PKPD modelling of drug-induced QTc interval prolongation in man: prediction from *in vitro* hERG binding and functional inhibition assays and conscious dog studies

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Abstract:

Background and Purpose: Functional measures of hERG channel inhibition have been prioritised as a screening tool for candidate molecules *in vitro*. However, it is unclear how these results can be translated to humans. Here we explore how drug binding and functional inhibition data *in vitro* relate to QT prolongation *in vivo*. Using cisapride, sotalol and moxifloxacin as paradigm compounds, we assess the relationship between drug concentrations, binding, functional measures and *in vivo* effects in preclinical species and humans.

Experimental Approach: Data on drug effects in hERG functional patch clamp, in hERG radio-labelled dofetilide displacement and QT interval in conscious dogs were analysed in parallel to identify potential correlations between pharmacological activity *in vitro* and *in vivo*.

Key results: Pharmacokinetic-pharmacodynamic modelling based on an Emax model was not possible due to large variability in the functional patch clamp assay. Dofetilide displacement revealed that binding curves appear to be unrelated to the *in vivo* potency estimates for QTc interval prolongation in dogs and humans. Mean *in vitro* estimates (SD) ranged from 99.9 nM for cisapride to 1030 μM for moxifloxacin.

Conclusions and implications: The lack of standardised protocols for *in vitro* assays leads to significant differences in experimental conditions, making the assessment of *in vitro-in vivo* correlations unreliable. Identification of an accurate safety window during the screening of candidate molecules requires a quantitative framework that disentangles system- from drug-specific properties under physiological conditions, enabling translation of results to humans. Similar considerations will be relevant for the comprehensive *in vitro* pro-arrhythmia assay initiative.

Tables of Links

LIGANDS	
astemizole	penicillin
cisapride	moxifloxacin
dofetilide	sotalol
G418 (geneticin)	streptomycin
MK-499	verapamil

TARGETS
Voltage-gated ion channels
Cav1.2
Kv11.1
Nav1.8

These Tables of Links list key voltage-gate ion channels and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015)

Abbreviations

BCA - Bicinchoninic acid

BSA - Bovine serum albumin

CHO - Chinese hamster ovary

DMEM - Dulbecco's Modified Eagle's Medium

EGTA - Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hERG – human Ether-à-go-go-related gene

HEK / HEK293 – Human embryonic kidney

I_{Kr} – rapid delayed rectifier current

I_{Ks} – slow delayed rectifier current

$I_{Ca,L}$ – L-type Ca2 channel current

I_{Na} –Na+ channel current

NPDE - normalized prediction distribution error

pcDNA3 – (plasmid) expression vector with the cytomegalovirus promoter and a neomycin-resistance marker.

PKPD – pharmacokinetic-pharmacodynamic

TdP –Torsade de Pointes

Introduction

During the last two decades a number of drugs have had to undergo labelling revision or market withdrawal due to post-marketing reports of sudden cardiac death linked to Torsade de Pointes (TdP) (Cavero et al., 2000; Haverkamp et al., 2000; Redfern et al., 2003; Shah and Hondeghem, 2005; Thomsen et al., 2006). Despite major efforts to screen compounds for their pro-arrhythmic activity *in vitro*, compounds still progress into clinical development with an unclear risk of QT interval prolongation in humans. (Chen et al., 2006; Ducroq et al., 2007; Gintant, 2008).

From a drug discovery perspective, pharmaceutical R&D has relied on *in vitro* human Ether-à-go-go-related gene (hERG) assays as a primary screening filter before *in vivo* experimental protocols are used to evaluate QT/QTc interval prolongation in pre-clinical species. Multiple approaches have been developed to evaluate drug effects on hERG current *in vitro*. Gintant et al (Gintant et al., 2006) proposed to divide them into two different classes based on whether experimental measures will reflect a direct or indirect effect on the hERG current. Indirect approaches include binding assays, assays measuring ionic flux changes, and assays detecting changes in membrane potential. An advantage of binding assays that measure displacement of potent, radiolabelled hERG ligands is that they provide a convenient screening method to detect drug-hERG channel interactions. These systems use intact cells or cell membranes from heterologous expression systems transfected with the hERG channel and potent, radiolabelled hERG ligands such as dofetilide (Diaz et al., 2004), methanesulfonanilide MK-499 (Wang et al., 2003) and astemizole (Chiu et al., 2004). Results obtained with the aforementioned indirect assays are considered to be less sensitive than those reported using direct functional measures of hERG current, but have the advantage of greater throughput, as compared to functional measures.

More recently, newer techniques have been developed to assess hERG currents in a more direct way (Dubin et al., 2005), but there is no evidence thus far of how the results from these assays correlate with the drug induced QT-interval prolongation in humans, even one considers compounds which show no affinity for other ion channels. In fact, regulatory agencies, academic researchers and pharmaceutical companies appear to have recognised the drawbacks of the existing approach for the evaluation of pro-arrhythmic properties based on a predominant focus on the hERG channel. As a result, at the conference of the Cardiac Safety Research Consortium (CSRC)-Health and Environmental Sciences Institute (HESI)-Food and Drug Administration (FDA) held in July 2013, a revision of ICH S7B and possible elimination of ICH-E14 were proposed. The proposal is aimed at shifting the focus from evaluating QT prolongation to evaluating pro-arrhythmic activity using a comprehensive *in vitro* pro-arrhythmia assay (CiPA) (Cavero et al., 2014; Sager et al., 2014, Fermini et al 2016). The approach seems however to overlook the importance of a stricter quantitative framework for the translation of *in vitro* findings and in particular of the potential differences between *in vitro* and *in vivo* concentration-effect (PKPD) relationships .

Here we evaluate whether a systematic correlation can be found between hERG binding and functional inhibition data *in vitro*. Subsequently, we attempt to assess how binding and functional inhibition data correlate with the underlying concentration-effect relationship *in vivo*, both in nonclinical species (dogs) and healthy human subjects (Chain A.S.Y & Dubois V.F.S. et al., 2013). Reference compounds with known clinical QT prolonging effects are used for the purposes of this evaluation, namely cisapride, sotalol and moxifloxacin. Evidence of such a correlation might support the use of hERG binding data in conjunction with PKPD relationships as a screening tool in early drug discovery. The concept might then be expanded to other ion channels. The ultimate goal of this investigation is therefore to assess the feasibility and translational value of binding

information as the basis for establishing *in vitro-in vivo* correlations for drugs with varying affinity for the hERG channel.

Limitations of the assessment of hERG channel blockade

Even though different functional assays are available for screening, the use of *in vitro* hERG inhibition is based on the assumption that any strong signal, i.e., hERG channel inhibition will be predictive of potential QT prolongation *in vivo*. Yet, none of the available pre-clinical *in vitro* and *in vivo* methods appear to fully predict the torsadogenic potential in humans (Hoffmann and Warner, 2006). Among other things, there has been limited attention to whether experimental conditions are representative of the physiological milieu in humans (e.g., low K^+ concentration, proteins, and low rate stimulation imitating bradycardia). Most importantly, the experiments are performed without taking into account the most likely range of drug exposure at the therapeutic dose levels. To better understand the implications of differences in experimental conditions, a brief overview of the hERG assays and their relevance for the evaluation of pro-arrhythmic effects is provided in the supplemental material.

Based on the aforementioned hERG channel properties, the use of IC_{50} values characterising the potential of a compound to block the hERG current provides a convenient way to compare compounds. However, it should be recognised that potency estimates represent an oversimplification of potentially complex time-, voltage-, and state-dependent processes. Part of this complexity relates to the fact that some compounds will bind in the open phase of the hERG channel ("open state blockers"), whereas others will bind when the ion channel is closed again, but the channel needs to be activated first. Also the association rate for the ion channel has an influence (Yu et al. 2015). Thus, the configuration of the voltage clamp waveform (the time at a certain voltage and the voltage steps) may affect the potency of the drug as well as the time course of inhibition and recovery, reflecting interactions with different states of the channel.

One of the main implications of such differences hERG binding and inhibition is the high incidence of false positive and false negative results in QT prolongation, as previously illustrated in the publications by Chiang et al.(2010), Laverty et al.(2011) and Mirams et al.(2014), all of which provide figures associated with the sensitivity and specificity of experimental protocols used for drug screening. With regard to false positive results, it should be highlighted that despite a considerable debate supporting the views that hERG inhibition in non-clinical assays are highly predictive of drug effects on the QT interval, exceptions exist which raise questions about the generalisability of such correlations (e.g., verapamil, which has a high potency for the hERG ion channel but does not prolong the QT interval *in vivo*) (Wallis, 2010). It has been suggested that false positive results are due to the actions of a compound on currents other than I_{Kr} such as $I_{Ca,L}$ (L-type Ca^{2+} channel current) or I_{Na} (Na^+ channel current) (Antzelevitch et al. 2004). On the other hand, false negative results in hERG assay data are linked to the fact that that various sequentially activated ion channels and transporters may affect the action potential duration. Dumotier et al. suggest that false negatives in hERG inhibition data arise from (i) effects of other ionic currents, (ii) additional effects such as hERG trafficking inhibition, (iii) drug accumulation in the ventricular myocardium, and (iv) drug metabolite effects on hERG current even if parent drug has no effect (Dumotier et al., 2008).

Another important limitation is the questionable accuracy of the so-called quantitative parameters. Usually, IC_{50} values are used to compare and rank compounds. Given the complexities associated

with hERG channel inhibition, conclusions drawn from such comparisons may not be accurate. This has potentially important implications for the selection of novel molecules. The bias caused by intrinsic mechanistic differences is often further compounded by other sources of variability in experimental protocols. For instance, temperature has been shown to affect IC_{50} values for some compounds (Kirsch et al., 2004; Yao et al., 2005). Differences in experimental procedures and techniques contribute to the range of IC_{50} values reported for any given compound in the literature. These discrepancies suggest that a relative bias remains for different protocol settings even when positive control standards are used to monitor assay sensitivity (Su et al., 2006).

In spite of the fact that different chemotypes can bind with high affinity to the hERG channel and changes in a functional group or structure of a given chemotype can alter the hERG binding profile significantly (Sanguinetti and Tristani-Firouzi, 2006; Polak et al., 2009, Eichenbaum et al., 2012), here we explore the notion of target occupancy as a screening parameter with potentially direct clinical meaning. Irrespective of whether a systematic relationship between binding and effect can be identified across compounds, we believe that assessment of the correlation between binding and the degree of hERG channel blockade will shed further light on the relevance of parameter estimates such as IC_{50} arising from functional assays *in vitro*.

Materials and methods

In vitro **displacement and functional assays**

1) [³H] dofetilide-isolated membrane displacement: Dofetilide, moxifloxacin, sotalol and cisapride were purchased from Sigma Aldrich (Zwijndrecht, the Netherlands. [³H] Dofetilide (specific activity 70.0 Ci mmol^{-1}) was purchased from Perkin Elmer (Groningen, the Netherlands). Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO). G418 (geneticin) was obtained from Stratagene (Cedar Creek, U.S.A.). All the other chemicals were of analytical grade and obtained from standard commercial sources. HEK293 cells stably expressing the hERG K$^+$ channel (hERG/HEK293) were kindly provided by Dr Eckhard Ficker (University of Cleveland, USA).

Cell culture: hERG/HEK293 cells were cultured in a humidified atmosphere at 37 °C and 7 % CO_2 in Dulbecco's Modified Eagle's Medium (DMEM), containing 10 % foetal calf serum, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 1.25 μg ml^{-1} G418. Cells were sub cultured twice a week (1:8). Then, the cells were sub cultured 1:10 and transferred to large 15-cm diameter plates for membrane preparation.

Membrane preparation: hERG/HEK293 cells were grown to 80 - 90 % confluence and detached from the plates by scraping them into 5 ml of PBS. Then, the detached cells were collected and centrifuged at 250 g for 10 min. The cell pellets were pooled and resuspended in 50 mM ice-cold Tris-HCl buffer containing 2 mM $MgCl_2$, pH 7.4. An UltraTurrax (Heidolph Instruments, Schwabach, Germany) was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 g in an Optima LE-80K ultracentrifuge (Beckman Coulter, Fullerton, CA) at 4°C for 20 min. The pellets were resuspended using similar procedures in ice-cold incubation buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 130 mM NaCl, 60 mM KCl, 0.8 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM glucose, 0.1 % BSA, pH 7.4) using

the UltraTurrax. Aliquots (125 or 250 µl) were stored at −80 C. The protein concentration of the membranes was measured using the BCA (bicinchoninic acid) method (Smith et al., 1985).

Equilibrium radio ligand binding assays: The [³H]dofetilide equilibrium binding assays for the hERG K⁺ channel were performed as described before (Chadwick et al., 1993; Finlayson et al., 2001; Chiu et al., 2004; Diaz et al., 2004) with minor modifications. In short, membrane aliquots containing 20 µg protein were incubated in a total volume of 100 µl incubation buffer at 25°C for 60 min. Radio ligand displacement experiments were conducted using a range of concentrations of the competing ligand in the presence of 5 nM [³H] dofetilide. At this concentration, total radio ligand binding did not exceed 10 % of the initial radio ligand added to prevent the ligand depletion. Nonspecific binding was determined in the presence of 10 µM astemizole and represented approximately 15 % of the total binding. [³H]dofetilide did not bind specifically to membranes prepared from empty HEK293 cells lacking the hERG K⁺ channel (data not shown). Total binding was determined in the presence of incubation buffer and was set at 100 % in all experiments, whereas non-specific binding was set at 0 %. Given the scope of the analysis, data were normalised as percentage (%) dissociation in order to ensure direct comparison across experiments and normalise for eventual differences in assay handling. Incubations were terminated by dilution with ice-cold wash buffer. Separation of bound from free radio ligand was performed by the rapid filtration through a 96-well GF/B filter plate using a Perkin Elmer Filtermate-harvester (Perkin Elmer, Groningen, The Netherlands). Filters were subsequently washed 12 times with ice-cold wash buffer. The filter-bound radioactivity was determined by scintillation spectrometry using the P-E 1450 Microbeta Wallac Trilux scintillation counter (Perkin Elmer) after addition of 25 µl microscint and 2 h extraction. The protocol was based on triplicates (n=3), which is considered standard practice for these experiments.

2) Whole cell patch-clamp: hERG patch clamp data from reference compounds cisapride, sotalol and moxifloxacin respectively were retrieved from TI-Pharma data repository. The hERG assays were all performed using kidney (HEK293) cell lines stably transfected with pcDNA3 vector expressing hERG (University of Wisconsin, USA). These cells are fully characterised (Zhou et al., 1998) and are the most widely used cells in functional isolated whole cell patch-clamp hERG assays. The extracellular solution consists of (mM): 150 NaCl; 1.8 CaCl2; 1 MgCl2; 5 glucose; 10 HEPES; at PH 7.4. KCl and assay voltage varied in each study (see Table 1 for details). Most protocols were run at 15 s intervals. The amplitude of tail current following the voltage step back to baseline was measured relative to holding potential. The signals were corrected for the averaged rundown observed during approximately 10-min exposure to vehicle solution. All values were given as % of control values for each concentration tested. All experiments were performed at room temperature.

Data analysis

1) [³H] dofetilide-isolated membrane displacement: An Imax (maximum inhibitory effect) model was used to describe the displacement of *[³H]* dofetilide (equation 2):

$$Displacement = I_0 + \frac{I_{max} - I_0 \times [C]}{IC_{50} + [C]} \qquad \text{Equation 2}$$

where *Displacement* represents the degree of [³H] dofetilide displacement. I_0 is the baseline dofetilide binding, *Imax* the maximum displacement and IC_{50} the concentration at which 50% displacement is observed. *C* is concentration of compound tested.

In contrast to functional measures, drug binding was expected to provide information about target occupancy or blockade, and as such reflect the differences in the affinity of each ligand for the hERG channel. Parameters of interest are expressed as inhibitory concentrations, but reflect the degree of *[³H]* dofetilide displacement: IC_{20}, IC_{50}, IC_{70} and IC_{80} represent the concentrations associated with 20%, 50%, 70% and 80% displacement. The concentrations of competing ligands were analysed using the non-linear regression in R. Nonlinear mixed effects modelling was not deemed necessary due to the number of samples and limited variability in the results obtained with the proposed protocol design. It should also be highlighted that no statistical hypothesis testing was performed to compare differences between compounds.

2*) Whole cell patch-clamp:* A sigmoid Imax model was chosen to describe the inhibitory drug effects on hERG channel (equation 3):

$$Inhibition = I_0 + \frac{I_{max} - I_0 \times [C]^\gamma}{IC_{50}{}^\gamma + [C]^\gamma} \qquad \text{Equation 3}$$

where *Inhibition* is the effect. I_0 is the baseline inhibition, *Imax* the maximum inhibition and IC_{50} the concentration at which 50% inhibition is observed. *C* is concentration of compound tested and γ describes the shape or steepness of the curve.

The analysis was performed using non-linear mixed effects modelling in NONMEM v.7.1.2 (ICON, Maryland, USA) running on a Windows PC. Model diagnostics were based on graphical and statistical criteria, including goodness of fit plots, visual predictive checks and normalized prediction distribution errors (NPDE). All three compounds were analysed concurrently, yielding a common parameter estimates for I_0, IC_{50} and Imax. Baseline and maximum inhibition were deemed to be comparable across the compounds and as such reflect the experimental conditions (i.e., system specific properties). By contrast, drug potency varied for each drug. Additive error terms were estimated separately where appropriate.

3) In vivo and clinical PKPD studies: Estimates of the effects of cisapride, sotalol and moxifloxacin on QT interval prolongation in dogs and healthy subjects, expressed in terms of the concentrations corresponding to a probability of QT interval prolongation $\geq$ 10 ms, were used as clinical reference for establishing potential correlations between *in vitro* and *in vivo* experiments. Full details of the experimental protocols and approval by the ethics committees can be found

elsewhere (Chain ASY, Dubois VFS et al., 2013). The pharmacokinetic-pharmacodynamic analysis was performed using a Bayesian hierarchical model in WinBUGS version 1.4.2 (Lunn et al., 2002). The model comprises three components, including an individual correction factor for RR interval (heart rate), an oscillatory component describing the circadian variation and a truncated Emax model, as shown by equation 4:

$$QT = QT_0 \cdot RR^{\alpha} + A \cdot \cos\left(\frac{2\pi}{24}(t - \phi)\right) + slope \cdot C \qquad \text{Equation 4}$$

where QT_0 [ms] is the intercept of the QT-RR relationship (for each individual), RR [s] is the interval between successive R waves, α is the individual heart rate correction factor, A [ms] is the amplitude of circadian rhythm, t is the clock time, Φ is the phase, slope [ms/concentration unit] is the linear pharmacodynamic relationship, and C is the observed or when not available predicted concentration of the drug at the time of QT measurements.

One of the advantages of this approach is the possibility of characterising drug effect in a quantitative manner and expressing it in terms of the probability relative to a clinically relevant threshold, irrespective of the baseline QTc values. For the purposes of our analysis, a threshold of $\geq$10ms increase in QT was used to assess the relationship between total plasma concentration and the probability of QT prolongation. This reference threshold was selected due to its clinical relevance with regard to increased risk of TdP. Moreover, there is some evidence that in humans and dogs, QT prolongation does not seem to correlate with baseline QT after correction for differences in heart rate. It should also be noted that plasma protein binding was assumed not to be restrictive for these compounds. Unbound drug concentrations would have yielded similar results, but different apparent parameter values would have been derived after correction for protein binding.

3) In vitro-in vivo correlation (IVIVC): The assessment of a potential correlation between binding, functional assay and the probability of QT interval prolongation in dogs and healthy subjects was based on graphical summaries, linear and log-linear regression techniques. To ensure normalisation of the results across different compounds and experimental protocols, the intercept of the log-linear regression obtained from the IC_{20}, IC_{50} and IC_{70} values of the [3H] dofetilide-isolated membrane binding assays was calculated and used as parameters of interest for the *in vitro* experiments. In principle, the intercept of this regression corresponds to the concentrations associated with the initial, detectable onset of displacement of [3H] dofetilide (Oinh). It was compared with the IC_{50} values obtained from hERG patch clamp assays and concentrations corresponding to 50% probability of QTc prolongation >10ms (i.e., CP50) in dogs and humans. All calculations were performed in R 2.12.1.

Each of the analysis described above was performed in an unblinded manner.

Results

[^{3}H] dofetilide-isolated membrane binding

All three compounds produced a concentration-dependent displacement of the specific [^{3}H] dofetilide binding. Even though experimental data was sampled in triplicates, variability was relatively low. The maximum standard deviation (SD) was 12.3%, with only 3 measurement points over 6%. The displacement curves were best described by a maximum inhibitory effect model. An overview of the concentration vs. displacement curves for all three compounds is presented in Figure 1 along with the functional assay results and concentration vs. probability of QT prolongation $\geq$ 10 ms. The model-predicted parameter estimates listed in **Table 2a**. Cisapride had the highest affinity to the hERG K$^+$ channel, displacing [^{3}H] dofetilide with an IC$_{50}$ value of 99.9 nM, whereas moxifloxacin exhibited the lowest affinity of 1030 μM. Sotalol showed displacement with IC$_{50}$ value of 56.4 μM.

hERG functional assay – whole cell patch clamp

Data from multiple whole cell patch-clamp experiments, including cisapride, sotalol and moxifloxacin were pooled and explored for consistency and homogeneity before data fitting. In contrast to the experimental results obtained from the[^{3}H] dofetilide-isolated membrane binding, functional hERG inhibition was considerably variable. Of note was the variability in the experimental data from cisapride, which had apparent IC$_{50}$ values varying > 1000 fold across protocols. The inhibition curves for all three compounds were best described by a sigmoid maximum inhibitory effect model (Figure 1). Goodness-of-fit plots showed that model predictions (PRED, IPRED) were able to describe the observed hERG inhibition. Conditional weighted residuals showed no trend for population predictions and only minor trends for individual concentration values. NPDEs were normally distributed with a 0 mean and 1 variance, without a trend (see figures S1 and S2 in the supplemental material).

Model-predicted IC$_{50}$ values ranged from 3.57 nM for cisapride, to 103 μM for sotalol and 227 μM for moxifloxacin. I$_0$ and Imax were found to be around physiologically plausible values, i.e., 6.4% and 95.7%, respectively. High inter-individual variability (60%) was found for IC$_{50}$ estimates, with a residual additive error of 23.3% for sotalol and moxifloxacin and 57.7% for cisapride (Table 2b). The shape parameter γ was close to 1. Despite the satisfactory model diagnostics, predicted individual inhibition curves could not be derived due to the poor precision of the parameters describing interindividual variability.

In vitro-in vivo correlation

In order to establish whether an *in vitro-in vivo* correlation exists between hERG binding, inhibition and clinically relevant changes in QT interval, the IC$_{20}$, IC$_{50}$, IC$_{70}$ and IC$_{80}$ values from the *in vitro* displacement assays (Table 2a) and the potency estimates from the functional assay using patch clamp (Table 2b) were compared to CP50 estimates previously described by Chain & Dubois et al. (Table 2c). For the sake of clarity, the reader is also advised to assess the overall relationship between drug concentration and probability of QT interval $\geq$ 10 ms (Figure 1).

Figure 2 shows that the slopes describing the displacement curves for cisapride, sotalol and moxifloxacin are of the same order of magnitude. However, no correlation was found between the concentrations at which displacement occurs (i.e., the beginning of linear portion of the curve) and the predicted CP50 values in dogs and humans. For cisapride, this value occurs at lower values

than the CP50 in humans, whereas for moxifloxacin and sotalol the onset of displacement occurs at values higher than the CP50 in dogs. Similarly, there was no clear pattern or correlation between IC_{50} estimates derived from hERG patch clamp and CP50 in dogs and humans. The IC_{50} of cisapride is reached around a 40-fold lower than the CP50 in humans, whereas sotalol and moxifloxacin have > 80-fold or >20-fold difference compared to the CP50 in humans and dogs, respectively. An overview of the parameters used to establish a potential *in vitro-in vivo* correlation is presented in Table 3.

Discussion

Functional inhibition of the hERG channel assays have been used systematically in drug development since the discovery of the link between drug-induced hERG inhibition and TdP. Typically, IC_{50} values in µM or nM range are compared to projected plasma concentrations to define a safety margin, i.e., the closer these values get the more the results are considered as a liability for QT interval prolongation in humans. As a screening tool, such these experiments have evolved to show high sensitivity to changes hERG function, irrespective of growing evidence about the differences between the degree of inhibition, QT prolongation and TdP (Wallis, 2010; Di Veroli et al., 2014).

This situation is not unique to the screening of candidate molecules, and often reflects common practice in experimental safety protocols used post-candidate selection (Sahota et al., 2015). In addition to the lack of standardisation for experimental procedures, a general feature in these protocols is the absence of information regarding the underlying concentration-effect relationships, which can be used as a denominator across experimental conditions and species. Our own group has shown that pharmacokinetic-pharmacodynamic relationships can be used to establish in a quantitative manner how changes in drug exposure relate to QT/QTc interval prolongation in pre-clinical species and in humans. Moreover, we have shown that by using appropriate parameterisation, it is possible to distinguish between drug and system-specific properties, making it clear how different drugs relate to each other. In fact, a model-based approach may allow one to disentangle pharmacokinetic from pharmacodynamic differences as well as other intrinsic or extrinsic factors contributing to variability in drug effects (Danhof et al., 2008).

The current investigation was aimed therefore at exploring whether binding and functional inhibition could be linked to QTc interval prolongation in pre-clinical species and in human using pharmacokinetic-pharmacodynamic modelling. Evidence of an *in vitro-in vivo* correlation for compounds with known QT prolonging effects may provide further insight into the role of differences in receptor density and binding kinetics and consequently facilitate the translation of early findings (Della Pasqua, 2013; France and Della Pasqua, 2015). In addition, evidence of such a correlation might provide the basis for experimental protocol standardisation, reducing the rate of false positive and false negative results, for which accurate figures are variable due to differences in experimental protocols and by the discontinuation of compounds (i.e., no clinical data is available for compounds with strong preclinical signal). This problem is illustrated by the case of verapamil, which shows a significant hERG signal, but does not produce QT interval prolongation (Chiang et al., 2010; Laverty et al., 2011; Mirams et al., 2014). If verapamil were developed according to current screening criteria, it might have been discontinued before reaching the clinic .

Clearly, our results contrast with previous publications in which a claim has been made about the predictive performance of the hERG functional assay (Gintant et al., 2006; Wallis, 2010).

However, it should be noted that in most investigations data have not been generated or compared in a systematic manner using a model-based approach. As can be seen from the summary results in Figure 2, it appears that not only the concentration at which effects become evident (i.e., onset of inhibition) varies between compounds, but also the relationship between binding and hERG inhibition. Moreover, these inflection points do not correlate with the predicted CP50 values in humans (i.e., Oinh vs. CP50 in Table 3).

Furthermore, it appears that variability in hERG patch clamp data is large and very sensitive to differences in experimental protocol conditions. Such variability affects the potency estimates and may lead to inaccurate ranking of candidate molecules when comparing functional assay results. Most importantly, potency estimates from whole cell patch clamp do not appear to be strictly predictive of the drug levels associated with QT/QTc interval prolongation in dogs and humans even if one takes into account the potential role of differences in plasma protein binding. Similar conclusions were also drawn by Watson and colleagues when comparing modelling results in cynomolgus monkeys with the concentration–response relationships of hERG current derived from *in vitro* patch clamp experiments (Watson et al., 2011).

Given this mismatch between functional measures *in vitro* and clinical effects, it would be of interest to establish whether binding information, as assessed by $[H^3]$-dofetilide displacement, bears any correlation with QT interval prolongation *in vivo*. Surprisingly, this turns out not to be the case, at least for these three compounds. Parameter estimates describing drug affinity for the hERG channel, show that displacement occurs at levels which are much higher than the concentrations associated with QT prolongation ≥ 10 ms in humans. Furthermore, maximum displacement occurs at very high concentrations, which may represent an important limitation for compounds with poor solubility.

A number of factors may explain these results. First, one should not ignore the fact that hERG ion-channels are overexpressed in HEK293 cells, which may cause a significant increase in the absolute amount of ligand required to block the available pool of binding sites, yielding apparent estimates which are unlikely to reflect *in vivo* conditions. Second, biophase equilibration kinetics and differences in drug-ion channel interaction may lead to variable signal transduction, yielding different results in functional assays and subsequently discrepancies in action potential duration and QT interval prolongation (Di Veroli et al., 2014, Mirams et al., 2015).Indeed, examples exist in the published literature, which illustrate the implications of delayed equilibration and slow drug-receptor interactions for the pharmacological effect vs. time profile of a compound *in vivo* and *in silico* conditions (Yassen et al., 2005; Durdagi et al., 2012; Lee et al., 2015). Another important point to consider explaining the observed discrepancies between *in vitro* and *in vivo* experimental data is that hERG blockade is one of a range of factors associated with pro-arrhythmia. Even though all three compounds are known to bind to hERG channels, it is clearly not the only mechanism underlying drug effects *in vivo*, as for instance in the case of cisapride, for which other ion channels are known to be involved in the observed QT prolonging effect in dogs and humans (Jonsson et al., 2012). Thus, an evaluation of the potential pro-arrhythmic effects of drugs based on hERG binding or current block alone may provide an incomplete view of a drug's effects on cardiac repolarisation processes.

In summary, it seems that even when considering concentration-effect relationships, there remains a translational gap between drug screening and QT prolongation in the clinic. It appears that screening and ranking procedures based on binding or potency estimates for inhibitory activity on single ion channels may be misleading, even for compounds with known activity on a single ion channel. Our endeavour to discriminate system vs. drug specific properties in a parametric manner

has not yielded the expected results, in that overall measures of target occupancy or inhibition *in vitro* do not seem to reflect or predict in a quantitative manner the magnitude of drug effects *in vivo* in dogs or in humans.

Undoubtedly, integrative approaches are needed that account for the multifactorial nature of the pro-arrhythmic effects *in vivo*. Among the available options, one should consider the use of a virtual population generator for human cardiomyocytes parameters, as proposed by Polak et al. (2012). The authors propose a computational system including simulations for the evaluation of proarrthymic potential (Tusscher et al., 2004; O'Hara et al. 2011), taking into account the influence of inter-individual variability in the parameters of interest. In fact, this concept has been recently used to predict the effects of domperidone (Mishra et al., 2014), illustrating how *in vitro*, and more specifically *in silico* simulations can be used in conjunction with physiologically-based pharmacokinetic models to predict drug effects in humans. Despite these promising results, some obvious limitations exist, which cannot be overlooked. None of the *in silico* models currently available account for the contribution of physiological factors such as body temperature, insulin/glucose homeostasis, changes in electrolytes or autonomic tone known to alter QT interval *in vivo* (van der Linde et al., 2008; Fossa, 2008). These models also ignore the contribution of differences in binding properties and biophase kinetics (Durdagi et al., 2012; Di Veroli et al., 2014; Lee et al., 2015). A comparable situation applies to the extrapolation of QT prolongation from controlled clinical trials to a real-life setting (Chain et al., 2013). This should be carefully considered by those supporting the CiPA working groups, who are currently responsible for the development and implementation of alternative guidelines for ICH S7B, which will ultimately guide the ranking of compounds in terms of their pro-arrhythmic risk (Cavero & Holzgrefe, 2015).

We acknowledge a few limitations in our research. First, it is worth reminding the reader that PKPD parameters should be independent from and uncorrelated with the dose- and/or experimental protocol design. We have analysed functional hERG assay data available to the TIPharma consortium, which included contributions from five large pharmaceutical companies. However, we cannot exclude the possibility that different protocol settings might have yielded different results. Despite the different protocols (Table 1), it remains unclear whether they represent the optimal experimental conditions for establishing *in vitro-in vivo* correlations. Also others have shown some degree of intra-lab variability (particularly over time) and inter-lab variability even when using the same protocols. The ongoing CiPA project is working closely with contributing scientists and vendors to establish standardised protocols for each/all of the ion channel assays under consideration. Similarly, it should be noted that the variability between *in vivo* pharmacokinetics and pharmacodynamics may contribute to potential inaccuracies in parameter estimates. In this respect, one should be aware that the use nonlinear mixed effects modelling can take into account the effect of interindividual differences in pharmacokinetics and pharmacodynamics. Moreover, it allows for discrimination of covariate effects from random variation.

Whereas it is evident that concentration-effect curves and IC_{50} values do not provide information on binding kinetics, PKPD modelling of these relationships may account for differences in the interaction between drugs and ion channels. Appropriate parameterisation and specific sampling requirements would apply to ensure identification of association/dissociation rate constants. The available experimental data were not suitable for such an approach. We anticipate that a similar mismatch between *in vitro* binding, functional assay and QT prolongation would have been observed if other PKPD indices were used, e.g., IC_{10}, IC_{20}. A first (or even second-order) rate constant may be required to describe biophase equilibration processes.

We are aware of the potential implication of differences in plasma protein binding for the characterisation of PKPD relationships. In fact, controversy regarding the correction for protein binding has been highlighted in previous publications (Gintant et al, 2006). Nevertheless, protein binding for the compounds under evaluation is known to be non-restrictive, i.e., the affinity of a compound for the plasma protein does not necessarily alter binding equilibrium relative to the hERG channel or other relevant target channel. We have summarised our results in terms of total concentration as conclusions would not have been different even after correcting for differences in protein binding. It should be noted that he compound with the highest protein binding (i.e., cisapride) has also a very high affinity for the hERG channel.

Lastly, we recognise that the results from three reference compounds are not sufficient to allow generalisation of the conclusions to a wide class of molecules, as it can be anticipated that changes in a functional group of a given chemotype can alter the hERG binding profile significantly (Polak et al., 2009).

In conclusion, sensitivity and selectivity criteria have driven the development of experimental protocols for the screening of the pro-arrhythmic potential of candidate molecules in early drug discovery. Whilst our investigation is limited to three reference compounds with known QT-prolonging effects in humans, the apparent potency obtained from *in vitro* assays are far higher than the estimates observed *in vivo* for the same drugs. The progression of molecules may be unintentionally stopped due to the discrepancies or lack of a systematic *in vitro-in vivo* correlation. Despite the emphasis on the relevance of these assays as a screening tool, our findings indicate that results from *in vitro* protocols are qualitative at best and cannot be used to define the probability a clinically relevant increase in the QTc interval.

Conflicts of interest: None declared.

Authors' contribution:

V.F.S.D performed the data analysis and wrote the manuscript

M.D. contributed to the research proposal and revision of the manuscript

O.D.P contributed to the research proposal, data analysis and revision of the manuscript

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Legends:

Table 1 Patch clamp assay data available for modelling. Data included in the current analysis are marked with an asterix (*). ** For the sake of completeness, IC_{50} values derived from the original experimental protocols are also presented along with the experimental protocol details. These estimates may differ from the values obtained by nonlinear mixed effects modelling, which was used to analyse the data in the current investigation.

Table 2a: Mean parameter estimates (90% confidence intervals) and derived pharmacokinetic-pharmacodynamic indices IC_{20}, IC_{70} and IC_{80}, i.e., the inhibitory concentrations associated with 20, 70 and 80% binding, respectively. Data (n=3 for each experimental point) from the equilibrium [^{3}H] dofetilide binding displacement assay was analysed using an Imax model. I_0 represents the percentage [^{3}H] dofetilide binding in the absence of a competing molecule.

Table 2b: Population parameter estimates for cisapride (n=11), moxifloxacin (n=28) and sotalol (n=8) in the hERG patch clamp assay. Experimental data obtained with the different compounds were analysed concomitantly. System-specific parameters (I_0, Imax and Hill coefficient (γ)) were unique to the experimental setting. Only IC_{50} varied for each compound. A separate additive error term was estimated for cisapride as residual variability in those experiments was significantly higher.

Table 2c: Population pharmacokinetic-pharmacodynamic parameter estimates along with 90% credible intervals describing the probability of QT interval prolongation $\geq$ 10 ms, as reported by Chain and Dubois et al., 2013. Data analysis was performed using a Bayesian hierarchical model, which comprises three components, namely: an individual correction factor for RR interval (heart rate), an oscillatory component describing the circadian variation and a truncated Emax model, which is parameterised in terms of a slope.

Table 3 Summary table for the comparison between *in vitro* (dofetilide displacement, functional assay) and *in vivo* (probabilities of QT prolongation *in vivo* in dogs and humans) data, where CP50D and CP50H is the concentration associated with a 50% probability of reaching $\geq$ 10msec increase of the QT interval in dogs and humans respectively. The onset of inhibition (Oinh) was obtained by log-linear regression using mean IC20, IC_{50} and IC70 estimates from the displacement assays. IC_{50} values are population parameter estimates of the nonlinear mixed effect modelling of patch clamp assays.

Figure 1: Overview of the concentration-effect relationships in *in vitro* (binding vs. functional hERG assay) and *in vivo* QT prolongation (in dogs and humans) for cisapride (top), moxifloxacin (middle) and sotalol (bottom). Left panels show the dofetilide displacement assay, mid panels display the functional hERG patch clamp assay, whereas the curves depicting the concentration vs. probability of reaching ≥10 msec QT prolongation in dogs (dotted line) and humans (solid line) are shown on the right panels (Chain and Dubois et al., 2013). In all plots the lines represent the model based population predictions and the symbols the observations. For group size details see Tables 2a, 2b and 2c.

Figure 2: Lack of correlation between *in vitro* (dofetilide displacement, functional assay) and *in vivo* (probabilities of QT prolongation *in vivo* in dogs and humans) data. Ideally, proportional differences should be found between these experiments, reflecting the relative differences in the potency of the different compounds. Data summaries include cisapride (blue, diamonds), sotalol (red, triangles) and moxifloxacin (dark green, dots). Log-linear regression (solid lines) of the estimates of IC_{20}, IC_{50} and IC_{70} obtained from the dofetilide displacement are compared with the IC_{50} values obtained from hERG patch clamp assays (open symbols) and *in vivo* (dashed vertical lines) and clinical (dot-dashed vertical lines) concentrations corresponding to 50% probability of QTc prolongation ≥10ms (i.e., CP50). The window associated with these concentrations (i.e., CP50) in dogs and humans is depicted using the blue, red and dark green shaded areas for cisapride, sotalol and moxifloxacin, respectively. For instance, for cisapride, IC50 in the binding assay indicates a potency of 99.9 nM. On the other hand potency in the hERG functional assay is much higher, i.e., 3.57 nM. These values contrast with CP50 estimate *in vivo* in dogs and in healthy subjects, which yield estimates of 2233.6 nM and 141.5 nM respectively. See table 3 for further details. Parameters describing the log linear regression for cisapride, sotalol and moxifloxacin were respectively, $y=-49.85+49.91*\log(x)$, $y=-196.08+52.22*\log(x)$, and $y=-220.46+45.96*\log(x)$.