

## RESEARCH PAPER

# Potential of E-4031-induced torsade de pointes by HMR1556 or ATX-II is not predicted by action potential short-term variability or triangulation

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**Background and purpose:** Torsade de pointes (TdP) can be induced by a reduction in cardiac repolarizing capacity. The aim of this study was to assess whether  $I_{Ks}$  blockade or enhancement of  $I_{Na}$  could potentiate TdP induced by  $I_{Kr}$  blockade and to investigate whether short-term variability (STV) or triangulation of action potentials preceded TdP.

**Experimental approach:** Experiments were performed in open-chest, pentobarbital-anaesthetized,  $\alpha_1$ -adrenoceptor-stimulated, male New Zealand White rabbits, which received three consecutive i.v. infusions of either the  $I_{Kr}$  blocker E-4031 (1, 3 and 10 nmol kg<sup>-1</sup> min<sup>-1</sup>), the  $I_{Ks}$  blocker HMR1556 (25, 75 and 250 nmol kg<sup>-1</sup> min<sup>-1</sup>) or E-4031 and HMR1556 combined. In a second study rabbits received either the same doses of E-4031, the  $I_{Na}$  enhancer, ATX-II (0.4, 1.2 and 4.0 nmol kg<sup>-1</sup>) or both of these drugs. ECGs and epicardial monophasic action potentials were recorded.

**Key results:** HMR1556 alone did not cause TdP but increased E-4031-induced TdP from 25 to 80%. ATX-II alone caused TdP in 38% of rabbits, as did E-4031; 75% of rabbits receiving both drugs had TdP. QT intervals were prolonged by all drugs but the extent of QT prolongation was not related to the occurrence of TdP. No changes in STV were detected and triangulation was only increased after TdP occurred.

**Conclusions and implications:** Giving modulators of ion channels in combination substantially increased TdP but, in this model, neither STV nor triangulation of action potentials could predict TdP.

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**Keywords:** ATX-II; E-4031; HMR1556;  $I_{Kr}$ ;  $I_{Ks}$ ;  $I_{Na}$ ; K<sup>+</sup> channel blockers; proarrhythmia; repolarization reserve; torsade de pointes

**Abbreviations:** APD<sub>90</sub>, action potential duration at 90% repolarization; ATX-II, sea anemone toxin; AV, atrio-ventricular;  $I_{Kr}$ , delayed rectifier potassium current;  $I_{Kr}$ , rapidly activating delayed rectifier potassium current;  $I_{Ks}$ , slowly activating delayed rectifier potassium current;  $I_{Na}$ , sodium current; MAP, monophasic action potential; STV, short-term variability; TdP, torsade de pointes

## Introduction

Torsade de pointes (TdP) is a polymorphic ventricular tachycardia, which is commonly associated with prolongation of the QT interval of the ECG. The electrophysiological mechanisms underlying this arrhythmia are not completely understood, but it has been proposed that TdP can occur when the repolarizing capacity of the cardiac ventricles is reduced (Roden, 1998). In the normal myocardium, several potassium currents contribute to repolarization (and hence the duration of the action potential and the QT interval),

and if one current is reduced others can compensate; this is known as repolarization reserve (Roden, 1998; Biliczki *et al.*, 2002). Drug-induced blockade of cardiac potassium channels is a known cause of TdP (Haverkamp *et al.*, 2000) and this may be associated with a reduction in repolarization reserve.

The delayed rectifier potassium current ( $I_K$ ) is the main repolarizing current terminating the ventricular action potential in most species. It is voltage- and time-dependent with a rapidly activating ( $I_{Kr}$ ) and slowly activating ( $I_{Ks}$ ) component (Sanguinetti and Jurkiewicz, 1990).  $I_{Kr}$  is blocked by drugs such as dofetilide and E-4031 (Sanguinetti and Jurkiewicz, 1990), whereas, HMR1556 is a potent inhibitor of  $I_{Ks}$  with 1000-fold selectivity for  $I_{Ks}$  over  $I_{Kr}$  (Gögelein *et al.*, 2000). *In vitro* work, which has examined the cardiac electrophysiological effects of these drugs in combination, supports the concept of repolarization reserve, in that, action

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potential prolongation by  $I_{Kr}$  blockade is potentiated when combined with an  $I_{Ks}$  blocker (Varro *et al.*, 2000; Biliczki *et al.*, 2002; Volders *et al.*, 2003; Jost *et al.*, 2005; So *et al.*, 2006). *In vivo* it has also been shown that HMR 1556 potentiated the QT prolongation induced by dofetilide, in the dog, but this was not associated with the occurrence of arrhythmias or of TdP (Nakashima *et al.*, 2004). The reason for the lack of effect of a reduction in repolarization reserve on the occurrence of TdP in this study may be that Nakashima *et al.* (2004) did not use an animal model in which drugs that block  $I_{Kr}$  have been shown to cause TdP.

Increasing inward currents can also contribute to delays in repolarization. DPI 201–106, the plant alkaloid veratridine, and the sea anemone toxin (ATX-II) are compounds that increase sodium current ( $I_{Na}$ ) (Ravens and Himmel, 1999). ATX-II, in particular, has a high potency for cardiac  $I_{Na}$  (Hanck and Sheets, 2007). *In vitro*, ATX-II prolonged repolarization and induced arrhythmias (Song *et al.*, 2004; Wu *et al.*, 2004, 2006; Milberg *et al.*, 2005) including TdP (Shimizu and Antzelevitch, 1997). Furthermore, ATX-II potentiated prolongation of the action potential caused by E-4031 in isolated guinea pig hearts (Wu *et al.*, 2004), and potentiated the proarrhythmic actions of several other  $I_{Kr}$  blockers in isolated rabbit hearts (Wu *et al.*, 2006). However, there is a lack of information on the effects of ATX-II on the induction of TdP *in vivo*.

In recent years there has been growing interest in the need to identify parameters that can predict the occurrence of TdP. It has been suggested that augmentation of triangulation of the action potential (Hondeghe *et al.*, 2001) and temporal dispersion of repolarization (instability) measured as short-term variability (STV) in the duration of a number of consecutive action potentials (Thomsen *et al.*, 2004), rather than QT prolongation *per se*, provide the proarrhythmic substrate and precede TdP. These factors have not yet been examined in open-chest,  $\alpha$ -adrenoceptor-stimulated anaesthetized rabbits.

The first aim of this study was, therefore, to test the hypothesis that TdP will occur when repolarization reserve is severely limited, either by combined blockade of  $I_{Kr}$  and  $I_{Ks}$  or by blockade of  $I_{Kr}$  in combination with prolongation of  $I_{Na}$ , in the  $\alpha_1$ -adrenoceptor-stimulated open-chest pentobarbital-anaesthetized rabbit, which has been well characterized as a model in which drugs that block  $I_{Kr}$  may cause TdP (Carlsson *et al.*, 1990; Batey and Coker, 2002). A second aim was to assess whether changes in beat-to-beat variability of repolarization or triangulation of the action potential could predict the occurrence of TdP in this model. Some of these results have been presented to the International Society for Heart Research (Michael *et al.*, 2006, 2007).

## Methods

### Animal preparation

All animal experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and conducted under the authority of Project Licence number 40/2548. Male New Zealand White rabbits (2.2 to 2.8 kg) were purchased from Harlan (Bicester, UK). Rabbits were anaesthetized by i.v. administration of  $\sim 30$  mg kg<sup>-1</sup> sodium

pentobarbital given 15 min after application of local anaesthetic cream (EMLA 5%) to a marginal ear vein. They were then prepared for the induction of drug-induced arrhythmias as described in detail previously (Batey and Coker, 2002; Farkas and Coker, 2002). ECG limb leads I, II and III were recorded simultaneously along with arterial blood pressure. At certain time points, blood gases, pH and K<sup>+</sup> were also measured. Rabbits were ventilated with room air at 38 strokes per min and a stroke volume of 5 to 6 ml kg<sup>-1</sup> body weight. After opening the chest, a positive end-expiratory pressure of 0.5 to 1 cm water was applied. In most experiments, an epicardial monophasic action potential (MAP) was recorded from the left ventricle. Details of all equipment and recording conditions have been published previously (Farkas and Coker, 2002), except that in the ATX study a Hugo Sachs Elektronik spring-loaded epicardial Ag-AgCl MAP electrode (Linton Instrumentation, Diss, Norfolk, UK) was used to record MAPs.

### Experimental protocols

The standard arrhythmia induction protocol consisted of three cycles of drug administration. In each cycle the  $\alpha$ -adrenoceptor agonist phenylephrine was infused at a rate of 75 nmol kg<sup>-1</sup> min<sup>-1</sup> for 15 min, then the dose of phenylephrine was increased to 150 nmol kg<sup>-1</sup> min<sup>-1</sup> for 3 min followed by further increases to 225 then 300 nmol kg<sup>-1</sup> min<sup>-1</sup> for 3 min each. Five minutes into the first cycle a concurrent infusion of one or more ion channel modulators was started. At the end of the cycle both infusions were switched off and there was a 10 min drug-free interval. The doses of phenylephrine used in the second and third cycle were the same as in the first cycle, whereas the rate of infusion of ion channel modulator was increased to threefold in the second cycle and tenfold in the third cycle. This drug infusion protocol has been illustrated in previous publications (Batey and Coker, 2002; Farkas and Coker, 2002). For some pilot experiments there were only two cycles of drug infusion with the rate of infusion of E-4031 being the same in both cycles.

In the first main study (HMR study), rabbits were assigned randomly to receive either E-4031 (1, 3 and 10 nmol kg<sup>-1</sup> min<sup>-1</sup>,  $n=8$ ), HMR1556 (25, 75 and 250 nmol kg<sup>-1</sup> min<sup>-1</sup>,  $n=7$ ), E-4031 and HMR1556 combined (doses as above,  $n=10$ ) or vehicle (saline infused at the same rate as E-4031 and polyethylene glycol 400 infused at the same rate as HMR1556,  $n=4$ ). In a second randomized study (ATX study), rabbits received either E-4031 (1, 3 and 10 nmol kg<sup>-1</sup> min<sup>-1</sup>,  $n=8$ ), ATX-II (0.4, 1.2 and 4.0 nmol kg<sup>-1</sup>,  $n=8$ ) or both drugs ( $n=8$ ). Each dose of ATX-II was given as a slow i.v. bolus over 1 to 2 min.

### ECG analysis and arrhythmia diagnosis

ECG intervals were measured manually using on-screen markers and taking the mean of four consecutive complexes. The PR, QRS and QT intervals were measured only in beats originating from the sino-atrial node that were not preceded or followed by ectopic beats or conduction block, in the manner described in Farkas *et al.* (2004). The QT interval was measured from the onset of the Q wave to where the T wave

returns to the isoelectric baseline (including any U wave, if present). In the majority of cases, the end of the T(U) wave was clearly visible, but in some cases the T wave overlapped the P wave of the next heartbeat. In the latter situation, the end of the T(U) wave was estimated by extrapolation to the isoelectric line under the P wave as described previously (Farkas *et al.*, 2004). At some time points ECG intervals could not be measured from all animals because of marked changes in morphology or arrhythmic activity.

Ventricular premature beats (VPBs), bigeminy, salvos and ventricular tachycardia were defined according to the Lambeth Conventions (Walker *et al.*, 1988). TdP was defined as a polymorphic ventricular tachycardia of four or more beats, where twisting of the QRS complex around the isoelectric baseline was visible in at least one ECG lead. It was also accompanied by a decline in arterial blood pressure towards zero with little pulsatile activity. Conduction block, either intraventricular, such as bundle branch block, or atrioventricular (AV) block was also identified and quantified.

#### QT interval correction

QT values were corrected for heart rate using a correction factor derived from baseline heart rate and QT intervals from each animal included in each study. The correction factor was based on that originally described by Carlsson *et al.* (1993) and subsequently modified by others (Batey and Coker, 2002; Farkas and Coker, 2002, 2003). For the HMR1556 study, the rate corrected QT interval (QTc) =  $QT - 0.655(RR - 238)$  and for the ATX study the formula was  $QTc = QT - 0.556(RR - 241)$  which corrected QT intervals to the mean baseline heart rate observed in that study.

#### Beat-to-beat variability of repolarization

To assess beat-to-beat variability of QT intervals, values for 30 consecutive beats of sinus origin were measured manually before the start of the experimental protocol, that is, at baseline and immediately before the first VPB. Poincaré plots were produced by plotting each value against the former value. STV was determined as the mean orthogonal distance from the diagonal to the points of the plots and calculated according to the formula described previously (Thomsen *et al.*, 2004; Detre *et al.*, 2005) where  $STV = \sum |D_{n+1} - D_n| / [30 \times \sqrt{2}]$  and  $D$  represents QT duration.

Further analysis of variability in repolarization was carried out on the MAP data. The epicardial MAP signal was analysed using Electrophysiology Data Recorder software (version 2.8; John Dempster, University of Strathclyde, Glasgow, UK). The Po-Ne-Mah data files (recorded at 1000 Hz) were imported into the Electrophysiology Data Recorder application, a low pass filter of 100 Hz was applied and any baseline drift was subtracted. MAP durations were measured at 30 and 90% repolarization. From MAP duration at 90% repolarization (APD<sub>90</sub>), Poincaré plots were constructed and STV calculated as described above at baseline, immediately before the first VPB, before the first episode of TdP (or at an equivalent time point in rabbits that did not have TdP) and during the third cycle of drug administration. Triangulation of the MAP (APD<sub>90</sub>–APD<sub>30</sub>), as defined by Hondeghem *et al.* (2001), was also calculated at these time

points. STV and triangulation data have only been presented from experiments in which these measurements could be obtained at all of the first three time points.

#### Pilot studies: dose selection and study design

It has been reported previously (Bril *et al.*, 1996) that the I<sub>Kr</sub> blocker E-4031 given at rates of 10 and 20 µg kg min<sup>-1</sup> (~20 and 40 nmol kg min<sup>-1</sup>) to pentobarbital-anaesthetized rabbits caused TdP in 70 and 90% of rabbits respectively. The aim of our preliminary studies was to find a dose of E-4031 that would cause TdP in approximately 20% of rabbits. Two consecutive cycles of administration of the same dose of E-4031 caused TdP in 100, 50, 50 and 20% of phenylephrine-stimulated rabbits which received 24 nmol kg<sup>-1</sup> min<sup>-1</sup> (n = 2), 12 nmol kg<sup>-1</sup> min<sup>-1</sup> (n = 2), 6 nmol kg<sup>-1</sup> min<sup>-1</sup> (n = 4) and 3 nmol kg<sup>-1</sup> min<sup>-1</sup> (n = 5) E-4031 respectively. The dose of 3 nmol kg<sup>-1</sup> min<sup>-1</sup> E-4031 was therefore chosen to be the middle dose in the sequence of three rising doses used in the combination studies.

As there was no published information on the effects of HMR1556 on TdP *in vivo*, our preliminary studies in anaesthetized rabbits focused on finding a range of doses that increased QT intervals without having any adverse effects on heart rate or blood pressure. Previous publications indicated that 1 mg kg<sup>-1</sup> (~2.5 µmol kg<sup>-1</sup>) i.v. HMR1556 increased QT intervals in anaesthetized pigs (Knobloch *et al.*, 2004) and dogs (Nakashima *et al.*, 2004). With consecutive infusions of 25, 75 and 250 nmol kg<sup>-1</sup> min<sup>-1</sup>, there was a 20% increase in the QTc interval with no further increase seen with 750 nmol kg<sup>-1</sup> min<sup>-1</sup> HMR1556. On the basis of plasma concentrations of HMR1556 measured by Nakashima *et al.* (2004) in their studies, and extrapolation from *in vitro* measurements of the potency and selectivity of this drug (Gögelein *et al.*, 2000; Thomas *et al.*, 2003; Volders *et al.*, 2003), infusions of 25, 75 and 250 nmol kg<sup>-1</sup> min<sup>-1</sup> were selected as doses that should block I<sub>Ks</sub> only.

There is also little published information on the effects of ATX-II *in vivo*. A bolus dose of 5 µg kg<sup>-1</sup> (~1 nmol kg<sup>-1</sup>) increased QTc intervals without altering heart rate or blood pressure in halothane-anaesthetized dogs (Miyamoto *et al.*, 2001). Based on this, pilot studies with i.v. bolus doses and infusions of ATX-II in the presence of saline instead of phenylephrine were performed. Infusions of ATX-II increased the QTc interval but also increased heart rate and decreased mean arterial blood pressure by the end of each cycle. In contrast, slow bolus doses of ATX-II (0.4, 1.2 and 4.0 nmol kg<sup>-1</sup>) had minimal effects on heart rate and blood pressure but did prolong QTc intervals and were therefore selected for the main study.

#### Statistics

Continuous data were expressed as mean ± s.e.mean. One-way ANOVA, with *post hoc* Tukey's tests, was used to compare haemodynamic and QT interval data at baseline and the 15 min time point in each cycle within and among groups. The General Linear Model with interaction was used to compare differences in profiles between treatment groups. The incidence of arrhythmias and conduction block were compared by Fisher's exact probability test. Kruskal–Wallis tests were used

to compare the durations of arrhythmias and conduction block. STV and triangulation were compared with one-way ANOVA (for three or more groups or time points) or using paired Student's *t*-tests for within group comparisons or unpaired Student's *t*-tests for between group comparisons. Differences were considered statistically significant when  $P < 0.05$ .

### Drugs

HMR1556 (a gift from Aventis Pharma, Frankfurt, Germany) was dissolved at  $3 \text{ mg ml}^{-1}$  in polyethylene glycol 400 each day. Stock solutions of E-4031 (purchased from Wako, Neuss, Germany) in normal saline (0.9% w/v NaCl in distilled  $\text{H}_2\text{O}$ ) were prepared in advance and stored in aliquots at  $-20^\circ\text{C}$ , then diluted to  $51 \mu\text{g ml}^{-1}$  with saline prior to use. ATX-II (purchased from Alomone Laboratories, Jerusalem, Israel) was dissolved at  $100 \mu\text{g ml}^{-1}$  in distilled water and stored at  $-20^\circ\text{C}$ , then diluted to  $40 \mu\text{g ml}^{-1}$  with saline before each experiment. *L*-Phenylephrine (Sigma, Poole, UK) was dissolved at  $1 \text{ mg ml}^{-1}$  in saline and prepared freshly each day.

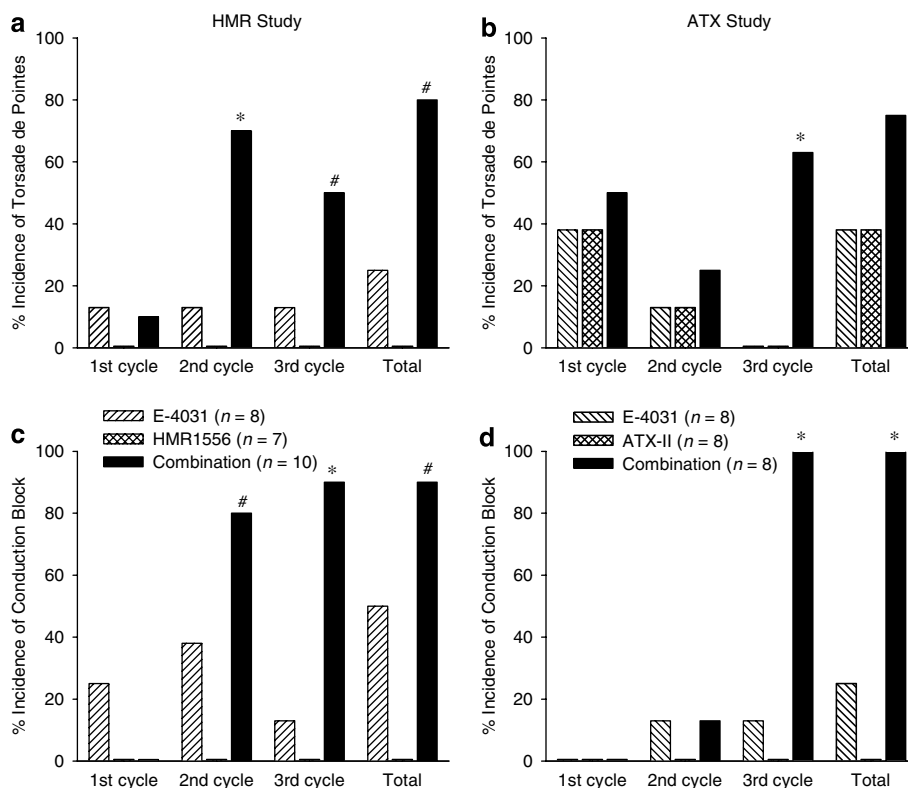
## Results

### Effects of HMR1556 and E-4031 alone and in combination on arrhythmias

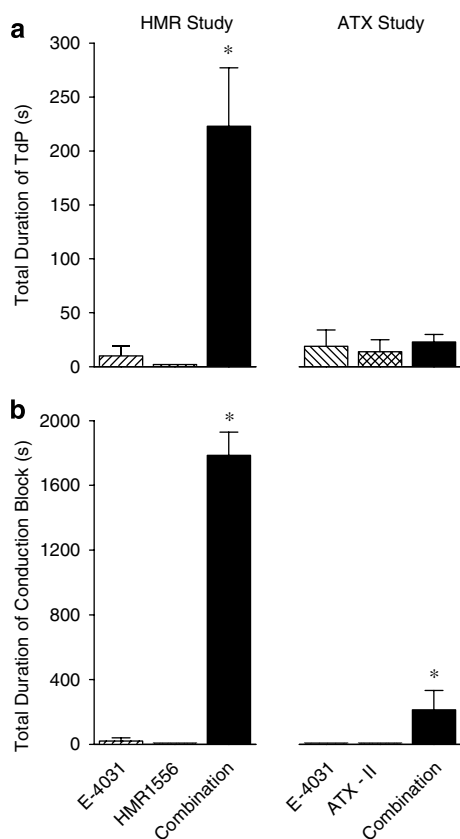
HMR1556 did not cause TdP itself but potentiated the torsadogenic action of E-4031. TdP was observed in a total of

75% of rabbits receiving both E-4031 and HMR1556 in combination, 25% of rabbits receiving E-4031 alone and in 0% of the rabbits receiving vehicle or HMR1556 alone. In the combination group, most TdP was observed in the second and third cycles (Figure 1a). Another feature noted in the combination group was that there tended to be more episodes of TdP and they lasted longer. Figure 2a illustrates the total duration of all episodes of TdP that occurred during the experimental protocol and emphasizes the highly significant increase in the group receiving the combination of both drugs compared to the others.

HMR1556 also potentiated the effects of E-4031 on other arrhythmias, such as salvos, ventricular tachycardia (monomorphic or polymorphic, but not showing the characteristics of TdP) and conduction block. For example, in the third cycle of drug administration, salvos and ventricular tachycardia occurred in 13% of rabbits receiving E-4031 alone, 14% of those receiving HMR1556 alone but in 88% of the rabbits receiving the combination of both drugs. Another rhythm disturbance that was observed frequently in the combination group was conduction block, but none of the rabbits given vehicle or HMR1556 alone had any conduction block (Figure 1c). The conduction block observed with E-4031 consisted of missing QRS complexes, and on average lasted 1 s in duration, whereas conduction block in the combination group was sustained (Figure 2b) with episodes of bundle branch block and 2:1 AV block being observed.



**Figure 1** Incidence of torsade de pointes (a and b) and conduction block (c and d) in the HMR1556 study (a and c) where anaesthetized rabbits received phenylephrine and either E-4031 (1, 3, 10  $\text{nmol kg}^{-1} \text{ min}^{-1}$ ), HMR1556 (25, 75, 250  $\text{nmol kg}^{-1} \text{ min}^{-1}$ ) or the combination of both drugs, and in the ATX study (b and d) where anaesthetized rabbits received phenylephrine and either E-4031 (1, 3, 10  $\text{nmol kg}^{-1} \text{ min}^{-1}$ ), ATX-II (0.4, 1.2, 4.0  $\text{nmol kg}^{-1}$ ) or the combination of both drugs. \* $P < 0.05$  compared to both other groups, # $P < 0.05$  compared to HMR1556 group, Fisher's Exact test. ATX-II, sea anemone toxin.



**Figure 2** Total duration of (a) torsade de pointes and (b) conduction block in the HMR1556 study where anaesthetized rabbits received phenylephrine and either E-4031 (1, 3, 10 nmol kg<sup>-1</sup> min<sup>-1</sup>), HMR1556 (25, 75, 250 nmol kg<sup>-1</sup> min<sup>-1</sup>) or the combination of both drugs, and in the ATX study where anaesthetized rabbits received phenylephrine and either E-4031 (1, 3, 10 nmol kg<sup>-1</sup> min<sup>-1</sup>), ATX-II (0.4, 1.2, 4.0 nmol kg<sup>-1</sup>) or the combination of both drugs. \**P* < 0.05 compared to both other groups, Kruskal–Wallis test. ATX-II, sea anemone toxin.

*Effects of ATX-II and E-4031 alone and in combination on arrhythmias*

ATX-II, administered alone, induced TdP in anaesthetized rabbits, and potentiated the torsadogenic effect of E-4031 during the third cycle of drug administration (Figure 1b). However, when the total incidence of TdP throughout the whole protocol is considered, ATX-II added to, rather than potentiated, the effects of E-4031. TdP was observed in a total of 75% of rabbits receiving ATX-II and E-4031 in combination, 38% of rabbits receiving ATX-II alone and in 38% of rabbits receiving E-4031 alone. With ATX-II alone and E-4031 alone most TdP occurred during the first cycle, whereas TdP was observed in all three cycles with the combination (Figure 1b). The total duration of all episodes of TdP is illustrated in Figure 2a. It is interesting to note that ATX-II did not potentiate the effects of E-4031 on the total duration of TdP.

ATX-II also potentiated the effects of E-4031 on other arrhythmias during the third cycle of drug administration. Salvos were observed in all rabbits given the combination, but only in 25% of rabbits receiving E-4031 alone or ATX-II alone. Ventricular tachycardia was not observed with ATX-II or E-4031 alone during the third cycle, but was seen in all

rabbits receiving the combination. Conduction block was never seen with ATX-II alone but was observed in some rabbits that received E-4031 alone and in all rabbits receiving the combination during the third cycle of drug administration (Figure 1d). The low incidence of E-4031-induced conduction block revealed the potentiation of the incidence of conduction block by ATX-II (Figure 1d). As in the HMR1556 study, conduction disturbances observed with E-4031 were brief. In contrast, conduction block that occurred in the presence of the combination of both ATX-II and E-4031 lasted longer (Figure 2b) and included both bundle branch block and 2:1 AV block. Interestingly, however, the total duration of conduction block with the combination of ATX-II and E-4031 was only about a tenth of that seen with combined administration of HMR1556 and E-4031 (Figure 2b).

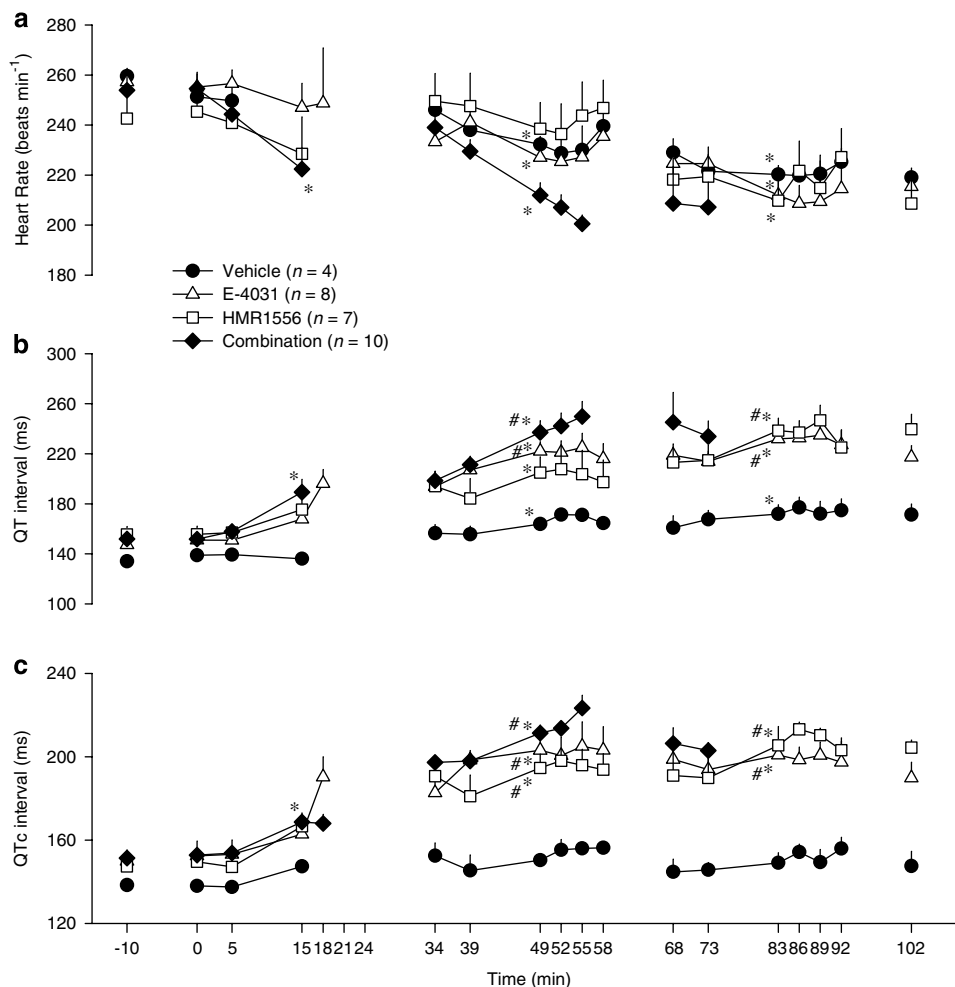
*Effects of HMR1556 and E-4031 alone and in combination on heart rate and QT intervals*

In contrast to the effects on arrhythmias, HMR1556 did not potentiate the effects of E-4031 on heart rate or QTc intervals. Heart rate declined over the course of the experimental protocol in all four treatment groups with no significant differences among the groups at the mid-point of each dosing cycle (Figure 3a). Within group analysis, however, revealed that the fall in heart rate reached significance during the first cycle of drug administration in the combination group, during the second cycle in the E-4031 and vehicle groups, but not until the third cycle in the group receiving HMR1556 alone (Figure 3a).

QT intervals increased progressively in the drug-treated groups (Figure 3b). In the vehicle group there were statistically significant, but modest, increases in the QT interval during the second and third cycles, but there were no significant differences in the QTc intervals (Figure 3c). In contrast, substantial increases in the QT and QTc interval occurred following administration of E-4031, HMR1556 and the combination of both drugs (Figure 3). Statistical comparison at the mid-point of the second cycle of drug administration indicated no significant differences in QTc intervals among the three groups that received the K<sup>+</sup> channels blockers, but the values in all three of these groups were significantly greater than those seen in the vehicle-treated rabbits which only received phenylephrine (Figure 3). When the rabbits that received K<sup>+</sup> channels blockers were divided into those that had TdP and those that did not, there was no significant difference in the change in QT intervals from baseline to the mid-point of the second cycle; 52 ± 6 and 47 ± 7% respectively. PR and QRS intervals were 65 ± 2 and 55 ± 1 ms at baseline in the vehicle group and remained similar throughout the course of the experiments. Values in the other groups were not significantly different from those in the vehicle group at any of the measured time points.

*Effects of ATX-II and E-4031 alone and in combination on heart rate and QT intervals*

Heart rate declined progressively over the course of the experimental protocol in all groups, falling significantly from



**Figure 3** (a) Heart rate, (b) QT intervals and (c) QTc intervals in anaesthetized rabbits in the HMR study which received phenylephrine in the presence of either E-4031 (1, 3, 10 nmol kg<sup>-1</sup> min<sup>-1</sup>), HMR1556 (25, 75, 250 nmol kg<sup>-1</sup> min<sup>-1</sup>) or the combination of both drugs. Values are expressed as mean  $\pm$  s.e.mean. \* $P < 0.05$  compared to baseline (-10 min), # $P < 0.05$  compared to vehicle group, one-way ANOVA. At some time points, there are no data or  $n$  is less than the stated values due to frequent arrhythmias which prevented accurate measurement of heart rate and ECG intervals.

baseline with E-4031 alone and the combination during the second cycle, but not until the third cycle with ATX-II alone. At the mid-points of the second and third cycles, heart rates were significantly lower in rabbits receiving the combination treatment compared with ATX-II alone (Figure 4a).

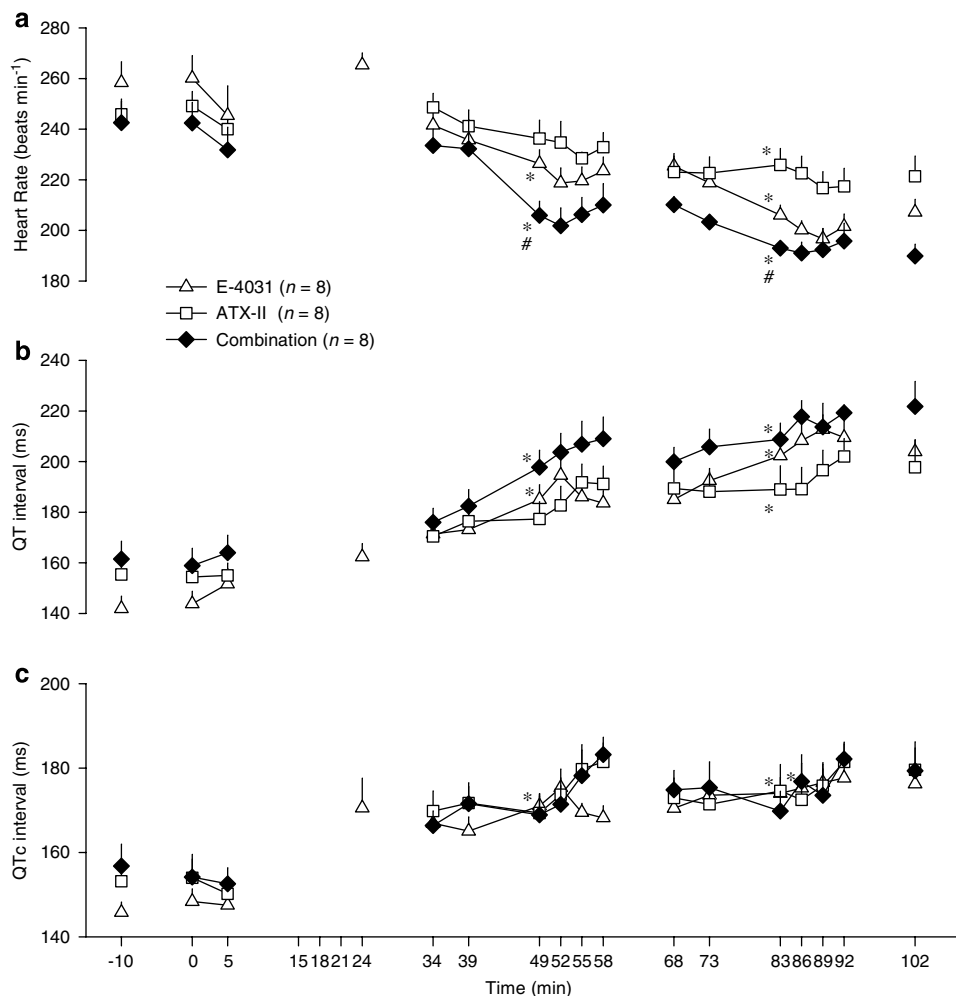
ATX-II did not potentiate the effects of E-4031 on QT or QTc intervals. Although QT intervals increased progressively and were significantly prolonged from baseline in all groups by the third cycle, there were no differences between groups at the mid-point of the second or third cycles (Figure 4b). The QT-prolonging effects of these drugs were diminished when intervals were rate-corrected. For example, in the second cycle, the QTc interval was only increased significantly from baseline in the E-4031 group. There were no significant differences in QTc intervals among the groups (Figure 4c). When the rabbits were divided into those that had TdP and those that did not, the change in QT intervals from baseline to the mid-point of the second cycle,  $23 \pm 5$  and  $24 \pm 5\%$  respectively, was similar.

PR intervals increased in all three groups as the experimental protocol progressed; from  $60 \pm 2$  ms at baseline to  $68 \pm 3$  ms by the mid-point of the third cycle in the ATX-II

group,  $60 \pm 2$  to  $72 \pm 3$  ms in the E-4031 group and  $57 \pm 3$  to  $76 \pm 4$  ms in the combination group, but there were no differences among the groups at either time point. Comparing the same time points, QRS intervals only increased in the groups receiving ATX-II alone and in combination with E-4031; from  $52 \pm 2$  to  $61 \pm 3$  ms and from  $56 \pm 3$  to  $70 \pm 3$  ms respectively. Again, there were no significant differences among the groups at either time point.

#### *Effects of HMR1556 and E-4031 alone and in combination on blood pressure, blood gases and K<sup>+</sup>*

Phenylephrine dose-dependently increased mean arterial blood pressure in all three drug administration cycles, with recovery to baseline values when the phenylephrine infusion was stopped (Figure 5a). There appeared to be some attenuation of the phenylephrine-induced increase in mean blood pressure in the middle of the second and third cycles although there were no significant differences among the groups at these time points. Detailed statistical analysis of each cycle as a whole revealed that in the third cycle giving both drugs in combination gave rise to a significantly



**Figure 4** (a) Heart rate, (b) QT intervals and (c) QTc intervals in anaesthetized rabbits in the ATX study which received phenylephrine in the presence of either E-4031 (1, 3, 10 nmol kg<sup>-1</sup> min<sup>-1</sup>), ATX-II (0.4, 1.2, 4.0 nmol kg<sup>-1</sup>) or the combination of both drugs. Values are expressed as mean  $\pm$  s.e.mean. \* $P$  < 0.05 compared to baseline (-10 min), # $P$  < 0.05 compared to ATX-II group, one-way ANOVA. At some time points, there are no data or  $n$  is less than the stated values due to frequent arrhythmias which prevented accurate measurement of heart rate and ECG intervals. ATX-II, sea anemone toxin.

different profile of mean arterial blood pressure values than those in the other treatment groups (Figure 5a). This latter effect is probably a consequence of the persistent conduction block reducing heart rate and therefore cardiac output.

Baseline blood gas, pH and K<sup>+</sup> values were  $PO_2 = 83 \pm 2$  mm Hg,  $PCO_2 = 31 \pm 1$  mm Hg, pH  $7.50 \pm 0.01$  units,  $K^+ = 1.79 \pm 0.05$  mmol l<sup>-1</sup> ( $n = 29$ ). During the course of the experiments there were no significant changes in  $PO_2$  or  $PCO_2$  and no differences in blood gas values among the groups at any time point. In the group that received the combination of E-4031 and HMR1556, blood K<sup>+</sup> values were  $1.81 \pm 0.08$ ,  $2.14 \pm 0.19$ ,  $3.12 \pm 0.37^*$  and  $4.03 \pm 0.29^*$  mmol l<sup>-1</sup> at baseline, end of the first cycle, end of the second cycle and end of the third cycle, respectively (\* $P$  < 0.05 compared to baseline). The corresponding pH values were  $7.50 \pm 0.02$ ,  $7.40 \pm 0.01^*$ ,  $7.30 \pm 0.01^*$  and  $7.20 \pm 0.02^*$  units (\* $P$  < 0.05 compared to baseline). Similar changes in pH and K<sup>+</sup> occurred in the other groups but there were no significant differences among the groups at any time point.

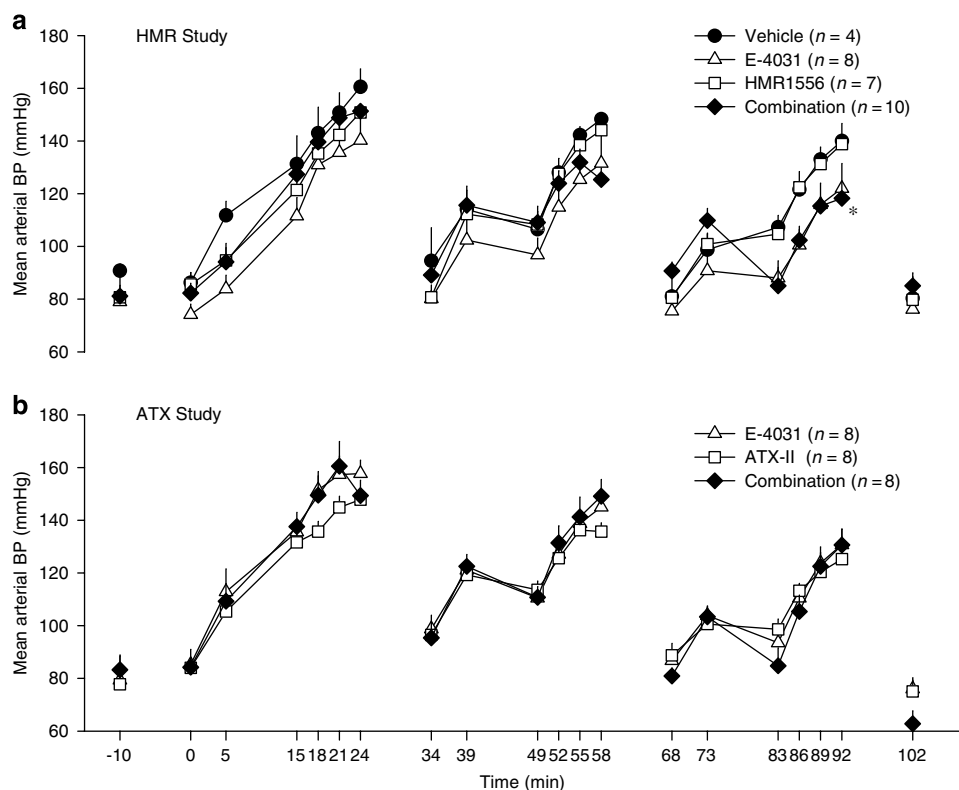
*Effects of ATX-II and E-4031 alone and in combination on blood pressure, blood gases and K<sup>+</sup>*

The changes in mean arterial blood pressure throughout the course of the experimental protocol in the ATX study were similar to those seen in the HMR study. Phenylephrine increased blood pressure in all three cycles and there were no differences among the groups at any time point (Figure 5b).

In the ATX study, baseline blood gas, pH and K<sup>+</sup> values were  $PO_2 = 85 \pm 2$  mm Hg,  $PCO_2 = 34 \pm 1$  mm Hg, pH  $7.49 \pm 0.01$  units,  $K^+ = 1.93 \pm 0.08$  mmol l<sup>-1</sup> ( $n = 24$ ). No significant changes in  $PO_2$  or  $PCO_2$  occurred, but pH decreased and K<sup>+</sup> increased in each group as the protocol progressed. The pattern and magnitude of these changes in K<sup>+</sup> and pH were similar to those seen in the HMR1556 study and there were no significant differences among the groups at any time point.

*Effects of ion channel modulators on variability of repolarization*

In the HMR1556 study, beat-to-beat variability of repolarization was assessed initially by calculating STV from Poincaré plots of QT intervals at baseline and immediately before the



**Figure 5** Mean arterial blood pressure (BP) in (a) the HMR1556 study where anaesthetized rabbits received phenylephrine and either E-4031 (1, 3, 10 nmol kg<sup>-1</sup> min<sup>-1</sup>), HMR1556 (25, 75, 250 nmol kg<sup>-1</sup> min<sup>-1</sup>) or the combination of both drugs, and in (b) the ATX study where anaesthetized rabbits received phenylephrine and either E-4031 (1, 3, 10 nmol kg<sup>-1</sup> min<sup>-1</sup>), ATX-II (0.4, 1.2, 4.0 nmol kg<sup>-1</sup>) or the combination of both drugs. Values are expressed as mean  $\pm$  s.e.mean. \* $P < 0.05$  for the profile of all the values in the third cycle in the combination group compared to the other groups, General Linear Model with interaction. To aid clarity, symbols for the within group statistical analyses have been omitted. At baseline and at the mid-point of each cycle there were no significant differences among groups, one-way ANOVA. ATX-II, sea anemone toxin.

first premature beat in each rabbit that received one or more K<sup>+</sup> channel blockers. These values were  $3.6 \pm 0.3$  and  $3.9 \pm 0.3$  ms in the E-4031 group,  $3.7 \pm 0.5$  and  $3.8 \pm 0.3$  ms in the HMR1556 group and  $3.6 \pm 0.3$  and  $4.1 \pm 0.7$  ms in the group given both drugs. There were no significant differences either within or between groups. When the data were sorted by whether or not TdP occurred, the STV values were  $3.6 \pm 0.3$  ms at baseline and  $3.9 \pm 0.2$  ms immediately before the first VPB in rabbits that did not have TdP ( $n = 15$ ). The corresponding values in the rabbits that did have TdP ( $n = 8$ ) were  $3.6 \pm 0.3$  and  $4.1 \pm 0.7$  ms. Again, there were no significant differences in these values either within or between those that had TdP and those that did not.

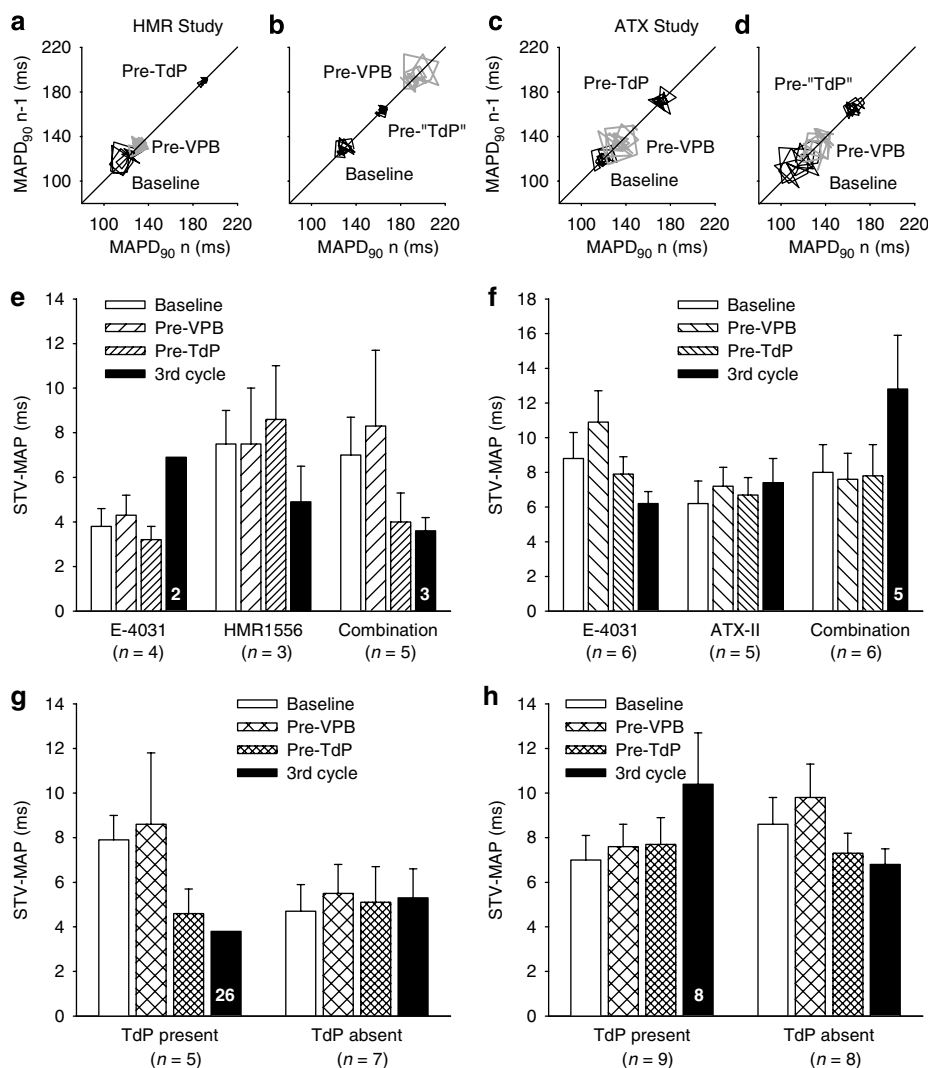
Further assessment of variability of repolarization focused on analysis of data obtained from measurements of epicardial MAPs. Figures 6a–d illustrate Poincaré plots of APD<sub>90</sub> from rabbits with and without TdP in both the HMR1556 and ATX studies. The STV of APD<sub>90</sub> was calculated at baseline, immediately before the first VPB, before the first episode of TdP (or at equivalent time points in those that did not have TdP) and during the third cycle of drug administration. In the HMR1556 study, neither of the K<sup>+</sup> channel blockers altered STV when given alone or in combination (Figure 6e). Similarly, in the ATX study, neither E-4031 nor ATX-II altered STV. Combined administration of ATX-II and E-4031 did appear to increase STV in the third cycle but this

apparent change was not significantly different (Figure 6f). Even when the data were plotted by whether or not TdP occurred there were no significant differences either within or between the groups in either the HMR1556 study (Figure 6g) or the ATX study (Figure 6h).

Possible changes in action potential morphology were assessed by calculating triangulation of the MAP (APD<sub>90</sub>–APD<sub>30</sub>) at the same time points as STV had been measured. In the HMR1556 study, no clear pattern of changes was observed within each drug treatment group (Figure 7a), although E-4031 tended to increase triangulation by the final time point. However, in the ATX study, E-4031 clearly increased triangulation in the third cycle when given alone or in combination with ATX-II (Figure 7b). Sorting the data from each study by whether or not TdP occurred produced differing results. In the HMR1556 study, triangulation was only increased in the third cycle in rabbits that had TdP (Figure 7c), whereas in the ATX study triangulation increased in the third cycle in rabbits in which TdP was present and in those in which it was absent (Figure 7d). It is important to note, however, that there were no changes in triangulation before TdP occurred, irrespective of how the data were plotted.

STV and triangulation data from both studies were pooled to increase the power of the comparisons between rabbits that had TdP and those that did not. This pooling of data





**Figure 6** (a–d) Examples of Poincaré plots of monophasic action potential (MAP) duration at 90% repolarization (MAPD<sub>90</sub>) at baseline, immediately prior to the first ventricular premature beat (Pre-VPB) and (a and c) before the first episode of torsade de pointes (Pre-TdP) or (b and d) at similar time points in rabbits that did not have TdP (Pre-'TdP'). (e–h) Mean ± s.e.mean values of short-term variability in MAPD<sub>90</sub> (STV-MAP) by treatment in (e) the HMR study and (f) the ATX study or by the presence or absence of TdP in (g) the HMR study and (h) the ATX study. Numbers in columns indicate n-values where these are less than the group value.

shows clearly that in this rabbit model no significant changes in STV occurred before the first VPB, before TdP or even in the third cycle of drug administration, in rabbits with TdP or in those without TdP (Figure 8a). In contrast, there were significant increases in triangulation but these were only seen towards the end of the experimental protocol (Figure 8b). Although there was greater triangulation at this time point in the rabbits that had TdP compared to those that did not have TdP, a significant increase did still occur in the latter group.

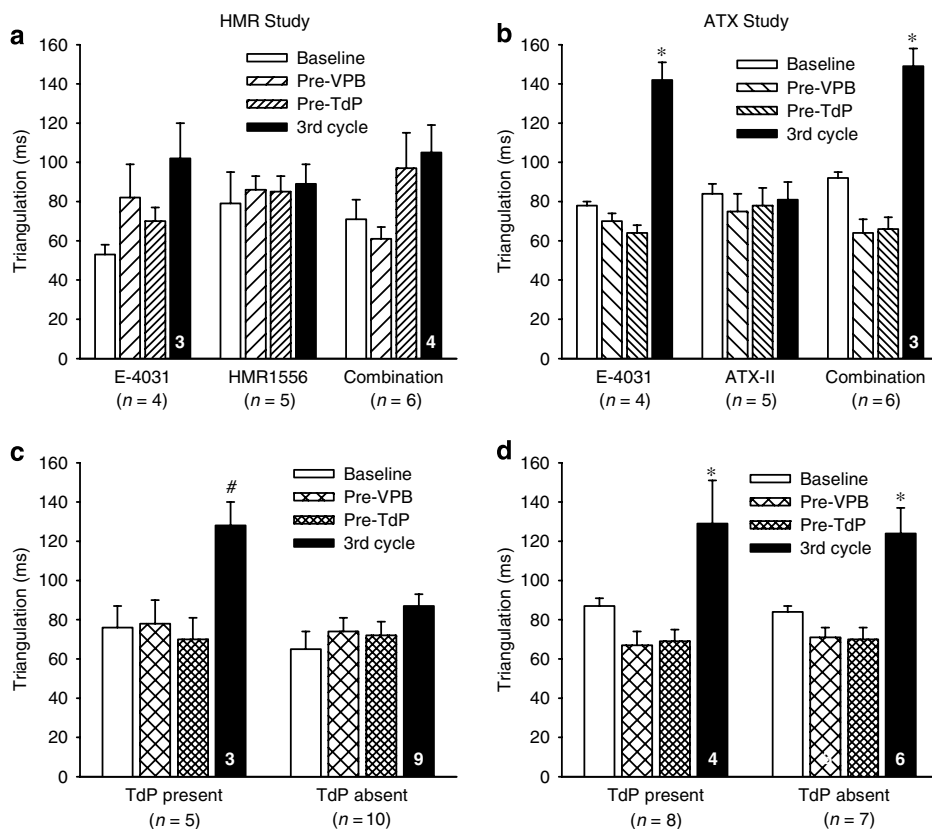
**Discussion**

The major novel findings of the present work are that (1) HMR1556 caused more potentiation of the torsadogenic action of E-4031 than ATX-II, at the doses studied; (2) ATX-II induced TdP *in vivo* and increased the proarrhythmic actions

of E-4031; (3) neither changes in STV of repolarization nor triangulation of the action potential could predict the occurrence of TdP in open-chest, α<sub>1</sub>-adrenoceptor-stimulated anaesthetized rabbits.

*Comparison of potentiation of the effects of I<sub>Kr</sub> blockade by blocking I<sub>Ks</sub> or prolonging I<sub>Na</sub>*

The I<sub>Ks</sub> blocker, HMR1556, potentiated the torsadogenic action of the I<sub>Kr</sub> blocker, E-4031, substantially increasing both the incidence and the duration of TdP. HMR1556 on its own prolonged repolarization but did not induce TdP. Prior to publication of our abstract on the HMR1556 study (Michael *et al.*, 2006), the *in vivo* cardiac electrophysiological effects of HMR1556 had only been reported in dog (Volders *et al.*, 2003; Nakashima *et al.*, 2004) and pig models (Knobloch *et al.*, 2004). A marked prolongation of the QT interval was observed in these species but HMR1556 was not



**Figure 7** Triangulation (MAPD<sub>90</sub>-MAPD<sub>30</sub>) displayed by treatment in (a) the HMR study and (b) the ATX study or by the presence or absence of TdP in (c) the HMR1556 study and (d) the ATX study. Values are mean  $\pm$  s.e.mean. \* $P < 0.05$  compared to all other time points, # $P < 0.05$  compared to Pre-TdP, one-way ANOVA. Numbers in columns indicate  $n$ -values where these are less than the group value.

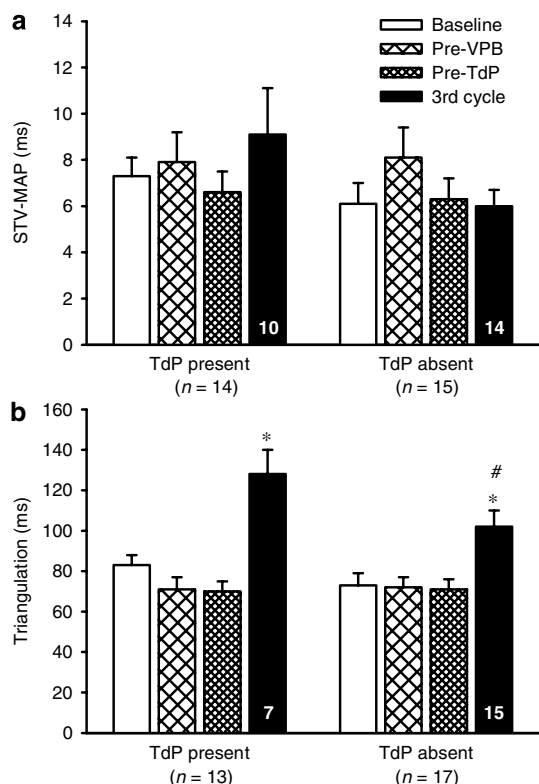
associated with the induction of TdP. A recently published paper, however, demonstrated that HMR1556 potentiated TdP induced by dofetilide in anaesthetized rabbits (Lengyel *et al.*, 2007). The effects on the incidence of TdP reported by Lengyel *et al.* (2007) are very similar to those reported here. HMR1556 itself did not cause TdP but increased the incidence of dofetilide-induced TdP from approximately 30 to 70%. In the present study, we have shown that HMR1556 also potentiated the total duration of episodes of E-4031-induced TdP.

A different pattern of changes was seen when ATX-II was combined with E-4031. First, unlike HMR1556, ATX-II given alone did cause TdP. Second, when the total incidence of TdP throughout the experimental protocol is considered, the effects of ATX-II and E-4031 were additive, whereas the interaction between HMR1556 and E-4031 was synergistic. Third, ATX-II did not increase the duration of TdP; in fact the duration of TdP with the combination of ATX-II and E-4031 was less than additive. Previously, it has been reported that ATX-II potentiated the proarrhythmic actions of  $I_{Kr}$  blockers *in vitro* (Wu *et al.*, 2006). The present work has demonstrated clearly that ATX-II can also cause TdP *in vivo* and potentiate some of the proarrhythmic actions of E-4031. In the third cycle of drug administration, ATX-II potentiated E-4031-induced salvos, VT, conduction block and TdP. At this time point, these arrhythmias were either absent or only occurred in one or two rabbits given either ATX-II or E-4031 alone. Thus the capacity to see substantial potentiation of

these arrhythmias existed. In their studies in isolated, AV-blocked, rabbit hearts, Wu *et al.* (2006) found that ATX-II given at a concentration which did not cause VT itself, significantly increased the incidence of VT induced by  $I_{Kr}$  blockers. It is possible that the interaction of ATX-II and E-4031 on the total incidence of TdP in our experiments appeared to be additive because each drug on its own already induced TdP in 38% of rabbits and thus the capacity to see significant potentiation was limited. However, this reasoning cannot be applied to the data on the duration of TdP, as unlike HMR1556, ATX-II did not increase the duration of TdP induced by E-4031. This suggests that there may be differences in the mechanisms by which episodes of TdP are sustained by drugs that prolong  $I_{Na}$  compared to those that block  $I_{Kr}$ .

#### *Influence of the extent of QT prolongation, hypokalaemia and conduction block*

In contrast to results from rabbit perfused heart experiments, where  $I_{Ks}$  blockers given alone did not increase action potential duration (So *et al.*, 2006) or QTc interval (Lengyel *et al.*, 2001), HMR1556 did prolong QT and QTc intervals in our *in vivo* experiments. This can be explained by the presence of intact sympathetic innervation *in vivo* and indicates that the rabbit, like dog and man (Volders *et al.*, 2003; Jost *et al.*, 2005), requires sympathetic influence to reveal the important contribution of  $I_{Ks}$  to repolarization



**Figure 8** Pooled data from both the HMR and ATX studies illustrating (a) short-term variability in MAPD<sub>90</sub> (STV-MAP) and (b) triangulation of the action potential in rabbits with TdP and those in which TdP was absent. Values are mean ± s.e.mean. \**P* < 0.05 compared to all other time points, one-way ANOVA, #*P* < 0.05 compared to TdP present, unpaired *t*-test. Numbers in columns indicate *n*-values where these are less than the group value.

reserve. This *in vivo* sympathetic drive may, however, limit the ability to see potentiation of repolarization. Previous *in vitro* studies on combined *I*<sub>Kr</sub> and *I*<sub>Ks</sub> blockade have shown synergistic increases in prolongation of repolarization, supporting the concept of repolarization reserve (Varro *et al.*, 2000; Biliczki *et al.*, 2002; Volders *et al.*, 2003; Jost *et al.*, 2005; So *et al.*, 2006). Similar findings have also been reported with combined *I*<sub>Na</sub> enhancement and *I*<sub>Kr</sub> block (Wu *et al.*, 2006). Such changes were not seen in the present *in vivo* studies. There were no significant differences in QT interval, QTc interval or APD<sub>90</sub> among the groups receiving ion channel modulators in either the HMR or ATX studies. In their recent studies in conscious dogs, Lengyel *et al.* (2007) reported additive rather than synergistic effects of combined *I*<sub>Kr</sub> and *I*<sub>Ks</sub> blockade on QTc prolongation. A recent commentary (Thomsen, 2007) on that paper suggested that the presence of adrenergic stimulation *in vivo* could explain why supra-additive effects on QT prolongation were not seen.

The lack of further prolongation of QT or QTc intervals with drug combinations in the present studies supports the hypothesis that there is no correlation between the extent of QT prolongation and the occurrence of TdP. It could be argued, however, that there was limited capacity to see potentiation of QT prolongation. In the HMR study, QTc intervals were increased substantially by about 50%, which

may be about the maximum increase that can be seen with these drugs before the development of persistent conduction block prevented measurement of ECG intervals. However, in the ATX study, the changes in QTc intervals were just under 25% with either ATX-II or E-4031 alone, but there was no further increase when both drugs were given together. Thus, despite there being capacity to see a further increase, it did not occur. Looking at the results overall, one group (HMR1556 alone) had a substantial increase in QTc intervals but no TdP, whereas the combination groups in both studies had similar incidences of TdP (75 and 80%) but differing extents of QTc prolongation (25 and 50%).

Hypokalaemia is a risk factor for the development of TdP, particularly with drugs that block *I*<sub>Kr</sub> as their potency is increased when extracellular K<sup>+</sup> concentrations are low (Yang and Roden, 1996). In the present study, blood K<sup>+</sup> concentrations were low (~2 mmol l<sup>-1</sup>) at baseline. Low K<sup>+</sup> concentrations have been reported previously in pentobarbital-anaesthetized rabbits (Barrett *et al.*, 1997; Barrett and Walker, 1998; Lightbown *et al.*, 2001; Farkas and Coker, 2002; Gil *et al.*, 2004). As discussed in our previous publications (Lightbown *et al.*, 2001; Farkas and Coker, 2002), the reduction of blood K<sup>+</sup> concentrations is a consequence of anaesthesia and is influenced by ventilation status as there is a correlation between PCO<sub>2</sub> and K<sup>+</sup>. As there were no differences in K<sup>+</sup> concentrations among the groups of rabbits that received E-4031, HMR1556, ATX-II or combinations, variations in the extent of hypokalaemia cannot explain the differences in the occurrence of TdP. The gradual rise in K<sup>+</sup> that occurred with time in all the rabbit groups also occurred previously in similar studies (Farkas and Coker, 2002, 2003). It was probably a consequence of continued administration of phenylephrine, an α-adrenoceptor agonists have been reported to cause K<sup>+</sup> release in anaesthetized rabbits (Coats, 1985).

Potentiation of conduction block was seen in both the HMR and ATX studies. Intraventricular block can develop as a consequence of extremely delayed repolarization, and 2:1 AV block can occur when the refractory period of the ventricular muscle is longer than the sino-atrial cycle length (Farkas *et al.*, 2004). As well as the anaesthetized α-adrenoceptor-stimulated rabbit model (Carlsson *et al.*, 1990; Batey and Coker, 2002), rabbit isolated perfused heart models with acute AV block (Eckardt *et al.*, 1998; Barrett *et al.*, 2001) have been used to study TdP. It may be that conduction block is a predisposing factor to TdP. However, ATX-II given alone did not cause any conduction block but did induce TdP. Thus conduction block *per se* is not essential for TdP to occur.

#### Predictors of TdP: STV of repolarization, action potential triangulation

The present results indicate that TdP cannot be predicted by increases in STV of the QT interval or epicardial APD<sub>90</sub> in this particular rabbit model. A lack of predictive value for STV of QT intervals has been reported previously in the chronic AV blocked dog model of TdP (Thomsen *et al.*, 2004), but increased STV of left ventricular monophasic APD<sub>90</sub> was found to be an excellent predictor of TdP in that study and

others (Thomsen *et al.*, 2004, 2006, 2007; Detre *et al.*, 2005). Recently, increased STV of QT intervals has been reported to predict TdP in conscious dogs and to 'parallel the increase in the incidence of TdP in anaesthetized rabbits' challenged with combined  $I_{Kr}$  and  $I_{Ks}$  blockade (Lengyel *et al.*, 2007). Despite finding similar potentiation of TdP with HMR1556 + E-4031 in the present studies as Lengyel *et al.* (2007) achieved with HMR1556 + dofetilide, no increases in STV were detected even when STV of APD<sub>90</sub> was calculated.

These divergent results raise the important question of how they may have occurred. There are two major differences between our rabbit model and that of Lengyel *et al.* (2007). First, our rabbits are open-chest and mechanically ventilated; second, they received phenylephrine. Our rabbits had lower mean arterial blood pressures (~80 vs ~100 mm Hg) and lower heart rates (~240 vs ~285 beats per min) at baseline, probably as a consequence of the deeper anaesthesia needed to perform a thoracotomy. This could have suppressed autonomic influences on the heart in our studies, which may be important in beat-to-beat changes in repolarization. The reduced heart rate *per se* is unlikely to be a factor as it has been shown in the chronic AV blocked dog model that it is the remodelling process rather than bradycardia which leads to increases in STV (Thomsen *et al.*, 2007). Alternatively, the continued administration of phenylephrine in our model may have an influence. A recent abstract suggests that QT variability could not predict TdP induced by phenylephrine + dofetilide in closed-chest anaesthetized rabbits (Orosz *et al.*, 2007). This agrees with our findings in open-chest anaesthetized rabbits.

Triangulation of the MAP was seen in these studies but only during the third cycle of drug administration. No changes were detected before the occurrence of VPBs or TdP. Neither HMR1556 nor ATX-II given alone caused any triangulation but ATX-II did cause TdP. It is clear from the present results that there is no association between triangulation and TdP in this model. There is, however, good evidence from *in vitro* studies that triangulation precedes TdP (Hondeghem *et al.*, 2001, 2003; Milberg *et al.*, 2005). Perhaps the much lower heart rates in perfused hearts *in vitro* allow greater development of triangulation at earlier time points. In rabbit Purkinje fibres, triangulation (and instability calculated in a similar manner to STV) induced by an  $I_{Kr}$  blocker was much greater at a lower stimulation rate (Lu *et al.*, 2006).

#### Limitations

In the present study, MAPs were only recorded from the epicardium of the left ventricle. It is possible that this area does not exhibit as much variability in repolarization as some other parts of the heart. For example, in the rabbit perfused left ventricular wedge preparation, QT prolonging drugs had greater effects on endocardium than epicardium (Liu *et al.*, 2006). Thus we cannot exclude the possibility, that in our *in vivo* model, changes in STV or triangulation of the action potential might have been detected from endocardial MAPs. It should also be noted that the present studies have focused on examining temporal dispersion of repolarization through measurement of STV. Although it has been

suggested recently that neither QT dispersion nor changes in  $T_{peak}-T_{end}$  of the QT interval predicted TdP in anaesthetized  $\alpha_1$ -adrenoceptor-stimulated rabbits (Orosz *et al.*, 2007), we cannot exclude the possibility that spatial dispersion of repolarization may predict TdP in our model.

## Conclusions

These studies have demonstrated that TdP induced by  $I_{Kr}$  blockade can be potentiated by prolonging  $I_{Na}$  or blocking  $I_{Ks}$ . The latter approach was more effective. In the particular model used here, the open-chest,  $\alpha$ -adrenoceptor-stimulated, anaesthetized rabbit, neither changes in STV of repolarization nor triangulation of the action potential could predict the occurrence of TdP. It is important that further studies are performed to assess the predictive power of STV of QT intervals, APD<sub>90</sub> and triangulation of the action potential in *in vivo* models of TdP.

## Acknowledgements

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## Conflict of interest

The authors state no conflict of interest.

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