anesthetized by an intraperitoneal injection of pentobarbital (60 mg/kg). With the animals restrained in a supine position, a tracheotomy was performed, and the animals were artificially ventilated with room air (90 counts/min, 5 ml/kg). Then the heart was quickly removed and its aorta was cannulated for perfusion of the coronary artery according to the Langendorff method with constant pressure (initial perfusion pressure: 60-70 mmHg). The heart was perfused at a temperature

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of $37.0 \pm 0.5^\circ\text{C}$ and a flow rate of 15 ± 5 ml/min with Krebs-Henseleit solution continuously aerated with a mixture of 95% O_2 and 5% CO_2 . The perfusion flow rate was measured with an electromagnetic flow meter (MFV2100, Nihon Kohden, Tokyo, Japan). Two electrocardiogram (ECG) electrodes were held lightly against the epicardium, the positive one on the left ventricle, the negative one on the right atrium. A monophasic action potential (MAP) recording electrode was attached to the wall of the left ventricle to obtain an epicardial MAP signal. A bipolar electrode was attached onto the right atrium for pacing (2 msec duration, 1.2-1.5 times the threshold voltage) by an electronic stimulator (SEN-3201, Nihon Kohden). The ECG and MAP were amplified with an RMP-6008 amplifier system (Nihon Kohden) and a DAM50 system (World Precision Instruments, Sarasota, FL, USA), respectively. All signals were collected on a computer with software

(WinVAS3 Version1.1, Physiotec, Tokyo, Japan) at 1 kHz and analyzed using the same software. The heart was allowed to stabilize for ≥ 30 min after being mounted on the Langendorff apparatus and studies were commenced after stabilizing the ECG and MAP signals.

Correction of ventricular repolarization period against RR interval

Fig. 1A illustrates the experimental procedure. To establish the relationship between the ventricular repolarization period (QT interval, MAP duration) and the RR interval over a wide range, five Langendorff hearts were initially perfused with zatebradine ($0.3 \mu\text{M}$) to reduce the heart rate. Zatebradine (0.03 , 0.1 , $0.3 \mu\text{M}$) showed a dose-dependent reduction in heart rate and $0.3 \mu\text{M}$ was selected as the treatment concentration because it caused continuous and stable reduction in heart rate by about 30% with-

(A) Relationship between QT/MAP₉₀ and RR

(B) Evaluation of test drugs

Fig. 1. Experimental protocol. (A) The protocol to find the relationship between the ventricular repolarization period and RR interval consisted of a minimum 30 min stabilization period once the heart perfusion was started, and then continuous infusion of zatebradine at $0.3 \mu\text{M}$ to reduce heart rate, followed by graded atrial pacing. (B) The protocol for the evaluation of test compounds consisted of a minimum 30 min stabilization period once the heart perfusion was started, and then measurement of pre-value, followed by 3 periods of perfusion of 15 min each for the vehicle control or test compound exposure at 3 ascending concentrations. In both experiments, ECG and MAP were monitored and recorded continuously throughout the experiment. At the horizontal bars, atrial pacing for 30 sec was performed to obtain each parameter under constant heart rate.

out affecting the actual repolarization period (Table 2). After stabilizing the heart rate under the continuous perfusion of zatebradine for at least 15 min, cardio-acceleration was achieved with right atrial pacing. The pacing at several rates was conducted for 30 sec each to fix the heart rate. The first pacing rate was set to slightly above each resting heart rate under continuous perfusion of zatebradine and the heart rate was accelerated according to every 20-msec shortening in the cycle length. Data samples were accepted only when the end of the T wave or the MAP had clearly returned to the baseline. As shown in Fig. 2B, the data of the MAP durations at 90% of the action potential repolarization (MAP_{90}) was available at broad pacing rates compared with that of QT interval, and so the plots of MAP_{90} (in sec) against RR (in sec) were fitted using the following fitting models to determine the most suitable equation:

Linear model: $MAP_{90} = a + b \times RR$

Parabolic model: $MAP_{90} = a + b \times RR^c$

Hyperbolic model: $MAP_{90} = a + b/RR$

Exponential model: $MAP_{90} = a + b \times e^{-c \times RR}$

The average MAP_{90} and RR values of five preparations ($n = 4$ or 5 at each RR interval: Fig. 2B) were used for calculating the constant value of each equation (b and c) by the least-squares regression method; a was an intercept of each equation. Then, the MAP_{90}/RR of each preparation was refitted by the determined equation shown in Table 1. For the comparative evaluation of the four equations, the fitting accuracy was assessed according to the minimum Akaike information criteria (AIC) (Akaike, 1974). When the AIC value was smallest, the model was considered to have fit the ventricular repolarization period against the

RR interval most effectively. The fitting procedure and AIC calculations were conducted using Origin 8 analysis software (Lightstone Corp., Tokyo, Japan).

Next, the MAP_{90} was corrected against the RR interval by two generic formulae from Bazett (1920) and Fridericia (1920) and the most effective model (exponential model) selected. The corrected MAP_{90} s were re-expressed to a reference cycle length ($RR_{ref} = 0.3$ sec) in order to compare the efficacies of the three correcting methods. The reference cycle length was determined according to the intrinsic heart rate of isolated guinea pig hearts (approximately 200 beats/min) (Table 2). The generic formulae and the exponential rate-correction methods used in this study were:

Corrected $MAP_{90}B = RR_{ref}^{0.5} \times MAP_{90}/RR_{raw}^{0.5}$ (Bazett)

Corrected $MAP_{90}F = RR_{ref}^{0.333} \times MAP_{90}/RR_{raw}^{0.333}$ (Fridericia)

Corrected $MAP_{90}E = MAP_{90} - b \times (e^{-c \times RR_{raw}} - e^{-c \times RR_{ref}})$ (Exponential model)

where MAP_{90} is the uncorrected raw MAP_{90} in sec, RR_{raw} is the RR interval associated with the raw MAP_{90} in sec, and RR_{ref} is the reference cycle length in sec.

Effects of test substances

Fig. 1B illustrates the experimental procedure. Before the application of the test substances, two basal recordings of both the sinus rhythm and atrial pacing at the rate of 240 beats/min (cycle length of 0.25 sec) for 30 sec were made 10 min apart as the control; the average of the two recordings was used as the baseline for each condition. After the second control recording, the first concentration of each test substance was perfused for 15 min. After sampling the data at sinus rhythm, atrial pacing was conducted for 30 sec to collect the data on the constant heart rate. The next concentration of the test substance was then perfused. Three consecutive concentrations were perfused cumulatively during each experiment. Solvent control (0.1% DMSO) was perfused 3 times to control preparations in the same manner as the test substance groups. The concentrations of test substances were 0.003, 0.03, and 0.3 μ M for E-4031; 10, 30, and 100 μ M for moxifloxacin; 0.01, 0.1, and 0.3 μ M for verapamil; and 0.03, 0.1, and 0.3 μ M for zatebradine.

The data obtained were analyzed during the 5 sec just before the start of pacing (sinus rhythm data) and the last 5 sec of each pacing sequence (pacing data). The parameters analyzed were heart rate, QT interval, RR interval at sinus rhythm, and MAP_{90} at atrial pacing ($MAP_{90(pacing)}$). The value of each parameter at each time point was the average of values analyzed from individual raw waveform obtained during the 5 sec. The QT interval at sinus

Table 1. Relative accuracy of fittings by linear, parabolic, hyperbolic and exponential models for MAP_{90} -RR plots in isolated guinea pig hearts under zatebradine (0.3 μ M) treatment and atrial pacing

Model	Equation	AIC
Linear	$MAP_{90} = a + 0.267 \times RR$	-151.7 ± 23.9
Parabolic	$MAP_{90} = a + 43.6 \times RR^{0.00197}$	-176.7 ± 30.4
Hyperbolic	$MAP_{90} = a - 0.0257/RR$	-173.1 ± 28.7
Exponential	$MAP_{90} = a - 0.283 \times e^{-5.54 \times RR}$	-186.5 ± 29.5

Values of Akaike's Information Criterion (AIC) are expressed as mean $\pm$ S.D. of five isolated hearts.

MAP_{90} : the duration of the monophasic action potential at 90% repolarization level. a : intercept of each equation.

Langendorff guinea pig heart for QT prediction

(A)

(B)

Fig. 2. Relationship between ventricular repolarization periods and RR interval in isolated guinea pig hearts. (A) Typical waveforms of ECG and MAP at several pacing rates (before pacing, cycle lengths of 400, 300 and 200 msec) under continuous perfusion of zatebradine at 0.3 μ M. The QT interval at cycle length of 200 msec could not be determined because the T wave overlapped the subsequent P wave. (B) QT-RR/MAP₉₀-RR relationships. $\blacktriangle$, $\bullet$: Average QT interval ($\blacktriangle$) and MAP₉₀ ($\bullet$) during graded atrial pacing under zatebradine (0.3 μ M) treatment (4 or 5 available data at each RR interval) from 5 preparations. Δ , $\circ$: Individual QT interval (Δ) and MAP₉₀ ($\circ$) at sinus rhythm after treatment of zatebradine (0, 0.03, 0.1, or 0.3 μ M) in 5 preparations. MAP₉₀: the duration of the monophasic action potential at 90% repolarization level. CL: cycle length.

Table 2. QTc interval, MAP_{90(pacing)}, and heart rate after application of test drugs or solvent in isolated guinea pig hearts

Treatment	Parameters		Baseline	Change from baseline		
				1st	2nd	3rd
Solvent	Concentration	(μ M)		-	-	-
	QTc	(sec)	0.191 $\pm$ 0.006	0.002 $\pm$ 0.001	0.001 $\pm$ 0.003	0.002 $\pm$ 0.004
	MAP _{90(pacing)}	(sec)	0.148 $\pm$ 0.002	0.000 $\pm$ 0.002	0.001 $\pm$ 0.004	0.001 $\pm$ 0.006
	Heart rate	(bpm)	202 $\pm$ 19	1 $\pm$ 6	-1 $\pm$ 7	-3 $\pm$ 8
E-4031	Concentration	(μ M)		0.003	0.03	0.3
	QTc	(sec)	0.188 $\pm$ 0.004	0.009 $\pm$ 0.002**	0.031 $\pm$ 0.010**	0.050 $\pm$ 0.014**
	MAP _{90(pacing)}	(sec)	0.149 $\pm$ 0.003	0.005 $\pm$ 0.002**	0.020 $\pm$ 0.005**	0.027 $\pm$ 0.005**
	Heart rate	(bpm)	216 $\pm$ 9	-3 $\pm$ 9	-33 $\pm$ 11**	-54 $\pm$ 11**
Moxifloxacin	Concentration	(μ M)		10	30	100
	QTc	(sec)	0.197 $\pm$ 0.010	0.008 $\pm$ 0.006	0.017 $\pm$ 0.007**	0.054 $\pm$ 0.010**
	MAP _{90(pacing)}	(sec)	0.156 $\pm$ 0.007	0.006 $\pm$ 0.003*	0.011 $\pm$ 0.004**	0.029 $\pm$ 0.005**
	Heart rate	(bpm)	198 $\pm$ 8	-8 $\pm$ 8	-19 $\pm$ 6**	-48 $\pm$ 17**
Verapamil	Concentration	(μ M)		0.01	0.1	0.3
	QTc	(sec)	0.188 $\pm$ 0.006	0.001 $\pm$ 0.004	-0.004 $\pm$ 0.005	-0.010 $\pm$ 0.010*
	MAP _{90(pacing)}	(sec)	0.148 $\pm$ 0.006	-0.001 $\pm$ 0.002	-0.004 $\pm$ 0.008	-0.011 $\pm$ 0.014
	Heart rate	(bpm)	193 $\pm$ 13	-6 $\pm$ 8	-8 $\pm$ 11	-24 $\pm$ 8**
Zatebradine	Concentration	(μ M)		0.03	0.1	0.3
	QTc	(sec)	0.194 $\pm$ 0.005	0.001 $\pm$ 0.002	0.001 $\pm$ 0.006	0.006 $\pm$ 0.005
	MAP _{90(pacing)}	(sec)	0.152 $\pm$ 0.004	0.000 $\pm$ 0.002	0.002 $\pm$ 0.001	0.003 $\pm$ 0.004
	Heart rate	(bpm)	195 $\pm$ 19	7 $\pm$ 8	-17 $\pm$ 12*	-60 $\pm$ 20**

Each value represents the mean $\pm$ S.D. of five isolated hearts except for the MAP_{90(pacing)} for moxifloxacin at 100 μ M (n = 3), and verapamil at 0.3 μ M (n = 4). MAP_{90(pacing)}: the duration of the monophasic action potential at 90% repolarization level at a cycle length of 0.25 sec, bpm: beats/min, QTc: QT interval corrected by the exponential equation, $QTc = QT + 0.283 \times (e^{-5.54 \times RR} - e^{-5.54 \times 0.3})$; QT, RR (in sec). *: P < 0.05, **: P < 0.01; statistically significant difference from the time-matched solvent control group.

rhythm was then corrected against the RR interval by the best equation determined in the first study (QTc interval). The QT correction equation was an exponential equation adjusted to the reference cycle of 0.3 sec as follows: $QTc = QT - b \times (e^{-c \times RR} - e^{-c \times 0.3})$, where QT and RR intervals are in sec.

Statistical analysis

To compare the three correction methods, linear regres-

sion equations for the corrected MAP₉₀ against the RR interval were obtained by the method of least squares, and the slope and coefficient of variation (CV) of the equations were calculated. When the slope was close to zero with a small CV, the correction method was considered to have most effectively eliminated the effect of heart rate change on the ventricular repolarization period. The slope and CV obtained from the exponential rate-correction were statistically compared with Bazett's or Fridericia's

formula using a *t*-test.

To evaluate the effects of the test compounds, the baseline values for each parameter were compared among the test groups using one-way analysis of variance (ANOVA) and changes from the baseline value were calculated. For a few groups, MAP_{90(pacing)} data could not be obtained in some of the preparations because of an impossibility to pace the heart. Since the number datasets available for all the groups was three or above, all values were statistically compared with the time-matched solvent control values using an unpaired *t*-test.

Values in the data are the mean $\pm$ S.D. These statistics were calculated using the SAS system (SAS Institute, Cary, NC, USA) and values of $P < 0.05$ were considered significant.

RESULTS

Correction of ventricular repolarization period against RR interval

Fig. 2 shows typical waveforms of ECG and MAP obtained from a single isolated heart and the relationship between ventricular repolarization periods (QT interval and MAP₉₀) and RR intervals obtained from five isolated hearts under zatebradine (0.3 μ M) treatment and the graded atrial pacing. Both QT interval and MAP₉₀ appeared to correlate well with RR interval in a like manner, but the available range of the MAP₉₀/RR relationship was wider than that of the QT/RR relationship because the T wave overlapped the start of the subsequent P wave at higher pacing rates. In addition, the tendency of repolarization periods against RR intervals obtained by atrial pacing was almost equivalent to that obtained by the pharmacological effect of zatebradine (0.03, 0.1 and 0.3 μ M) (Fig. 2B). These findings suggest that it would be reasonable to correct the QT interval at sinus rhythm using a formula established from the MAP₉₀/RR relationship at pacing. Therefore, the MAP₉₀/RR relationship under atrial pacing was fit to four models (linear, parabolic, hyperbolic or exponential) to determine a suitable fitting equation (Fig. 3). The four models were ranked based on AIC values resulting in Exponential < Parabolic < Hyperbolic < Linear (Table 1), indicating that the exponential showed the most appropriate fitting among the four.

In order to compare the correcting ability of the exponential equation with that of generic formulae widely used for guinea pig heart, the MAP₉₀-RR data were expressed as the corrected MAP₉₀ against RR from the application of two generic formulae (Bazett's and Fridericia's) and the exponential rate-correction equation (Fig. 4). The slope of the linear regression obtained from the exponential

(0.0027 ± 0.0183) showed less variability and was closer to zero than from Fridericia's (0.1009 ± 0.0350), but not from Bazett's (0.0115 ± 0.0225). The CV of the exponential ($0.9 \pm 0.3\%$) was significantly smaller than Bazett's ($2.2 \pm 0.7\%$) and Fridericia's ($2.6 \pm 0.8\%$). These indicate that the present exponential correction method showed superior correcting ability than traditional formulae.

Effects of E-4031, moxifloxacin, verapamil and zatebradine

There were no significant differences among test groups of baseline values in any of the parameters measured. The effects of the test drugs on QTc interval, MAP_{90(pacing)} and heart rate are shown in Table 2. The QTc interval was obtained using the exponential rate-correction method established in this study. Three applications of the solvent control (0.1% DMSO) showed no effects on any parameters tested. E-4031 significantly prolonged QTc interval and MAP_{90(pacing)} at 0.003 μ M and above in a dose-dependent manner. The prolongations from the baseline of QTc interval and MAP_{90(pacing)} were 4.7% and 3.6% at 0.003 μ M, 16.5% and 13.5% at 0.03 μ M, and 26.6% and 18.2% at 0.3 μ M, respectively. Moxifloxacin showed no effects on any parameters tested at 1 μ M (data not shown, $n = 5$), but significantly prolonged MAP_{90(pacing)} at 10, 30 and 100 μ M by 3.7%, 7.2% and 18.8%, respectively, from the baseline and QTc interval at 30 and 100 μ M by 8.5% and 27.4%, respectively, in a dose-response manner. In addition to the QTc/MAP_{90(pacing)} prolongations, both E-4031 and moxifloxacin also decreased the heart rate dose-dependently. Verapamil showed no effects on any parameters up to 0.1 μ M, but decreased heart rate and shortened QTc interval at 0.3 μ M, possibly caused by Ca²⁺ channel blocking activity. Zatebradine, an I_h channel blocker, decreased the heart rate at 0.1 μ M and above in a dose-dependent relationship without affecting QTc interval and MAP_{90(pacing)}. No arrhythmias developed from any of the test drugs or solvent control. Atrial pacing was not possible in 2/5 and 1/5 of the preparations of perfused moxifloxacin at 100 μ M and verapamil at 0.3 μ M, respectively, probably because each refractory period was longer than the interstimulus interval.

Comparison of MAP_{90(pacing)} prolongation in guinea pig heart with hERG inhibition and clinical outcomes regarding QT prolongation

Table 3 shows the minimum MAP_{90(pacing)}-prolonging concentrations of E-4031 and moxifloxacin in guinea pig hearts compared with 50% inhibitory concentrations in hERG (Zhou *et al.*, 1998; Kirsch *et al.*, 2004; Chen *et al.*, 2005; Lacroix *et al.*, 2003) and observations associ-

Fig. 3. Plots of MAP_{90} versus RR relation obtained from a single isolated heart fitted with four models; linear, parabolic, hyperbolic and exponential. Solid line is the regression line and each equation is shown in the figure. MAP_{90} : the duration of the monophasic action potential at 90% repolarization level.

ated with QT prolongation in clinic (Omata *et al.*, 2005; Balfour and Wiseman, 1999; Démolis *et al.*, 2000). Verapamil and zatebradine are not shown because QT interval prolongation was not observed in this study or in human (Gonzalez-Gomez *et al.*, 1988; Franke *et al.*, 1987); only hERG inhibitory potency of verapamil is shown (Zhang *et al.*, 1999; Kirsch *et al.*, 2004). In the comparison with hERG inhibitory potency, E-4031 and moxifloxacin showed a good relationship, but verapamil failed to link with hERG. On the other hand, the potent $MAP_{90(pacing)}$ observed in guinea pig hearts was consistent with clinical QT outcomes for all of the four test drugs.

DISCUSSION

The present study aimed to illustrate the utility of an *ex vivo* assay for detecting QT liability of drugs recently in the limelight, the isolated perfused guinea pig heart mod-

el using the Langendorff method. Concerning the relationship between ventricular repolarization period and RR interval, an exponential equation showed excellent rate-correcting ability. When corrected using the exponential equation, the QT-prolonging drugs tested showed dose-dependent prolongations in QTc interval and $MAP_{90(pacing)}$, whereas the non-QT-prolonging drugs did not. These results were consistent with clinical observations, suggesting that the Langendorff heart model with the present suitable correction method would be useful for predicting clinical QT liability of drugs.

Ventricular MAP duration is considered to be related to QT interval in anesthetized guinea pigs (Takahara *et al.*, 2005; Tabo *et al.*, 2007) and was found to be so in the present Langendorff study. Since MAP_{90} was more available than QT interval at higher pacing rates, we used a wide range relationship of MAP_{90}/RR in order to determine the appropriate correction method. Among the four

Fig. 4. Corrected MAP₉₀ against RR interval. A: Typical plot for a single isolated heart (same preparation as in Fig. 3). The corrected MAP₉₀ was derived from an exponential rate-correction (▲: MAP₉₀E = MAP₉₀ + 0.283 × (e^{-5.54×RR} - e^{-5.54×0.3})), Bazett's formula (△: MAP₉₀B = 0.3^{0.5} × MAP₉₀/RR^{0.5}), or Fridericia's formula (○: MAP₉₀F = 0.3^{0.333} × MAP₉₀/RR^{0.333}) at a reference cycle length of 0.3 sec (200 bpm). The regression line for exponential rate-correction is shown in the figure. B: Slopes and CV of linear regression line for corrected MAP₉₀-RR plots from exponential rate-correction, Bazett's, and Fridericia's formulae. Data are plotted for each preparation (n = 5) and values are the mean ± S.D. P < 0.01: Significantly different from exponential rate-correction by unpaired *t*-test. N.S.: Not significantly different from exponential rate-correction by unpaired *t*-test. MAP₉₀: the duration of the monophasic action potential at 90% repolarization level. CV: coefficient of variations.

Table 3. MAP_{90(pacing)}-prolonging concentrations of test drugs in guinea pig hearts, including free plasma concentrations associated with QT prolongation in clinic and hERG channel inhibition

	MAP _{90(pacing)} -prolonging conc. in guinea pig hearts (μM)	Free plasma conc. at clinical QT prolongation ^a (μM)	hERG ^a IC ₅₀ (μM)
E-4031	0.003	0.001 ^b	0.0077-0.017 ^f
Moxifloxacin	10	3.2-4.2 ^c	35.7-74.7 ^g
Verapamil	-	- ^d	0.143-0.37 ^h
Zatebradine	-	- ^e	N.D.

MAP_{90(pacing)}: the duration of the monophasic action potential at 90% repolarization level at a cycle length of 0.25 sec, -: No QT prolongation, N.D.: No data available.

^a Includes previously reported data, ^b Omata *et al.*, 2005, ^c Balfour and Wiseman, 1999 and Démolis *et al.*, 2000, ^d Gonzalez-Gomez *et al.*, 1988, ^e Franke *et al.*, 1987, ^f Zhou *et al.*, 1998 and Kirsch *et al.*, 2004, ^g Lacroix *et al.*, 2003 and Chen *et al.*, 2005, ^h Zhang *et al.*, 1999 and Kirsch *et al.*, 2004.

fitting models we applied, similarly tested in other studies (Matsunaga *et al.*, 1997; Stubhan *et al.*, 2008), the exponential model fit the MAP₉₀ against the RR interval most effectively with the smallest AIC values for the fitting line. On the other hand, Cheng *et al.* (2006) reported that linear and parabolic models fit the QT/RR relationship in isolated guinea pig hearts, while the exponential one did not. The difference from the current result was not clear, but the available RR range to fit models in Cheng's study (286-375 msec) was apparently narrower than that in this study (200-460 msec), which might affect the discrepancy. Bazett's and Fridericia's correction formulae have been generally used for QT rate-correction in guinea pig hearts (Hamlin *et al.*, 2004; Testai *et al.*, 2004), although the propriety has not necessarily been verified. Based on linear regression analysis of the corrected MAP₉₀ against the RR interval, the present exponential equation dissociated the RR influence better than Bazett's or Fridericia's with less slope and the lowest CV for the linear regression. As with the present isolated heart, an exponential model was also found to be most suitable for QT rate-correction in dogs and human (Matsunaga *et al.*, 1997; Sarma *et al.*, 1984). This is the first report to demonstrate that the exponential rate-correction method corrected the QT/MAP₉₀ against RR more effectively than the widely-used generic formulae in isolated guinea pig heart.

Moxifloxacin, one of the few available positive compounds for the clinical "Thorough QT/QTc Study" assessing the QT-prolonging potential of drugs (Shah, 2005), was used because it showed a slight but significant QTc interval prolongation at therapeutic doses in clinic (Balfour and Wiseman, 1999; Démolis *et al.*, 2000). Moxifloxacin caused dose-dependent increases in QTc interval and

MAP_{90(pacing)} at 10 or 30 μM and above in the present study. Since the IC₅₀ value of moxifloxacin on hERG currents is 35.7-74.7 μM (Lacroix *et al.*, 2003; Chen *et al.*, 2005), the QTc/MAP_{90(pacing)}-prolonging effects would be elicited by the I_{Kr}/hERG channel blockade. The increase in MAP_{90(pacing)} at 10 μM (3.7%) was comparable to the increases in QTc interval (4.0%) observed in humans at therapeutic doses of 400 mg, which attained a peak level of 1.3-1.7 $\mu\text{g/ml}$ (3.2-4.2 μM) of free plasma concentration (Balfour and Wiseman, 1999; Démolis *et al.*, 2000). Therefore, the sensitivity of the present model was very close to sensitivity in clinic, suggesting that this model is sufficiently sensitive to detect the clinical QT-prolonging risk of drugs.

Since cardiac repolarization period is controlled by various cardiac ion channel activities such as I_{Kr} and also I_{Na}, I_{Ca}, multi-channel blockers sometimes show discrepancies between I_{Kr}/hERG inhibitory activity and effects on ventricular repolarization period (Redfern *et al.*, 2003; Tabo *et al.*, 2007, 2008a, 2008b). Indeed, verapamil, a multi-channel blocker, has hERG inhibitory potential with an IC₅₀ value of 0.143-0.37 μM (Zhang *et al.*, 1999; Kirsch *et al.*, 2004), but shortened QTc interval at 0.3 μM . On the other hand, E-4031, a selective I_{Kr} channel blocker, showed significant prolongations in QTc interval and MAP_{90(pacing)} at 0.003 μM in guinea pig hearts, nearly consistent with its potent blockade of hERG (IC₅₀ of 0.0077-0.017 μM) (Zhou *et al.*, 1998; Kirsch *et al.*, 2004). A hERG assay using the patch-clamp method is known to be the most sensitive *in vitro* assay for evaluation of the risk of QT interval prolongation (Webster *et al.*, 2002) and is widely used for QT screening; however, the hERG assay has been known to occasionally show false positive sig-

Langendorff guinea pig heart for QT prediction

nals, as mentioned above. Thus, the present Langendorff heart model would be desirable for accurately assessing the QT risk of drugs in early-stage development.

There are some limitations in this study. First, the present guinea pig Langendorff heart model could not include the influences of hormonal effect, autonomic nervous activity, etc., which are known to affect the QT-prolonging potential of drugs *in vivo* (Harada *et al.*, 2005; Nakamura *et al.*, 2007; Kurokawa *et al.*, 2008; Kakusaka *et al.*, 2009). On the other hand, as an advantage, the present *ex vivo* model could evaluate the direct effects of drugs on cardiac repolarization period without these confusing influences. Next, test drugs were perfused only for 15 min in this study. The short exposure might make it difficult to detect the action of drugs which are slow to diffuse into tissues (e.g. astemizole, terfenadine) (Cavero *et al.*, 1999). When such a drug is evaluated, another experimental condition in which cardiac parameters are stable for a longer period is recommended. Finally, the correcting ability of the exponential QT correction formula established in this study showed somewhat individual variation (Fig. 4B). The variation was probably caused by the present methodological procedure in which a new correction formula was established from the average data of five preparations. The best way to minimize the variation would be to create individual correction formulae for each preparation by applying several bradycardiac or tachycardiac drugs before evaluating test substances; however, this would be practically difficult. In spite of this limitation, the individual variation of correcting ability in the exponential formula was smaller than those in Bazett and Fridericia which have been widely used in guinea pig hearts (Hamlin *et al.*, 2004; Hauser *et al.*, 2005) and thus the present exponential formula would be better utilized rather than the generally used formulae.

In conclusion, the present study demonstrates that the guinea pig isolated Langendorff heart model has suitable reliability and sensitivity for clarifying drug-induced QT interval prolongation. Regarding a QT correcting method, general correction formulae such as Bazett's and Fridericia's have been applied to guinea pig models, but, in the present study, an exponential equation was most effective among the several methods tested. The present model accurately assesses QT risk requiring only a small amount of test article and thus is suitable for evaluating compounds in early-stage drug development.

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