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Evaluation of the Rabbit Purkinje Fibre Assay as an *in vitro* Tool for Assessing the Risk of Drug-Induced Torsades de Pointes in Humans

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Abstract

Background: The issue of drug-induced QT interval prolongation and torsades de pointes represents a major concern for pharmaceutical development. In this investigation, we examined the value of the isolated rabbit Purkinje fibre as an *in vitro* action potential (AP) assay to predict the potential of drugs to induce these undesirable adverse effects.

Methods: First, we categorised the proarrhythmic risk of 26 medicinal products based on proportional reporting ratios for these two adverse events recorded in a US FDA database (Spontaneous Reporting System/Adverse Event Reporting System). Second, we measured drug effects on AP in rabbit Purkinje fibres. Finally, the results of the two analyses were compared to evaluate the predictive value of the *in vitro* assay.

Results: Analysis of the clinical data classified the drugs into 14 positive, 7 negative and 5 questionable for proarrhythmic risk. Based on *in vitro* electrophysiological profiles, the drugs were grouped into four categories: (i) profile 1 drugs prolong repolarisation without slowing depolarisation; (ii) profile 2 drugs prolong repolarisation and also slow depolarisation; (iii) profile 3 drugs shorten repolarisation; and (iv) profile 4 drugs are without effects. All 14 clinical-positive drugs fell into profiles 1 or 2 (prolongers) with low safety margins (except probucol, which showed no effect, probably because of its low solubility). Clinical-negative drugs belonged mostly to profiles 3 or 4 (non-prolongers) [except clemastine and amlodipine, which were prolongers but had large safety margins]. Clinical-questionable drugs either did not prolong or prolonged slightly but produced additional electrophysiological effects opposing prolongation.

Conclusion: The rabbit Purkinje fibre is a valuable assay for evaluating the proarrhythmic liability of pharmaceuticals as it can reveal complex electrophysiological profiles that modulate repolarisation delay.

Background

In recent years, health authorities and drug companies have been facing an increasingly sensitive safety issue: the induction by various pharmaceuticals of torsade de pointes, a potentially lethal ventricular arrhythmia. Since the early 1970s, it has been known that anti-arrhythmic drugs, particularly those belonging to class III such as sotalol, can promote or facilitate the induction of torsades de pointes as a consequence of their mechanism of action.[1] However, it is now recognised that such a life-threatening adverse effect is also associated with the therapeutic use of diverse non-cardiovascular drugs.[2] Therefore, a major challenge for drug developers is to adopt non-clinical testing strategies that detect as early as possible the proarrhythmic potential of new compounds.

Prolongation of the QT interval of the ECG ('QT prolongation') is a generally used, albeit still controversial, precursor surrogate marker of torsades de pointes. A number of non-cardiovascular drugs prolong the QT interval through an undesired ancillary pharmacological property, commonly inhibiting a specific cardiac ion channel, KCNH2, which is encoded by the human ether-a-go-go (h*ERG*) gene in humans and is responsible for the rapid delayed-rectifier potassium current (I_{Kr}) in the myocardium.[2] Therefore, nowadays, non-clinical strategies designed to evaluate proarrhythmic risk usually begin with the *in vitro* quantification of the interaction of a test compound with KCNH2, generally using patch-clamp electrophysiology. Redfern et al.[3] have discussed the clinical predictive value of such data and they concluded that the 'I_{Kr} safety margin' (concentration inhibiting I_{Kr} normalised to free therapeutic concentration) is typically small for compounds causing QT prolongation and large for others. However, exceptions were noted as some drugs that are not considered proarrhythmic have small margins (verapamil, fluoxetine, tamoxifen), while others cause QT prolongation and torsades de pointes despite large margins (sotalol, pimozide).

It should be possible to gain further insight into this discrepancy by investigating the overall electrophysiological profile of compounds in integrated heart preparations. *In vitro* action potential (AP) assays, such as the superfused rabbit cardiac Purkinje fibre, were recommended in the 1997 'Points-to-consider' document from the European Committee for Proprietary Medicinal Products.[4] However, the clinical predictive value of AP tests remains controversial because of the lack of extensive studies performed with a sufficient number of drugs from various therapeutic classes.

The work reported here presents such an evaluation with 26 drugs. In the first part, we determine the clinical proarrhythmic liability of the selected drugs by mining the postmarketing data found in the US FDA clinical adverse event (AE) database, the Spontaneous Reporting System/Adverse Even Reporting System (SRS/AERS) database. With this approach, which is innovative for the evaluation of an *in vitro* test system, clinical safety signals are identified through assessment of all AE reports in the SRS/AERS database, rather than just the limited information derived from published case reports. In the second part, we describe the actions of the 26 drugs on APs in rabbit Purkinje fibres and we show that this model provides a valuable *in vitro* assay to assess the clinical liability.

Methods

Calculation of Proportional Reporting Ratios

The proportional reporting ratio (PRR) detects possible safety signals in AE data by following an approach similar to the proportional mortality ratio, where no absolute event rate is known but the fraction of all deaths due to a specific cause in a test population can be calculated and compared with the corresponding fraction in a reference population. A safety signal is generated if the proportion of a selected AE for a drug of interest is larger than the proportion seen in the comparison group of drugs and if it meets the following criteria (PRR ≥ 2; $\chi^2 \geq 4$; number of reports ≥ 3).[5]

PRRs were calculated with the software Qscan™ FDA (DrugLogic, Preston, Virginia, USA), which accesses the large number of AEs in the SRS/AERS database publicly available through the Freedom of Information Act. The Qscan™ AE denominations used were ('electrocardiogram qt prolonged' OR 'electrocardiogram qt corrected interval prolonged') and ('torsades de pointes'). It should be noted that

	Actual number of reported cases			Proportion of cases	PRR	Expected number of cases
	AE_Y	other AEs	all AEs	AE_Y		
Drug or class X	A	C	A + C	P1 = A/(A + C)	PRR = P1/P2	E = (A + C) x P2
All drugs or subgroup	B	D	B + D	P2 = B/(B + D)		

Fig. 1. Formulas for calculation and evaluation of the proportional reporting ratio (PRR). **AE** = adverse event.

inclusion of an AE in either QT prolongation group is not related to a specific magnitude of prolongation but only to the reporter's judgement that clinically significant prolongation was observed. Figure 1 illustrates how PRRs are derived for a specific drug or class of drugs (D_X) and a selected AE (AE_Y). First, P1 is calculated, which is the frequency of occurrence of AE_Y relative to all reported AEs for D_X. Then, this value is divided by P2, the corresponding frequency calculated for all database drugs (or a particular drug subgroup). If the resulting PRR of AE_Y for D_X is >1, AE_Y occurs more frequently with D_X than with the ensemble of marketed drugs (or a particular subgroup) whereas, if PRR is <1, AE_Y occurs less frequently. Finally, the statistical significance of the signal is assessed by comparing the observed number of reports to its expected value (E), using the χ^2 test with 1 degree of freedom.

Electrophysiological Study in Rabbit Purkinje Fibres

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996). The Purkinje fibre preparation is adapted from that described by Adamantidis et al.[6] Female New Zealand white rabbits (1.5–4.0kg) are euthanised by cervical dislocation. The hearts are quickly excised and placed in a potassium- and glucose-enriched extracellular solution oxygenated with 95% O_2/5% CO_2 at room temperature (in mmol/L: NaCl 108.2; KCl 27; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 0.9; $NaHCO_3$ 24; glucose 55; and pH 7.35 ± 0.1 with NaOH or HCl). Purkinje fibres are dissected while keeping attached small pieces of ventricular muscle to prevent damage to the fibres. They are divided into 2–3 pieces, pinned through the ventricular muscle to the silicone bottom of the experimental chambers (2.5mL), and superfused at 2.5 mL/minute with oxygenated and enriched extracellular solution at 38.0 ± 0.5 °C. After 1 hour, the preparations are superfused with oxygenated normal extracellular solution (in mmol/L: NaCl 118; KCl 4; $CaCl_2$ 1.8; $MgCl_2$ 1; NaH_2PO_4 0.9; $NaHCO_3$ 24; glucose 11; and pH 7.35 ± 0.1 with NaOH or HCl).

Transmembrane voltage is recorded with a glass microelectrode having a tip resistance of 10–20MΩ when filled with 3 mol/L KCl. The preparation is electrically stimulated with a bipolar stainless-steel electrode to induce firing of APs. After 30 minutes of stabilisation at 2Hz (120 beats/min), the frequency is maintained at 1Hz (60 beats/min) until the APs are stable for at least 45 minutes. Thereafter, three sets of APs are recorded during 2-minute periods at each of three frequencies (0.2Hz, 1Hz and 2Hz); this is repeated 4–5 times. The last ten APs of each period are recorded since, in our hands, AP parameters have reached steady state after 1 minute at all three frequencies. At this point, if a preparation fulfills the quality parameters outlined in the next section, the following testing protocol is applied.

First, the solvent employed to prepare the test compound is added to the superfusion solution at the constant concentration used for the remainder of the experiment. After 15–18 minutes, ten APs are recorded at a stimulation frequency of 1Hz (60 beats/min). Then, the frequency is reduced to 0.2Hz (12 beats/min) for 2 minutes and ten APs are recorded. After 6 minutes of recovery at 1Hz, the frequency is increased to 2Hz (120 beats/min) for 2 minutes and ten APs are recorded. Finally, the frequency is returned to 1Hz for 2 minutes. Thereafter, the test compound is applied at the lowest concentration and the manoeuvres performed in solvent are replicated. After 30 minutes, the next highest concentration is tested; this is repeated for up to six concentrations.

In order to assess the viability and responsiveness of preparations where no major changes in AP parameters are observed with a test compound, a final 15-minute perfusion of 10 μmol/L quinidine is performed.

Parameters Measured, Quality Control, Statistics and Thresholds

The acquisition software calculates the following AP parameters: resting membrane potential (RMP), maximal rate of rise of phase 0 depolarisation (V_{max}) and duration at 70% and 90% repolarisation (APD_{70} and APD_{90}, respectively). Preparations are discarded at the end of the stabilisation period if one of the following occurs: (i) the AP parameters are not stable at each stimulation frequency; (ii) the preparation exhibits spontaneous activity (i.e. untriggered APs at any stimulation frequency); (iii) RMP >−78mV; (iv) V_{max} <200V/s or V_{max} >800V/s at 1 or 2Hz; (v) APD_{90} <200ms or APD_{90} >500ms at 1Hz; and (vi) APD_{90} <10% longer at 0.2Hz than at 1Hz. Drug effects are calculated as percent changes relative to the baseline (solvent) period for each fibre because this is the standard method used to partially correct for the fact that larger baseline values lead to larger effects.[7] When early-after-depolarisations (EADs) are observed, the number of distinct fibres exhibiting EADs is noted for each concentration. Triangulation (T) and reverse-use-dependence are calculated at C_P, which is either the concentration producing maximal prolongation of APD_{90} (for drugs producing significant prolongation) or the maximal concentration tested (for other drugs), using the following formulas [equation 1].

$$T = \left(\frac{(APD_{90}[Cp,1-Hz]-APD_{70}[Cp,1-Hz])}{(APD_{90}[S,1-Hz]-APD_{70}[S,1-Hz])}\right) - 1$$

$$RUD = \left(\frac{(APD_{90}[Cp,0.2-Hz]-APD_{90}[S,0.2-Hz])-(APD_{90}[Cp,1-Hz]-APD_{90}[S,1-Hz]}{(APD_{90}[S,1-Hz])}\right)$$

(Eq. 1)

where S is solvent and RUD is reverse-use dependence.

In order to minimise animal use, drug effects are compared with historical control data rather than parallel time-matched groups. Control data were obtained by measuring the responses of fibres perfused with normal extracellular solution only or containing 0.1% dimethyl sulphoxide (DMSO), and threshold effects were selected based on the 95% confidence interval of the control data: >20% for increases in APD_{90}, >5% for decreases in APD_{90} and >25% for decreases in V_{max}. Thereafter, for each group of fibres perfused with a test drug, the individual largest percent increases in APD_{90} (irrespective of drug concentration) are compared with the corresponding data in the control set, using a Mann-Whitney U-test (one-tailed); changes are considered drug-related for p-values <0.05. A similar evaluation is performed for the largest percent decreases in APD_{90} and V_{max}. Drug effects at each tested concentration are then calculated as the mean percent changes and the appropriate threshold concentrations are linearly extrapolated: EC_{20} [APD_{90}] for concentration producing 20% increase in APD_{90}; EC_5 [APD_{90}] for that producing 5% decrease in APD_{90}; EC_{25} [V_{max}] for that producing 25% decrease in V_{max}.

Test Articles and Concentrations

Table I lists the 26 drugs, the therapeutic classes used in the PRR evaluation, the suppliers of the substances and the solvent used in the *in vitro* assay. For each compound, steady state solubility in saline solution (in mmol/L: NaCl 150; KCl 4; $CaCl_2$ 1.2; $MgCl_2$ 1; 4-2-hydroxyethyl-1-piperazine ethane sulphonic acid (HEPES) 10; and pH 7.4 with NaOH; 0.1% DMSO) is measured by high performance liquid chromatography (HPLC) after filtration of an 8 mg/mL ultrasonicated aliquot that was previously left to equilibrate at room temperature for 48 hours. The maximum concentration (C_{max}) to be tested in the electrophysiology assay is then selected on a logarithmic scale based on the solubility (S) as:

- S > 100 μmol/L: C_{max} = 100 μmol/L unless higher values are needed to provide an ample margin above the human plasma concentrations achieved at the efficacious dose;
- S < 10 μmol/L: C_{max} = 10 μmol/L but care is taken to note any obvious precipitation;
- S between 10–100 μmol/L: C_{max} is selected as close to S as possible.

In some cases, the selected C_{max} could not be reached during the *in vitro* assay because electrophysiological effects of lower concentrations

Table I. Selected drugs, therapeutic classes, supplier and solvent[a]

Name	Class	Supplier	Solvent
Amiodarone	Antiarrhythmics	Sigma	DMSO#
Amlodipine	Calcium channel antagonists	F.Hoffmann-La Roche	DMSO#
Amoxicillin	Penicillins with extended spectrum	USB Corp.	DMSO
Astemizole	Histamine H_1 receptor antagonists (antihistamines)	Sigma	DMSO#
Bepridil	Calcium channel antagonists	Sigma	DMSO
Captopril	ACE inhibitors	Fluka	H_2O
Cisapride	Drugs for functional bowel disorders	Research Diagnostics	DMSO#
Clemastine	Antihistamines	Sigma	DMSO
Dofetilide	Antiarrhythmics	F.Hoffmann-La Roche	DMSO#
Erythromycin	Macrolide antibacterials	ICN Biomedicals	DMSO
Fluoxetine	Antidepressants	Sigma	H_2O
Haloperidol	Antipsychotics	Sigma	DMSO
Ibuprofen	NSAIDs	ICN Biomedicals	DMSO
Isradipine	Calcium channel antagonists	Research Diagnostics	DMSO
Nifedipine	Calcium channel antagonists	Calbiochem	DMSO#
Norfloxacin	Fluoroquinolone antibacterials	Sigma	DMSO
Pimozide	Antipsychotics	Sigma	DMSO
Probucol	Serum lipid-lowering agents	Sigma	DMSO
Quinidine	Antiarrhythmics	Aldrich	DMSO#
Simvastatin	Serum lipid-lowering agents	LKT Laboratories	DMSO
Sotalol	Antiarrhythmics	Bristol-Myers Squibb	H_2O
Sparfloxacin	Fluoroquinolone antibacterials	F.Hoffmann-La Roche	DMSO#
Tamoxifen	Hormone antagonists	Research Diagnostics	DMSO
Terfenadine	Antihistamines	Sigma	DMSO#
Thioridazine	Antipsychotics	Research Diagnostics	DMSO
Verapamil	Calcium channel antagonists	Fluka	H_2O

a The compounds were prepared as stock solutions in 100% solvent. If dimethyl sulphoxide (DMSO) was used, the test solutions were prepared such that the DMSO concentration was 0.1% throughout the experiment or, for early compounds (marked with #), increased in parallel with the compound concentration up to a maximum of 0.1%. In the latter case, the solvent was not added alone to the preparation before the first concentration of test compound.

were of sufficient magnitude to preclude further testing (see 'Results').

Results

Clinical Data and Classification

PRRs for QT prolongation and torsades de pointes were calculated between June 2003 and June 2004 for each of the 26 selected drugs relative to all other drugs in the database. For torsades de pointes, PRRs were also calculated for each drug relative to all other members of its therapeutic class and for each therapeutic class relative to all other drugs. Based on this analysis and on clinical data available in the scientific literature, the 26 drugs can be divided into three groups (table II):

- **Positive:** Fourteen drugs with PRR values >2 for both QT prolongation and torsades de pointes. For each of these drugs, the numbers of AE reports concerning QT prolongation or torsades de pointes are more than twice those expected based on the 'normal' frequencies of such reports. In all 14 cases, a safety signal exists when using standard statistical criteria. Although the PRR evaluates disproportionality and cannot be used to classify drugs for their relative risk, these results also suggest that some drugs generate stronger safety signals and may have higher risks (e.g. bepridil, dofetilide) than others (e.g. thioridazine, haloperidol).
- **Negative:** Seven drugs with a PRR value close to or <1 for both QT prolongation and torsades de

Table II. Proportional reporting ratios (PRRs) of QT prolongation and torsades de pointes for 26 drugs[a]

Group	Drug no.	name	PRR			
			QT	torsades de pointes		
			drug/all	drug/all	drug/class	class/all
Positive	1	Amiodarone	5.7[b]	13[b]	1.3[b]	13[b]
	2	Astemizole	22[b]	13[b]	3.0[b]	5.0[b]
	3	Bepridil	55[b]	92[b]	49[b]	2.2[b]
	4	Cisapride	31[b]	25[b]	16[b]	1.7[b]
	5	Dofetilide	63[b]	20[b]	2.1[b]	13[b]
	6	Erythromycin	7.7[b]	11[b]	2.1[b]	7.4[b]
	7	Haloperidol	3.4[b]	5.8[b]	3.1[b]	2.1[b]
	8	Pimozide	35[b]	22[b]	11[b]	2.1[b]
	9	Probucol	34[b]	6.7[b]	5.4[b]	1.3[b]
	10	Quinidine	8.6[b]	15[b]	1.5[b]	13[b]
	11	Sotalol	18[b]	45[b]	3.9[b]	13[b]
	12	Sparfloxacin	11[b]	15[b]	4.6[b]	3.6[b]
	13	Terfenadine	12[b]	8.1[b]	2.3[b]	5.0[b]
	14	Thioridazine	5.5[b]	3.6[b]	1.9[b]	2.1[b]
Questionable	15	Captopril	1.8[b]	3.2[b]	1.8[b]	2.1[b]
	16	Fluoxetine	1.9[b]	1.8[b]	1.4[b]	1.6[b]
	17	Nifedipine	1.3[c]	0.94	0.43[b]	2.2[b]
	18	Norfloxacin	1.7	3.9[b]	1.2	3.6[b]
	19	Verapamil	1.6[b]	1.4	0.62[b]	2.2[b]
Negative	20	Amlodipine	1.2[c]	1.5[b]	0.73[c]	2.2[b]
	21	Amoxicillin	1.4	1.1	2.2[b]	0.93
	22	Clemastine	1.2	0.40	0.57	5.0[b]
	23	Ibuprofen	0.53[b]	0.48[b]	1.2	0.39[b]
	24	Isradipine	1.4	0	0	2.2[b]
	25	Simvastatin	1.1	1.5[c]	1.3	1.3[c]
	26	Tamoxifen	0.56	0.45	0.40	0.72

a For torsades de pointes, the PRRs were calculated for each drug relative to all other drugs in the database as well as relative to all other members of the same therapeutic class. In addition, the PRR was also calculated for each therapeutic class relative to all other drugs in the database. The numbering of drugs is used in figure 2.

b $p < 0.01$.

c $p < 0.05$.

pointes. This indicates that the proportions of QT/torsades de pointes AEs for these agents are similar to or below those reported for the 'average drug'. Additionally, there are no indications in the scientific literature that these seven drugs may produce clinical QT prolongation or torsades de pointes.

- **Questionable:** Five drugs generate an unclear clinical dataset either because they have a significant clinical signal for only one of the two AEs or because a discrepancy exists between PRR data and published clinical data. Hence, captopril and norfloxacin have high PRRs for torsades de pointes (3.2 and 3.9, respectively), but their PRRs for QT prolongation are below the significance level (1.8 and 1.7, respectively). Fluoxetine, nifedipine and verapamil have PRRs between 1 and 2 for both AEs, indicating that these drugs generate no signal for either. Nevertheless, published clinical data exist for the three drugs that suggest a risk of QT prolongation and/or torsades de pointes.[8-12]

The PRRs for QT prolongation and torsades de pointes are plotted in figure 2. For the clinically positive drugs, a good correlation is found between the two parameters.

Non-Clinical Data and Classification

The quantitative data for the effects of the 26 compounds on rabbit Purkinje APs are shown in table III and table IV. Based on qualitative aspects of their actions, we assigned these compounds to four different profiles.

Profile 1: Compounds that Prolong Action Potential Duration at 90% Repolarisation (APD_{90}) Without Modifying the Maximum Rate of Rise of Phase 0 Depolarisation (V_{max})

Among the nine compounds in this group, seven (cisapride, clemastine, dofetilide, erythromycin, haloperidol, sotalol, sparfloxacin) produce marked prolongation (up to ~200 to ~700%) and two (amiodarone, captopril) produce less than ~30% prolongation at all tested concentrations. All compounds except captopril cause significant triangulation of the APs. Similarly, all but amiodarone prolong with a significant reverse-use-dependence.

Clemastine is a representative drug that prolongs APs markedly (figure 3). The increase in APD_{90} is concentration- and rate-dependent (figure 3a) and results from a prolongation of phase 2 of the AP associated with a slowing of phase 3 (inset). In addition, clemastine induces a concentration-dependent increase in the occurrence of EADs at 0.2Hz (figure 3b). EADs were also observed with haloperidol (0.2Hz only), with cisapride, dofetilide and sparfloxacin (0.2Hz and 1Hz), but not with erythromycin or sotalol.

Captopril prolongs APs minimally (figure 4). As for clemastine, the increase in APD_{90} is rate-dependent; however, contrary to clemastine, the increase reaches a maximum with increasing captopril concentrations and then declines. Captopril (or amiodarone) did not induce EADs at any concentration or pacing frequency tested.

Profile 2: Compounds that Prolong APD_{90} and Reduce V_{max}

Among the nine compounds in this group, one (quinidine) produces marked maximal prolongation (~330%), three (amlodipine, bepridil, thioridazine) lead to minimal prolongation (<40%) and five (astemizole, fluoxetine, pimozide, terfenadine, verapamil) reach intermediate levels (~75 to ~175%). The V_{max} reductions range from ~40% (amlodipine, bepridil, pimozide, quinidine) to ~95% (astemizole, verapamil). Only quinidine (at 0.2Hz) and pimozide (at 0.2Hz and 1Hz) induced concentration-dependent increases in the occurrence of EADs. All compounds cause significant triangulation of APs and all

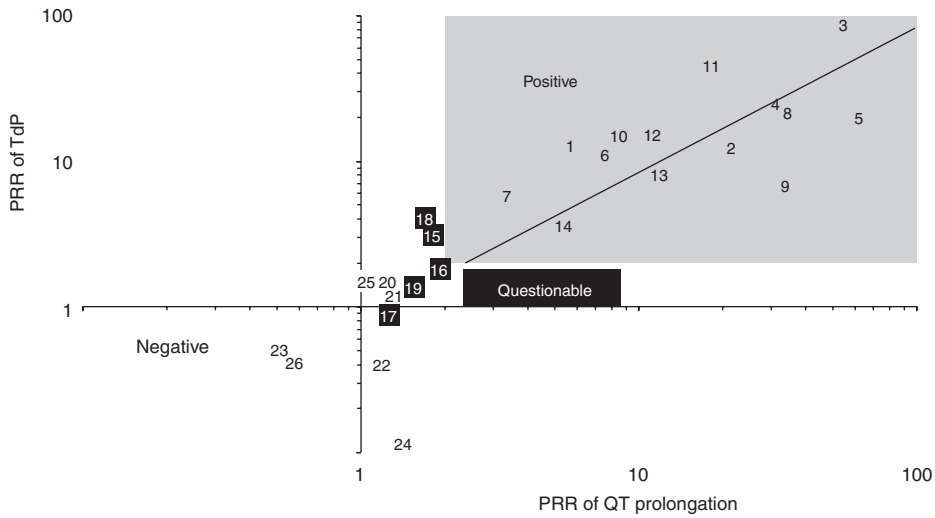

Fig. 2. Correlation between the proportional reporting ratios (PRR) for QT prolongation and for torsades de pointes (TdP). Numbers refer to the drug number in table II. The straight line is a linear fit to only those data points >2 on both axes, which represent drugs with a signal for both adverse events (Spearman non-parametric correlation coefficient = 0.75). White numbers on black indicate questionable compounds (see Results section 'Clinical Data and Classification').

Table III. Effects of the 26 selected compounds on cardiac action potentials (maximum rate of rise of phase 0 depolarisation [V_{max}]). Compounds are grouped in four profiles (first column) as described in the 'Results' section. For baseline (BL), the values are in V/s; for the test concentrations (C1–C5), the values are in % change from BL. The first value corresponds to the 1Hz stimulation rate and the second value to 2Hz

Profile	Drug name	Solubility (μmol/L)	No. of fibres	Concentrations tested (μmol/L)	V_{max} (1Hz/2Hz)							
					BL	C1	C2	C3	C4	C5	p-value	EC_{25}
1	Amiodarone	<1.5	7	0.01, 0.1, 1, 10, 30#	385/382	3/−5	2/2	9/8	4/−2	−1/−6	-/-	na/na
	Captopril	>41 000	6	0.1, 1, 10, 30, 100	312/301	−8/−4	−5/−2	−2/−3	−6/−2	−7/0	-/-	na/na
	Cisapride	<2.1	4–7	0.003, 0.01, 0.1, 1, 3‡	499/493	−5/−5	−6/−8	2/2	−6/−4	−17/−32	-/-	na/(2.5)
	Clemastine	70	7	0.01, 0.03, 0.1, 0.3, 1‡	371/349	−5/1	−1/2	−3/3	−3/0	−4/−9	-/-	na/na
	Dofetilide	530	7	0.001, 0.003, 0.01, 0.3‡	277/271	−1/1	1/1	−6/−13	−9/−9		-/-	na/na
	Erythromycin	24 000§	6	0.1, 1, 10, 30, 100‡	532/487	−4/3	−3/2	−6/−3	−2/−1	−5/−2	-/-	na/na
	Haloperidol	74	6	0.003, 0.01, 0.03, 0.1, 0.3‡	334/324	9/−1	−3/−2	−5/−4	−5/−5	−6/−20	-/-	na/na
	Sotalol	37 000	6	0.1, 1, 10, 30‡	379/368	−3/−1	−1/−9	−17/−15	−11/−20		-/-	na/na
	Sparfloxacin	775	6	1, 3, 10, 30, 100‡	618/568	−6/6	−5/10	−3/0	−17/−1	−15/9	-/-	na/na
2	Amlodipine	12 700	8	0.01,0.1,1,10,30	374/371	1/0	2/4	1/2	−3/−8	−35/−43	-/*	(24)/20
	Astemizole	<2.2	6	0.01, 0.1, 1, 10	360/359	1/0	4/−1	−9/−25	−68/−97		**/**	3.4/1.0
	Bepridil	1600	7	0.03, 0.1, 0.3, 1, 3‡	354/354	−5/−5	−3/−4	−3/−6	−6/−11	−20/−44	-/*	na/2.0
	Fluoxetine	5900	7	0.01, 0.1, 1, 10‡	314/310	2/−6	−3/1	1/−4	−28/−55		-/*	(9.1)/4.7
	Pimozide	<2.2	7	0.03, 0.1, 0.3, 1, 3‡	336/324	5/−1	−8/−14	−12/−20	−22/−36	−33/−40	*/*	1.6/0.52
	Quinidine	2000	6	0.1, 0.3, 1, 3, 10‡	534/515	2/−1	2/0	−1/−7	−2/−6	−24/−39	-/*	(10)/7.1
	Terfenadine	<2.1	5–6	0.1, 0.3, 1, 3, 10	353/341	−11/−13	3/1	−13/−25	−7/−31	−39/−56	-/*	(6.9)/1.0
	Thioridazine	46	6	0.03, 0.1, 0.3, 1, 3‡	408/387	−1/1	−3/−1	−16/−16	−29/−58	−69/−82	**/**	0.78/0.44
	Verapamil	32 000	6	0.01, 0.1, 1, 10, 30‡	287/285	0/0	6/2	5/−1	−40/−54	−72/−96	*/*	7.0/5.0
3	Isradipine	5.4	7	0.01, 0.1, 1, 3, 10	477/454	−4/0	1/3	−1/2	−4/1	−17/−18	-/-	na/na
	Nifedipine	176	7	0.1, 1, 3, 10, 30	263/267	2/0	0/1	1/3	1/1	−5/−3	-/-	na/na
	Simvastatin	<2.5	6	0.01, 0.1, 1, 3, 10	427/427	0/−6	−8/−9	−12/−12	−17/−19	−54/−48	*/*	4.5/4.4
	Tamoxifen	3	6	0.01, 0.1, 1, 10	435/416	0/4	−2/2	0/3	3/0		-/-	na/na
4	Amoxicillin	13 000	7	3, 30, 100, 300, 1000	429/419	1/2	2/1	−2/12	9/10	5/5	-/-	na/na
	Ibuprofen	3700	7	0.1, 1, 10, 30, 300	447/422	2/10	−2/3	−4/9	−1/2	−8/−2	-/-	na/na
	Norfloxacin	2200	7	1, 3, 10, 30, 100	424/416	5/4	1/−2	−6/−5	−5/−15	4/−9	-/-	na/na
	Probucol	<1.9	7	0.01, 0.1, 1, 3, 10	369/375	−1/−3	2/1	−2/−4	−2/−7	−4/−7	-/-	na/na

EC_{25} = concentration reducing V_{max} by 25% (values are in parenthesis when statistical significance is not achieved); **na** = not achieved; **§** indicates solubility value based on published data;[13] **#** indicates error in selecting the maximal concentration; **‡** indicates target maximal concentration could not be reached because of major effects at lower concentrations. * $p < 0.05$; ** $p < 0.01$.

Table IV. Effects of the 26 selected compounds on cardiac action potentials (action potential duration at 90% repolarisation [APD_{90}]). For baseline (BL), the values are in ms; for the test concentrations (C1–C5), the values are in % change from BL. The first value corresponds to the 1Hz stimulation rate; the second value to 0.2Hz. Drug names and APD_{90} values shown in italics indicate the occurrence of early-after-depolarisations

Profile	Drug name	Concentrations tested	APD_{90} (1Hz/0.2Hz)								T (%)	RUD (%)
		(μmol/L)	BL	C1	C2	C3	C4	C5	p-value	EC_{20} or EC_5¥		
1	Amiodarone	0.01, 0.1, 1, 10, 30#	257/330	7/9	10/14	13/16	14/19	15/16	*/–	na/10	34*	9
	Captopril	0.1, 1, 10, 30, 100	300/484	9/9	13/11	15/30	19/32	2/21	–/*	30/5.2	9	33**
	Cisapride	0.003, 0.01, 0.1, 1, 3‡	262/320	13/18	28/38	38/59	107/*456*	*249*/*715*	**/**	0.006/0.004	72**	639**
	Clemastine	0.01, 0.03, 0.1, 0.3, 1‡	265/383	2/6	8/15	20/*22*	50/*136*	77/*388*	**/**	0.1/0.08	224**	425**
	Dofetilide	0.001, 0.003, 0.01, 0.3‡	318/452	26/44	61/127	*140*/*340*	*233*/*546*		**/**	<0.001	59**	476**
	Erythromycin	0.1, 1, 10, 30, 100‡	255/377	2/–5	5/6	12/23	33/85	87/221	**/**	18/8.6	159**	235**
	Haloperidol	0.003, 0.01, 0.03, 0.1, 0.3‡	257/336	5/5	11/13	22/44	50/*144*	96/*267*	**/**	0.026/0.014	221**	259**
	Sotalol	0.1, 1, 10, 30‡	255/348	7/14	12/25	55/139	144/405		**/**	2.6/0.61	107**	401**
	Sparfloxacin	1, 3, 10, 30, 100‡	293/397	6/14	5/11	35/99	77/264	*209*/*651*	**/**	6.6/3.7	69*	577**
2	Amlodipine	0.01,0.1,1,10,30	212/303	4/9	8/13	11/15	29/33	25/16	**/**	5.7/3.4	84**	11
	Astemizole	0.01, 0.1, 1, 10	256/343	8/16	21/50	69/150	15/66		**/**	0.95/0.02	93**	138**
	Bepridil	0.03, 0.1, 0.3, 1, 3‡	243/310	5/3	5/4	14/11	23/21	25/23	**/–	0.80/0.95	159**	4
	Fluoxetine	0.01, 0.1, 1, 10‡	309/467	11/3	22/17	36/72	31/66		**/**	0.08/0.15	80**	77**
	Pimozide	0.03, 0.1, 0.3, 1, 3‡	369/469	8/14	27/*54*	*49*/*169*	*72*/*162*	46/*174*	**/**	0.075/0.040	107**	162**
	Quinidine	0.1, 0.3, 1, 3, 10‡	251/309	15/22	22/35	35/71	55/152	72/*330*	**/**	0.24/0.092	108*	342**
	Terfenadine	0.1, 0.3, 1, 3, 10	295/454	8/6	9/20	17/28	16/69	49/94	*/*	0.30/3.8	184**	70*
	Thioridazine	0.03, 0.1, 0.3, 1, 3‡	314/384	8/6	15/15	20/30	22/40	19/33	**/**	0.30/0.17	78**	28**
	Verapamil	0.01, 0.1, 1, 10, 30‡	280/420	4/5	9/25	31/83	46/58	27/8	**/**	0.56/0.08	90**	98**
3	Isradipine	0.01, 0.1, 1, 3, 10	250/320	2/1	–2/–3	–17/–24	–33/–40	–48/–56	–/**¥	0.27/0.18¥	7	
	Nifedipine	0.1, 1, 3, 10, 30	290/397	1/–6	–9/–18	–20/–31	–34/–44	–50/–58	–/**¥	0.65/0.09¥	0	
	Simvastatin	0.01, 0.1, 1, 3, 10	272/414	4/–3	5/1	–11/–29	–16/–37	–24/–41	–/**¥	0.66/0.28¥	26	
	Tamoxifen	0.01, 0.1, 1, 10	250/356	1/0	4/1	8/6	2/–8		–/*¥	na/8.2¥	30	
4	Amoxicillin	3, 30, 100, 300, 1000	236/302	5/7	8/12	12/15	12/14	15/21	–/–	na/(880)	5	
	Ibuprofen	0.1, 1, 10, 30, 300	257/315	8/5	4/6	5/10	5/12	2/–1	–/–	na/na	6	
	Norfloxacin	1, 3, 10, 30, 100	264/361	2/–2	3/0	4/3	6/10	8/3	–/–	na/na	4	
	Probucol	0.01, 0.1, 1, 3, 10	245/325	5/5	7/6	10/7	14/14	17/11	–/–	na/na	13	

EC_{20} = concentration increasing APD_{90} by 20% (values are in parenthesis when statistical significance is not achieved); **EC_5** = concentration decreasing APD_{90} by 5%; **na** = not achieved; **RUD** = reverse-use dependence; **T** = triangulation. **#** indicates error in selecting the maximal concentration; **¥** indicates p-value and EC_5 are for APD_{90} decrease (profile 3 drugs); **‡** indicates target maximal concentration could not be reached because of major effects at lower concentrations. * $p < 0.05$; ** $p < 0.01$.

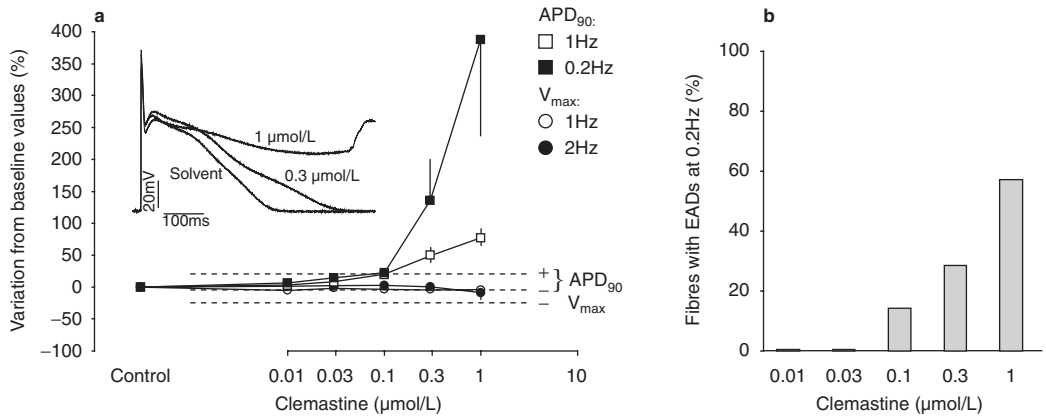

Fig. 3. Effects of clemastine on cardiac action potentials. (**a**) Effects on APD_{90} and V_{max} at stimulation frequencies of 2, 1 and 0.2Hz. Data points are mean ± standard error of the mean (SEM) but, for clarity, only SEM >10 are shown; the dashed lines show the limits of normal variations. Inset: representative APs at 0.2Hz in a fibre treated with clemastine. (**b**) Occurrence of EADs at 0.2Hz. n = 7 for both panels. **APD_{90}** = action potential duration at 90% repolarisation; **EAD** = early-after-depolarisation; **V_{max}** = maximum rate of rise of phase 0 depolarisation.

compounds except amlodipine and bepridil prolong with a significant reverse-use-dependence.

Quinidine and terfenadine prolong APD_{90} in a rate-dependent manner with a monotonic concentration-dependence and they reduce V_{max} at the highest concentrations (figure 5a). In contrast, verapamil produces a rate-dependent increase in APD_{90} that reaches a peak with increasing concentrations and then declines (figure 5b). The remaining drugs in this group also produce a limited AP prolongation, but the waning of prolongation at high concentrations is seen only with astemizole and amlodipine, and not with bepridil or pimozide. The increase in APD_{90} results from a prolongation of phase 2 of the AP associated with a slowing of phase 3; in addition, the waning of prolongation results from a depression of phase 2 (insets).

Profile 3: Compounds that Shorten APD_{90}

Among the four compounds in this group, three (isradipine, nifedipine, tamoxifen) produce only AP shortening and one (simvastatin) also decreases V_{max} to ~50%. None of the four profile 3 drugs induced EADs at any concentration or pacing frequency tested and none caused significant triangulation.

Profile 4: Compounds Without Effects on APs

Four compounds (amoxicillin, ibuprofen, norfloxacin, probucol) produce no effects on V_{max} or APD_{90}. They did not induce EADs at any concentration and did not cause significant triangulation.

Discussion

Assessment of Clinical Risk

Our first concern in designing the study was to provide an objective and standardised measurement of the clinical proarrhythmic liability of a large number of marketed drugs from various therapeutic classes. In previously published evaluations of other *in vitro* assays, the clinical liability of reference compounds was usually evaluated by counting the number of case reports found in the scientific literature. However, published reports are likely to represent a small, highly selected fraction of the total number of AEs and this fraction may vary widely between drugs, a fact which could lead to biased evaluations of their liability. The SRS/AERS database represents a larger repository of publicly accessible clinical safety information than the scientific literature. Analysis of this database using the PRR methodology allows an extensive assessment of the occurrence of AEs.

Discrepancies in the proarrhythmic signals obtained from the literature or the SRS/AERS database are immediately apparent. Some proarrhythmic drugs are more 'under-published' than others: pimozide (3 reports published; 12 in the database) or

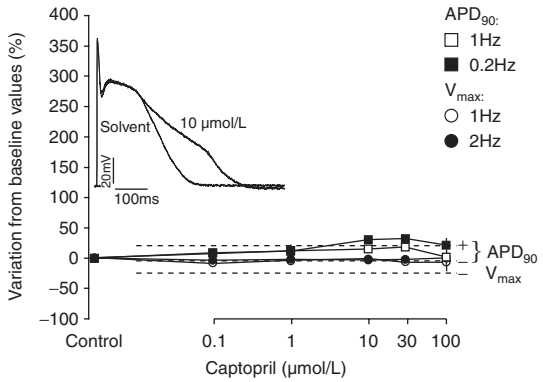

Fig. 4. Effects of captopril on cardiac action potentials (APD_{90} and V_{max}) at stimulation frequencies of 2, 1 and 0.2Hz (n = 6). Data points are mean ± standard error of the mean (SEM) but, for clarity, only SEM >10 are shown; the dashed lines show the limits of normal variations. Inset: representative APs at 0.2Hz in a fibre treated with captopril. **APD_{90}** = action potential duration at 90% repolarisation; **V_{max}** = maximum rate of rise of phase 0 depolarisation.

thioridazine (5 published; 21 in the database), compared with sparfloxacin (3 published; 5 in the database) or haloperidol (>30 published; 66 in the database). Furthermore, drugs sometimes considered as having a moderate risk actually generate a very large excess proportion of torsades de pointes reports: pimozide (PRR = 22) or sparfloxacin (PRR = 15). Conversely, some drugs customarily thought to carry an inherently high risk generate only a 'small' excess of torsades de pointes reports: thioridazine (PRR = 3.6) or haloperidol (PRR = 5.8). As a consequence, 'conventional wisdom' may not dependably reflect the cardiac threat inherent in the use of many drugs. It may be interesting to re-examine recent evaluations of other *in vitro* assays using the analysis of the clinical risk proposed here. Indeed, such a re-evaluation of the value of the I_{Kr} assay was performed very recently using the International Drug Monitoring Program of the WHO, which confirmed an association between 'anti-hERG activity' and risk of reports of serious ventricular arrhythmias and sudden death.[14]

The PRR analysis of the SRS/AERS database for the 26 selected drugs reveals that all drugs having a signal of QT prolongation (PRR >2) also have a signal of torsades de pointes; furthermore, a correlation exists between the magnitudes of the two safety signals (i.e. the PRRs). This observation substantiates the often-questioned notion that drug-induced QT prolongation is a surrogate marker of a risk of torsades de pointes. However, our PRR analysis does not allow any conclusions to be drawn regarding a relation between the amplitude of drug-induced QT prolongation (in ms or %) and the occurrence of torsades de pointes because, in the FDA database, quantitative data regarding ECG intervals are not used for the designation of AEs (these data are often not even reported).

Non-Clinical Assessment

We focused the analysis of drug effects on four AP parameters (APD_{90}, V_{max}, triangulation and re-

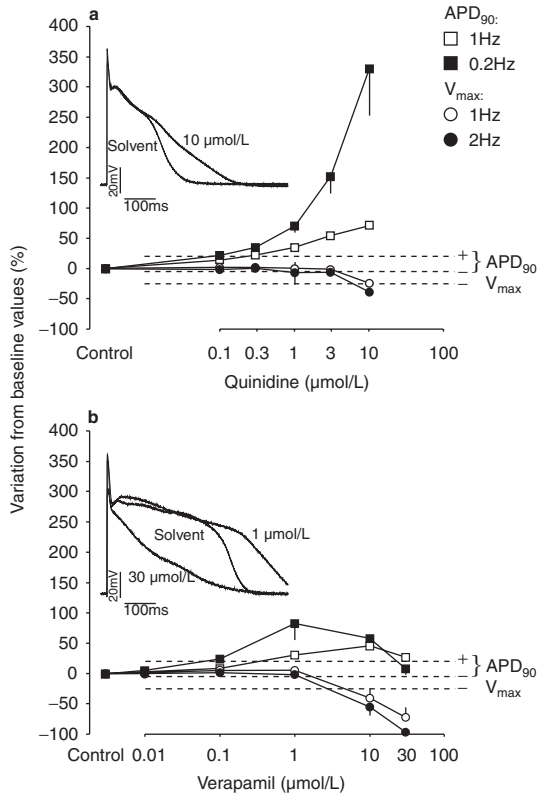

Fig. 5. Effects of quinidine (**a**) and verapamil (**b**) on cardiac action potentials (APD_{90} and V_{max}) at stimulation frequencies of 2, 1 and 0.2Hz. Data points are mean ± standard error of the mean (SEM) but, for clarity, only SEM >10 are shown; the dashed lines show the limits of normal variations. Insets: representative APs at 0.2Hz in a fibre treated with terfenadine (**a**) or verapamil (**b**). n = 6 for both panels. **APD_{90}** = action potential duration at 90% repolarisation; **V_{max}** = maximum rate of rise of phase 0 depolarisation.

verse-use-dependence). Increases in APD_{90} are clearly associated with a risk of QT prolongation *in vivo*, whereas decreases in APD_{90} hint at a risk of reduced contractile force, among others. Decreases in V_{max} suggest a block of cardiac sodium currents, which is often associated with QRS prolongation *in vivo*. Finally, triangulation and reverse-use-dependence have been suggested as valuable parameters for detecting proarrhythmic compounds.[15]

Based on this analysis, we propose to classify drugs as profile 1 (prolong APD_{90} exclusively), profile 2 (prolong APD_{90} and decrease V_{max}), profile 3 (shorten APD_{90} in isolation or with reduced V_{max}) or profile 4 (no measurable electrophysiological effect). Most drugs in profiles 1 and 2 have been shown in separate studies to block I_{Kr},[3] which is likely to be the main mechanism by which they prolong cardiac APD_{90}. This interpretation can probably be extended to the drugs for which such block has not yet been reported (amlodipine, captopril, clemastine), although the same functional consequences can theoretically be obtained either by blocking other potassium channels or by enhancing currents through cardiac sodium channels (I_{Na}) or L-type calcium channels (I_{CaL}).

Blocking I_{Na} probably mediates the reduction in V_{max} seen with profile 2 drugs and with simvastatin. Such a block may secondarily shorten APD_{90}, either by a direct reduction of the late sodium current during the plateau phase and/or by a change in the kinetics and magnitude of other late currents following the slowing of the initial depolarisation.[16] This mechanism could explain the limited APD_{90} prolongation seen with many profile 2 drugs as well as the shortening observed with simvastatin. However, I_{CaL} block or activation of adenosine triphosphate-dependent potassium channels by the test drug could also contribute to APD_{90} shortening.

Indeed, three drugs in profile 3 shorten APD_{90} without decreasing V_{max} (isradipine, nifedipine, tamoxifen). This dissociation suggests that I_{Na} block is not the basis of APD_{90} shortening for these drugs. Of course, I_{CaL} block is a likely mechanism of APD_{90} shortening for isradipine and nifedipine. Whether tamoxifen also acts through calcium channels is not known, but undesired block of these channels seems more probable than undesired activation of potassium channels. Amiodarone and captopril inhibit I_{CaL},[17,18] which probably accounts for the minimal prolongation also seen with these profile 1 drugs.

Relation between Non-Clinical Data and Clinical Risk

The predictive value of the rabbit Purkinje fibre assay can be judged by comparing the safety signals derived from the *in vitro* test with those from the clinical assessment. The *in vitro* safety signal for QT prolongation and torsades de pointes was quantified as the margin between the first concentration producing significant prolongation of APs (EC_{20} [APD_{90}] of table III and table IV) and drug concentrations derived from published clinical studies using the highest therapeutic dose. In order to find the best relation, three related drug concentrations were considered: (i) the maximal total plasma concentration; (ii) the maximal free concentration calculated with published protein binding values (PB); and (iii) the maximal effective concentration based on corrected PB. The proposed correction of protein binding consists in limiting high values to 90%, which is equivalent to setting the effective concentration to be at least 10% of the total concentration. This correction is based on the fact that the correlation between *in vitro* drug effects and plasma concentrations generally holds well for low protein-binding drugs (PB <90%) but tends to underestimate the effects of some high protein-bound compounds (PB >90%) [E. Ertel, unpublished observations]. For the high protein-bound compounds, this underestimation may somehow be linked to the large relative changes in free concentrations associated with small variations in protein binding or to the high tissue accumulation of such compounds, resulting in locally effective concentrations higher than free plasma concentrations. In particular, myocardial concentrations of haloperidol,[19] terfenadine,[20] bepridil,[21] amiodarone[22] and, above all, astemizole[23] are substantially greater than circulating concentrations and safety margins based on free plasma concentrations would be greatly overestimated.

Table V shows the relation between the clinical and non-clinical classifications. Thirteen of the 14 compounds with an excess of torsades de pointes and QT prolongation reports, based on the PRR analysis, belong to profiles 1 and 2 (prolongation).

Table V. Predictive value of the rabbit Purkinje fibre assay for assessing the risk of QT prolongation and torsades de pointes in humans. Unless otherwise indicated[a], plasma protein binding values (PB) and *in vivo* plasma concentrations used for the safety margins are from DRUGDEX®[24] and agree with the values used in Redfern et al.[3] Significant prolongation of APD90 is selected as >20% based on the 95% confidence interval of the control data (see 'Methods' section)

Clinical group	*In vitro* profile	Drug name	PB (%)	Margin (20% APD_{90} increase)		
				total	free	90%
Positive	1	Amiodarone IV/PO	95.6[b]	0.25/2.3	5.7/53	2.5/23
		Cisapride	98	0.013[c]	0.65[c]	0.13[c]
		Dofetilide	60	0.15	0.37	0.37
		Erythromycin IV/PO	75	0.25/1.2	1.0/4.6	1.0/4.6
		Haloperidol	90	0.10[d]	1.0[d]	1.0[d]
		Sotalol	10	0.051	0.056	0.056
		Sparfloxacin	45	0.74	1.3	1.3
	2	Astemizole	97	2.3	77	23
		Bepridil	99.7	0.082[e]	27[e]	0.82[e]
		Pimozide	99	0.82	82	8.2
		Quinidine	80	0.010	0.014	0.014
		Terfenadine	97	1.8	59	18
		Thioridazine	97.5[f]	0.057	2.3	0.57
	4	Probucol	99	>0.16	>17	>1.7
Questionable	1	Captopril	25	0.75	1.0	1.0
	2	Fluoxetine	94.5	0.023[g]	0.42[g]	0.23[g]
		Verapamil	90	0.10	1.0	1.0
	3	Nifedipine	90[h]	>100	>1000	>1000
	4	Norfloxacin	10	>13	>15	>15
Negative	1	Clemastine	95[i]	20	390	195
	2	Amlodipine	93	87	1260	872
	3	Isradipine	95	>435	>8700	>4350
		Simvastatin	95	>33	>660	>330
		Tamoxifen	98	>8	>415	>83
	4	Amoxicillin	15	>37	>43	>43
		Ibuprofen	99	>1.1	>110	>11

a Differences between the data used for this table and that found in Redfern et al.[3] are listed in footnotes b–i.

b Amiodarone PB.[24]

c Cisapride has therapeutic concentrations of 80 ng/mL and 80% higher following metabolic inhibition.[25]

d Haloperidol has 95% higher plasma concentrations with metabolic inhibition.[26]

e Bepridil has therapeutic concentrations of 3600 mg/mL (Physician's Desk Reference).

f Thioridazine PB.[27]

g Fluoxetine has therapeutic concentrations of 500–700 ng/mL and 57% higher following metabolic inhibition.[28]

h Nifedipine PB.[24]

i Clemastine PB (Swiss Compendium of Drugs).

APD_{90} = action potential duration at 90% repolarisation; **nd** = not defined; **IV** = intravenous; **PO** = per os; **SI** = separation index calculated as EC_{25} [V_{max}] (concentration reducing V_{max} by 25%) divided by EC_{max} [APD_{90}] (concentration where maximal prolongation of APD_{90} first occurs); **V_{max}** = maximum rate of rise of phase 0 depolarisation; **#** reduction of V_{max} by cisapride was not statistically signifiant.

The only exception is probucol, which belongs to profile 4 (no effect). For most drugs, the safety margin is small (<10) between therapeutic plasma concentrations (total, free or corrected) and concentrations producing prolongation *in vitro*. Exceptions are amiodarone (oral), astemizole, bepridil, pimo-

zide and terfenadine, for which the safety margin based on free plasma concentrations remains fairly high (27–82). Since these compounds exhibit very high protein binding, the margins become <25 with corrected plasma concentrations.

Probucol, a clinically positive compound, is not expected to be in profile 4. However, because of limited solubility (<2 μmol/L), we have only tested this drug up to a maximum of 10 μmol/L, which provides little margin above therapeutic concentrations of the drug (table V). Plasma concentrations of probucol can reach substantial values (~60 μmol/L), its protein binding is very high (>99%) and its pharmacokinetic profile suggests slow and considerable tissue accumulation (human steady state plasma concentrations are reached over a month after starting therapy and washout upon drug discontinuation requires many months[24]). These observations suggest that compounds with no effects *in vitro* should not be considered safe if the highest testable concentration cannot provide a sufficient safety margin above therapeutic concentrations. Based on the safety margins of the other clinically positive compounds and by analogy with the analysis of I_{Kr} data by Redfern et al.,[3] we would propose to target a 30-fold margin. Such compounds need very careful re-evaluation *in vivo*, preferably using repeated drug administration and reaching supra-therapeutic plasma concentrations. In monkeys, this approach revealed QT prolongation with probucol after 8 weeks of administration at peak plasma concentrations of 120–150 μmol/L.[29]

Five of the seven drugs without an excess of torsades de pointes or QT prolongation reports, based on the PRR analysis, belong (as could be expected) to *in vitro* profile 3 (AP shortening) or to profile 4 (no effect). The other two drugs belong to profile 1 (clemastine) and profile 2 (amlodipine). In rabbit Purkinje fibres, clemastine markedly prolongs APD_{90} and induces EADs, suggesting that it could be proarrhythmic in humans. However, there is no evidence of such a risk in the PRR analysis, which shows clemastine to be a 'torsades de pointes safe' drug (PRR = 0.4) despite belonging to the most proarrhythmic therapeutic class (PRR = 5 for antihistamines). There are also no published data suggesting a QT/torsades de pointes liability for this drug. This apparent discrepancy between *in vitro* and clinical data is explained by the very large 'safety margin' between plasma concentrations required for therapeutic efficacy and concentrations producing *in vitro* prolongation (≥200-fold). This finding is essential for safety pharmacology since it demonstrates that safety margins are meaningful parameters to consider when gauging the proarrhythmic potential of a drug. Amlodipine also prolongs APs *in vitro* despite the absence of any clinical signal but for this drug as well, the prolongation is not expected to result in clinical risk as the safety margin is even larger (≥800-fold).

The five drugs in the 'questionable' group distribute across four *in vitro* profiles. Norfloxacin is a profile 4 compound, consistent with the absence of a PRR signal for QT prolongation as well as of any published data suggesting a proarrhythmic liability for this drug. The presence of a PRR signal for torsades de pointes (3.9, resulting from only ten reports) without a signal for QT prolongation cannot be explained based on available information.

Nifedipine is a profile 3 compound, consistent with the absence of PRR signals for QT prolongation or torsades de pointes. We had labelled nifedipine as 'questionable' on the basis of two published case reports of torsades de pointes. However, as already discussed by Redfern et al.,[3] it is unlikely that these events suggest a direct proarrhythmic liability for nifedipine, as they both occurred in patients with cardiovascular disease and are attributable to myocardial ischaemia caused by coronary steal (where arteries in healthy tissue dilate more than in diseased tissue, such that blood is shunted away from the area that needs it most). It is worth noting that only two reports of torsades de pointes in patients taking nifedipine have been published in over 30 years of clinical use of this drug. In contrast, 29 spontaneous reports are found in the FDA database, which results in a PRR of only 0.94, suggesting that they represent the background event rate. Therefore, cases of torsades de pointes in patients receiving nifedipine are actually highly underpublished in the scientific literature and a 15-fold higher number of published cases would still not indicate a relevant risk for this drug. This observation underscores the advantages of the PRR analysis as it demonstrates that relying on published case

reports to label a drug as 'possibly proarrhythmic' should be avoided.

Captopril, the next 'clinically questionable' compound, belongs to *in vitro* profile 1 as it prolongs APD_{90} without affecting V_{max}. However, the prolongation remains small because of a flat concentration-response curve (<32% compared with >100% for all other profile 1 compounds except amiodarone). Furthermore, captopril is the only prolonging compound that does not triangulate APs. The basis for this pattern is not clear, as limited data exist regarding ion channel effects of captopril. At >300 μmol/L, this compound blocks I_{CaL} and I_{Na} and it increases the delayed rectifier potassium current;[18] these actions could explain a shortening of APs at high concentrations. In contrast, there is no known basis for an initial lengthening and a definitive measurement of the effects of captopril on I_{Kr} would be useful. The small magnitude of the prolongation observed in the rabbit Purkinje fibres may explain the absence of a PRR signal for QT prolongation, but the PRR for torsades de pointes (3.2) is based on a total of 54 reports (a fairly large number). However, patients taking captopril have underlying cardiac diseases, which may in themselves precipitate arrhythmias. Consistent with this idea, the PRR for torsades de pointes for captopril relative to other ACE inhibitors (1.8) is not indicative of a signal for this particular compound in its class whereas the PRR of ACE inhibitors relative to all drugs (2.1) suggests a signal for the entire drug class. We propose that these observations may indicate an increased baseline occurrence of torsades de pointes in the relevant patient population, independent of treatment, since there is no known basis for a class effect of ACE inhibitors in precipitating cardiac arrhythmias. If anything, ACE inhibition is considered to be protective.[30-32]

The last two compounds in the 'clinically questionable' group are fluoxetine and verapamil, which belong to *in vitro* profile 2. Like nifedipine, these compounds were deemed 'questionable' in the absence of signals in the PRR analysis, based on published case reports of QT prolongation and torsades de pointes. Although they are from different chemical and therapeutic classes, the two drugs produce similar *in vitro* effects, as they prolong APD_{90} with a biphasic concentration effect relation (~80% maximal prolongation) and they markedly slow V_{max}. Contrary to captopril, these effects are consistent with published data regarding effects on ion channels: fluoxetine and verapamil block I_{Kr} (inhibitory concentration $[IC]_{50}$ ~1.5 and 0.14 μmol/L[33,34]), I_{CaL} (IC_{50} ~5.4 and 1.7 μmol/L[35,36]) and I_{Na} (IC_{50} ~10–20 and 22 μmol/L[37,38]). This pattern of ion channel effects should lead to the observed outcome on AP morphology. Yet, despite the observed prolongation and potent I_{Kr} block, fluoxetine and verapamil do not appear to have a clinical QT/torsades de pointes signal according to the PRR analysis. This well known discrepancy is usually explained by the idea that concomitant block of I_{CaL} counteracts functionally the deleterious effect of I_{Kr} block.

Added Value of the Rabbit Purkinje Fibre Assay

A self-limiting prolongation of cardiac APs has been proposed often as a possible mechanism for safe, or at least safer, prolongation. Indeed, four drugs that we tested show this pattern and have either no signal (amlodipine, fluoxetine, verapamil) or a questionable signal (captopril) in the PRR analysis. Yet, another five drugs show a similar pattern but exhibit a signal in the PRR analysis (amiodarone, bepridil, thioridazine, astemizole, pimozide). Block of I_{CaL} has been reported for amiodarone (IC_{50} ~0.36–5.8 μmol/L[17]), bepridil (IC_{50} ~0.5 μmol/L[39]) and pimozide (IC_{50} ~0.2 μmol/L[40]), and this could contribute to the limited APD_{90} prolongation observed with these drugs. Block of I_{CaL} has also been demonstrated for terfenadine (IC_{50} ~0.14 μmol/L[41]), but prolongation does not reach a clear maximum for this drug. For astemizole and thioridazine, this type of block has not been reported. Therefore, concomitant I_{CaL} block does not always counteract I_{Kr} block, from a proarrhythmic point of view, and the different proarrhythmic liabilities of profile 1 and profile 2 drugs probably stem from different relative blocking potencies on human channels confounded by the presence of further electrophysiological actions (e.g. block of I_{Na} or of the slow-delayed-rectifier potassium current [I_{Ks}]).

Saturation and/or reversal of APD_{90} prolongation at high concentrations could result from block of

either I_{CaL} or I_{Na} but compounds maximising prolongation before reducing V_{max} would be expected to block I_{CaL} before I_{Na}. We examined the separation between EC_{25} [V_{max}] and EC_{max} [APD_{90}] (the concentration where maximal prolongation of APD_{90} first occurs) by calculating a separation index (SI) as the ratio of these two values (table V). For most PRR-positive drugs (astemizole, bepridil, cisapride, pimozide, quinidine, terfenadine, thioridazine) this index is <1, whereas negative or questionable drugs (captopril, fluoxetine, verapamil, amlodipine) have indices >2. Only amiodarone has a large index despite high PRR values for torsades de pointes and QT prolongation. However, it is noteworthy that amiodarone is considered to be one of the less proarrhythmic of the class III antiarrhythmic drugs. Thus it seems that profile 1 or profile 2 drugs for which a self-limiting prolongation is due, at least initially, to block of I_{CaL} only (high index) are safer than those for which reversal is due to, or occurs simultaneously with, I_{Na} block. It will be interesting to see whether this observation holds true as new drugs are evaluated.

Although triangulation and reverse-use-dependence are valuable parameters for detecting proarrhythmic compounds in isolated rabbit hearts[15] and dog Purkinje fibres,[42] the value of these parameters in the rabbit Purkinje fibre assay remains uncertain. In the drug set tested here, all prolonging drugs (profiles 1 and 2) prolong in a reverse-use-dependent manner and triangulate the AP, except for captopril (no triangulation) and amiodarone/amlodipine/bepridil (no reverse-use-dependence). These observations may suggest that AP prolongation is safer if it occurs without triangulation (captopril), but confirmation of this awaits testing more such compounds. Whether a lack of reverse-use-dependence is of value is doubtful: amlodipine prolongs with too high a safety margin to help in this assessment, amiodarone may be safer than other class III agents but remains proarrhythmic, and bepridil is markedly proarrhythmic. It is worth noting that in the isolated rabbit heart, amiodarone and bepridil prolong APs without triangulation, reverse-use-dependence or instability, whereas in the dog Purkinje fibre, bepridil triangulates without prolonging APs. Obviously, these two compounds are difficult to evaluate in AP assays.

A final added value of AP assays such as the rabbit Purkinje fibre is the possibility of observing AP shortening by some compounds (profile 3). Action potential shorteners could potentially reduce cardiac contractility *in vivo* if plasma concentrations reached sufficient levels. Based on clinical pharmacokinetic data, this may happen with nifedipine (margin of ~3 from free plasma concentrations to the concentration producing 5% APD_{90} shortening) but it seems less likely with simvastatin (margin of 10–20) and quite unlikely with isradipine or tamoxifen (margins >70). Indeed, among these four drugs, only nifedipine has been reported to induce negative inotropy in humans, albeit only upon intravenous administration.[43]

Limitations of the Rabbit Purkinje Fibre Assay

The rabbit Purkinje fibre assay shares some of the limitations common to many preclinical models used in safety pharmacology and toxicology. Foremost are the differences between the model and the clinical endpoint. In the case of proarrhythmic risk, the aim is to evaluate drug effects on 'susceptible' human ventricular muscle. However, this is not a tissue commonly available for study as even clinical studies generally evaluate QT prolongation in healthy subjects. Therefore, differences from the relevant human tissue are unavoidable in all preclinical models (e.g. species, tissue, membrane current density, etc.). The goal of a study such as ours is to show that, despite these differences, a model can provide data useful for decision making.

Female gender is a major risk factor for drug-induced QT prolongation and torsades de pointes in humans and in rabbits, apparently as a result of gender differences in ion current densities.[44,45] Therefore, to optimise our assay for sensitivity, we elected to use Purkinje fibres from female rabbits only. However, there is a risk of incorrectly categorising a compound that would prolong more extensively in males, although no such example is presently known.

Many examples exist of drug metabolites responsible for most of the toxicity of the parent drug, particularly in relation to liver toxicity. We know of no such example in the case of proarrhythmic risk, but this possibility remains a subject of concern since metabolites may possess electrophysiological

profiles that are similar to (metabolites of astemizole and halofantrine) or very different from (fexofenadine, the metabolite of terfenadine) the profile of their parent drug. Indeed, metabolites of probucol could be responsible for the clinical proarrhythmic propensity of this drug despite its apparent innocuousness in tests performed *in vitro*. With the rabbit Purkinje fibre assay, as with other electrophysiological *in vitro* assays, metabolites need to be identified and tested separately.

Conclusion

The rabbit Purkinje fibre assay is a useful *in vitro* safety pharmacology tool to refine the assessment of proarrhythmic risk initiated with a standard I_{Kr} assay[3] because it documents the complex electrophysiological profile carried by some compounds. An analysis of the concentration-dependence of AP prolongation in comparison to therapeutic concentrations shows that the numbers of false negatives (probucol in this study) and false positives (fluoxetine, verapamil and possibly captopril) are small. This concordance is also better than that obtained using dog Purkinje fibres, possibly because fibres from rabbits are more sensitive to drug-induced AP prolongation than fibres from other species.[46] In an earlier study, performed with fewer compounds and looking only at AP prolongation, Gintant et al.[47] could not reveal the proarrhythmic potential of terfenadine. Champeroux et al.[42] recently proposed a complex algorithm for revealing, in dog fibres, the proarrhythmic risk of this drug (but not its QT prolongation potential). These observations render the use of Purkinje fibres from dogs ethically questionable as compared to those from rabbits. In general, the benefits of the Purkinje fibre assay would be to confirm, refine and extend the liability profile of compounds detected with an I_{Kr} assay and to evaluate the possible mitigation of I_{Kr} block through the modulation of other ion channels shaping the AP. In addition, the assay can reveal cardiac liabilities other than QT prolongation. Thus, when combined with a standard I_{Kr} assay and ECG data from animals, the rabbit Purkinje fibre assay would help achieve a more complete assessment of the electrophysiological profile and proarrhythmic potential of pharmaceuticals.

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