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BACKGROUND AND PURPOSE

Ventricular arrhythmias induced by human ether-a-go-go related gene (hERG; $K_v11.1$ channel) blockers are a consequence of alterations in ventricular repolarisation in association with high-frequency (HF) oscillations, which act as a primary trigger; the autonomic nervous system plays a modulatory role. In the present study, we investigated the role of β_1-adrenoceptors in the HF relationship between magnitude of heart rate and QT interval changes within discrete 10 s intervals (sorted into 5 bpm heart rate increments) and its implications for torsadogenic hERG blockers.

EXPERIMENTAL APPROACH

The HF relationship was studied under conditions of autonomic blockade with atenolol (β_1-adrenoceptor blocker) in the absence or presence of five hERG blockers in beagle dogs. In total, the effects of 14 hERG blockers on the HF relationship were investigated.

KEY RESULTS

All the torsadogenic hERG blockers tested caused a vertical shift in the HF relationship, while hERG blockers associated with a low risk of Torsades de Pointes did not cause any vertical shift. Atenolol completely prevented the effects four torsadogenic agents (quinidine, thioridazine, risperidone and terfenadine) on the HF relationship, but only partially reduced those of dofetilide, leading to the characterization of two types of torsadogenic agent.

CONCLUSIONS AND IMPLICATIONS

Analysis of the vertical shift in the HF relationship demonstrated that signs of transient sympathetic activation during HF oscillations in the presence of torsadogenic hERG blockers are mediated by β_1-adrenoceptors. We suggest the HF relationship as a new biomarker for assessing Torsades de pointes liability, with potential implications in both preclinical studies and the clinic.

Abbreviations

APD, action potential duration; BBB, bundle branch block; BVR, beat-to-beat variability of ventricular repolarisation; HF, high frequency; HR, heart rate; STV_{QT}, short term QT interval variability; TdP, Torsades de Pointes

Tables of Links

TARGETS
GPCRs[a]
β1-adrenoceptor
Ion channels[b]
hERG (Kv11.1)

LIGANDS			
Atenolol	Haloperidol	Quinidine	Terfenadine
Cisapride	Nicardipine	Risperidone	Thioridazine
Dofetilide	Phenytoin	Sotalol	Verapamil

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Torsades de Pointes (TdP) is a malignant form of ventricular arrhythmias responsible for sudden death in various forms of long QT (LQT) syndrome (Shimizu and Antzelevitch, 1999) and in patients treated with molecules known to block the human ether-a-go-go related gene (hERG; also known as the K_v11.1 channel) (Redfern *et al.*, 2003). This latter effect has become a major issue in drug development as it is difficult to predict whether a substance will have this property (Hancox *et al.*, 2008; Abi-Gerges *et al.*, 2011; Nalos *et al.*, 2012; Di Veroli *et al.*, 2014). It is now known that an ability to block cardiac hERG is not the only determinant for triggering TdP. However, one of the major difficulties in identifying these harmful molecules arises from the fact that the propensity of a drug to induce TdP does not fit with a unique and common electrophysiological profile. Alternatively, the autonomic nervous system has been reported to play a critical role in ventricular arrhythmia liability of drugs, causing QT prolongation through abnormalities in the restoration of the QT/RR relationship (Fossa, 2008). In a recent study, we showed that the autonomic high-frequency (HF) oscillations in the amplitude of heart rate (HR) play a major role in beat-to-beat variability of ventricular repolarisation (BVR) and are one of the mechanisms responsible for dofetilide-induced TdP (Champeroux *et al.*, 2015). These HF oscillations result from alternate sequences of parasympathetic activation and deactivation occurring within a very short period of a few seconds (Pagani *et al.*, 1986). These fast changes in beat-to-beat HR immediately influence QT-interval duration through the well-known inverse relationship between these parameters. In parallel, the sympathetic component of autonomic control is clearly involved, through β_1-adrenoceptors, in the mechanisms of TdP triggering in the LQT_1 syndrome, the most prevalent form of inherited LQT syndrome; β-blocker therapy effectively prevents TdP in LQT_1 patients (Viitasalo *et al.*, 2006). Consequently, we studied the role of β_1 adrenoceptors in HF oscillations and its consequences on QT-interval variability in the context of QT prolongation by hERG blockers. This study was conducted in beagle dogs. This latter species has the advantage of exhibiting an autonomic balance close to that of humans with a predominant vagal tone (Pagani *et al.*, 1986). Moreover, this species is the main species employed in preclinical studies to investigate cardiac safety issues of new drug candidates.

Methods

All experiments were subjected to ethical review (ethical committee n° CEEA-111) according to 2010/63/UE animal welfare European directive. Studies were undertaken following the 3-R rule (replacement, reduction and refinement) including standard operating procedures for environmental enrichment and animal welfare in a (Good Laboratory Practices) GLP testing facility. Reporting of experiments follows the ARRIVE guidelines (Kilkenny *et al.*, 2010).

Electrocardiogram recordings in conscious dogs

Adult male and female (8–24 months old, 10–15 kg, Centre d'Elevage du Domaine des Souches – Mezilles, France) beagle dogs were instrumented with radio telemetry transmitters (Data Sciences International, Saint Paul, USA) as described elsewhere (Champeroux *et al.*, 2013). After left thoracotomy, one electrode was sutured directly to the left ventricular epicardium near the apex while the second electrode was sutured to the pericardium above the right atrium to approximate a limb Lead II ECG. Analgesic treatment with buprenorphine/meloxicam was given before surgery and continued for a minimum of 2 days to alleviate any postoperative pain. A minimum period of 3 weeks was allowed for recovery from the surgery. Animals were housed in individual stainless-steel cages for telemetry recordings. Outside of recording periods, animals were housed in pens with groups of six animals maximum. Environmental parameters were recorded continuously and maintained within a fixed-range, room temperature at 15–21°C and 45–65% relative humidity. The artificial day/night cycle was 12 h light and 12 h dark with lights on at 07:30 h. Drinking water was provided *ad libitum*. Solid diet (300 g) was given daily in the morning. All dosing was performed between 15:00 h and 15: 30 h. ECGs were recorded continuously for a minimum of 2 h before dosing up to 24 h post-dose. ECG waveforms were continuously recorded at a sampling rate of 500 Hz using the ART™ acquisition software release 4.1. (Data Sciences International, New Brighton, MN, United States). QT interval and HR values were calculated according to an automated procedure from a beat-to-beat analysis using internal software developed in RPL (RS/1 programming language, RS/1 release 6.3, Applied Materials, Santa Clara, CA, USA), GLP validated. Location of cardiac waves was performed

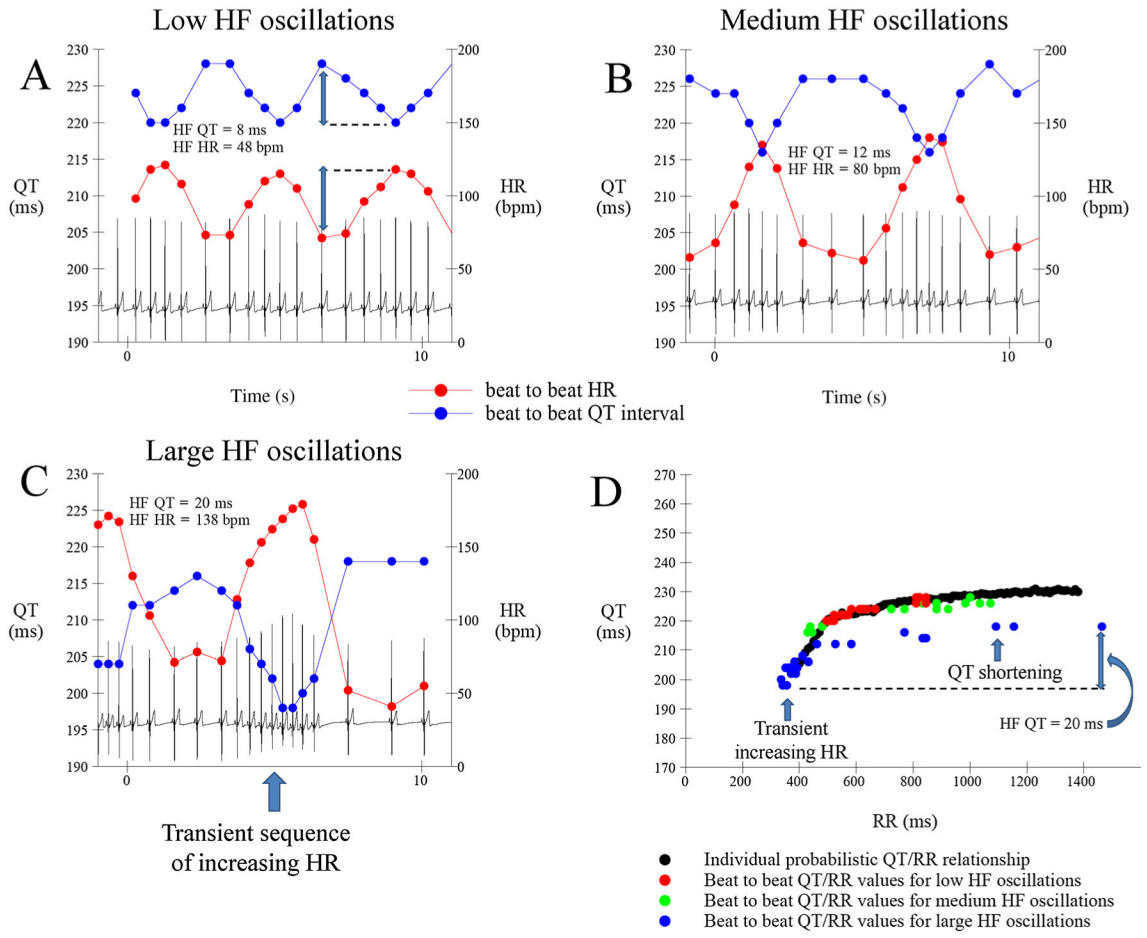

Figure 1

(A, B, C) Examples of low, medium and large high-frequency (HF) oscillations, respectively. All ECG examples presented were collected from the same dog in free-treatment conditions. Arrows in (A) depict examples of calculations of magnitude of QT interval HF oscillations (called HF QT) and heart rate (HR) HF oscillations (called HF HR). (D) Beat-to-beat QT/RR values collected during these low, medium and large HF oscillations were plotted versus the individual mean QT/RR relationship calculated over a 24 h period in this dog according to the probabilistic method (Holzgrefe *et al.*, 2014). This graph demonstrates the well-known rate dependence of QT interval upon RR interval during HF oscillations. For large HF oscillations, signs of increasing heart rate associated with QT interval shortening are observed.

according to procedures described by Ettinger and Suter (1970). Validation of correct location of cardiac wave markers was performed according to a standardised procedure which covered the whole 24-hour period. In most cases, the percentage of errors in location of the end of the T wave was less than 3%.

HF oscillations

The term 'HF oscillations' refers to the magnitude of HR and QT interval changes within discrete 10 s intervals, that is, the maximum period of HF rhythms (0.1 Hz) of the autonomic nervous system (Champeroux *et al.*, 2013). The magnitude of HF oscillations was calculated from the difference between maximum and minimum HR or QT interval values noted within each 10 s sequence as previously described (Champeroux *et al.*, 2015, see Figure 1A for a typical example of determination of HF oscillations).

HF relationship

The term 'HF relationship' refers to the relationship between HR oscillations (HF HR) and QT interval oscillations (HF QT). Building this relationship was based on a close methodological approach to that described earlier for the QT/RR relationship (Holzgrefe *et al.*, 2014). This analysis consists in sorting all pairs of HR HF oscillations by increasing increments of 5 bpm (beats min^{-1}) over a selected time range. Subsequently, mean magnitude of HF QT was calculated for each 5 bpm HR HF oscillations increment. A minimum of 10 pairs was required for calculations per increment. Respiratory sinus arrhythmia occurs naturally during vagal HF oscillations, and their rhythmic feature constitutes the fundamental principle for measurement of vagal HF rhythms from spectral analysis of HR variability (Pagani *et al.*, 1986). This respiratory sinus arrhythmia does not usually cause pauses with an RR interval larger than 2000 ms in dogs. In this species, they can exceptionally degenerate into

larger and non-rhythmic pauses (>2000 ms). Consequently, 10 s sequences with less than five beats were excluded from analysis to avoid inclusion of sequences with incomplete HF cycles. Finally, 10 s sequences with the presence of arrhythmias and the two directly adjacent sequences were also excluded from analysis. The HF relationship was constructed from data collected over a time period ranging between 3 and 8 h depending on the duration of the drug effects. Consequently, the time range selected might be different depending on dose levels. The time ranges selected are reported in the figure legends. A minimum of 3 h is necessary to allow the collection of a convenient amount of data for a good definition of the HF oscillations relationship. Narrow time ranges truncate the HF relationship at both extremes. Finally, we compared HF relationships derived from HR oscillations with that derived from RR interval oscillations. This comparison showed that constructing the HF relationship from RR interval oscillations is not appropriate for revealing increases in QT interval oscillations during large HF oscillations. Explanations are provided as supplemental data (Figure S1).

Statistical procedures

Statistics were processed using GLP-validated RS/1 computer procedures (release 6.3, Applied Materials). Experiments were conducted following randomised cross-over study designs in groups of six animals (three males and three females). HF relationships were constructed individually. The extent of the HF relationships might slightly differ between animals. Only increments in HR HF oscillations common to all animals were used for constructing mean relationships and statistical comparisons for fixed increments. Drug-induced effects on HF oscillations were compared with those of vehicle for each increment in HF relationships using an ANOVA followed by an LSD test (Fisher's Least Significant Difference test) in the case of multiple comparisons. Experiments with increasing dose levels were conducted separately on different groups of animals at different periods for each dose level (hERG blocker vs. vehicle). This explains some small changes in variability (SEM) between vehicle sessions depending on dose levels. Experiments with atenolol were undertaken on the same group of animals (hERG blocker alone, hERG blocker + atenolol vs. vehicle).

Drugs

Dofetilide was purchased from Sequoia Research Product Ltd. (Pangbourne, UK). This molecule was dissolved in water. Atenolol (1 mg·kg^{-1}, i.v.) was used for blockade of β_1 adrenoceptors. Atenolol was purchased from Sigma-Aldrich (Saint Quentin, France). This agent was dissolved in saline. Other hERG blockers were dissolved in water or 0.5% methylcellulose. Dose levels reported in the present study were chosen on the basis of preliminary trials in two animals for ethical reasons. Dose levels were increased according to a semi-log progression until evidence of QT prolongation and/or a vertical shift in the HF relationship was obtained. Dose progression was stopped after the first signs of intolerance, for drugs devoid of effects on the latter parameters. The effects of hERG blockers were studied on groups of six animals. For agents devoid of effect on the HF relationship, we tested the highest dose level only on a group of six animals, except verapamil which was tested at three dose levels.

Results

HF oscillations in beagle dogs

Detailed inspection of typical ECG traces collected in free treatment conditions from the same dog during HF oscillations revealed that QT interval oscillations are lower for low HR oscillations (Figure 1A) when compared with medium HF oscillations (Figure 1B) and larger for large HF oscillations (Figure 1C). Plotting the beat-to-beat HR and QT interval values versus the

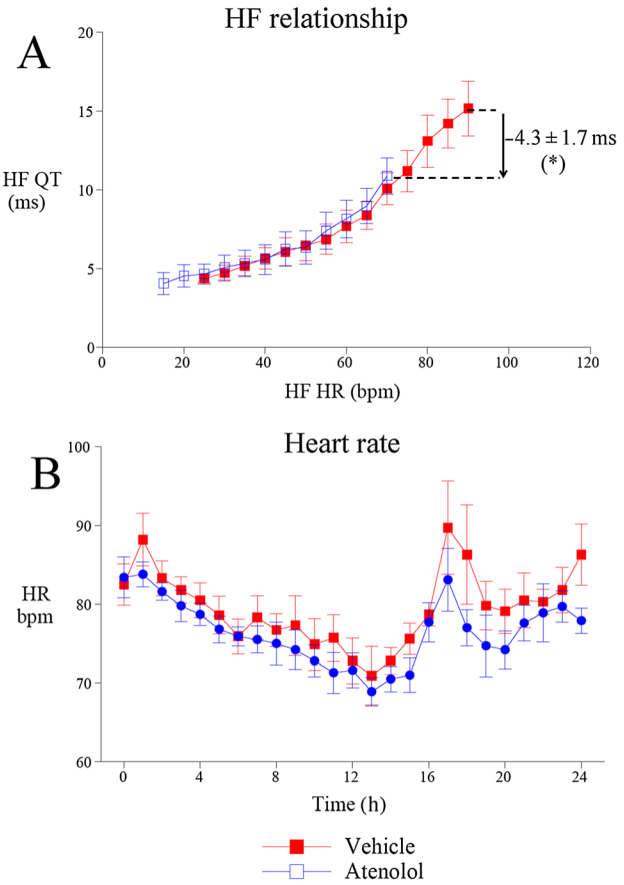

Figure 2

(A) Atenolol (1 mg·kg^{-1}, i.v.) caused a moderate shift of high-frequency (HF) oscillations towards lower levels. As a consequence, it decreased by −4.3 ± 1.7 ms ($P \leq 0.05$) the QT interval of HF oscillations for medium and large oscillations when compared with vehicle. Time period used for building relationships: 1 to 7 h post dosing. (B) Time course of heart rate over a 24 h period following the administration of atenolol compared with vehicle. In this experiment, the mean heart rate calculated over 1 h periods remained unchanged after the administration of atenolol ($n = 6$, $P > 0.05$, when compared with vehicle). Data are presented as mean values ± SEM.

individual mean QT/RR relationship revealed that beat-to-beat changes in QT interval follow the mean QT/RR relationship in accordance with the well-known rate dependence of QT interval upon RR interval (Figure 1D). It should be noted that large HF oscillations corresponded to HF oscillations including transient sequences of increasing HR visible from both the ECG trace and from the comparison with the mean QT/RR relationship. They were characterized by the presence of short RR intervals coexisting with large RR intervals. Because of the curvature of the QT/RR relationship for low RR intervals, the magnitude of the QT interval changes during HF oscillations was increased. As shown in Figure 1D, large HF oscillations were associated with QT shortening for high RR interval values (plateau of the QT/RR relationship).

Effect of atenolol on the HF relationship

In vehicle sessions, the HF relationship confirmed that HF QT oscillations were progressively increased when HF HR oscillations were increased (Figure 2A). Blockade of β_1-adrenoceptors by atenolol reduced the largest HF oscillations of HR (70 bpm with atenolol instead of 90 bpm with vehicle, Figure 2A). The main consequence was a decrease in HF QT oscillations by -4.3 ± 1.7 ms ($P \leq 0.05$) for the largest HF HR oscillations when compared with vehicle. In the experiment reported, atenolol (1 mg·kg^{-1}, i.v.) had no statistically significant effect on mean HR calculated over a 1 h period when compared with vehicle (Figure 2B). This demonstrates that the basal level of sympathetic activation was low in this experiment. This finding is not unimportant. Indeed, the lack of changes in mean HR suggests that the HF relationship could allow detection of signs of transient sympathetic activation even in conditions where mean HR remains unchanged.

β_1-adrenoceptors contribute to the effects of the torsadogenic hERG blockers on the HF relationship

As in cynomolgus monkeys (Champeroux *et al.*, 2015), dofetilide (1 mg·kg^{-1}, p.o.) caused a vertical shift of the relationship when compared with vehicle (Figure 3A: vertical shift for large HF oscillations: +21.7 ± 3.6 ms, $P \leq 0.01$). The vertical shift induced by dofetilide was still statistically significant in the presence of atenolol when compared with vehicle. Dofetilide effects alone or in the presence of atenolol were not statistically different ($P > 0.05$, dofetilide alone vs, dofetilide + atenolol) for the same HF oscillations increments, but atenolol induced a decrease in vertical shift for the largest HF oscillations (-15.3 ± 6.3 ms, $P \leq 0.05$, dofetilide alone vs. dofetilide + atenolol). Sotalol (30 mg·kg^{-1}, p.o.) had a similar profile with regard to its intrinsic effects on β_1-adrenoceptors (Figure 3B). However, the vertical shift was less pronounced and not statistically significant ($P > 0.05$) when compared with vehicle except for the largest HF oscillations (+3.8 ± 0.5 ms, $P \leq 0.05$, sotalol vs. vehicle). Four other torsadogenic hERG blockers were tested in the absence and presence of atenolol: thioridazine, quinidine, risperidone and terfenadine (Redfern *et al.*, 2003; Vieweg *et al.*, 2008). All these hERG blockers induced a vertical shift in the HF relationship (Figure 4) except terfenadine, which induced a vertical shift for low/medium HF oscillations only. Atenolol suppressed these shifts in all cases ($P > 0.05$, when compared with vehicle sessions). Three other hERG blockers were also

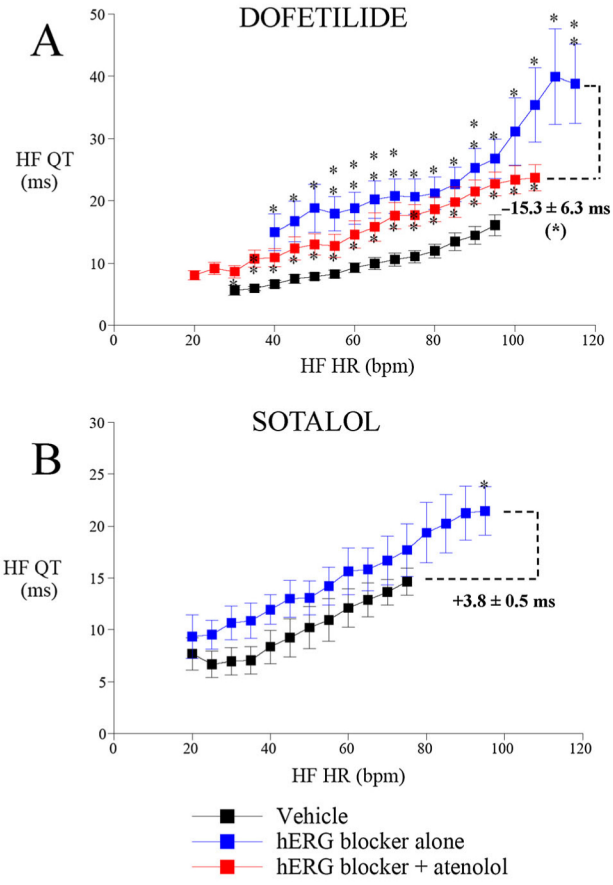

Figure 3

(A) Dofetilide (1 mg·kg^{-1}, p.o.) caused a large vertical shift in the high-frequency (HF) relationship for low, medium and large HF oscillations. Vertical shift induced by dofetilide still remains statistically significant in the presence of atenolol (1 mg·kg^{-1}, i.v.) when compared with vehicle. Atenolol tended to reduce this effect, but dofetilide effects alone or in the presence of atenolol were not statistically different ($P > 0.05$, dofetilide alone versus dofetilide + atenolol) except a decrease in vertical shift for large HF oscillations (-15.3 ± 6.3 ms, $P \leq 0.05$). (B) Sotalol (30 mg·kg^{-1}, p.o.) had a similar profile with regard to its intrinsic β_1 adrenoceptors properties. However, the vertical shift was less pronounced and not statistically significant ($P > 0.05$) when compared with vehicle except for the largest HF oscillations (+3.8 ± 0.5 ms). The common pattern of these two torsadogenic hERG blockers is their ability to cause a residual vertical shift in the HF relationship even in the presence of blockade of β_1 adrenoceptors. Time period used for building relationships: 2 to 9 h post dosing for dofetilide, 1 to 5 h post dosing for sotalol. Data are presented as mean values ± SEM (n = 6, *: $P \leq 0.05$, **: $P \leq 0.01$ when compared with vehicle).

found to cause vertical shifts in the HF relationship: cisapride, haloperidol and moxifloxacin (Figures S7–S9).In contrast, five other hERG blockers did not induce any vertical shift of the HF relationship: ciprofloxacin, ebastine, phenytoin, nicardipine and verapamil (Figures S10–S11). HF relationships were produced at increasing doses for nine hERG blockers (Figures S2–S10). Table 1 reports a summary of dose levels at which a vertical shift and first changes in HF HR

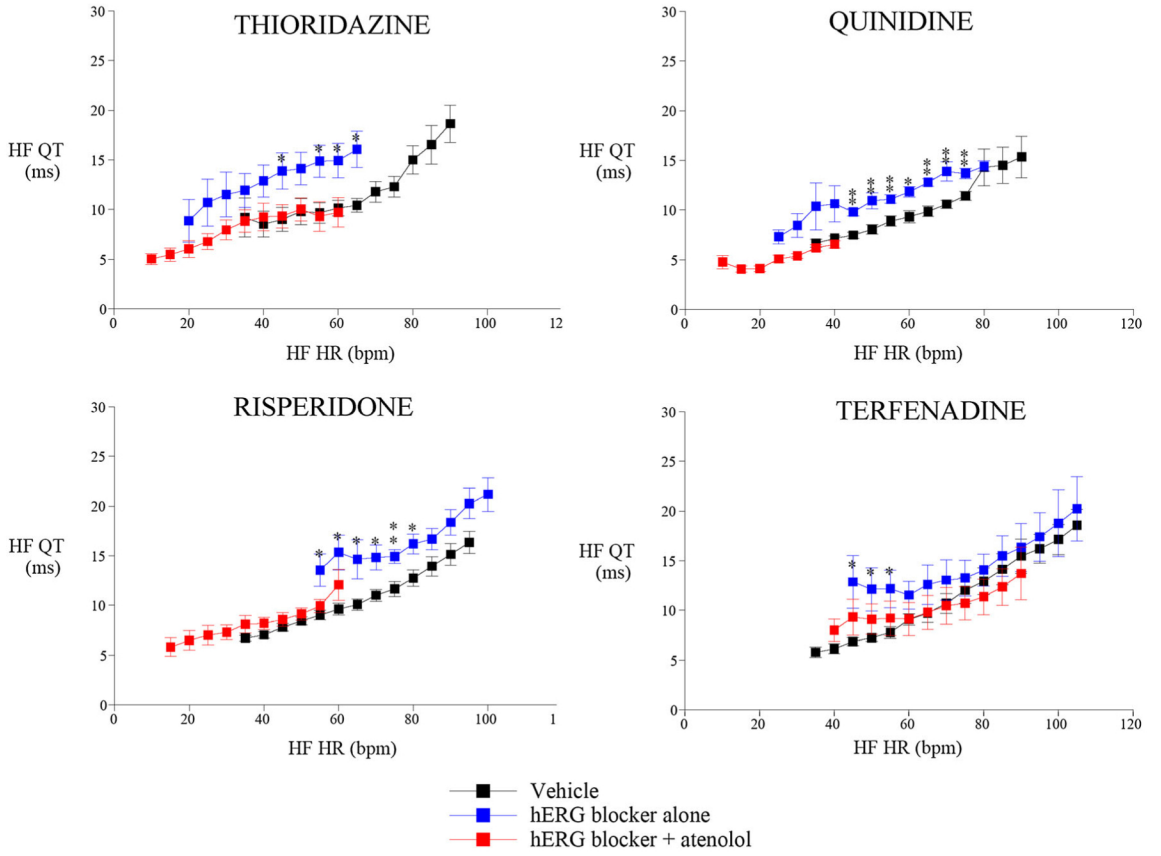

Figure 4

Thioridazine (20 mg·kg^{-1}, p.o.), quinidine (30 mg·kg^{-1}, p.o.), risperidone (1 mg·kg^{-1}, p.o.) and terfenadine (30 mg·kg^{-1}, p.o.) caused vertical shifts in the high-frequency (HF) relationship. Atenolol (1 mg·kg^{-1}, i.v.) suppressed these shifts in all cases ($P > 0.05$, when compared with vehicle sessions). The common pattern of these torsadogenic hERG blockers was an absence of residual vertical shift during blockade of β_1-adrenoceptors. Data are presented as mean values ± SEM ($n = 6$ per molecule, *: $P \leq 0.05$, **: $P \leq 0.01$ when compared with vehicle).

oscillations respectively were observed. Effects on QTc and mean HR associated with effects on HF HR oscillations are also provided. In four cases (thioridazine, quinidine, haloperidol and cisapride), effects on HR HF oscillations were observed at lower doses than the first dose producing a vertical shift in the HF relationship (Table 1). Of all the hERG blockers tested that caused a vertical shift, none caused a vertical shift before causing a shift in the HF relationship towards larger HF HR oscillations and an increase in HR HF oscillations. Moxifloxacin was the only hERG blocker that caused a vertical shift and decreased the HF oscillations. Among the quinolones, moxifloxacin was associated with the greatest risk of TdP, whereas ciprofloxacin was associated with the lowest TdP rate (Briasoulis *et al.*, 2011), this latter molecule being devoid of an effect on the HF relationship. hERG blockers associated with a low risk for TdP (phenytoin, ebastine, ciprofloxacin, verapamil and nicardipine) were unable to produce a vertical shift in the HF relationship or an increase in HR HF oscillations (Table 1).

Arrhythmic properties of dofetilide in dogs

Analyses of ECG traces in dofetilide-treated dogs confirmed the presence of large HF oscillations. These large HF oscillations were characterized by transient rhythmic sequences of increasing HR followed by a large pause (Figure 5A). This pattern was observed in all animals injected with dofetilide. In four out of six dogs, these transient sequences of increasing HR were associated with conduction disturbances originating from the conductive network. These conduction disturbances were characterized by bundle branch blocks (BBB) systematically seen during the first beats of increasing HR sequences (Figure 5B, Figure S12 for a zoomed view). Atenolol did not prevent the occurrence of BBB during HF oscillations. However, atenolol did suppress the dofetilide-induced premature ventricular beats seen in three out of six dogs (Figure S13).

Discussion

The HF relationship

Changes in beat-to-beat QT interval duration follow an inverse relationship to beat-to-beat HR changes in accordance with the well-known rate-dependence of QT interval upon HR. This relationship is very well preserved even for very short 10 s sampling sequences (Figure 1D, Holzgrefe *et al.*, 2007). The HF oscillations are a direct consequence of this

Table 1

Summary of the effects of 14 hERG blockers on HF relationship. This table reports the first dose level (in mg·kg^{-1}) at which a vertical shift of HF relationship was observed (VS) in comparison with the first dose at which an increase in HR HF oscillations was found (Champeroux *et al.*, 2015)

hERG blockers	VS (mg·kg$_{-1}$)	HF HR (mg·kg$_{-1}$)	HR (bpm)	QTc (ms)	Puurkinje profile
Dofetilide[a]	0.1	0.1	+9.4 ± 3.4 (**)	+24.5 ± 5.2 (**)	A1
DL sotalol	30	30	NS	+36.8 ± 4.6 (**)	A1
Quinidine	30	10	NS	+11.3 ± 2.1 (*)	A2
Terfenadine[a]	30	30	+11.9 ± 3.8 (**)	+18.5 ± 3.2 (**)	A2
Cisapride	6	2	+15.3 ± 3.4 (**)	+7.8 ± 3.2 (*)	A2
Thioridazine	20	1.5	+10.3 ± 2 (*)	NS	A2
Haloperidol	10	1	+16.3 + 16.3 ± 6(**)	NS	A2
Risperidone[a]	1	1	+10.1 + 4.3(*)	+18.4 ± 4.4 (**)	B
Moxifloxacin	90	Decrease (90)	NS	+41.6 ± 6.8 (**)	B
Ciprofloxacin	NS (100)	NS (100)	NS	+7.4 ± 3.7(*)	C
Ebastine	NS (30)	Decrease (90)	NS	+14.2 ± 5.5(**)	C
Phenytoin	NS (100)	NS (100)	+14.5 ± 4.3(*)	NS	C
Nicardipine	NS (30)	Decrease (30)	+86.3 ± 4.2(**)	−32.5 ± 4.1(**)	C
Verapamil	NS (30)	Decrease (30)	+17.6 ± 4.3(**)	−17.3 ± 3.7(**)	C

[a]No lower dose levels were tested on groups of six animals with these molecules. Maximum effects on mean heart rate and QTc (probabilistic method) found at the first dose producing an increase in HR HF oscillations were provided in parallel. Data are presented as mean values ± SEM ($n = 6$, NS: $P > 0.05$,
*$P \leq 0.05$,
**$P \leq 0.01$ when compared with vehicle). Purkinje profile: electrophysiological profile defined from isolated Purkinje fibres experiments (Champeroux *et al.*, 2005).

rate-dependency. The main source of fast variations in cardiac rhythm is driven by the parasympathetic nervous system through rhythmic cycles of activation/deactivation. This limb of the autonomic nervous system is responsible for very fast changes in beat-to-beat HR generating rhythmic oscillations at a frequency greater than 0.1 Hz, that is, a time period less than 10 s (Pagani *et al.*, 1986; Champeroux *et al.*, 2013). The larger HF oscillations of the HF relationship are characterized by transient sequences of increasing HR coexisting with parasympathetic-mediated HR oscillations. These transient sequences of increasing HR during large HF oscillations are mediated by β_1-adrenoceptors, as large HF oscillations were suppressed in the presence of atenolol. We noted that large HF oscillations were associated with QT shortening for high RR interval values, that is, during the plateau of the QT/RR relationship. This finding suggests that the hysteresis phenomenon (Pelchovitz *et al.*, 2012) might play a role during large HF oscillations. This phenomenon has been largely studied in man in particular during exercise sessions; it involves successive phases. During exercise, the parasympathetic system is deactivated, which is followed by activation of the sympathetic system. During recovery from the exercise phase, there is an early parasympathetic reactivation with persistent, but declining, sympathetic excitation. During this particular phase of the hysteresis phenomenon, both systems are active, and this phase is associated with a QT shortening (Pelchovitz *et al.*, 2012) as we observed during large HF oscillations in dogs. Interestingly, increased pro-arrhythmic risk has been reported in women when compared with men (healthy subjects) because of a greater QT interval during the recovery phase of the hysteresis phenomenon (Chauhan *et al.*, 2002).

β_1-adrenoceptors contribute to the effects of the torsadogenic hERG on the HF relationship

Several hERG blockers in our dataset were found to increase HF QT oscillations either by prolonging the relationship towards large HF HR oscillations and/or by causing a vertical shift in the HF relationship depending on dose. Both mechanisms involve β_1-adrenoceptors because they were fully or partially prevented by atenolol depending on the torsadogenic drug being studied. As previously found after temporal analysis under full autonomic blockade (Champeroux *et al.*, 2015), a residual vertical shift in the HF relationship was also visible with dofetilide during β_1-adrenoceptor blockade. This result strongly supports a previously proposed hypothesis that this component of QT interval variability is a consequence of beat-to-beat variability of repolarisation (BVR). This hypothesis accords with the increases in BVR described earlier with several hERG blockers in *in vitro* models of cardiomyocytes (Abi-Gerges *et al.*, 2010; Oros *et al.*, 2010) and in *ex. vivo* preparations of isolated perfused heart (Hondeghem *et al.*, 2001). For four torsadogenic hERG blockers (thioridazine, quinidine, risperidone and terfenadine), the vertical shift was fully prevented by β_1-

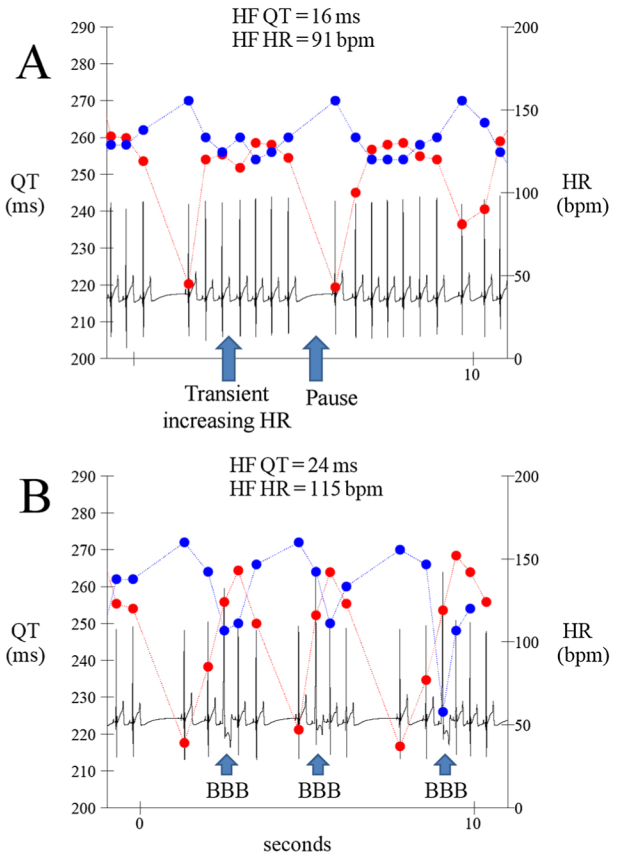

Figure 5

Examples of ECG traces collected from a beagle dog administered dofetilide (1 mg·kg^{-1}, p.o.) showing: (A) transient rhythmic sequences of increasing heart rate followed by a pause. (B) Left bundle branch blocks systematically seen during the first beats of transient sequences of increasing heart rate.

adrenoceptor blockade. Because the sympathetic system alone does not cause any significant vertical shift in medium and high ranges of HR HF oscillations, these data also strongly support a synergistic mechanism between BVR and the sympathetic nervous system. An analysis of the dose-dependence of torsadogenic hERG blockers effects on HR HF oscillations and vertical shift of the HF relationship demonstrated that both effects can be dissociated, also supporting the hypothesis that the first, initial effect (i.e. β_1-adrenoceptor stimulation) has a synergistic action on the second one (increase in BVR). Such a synergistic effect of the sympathetic system on QT interval variability has already been reported in patients with congenital LQT syndromes infused with adrenaline (Satomi *et al.*, 2005).

Two different profiles for the effects of torsadogenic hERG blockers on the HF relationship

When sotalol was used as a β_1-adrenoceptor blocking agent, it was found that BVR properties related to its electrophysiological profile can alone be responsible for an increase in QT interval variability only in the presence of parasympathetic-mediated HF oscillations. With dofetilide, they constitute a subgroup of torsadogenic drugs with the common property that they can still cause a vertical shift in the HF relationship even when the β_1-adrenoceptors are blocked. As stated previously, multiple ion channels are likely to be involved in this increase in BVR. In the case of dofetilide or sotalol, the late Na^+ current (late I_{Na}) has been demonstrated to have a role in the BVR and it was established that it is closely linked with the residual shift in the HF relationship. Indeed, this late Na^+ current is increased by these two hERG blockers (Yang *et al.*, 2014) and contributes to the enhancement of their lengthening effect on action potential duration (APD). Moreover, this late I_{Na} increase also contributes to the enhancement of the reverse rate-dependent effect on APD mediated by these drugs. This synergistic effect on APD and reverse rate-dependence was reported to enhance BVR at low pacing rates in particular and may be responsible for triggering TdP in isolated, paced rabbit hearts (Wu *et al.*, 2011). Furthermore, an increase in late I_{Na} has been proposed to facilitate early and delayed after depolarisations, triggered arrhythmias and cellular Ca^{2+} loading, causing in turn an increase in spatial and temporal dispersion of ventricular repolarisation (Shryock *et al.*, 2013). The other four torsadogenic hERG blockers tested in the presence of atenolol (thioridazine, quinidine, haloperidol and cisapride) do not have the same electrophysiological profile. They can be differentiated from the first subgroup by the fact that no residual vertical shift in the HF relationship was observed after blockade of β_1-adrenoceptors. These findings strongly support a role for specific ion channels in addition to hERG blocking properties as a major determinant for the arrhythmic risk of hERG blockers in the presence of autonomic HF oscillations. This subdivision of hERG blockers into two subgroups fits well with the identification of two electrophysiological profiles (named A1 and A2 profiles) drawn from isolated canine Purkinje fibre experiments (Champeroux *et al.*, 2005), which were later confirmed in patch-clamp assays (Champeroux *et al.*, 2011). In these previous studies, the electrophysiological profile of this second subgroup was associated with common properties of fast I_{Na} current inhibition. However, the specific arrhythmic effect involving multiple ion channels attributed to the second subgroup, the most numerous, remains to be confirmed. This conclusion fully supports the CIPA process (CIPA: Comprehensive In vitro Proarrhythmia Assay) for better understanding and predicting the risk of drug-induced TdP (Cavero and Holzgrefe, 2014).

Mechanisms of BBB and transient increases in heart rate

The conduction disturbances seen with dofetilide are characterized by BBB occurring during the first beats of transient sequences of increasing HR. A lengthening of the ventricular repolarisation induced by dofetilide could be responsible for conduction disturbances in the absence of autonomic control (Farkas *et al.*, 2006). Indeed, while repolarisation is incomplete in the presence of prolonged action potential duration, recovery of the voltage-dependent sodium channels from the inactivated state is slowed, which results in reduced conduction (Carmeliet, 1999). Such conduction disturbances are known to occur in various pathological situations in association with QT prolongation. They are triggered when HR is increased after a long pause so that an action

potential arises before repolarisation of the preceding action potential has finished (Boyden, 1996). Furthermore, it is well documented that a long cycle length or pause increases the effect of hERG blockers on action potential duration. This conduction slowing being effective after a long pause is why BBB were triggered during the first beats of transient sequences of increased HR following a pause. Purkinje fibres in the conductive network are particularly sensitive to this mechanism and the lengthening effects of dofetilide on action potential duration (Champeroux *et al.*, 2005). In the present study, we demonstrated that the mechanism responsible for these transient sequences of increased HR involve transient sympathetic stimulation during HF oscillations in the presence of torsadogenic hERG blockers. Increased sympathetic activity introduces further favourable conditions for triggering ventricular arrhythmic events (Baumert *et al.*, 2011; Leenhardt *et al.*, 2012). Preceding ventricular arrhythmias were reported as increasing the probability of TdP occurring (Farkas *et al.*, 2010). Large pauses seen in the presence of dofetilide were rhythmic and are thus mediated by vagal activity. Vagal nerve activity has been demonstrated to play an essential role in the generation of hERG-induced TdP (Farkas *et al.*, 2008). Such a pattern, that is, a large pause following an increase in heart rate, has been previously described in patients with acquired (Locati *et al.*, 1995) or congenital (Noda *et al.*, 2004) LQT syndromes. In these latter situations, a marked pause precedes the onset of TdP. A review of case studies from the literature showed that the majority (74%) of spontaneous TdP seen in congenital LQT syndromes were pause-dependent (Viskin *et al.*, 2000). We also reported this pattern for dofetilide-induced TdP in cynomolgus monkeys (Champeroux *et al.*, 2015). Noteworthy signs of slowed conduction associated with premature ventricular beats have also been recorded in this latter model of TdP (Figures S14–S16). Dofetilide did not produce TdP in healthy beagle dogs, in accordance with the fact that this species requires ventricular remodelling (such as chronic atrioventricular block) as a prerequisite for TdP induction (Dunnink *et al.*, 2012). This mechanism responsible for these transient sequences of sympathetic activation and increased HR during HF oscillations is triggered even under conditions of mild QT prolongation, because an increase in HR HF oscillations was found with all the torsadogenic agents tested under conditions of moderate QT prolongation. The results with thioridazine show that this phenomenon can occur even in conditions of apparent absence of QT prolongation, a situation where the sympathetic system itself was found to mask thioridazine-induced QT prolongation (Champeroux *et al.*, 2010). Conversely, QT prolongation is not systematically associated with transient sequences of sympathetic activation and increased HR. The results with moxifloxacin that induces a marked QT prolongation in dogs illustrate such a situation. In conclusion, this study puts known arrhythmic mechanisms into the context of autonomic HF oscillations. This part of the discussion is summarised in the Figure 6.

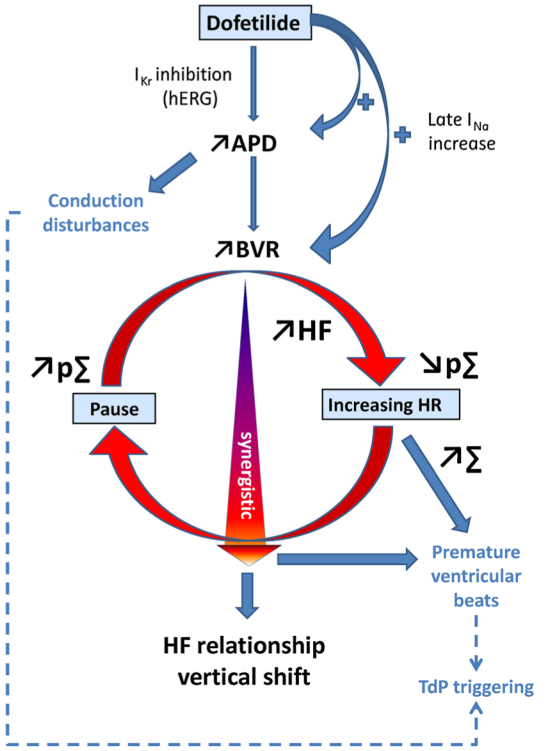

Figure 6

This figure puts known arrhythmic mechanisms of dofetilide into the context of high-frequency (HF) oscillations. The blockade of hERG (K_v11.1) channels causes prolongation of the action potential duration (APD). This effect is enhanced by an increase in I_{Na}. Both effects contribute to an increase in beat to beat variability of ventricular repolarisation (BVR). A sympathetic (Σ) driven increase in heart rate (HR) followed by a large pause precedes the onset of Torsades de Pointes (TdP). TdP are triggered in the presence of increased BVR through a synergistic action with parasympathetic (pΣ) HF oscillations, sympathetic driven ventricular premature beats and conduction disturbances.

Implications

Analysis of vertical shift in the HF relationship seems to be an interesting biomarker for TdP liability. This biomarker is clearly more sensitive than the short-term QT interval variability, that is, STV_{QT} (Thomsen *et al.*, 2004) that was found to be positive in dogs with only a limited number of torsadogenic hERG blockers (Champeroux *et al.*, 2015). When compared with HR HF oscillations, it offers the advantage of making reference to QT interval variability. In parallel, HR HF oscillations can be also considered to have potential as a sensitive tool for detection of signs of mild sympathetic activation without or with little impact on HR. One of the major interests of both biomarkers lies in its applicability to safety pharmacology in preclinical studies conducted in healthy animals. This point is essential because specific and reliable biomarkers are still currently lacking for such studies, QT prolongation is now considered a poor indicator of TdP liability. Moreover, the close link between this biomarker and the role of HF oscillations should affect current and future efforts made for designing standardised *in vitro* assays dedicated to prediction of drug-induced arrhythmic risk. Indeed, *in vitro* models are designed exclusively under fixed pacing rates conditions. Our results strongly suggest that *in vitro* experiments designed with oscillatory pacing rates might be more suitable for a detection of arrhythmic profiles, as previously proposed (Green *et al.*, 2011). The same recommendation can be applied to *in silico* modelling derived from multiple ion channel, patch-clamp assays. For clinical applications, numerous studies have been conducted with the

aim of finding sensitive and early biomarkers in various cardiovascular diseases. This new biomarker is likely to provide sensitive information, more sensitive than STV_{QT}, in particular in the context of increased sympathetic activity, probable increased QT interval variability and/or LQT syndrome (Shamsuzzaman *et al.*, 2003; Hinterseer *et al.*, 2009). Finally, by demonstrating a synergistic action on QT interval variability involving the sympathetic system, this study fully supports therapeutic strategies leading to a reduction in sympathetic activity, such as left cardiac sympathetic denervation (Bos *et al.*, 2013) and β-blocker therapy (Moss *et al.*, 2000; Viitasalo *et al.*, 2006) in LQT syndromes.

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Conflict of interest

The authors state no conflict of interest.

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