OPINION

Evolution of strategies to improve preclinical cardiac safety testing

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Abstract | The early and efficient assessment of cardiac safety liabilities is essential to confidently advance novel drug candidates. This article discusses evolving mechanistically based preclinical strategies for detecting drug-induced electrophysiological and structural cardiotoxicity using *in vitro* human ion channel assays, human-based *in silico* reconstructions and human stem cell-derived cardiomyocytes. These strategies represent a paradigm shift from current approaches, which rely on simplistic *in vitro* assays that measure blockade of the K_v11.1 current (also known as the hERG current or I_{Kr}) and on the use of non-human cells or tissues. These new strategies have the potential to improve sensitivity and specificity in the early detection of genuine cardiotoxicity risks, thereby reducing the likelihood of mistakenly discarding viable drug candidates and speeding the progression of worthy drugs into clinical trials.

Ideally, preclinical cardiotoxicity screening efforts should efficiently detect unsafe compounds sufficiently early on in the drug discovery process to influence candidate drug selection, thus reducing later-stage attrition, risks to participants in clinical studies, development time and costs. However, over-simplified and highly sensitive (but not specific) approaches for the early detection of cardiac safety liabilities can result in unwarranted attrition of novel drug candidates owing to false-positive findings. The risk of such unwarranted attrition may also be increased by a lack of concordance between the effects of compounds in animals (or animal-derived tissues) and those in humans.

At present, early screening assays typically involve simple, reductionist *in vitro* systems and surrogate markers for cardiac liabilities in animals or animal-derived tissues or organs. A strong focus remains on avoiding a rare, drug-induced, life-threatening ventricular arrhythmia termed torsades de pointes (TdP, 'twisting of the points')[1–4]. TdP is associated with delayed repolarization, a surrogate marker of proarrhythmia that is observed as prolongation of the QT interval on a surface electrocardiogram (ECG).

Blockade of the K_v11.1 current (also known as the hERG current or I_{Kr}, a repolarizing potassium current carried by the pore-forming α-subunit of the voltage-gated potassium channel K_v11.1 (REFS 5–8), commonly called the hERG channel, which is encoded by *KCNH2*) is recognized as a predominant mechanism responsible for the drug-induced delayed repolarization that is linked to TdP (FIG. 1). Consequently, emphasis on detecting I_{Kr} blockade early in drug discovery has, along with mandated clinical thorough QT (TQT) studies to quantify QTc (the QT interval corrected for changes in heart rate) prolongation later in drug development, contributed to the successful removal of TdP risk for new chemical entities. However, there is concern that the lack of specificity of such studies may lead to potential drug candidates that otherwise have desirable properties being wrongly discarded from drug pipelines.

Contractile and structural cardiotoxicity, such as those seen with several kinase-targeted cancer drugs[9–12], represent another growing safety concern. Contractile and structural cardiotoxicity in non-human species may prevent the progression of promising compounds, despite known species-related differences in contractile function and calcium handling. As less is known about the mechanisms responsible for these more varied and complex toxicities, more-comprehensive multi-parametric approaches using phenotypic cellular assessments are more appropriate (and informative) to detect contractile and structural cardiotoxicity. Ideally, such effects would be detected using *in vitro* preclinical assays with human-derived tissues, before the use of more costly, longer-term animal toxicity studies.

This article discusses evolving human-based preclinical cardiotoxicity-testing strategies that are based on mechanistic assessments in the context of the simplest functional unit in the heart, the ventricular myocyte. The recently proposed Comprehensive *in vitro* Proarrhythmia Assay (CiPA) schema for the preclinical evaluation of proarrhythmic liabilities is provided as an example[13,14]. The CiPA proposes to define proarrhythmic risk based on *in silico* reconstructions of human ventricular electrical activity. These reconstructions integrate drug effects on multiple human cardiac currents and the results are confirmed with human stem cell-derived cardiomyocytes (hSC-CMs). This approach represents a paradigm shift away from approaches that are reliant on pragmatic, reductionist (often single parameter) preclinical non-human assays that use surrogate markers and approaches that use integrated responses derived from poorly characterized, non-human preparations that are reliant on downstream surrogate markers of proarrhythmia (such as QT prolongation). hSC-CMs can also be used to assess structural and contractile cardiotoxicity based on multi-parametric, integrated 'signatures' of functional, phenotypic responses derived from high-content screening. We assert that these approaches using human channels and *in silico* reconstructions of human cellular proarrhythmia and hSC-CMs will reduce the inappropriate premature discontinuation of new chemical entities during drug discovery, reduce reliance on clinical surrogate markers during later-stage drug development (such as QT prolongation) and enable the rapid and efficient development of safer novel drugs.

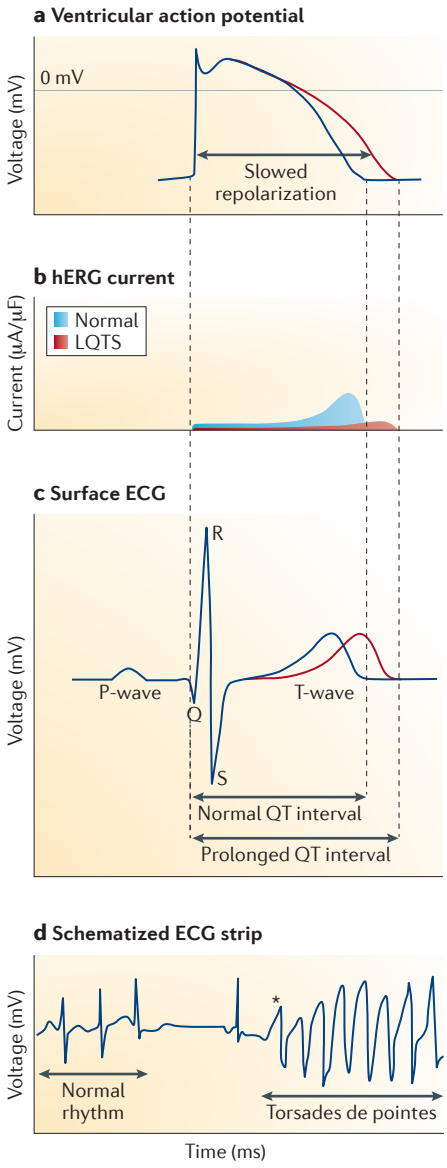

Figure 1 | **Link between delayed repolarization and torsades de pointes proarrhythmia.** **a** | The slowing of ventricular repolarization on a cellular level results from a decrease in net outward current. **b** | This change in current may occur as a result of a reduction in hERG current (I_{Kr}) owing to I_{Kr} blockade by a drug or inherited channelopathies such as the long QT syndromes (LQTS). **c** | Although the relationship between ventricular repolarization and torsade de pointes (TdP) is complex, on a cellular level, delayed ventricular repolarization leads to prolongation of the QT interval, which can be observed with a surface electrocardiogram (ECG). **d** | Prominent QT prolongation in the presence of an appropriate electrophysiological substrate and rhythm may further dysregulate repolarization, giving rise to a premature ventricular beat before the end of the T wave (indicated with an asterisk) to initiate TdP. Figure is reproduced with permission from REF. 174, Frontiers (http://dx.doi.org/10.3389/fphar.2010.00137).

Current proarrhythmia testing approaches

The evolution, limitations and learnings of the International Committee on Harmonization S7b (preclinical) and E14 (clinical) guidelines. The late 1980s and early 1990s witnessed a growing awareness of the potential of non-cardiovascular drugs to cause TdP[15]. Arguably, these concerns took hold between 1988 and 1991, when three drugs (prenylamine, lidoflazine and torodiline) were withdrawn from markets in the United Kingdom and elsewhere in Europe owing to their association with QT prolongation and TdP. Further concerns were raised by QT prolongation caused by drugs from additional therapeutic classes (halofantrine, an antimalarial drug, cisapride, a gastric prokinetic drug and terfenadine, an antihistamine) and by QT prolongation and increased mortality in patients treated with D-sotalol after myocardial infarction (the SWORD study). In response to these concerns, the European Committee for Proprietary Medicinal Products (CPMP) proposed a preclinical approach to evaluate the propensity of a drug to delay ventricular repolarization. This approach included an *in vitro* assessment of action potential prolongation along with early after-depolarizations (EADs) and triggered activity (forms of altered or interrupted repolarization) in appropriate animal tissues and species[16]. Subsequent studies emphasized the link between delayed ventricular repolarization and blockade of I_{Kr} by various non-cardiovascular drugs[7,17,18] and the relationship between a congenital long QT syndrome (namely LQT2) and reduced I_{Kr}[19].

These findings led to the inclusion of the functional I_{Kr} assay in the 2005 International Committee on Harmonization (ICH) S7B regulatory drug development guidance document (*The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals*)[20]. The evolution of this preclinical surrogate proarrhythmia marker was paralleled by the codification of QT prolongation in the ICH E14 clinical guidance (*The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs*)[21]. The goal of this clinical guidance was similar to that of S7B ("to assess the potential of a drug to delay cardiac repolarisation") but is applied to clinical studies of the QTc interval rather than non-clinical studies. This guidance established the TQT study, a carefully controlled clinical assay to assess drug-induced QTc prolongation at therapeutic and supratherapeutic exposure levels. Small increases in the QTc interval (that is, >10 milliseconds for the placebo-adjusted change from baseline (upper bound of the 90% confidence interval)) trigger regulatory concern. However, defining proarrhythmic liability based on an arbitrary threshold for clinical QT prolongation leads to a dichotomous classification of risk that is often misunderstood and not useful in defining risk–benefit ratios of new drugs.

Since its inclusion in the ICH S7B guidance, the I_{Kr} assay has assumed a prominent role as gatekeeper in a frontloading screening approach designed to reduce later-stage compound attrition[22–24]. This strategy was bolstered by the commercial availability of cell lines with stable and heterologous hERG channel expression, the development of higher-throughput automated planar patch-clamp platforms, a growing list of drugs shown to block I_{Kr} and the desire to avoid the cost and potential negative consequences of a positive TQT study (including additional clinical monitoring during clinical development and the labelling implications should the study be positive). In some cases, structure-based computer models to detect hERG-based liabilities have also been used to attempt to avoid any hint of I_{Kr} blockade even before compound synthesis.

As a consequence of positioning the I_{Kr} assay early in drug discovery, countless compounds are deprioritized (or discarded) early on. De Ponti[25] estimated that "as many as 60% of new molecular entities developed as potential therapeutic agents, when assayed for hERG blocking liability, test positive and are thus abandoned early in development." This would be fine if I_{Kr} blockade reliably predicted proarrhythmia, but this is not the case, as mechanistic and translational studies demonstrate that I_{Kr} blockade alone is not highly specific for predicting either delayed repolarization or clinical proarrhythmia[26] (see below). Although it might be possible to 'design out' this property, this strategy incurs added costs and project delays and unnecessary animal testing, as well as the potential loss of efficacy or other favourable compound attributes (for example, target specificity). False-negative determinations also need to be considered; Kramer *et al.*[27] identified 6 false negatives in a total of 55 torsadogenic drugs when the drugs were classified based only on their effects on I_{Kr}. Finally, drug-induced QT prolongation is not specific for

arrhythmogenesis. For example, alfuzosin, phenobarbital and ranolazine prolong the QT interval clinically but are not typically linked with TdP[28,29]. Similarly, small increases in the human QTc interval commonly result in the premature termination of drug development programmes (or in label warnings), even though a number of these drugs are unlikely to be proarrhythmic.

Limitations of I_{Kr} and QT assays to define proarrhythmic risk. On a cellular level, cardiac repolarization is a complex process representing a fine balance of multiple time- and voltage-dependent inward and outward currents (FIG. 2a). At an organ level, differences in current densities within the ventricles are responsible for the heterogeneity of ventricular repolarization that gives rise to the QT interval. Recent data suggest that drugs affect more cardiac currents than previously expected: automated patch-clamp systems have demonstrated the promiscuity of multiple non-cardiovascular drugs, including blockade of cardiac I_{Kr}, I_{CaL} (the L-type Ca^{2+} current) and I_{Na} (a Na^+ current)[27,30] (FIG. 2b). Together, these findings demonstrate that a single-current 'I_{Kr}-centric' approach fails to reflect a drug's integrated effect on the net outward current, which ultimately drives repolarization.

The term 'repolarization reserve' has been used to define the ability of ventricular cells to sustain cardiac repolarization in the face of drug effects on single or multiple currents[31,32]. This concept is related to 'multi-channel block' or 'multi-ion channel effects' — terms that are used to describe the complex electrophysiological effects of drugs that result from the blockade of multiple cardiac currents[33,34]. For example, despite being a potent blocker of I_{Kr}, verapamil does not cause meaningful QT prolongation because of its concomitant blockade of the depolarizing inward calcium current (I_{CaL}, through blockade of the calcium channel subunit $Ca_v1.2$), which mitigates the effects of reduced I_{Kr} outward current[35,36]. Similarly, ranolazine elicits QT prolongation consistent with blocking the repolarizing I_{Kr} but does not elicit proarrhythmia, most probably because of concomitant blockade of the depolarizing late inward sodium current (I_{NaLate}, which flows through the sodium channel subunit $Na_v1.5$)[37]. An *in vitro* study demonstrated that prolongation of Purkinje fibre action potentials by the I_{Kr}-blocking drug dofetilide was reduced by the concomitant administration of nifedipine (owing to I_{CaL} blockade) or lidocaine (owing to blockade of I_{NaLate}), demonstrating the role of multi-channel blockade[34] in modulating repolarization. An *in silico* reconstruction demonstrating the role of multi-channel blockade in modulating the effects of repolarization is shown in FIG. 3. Such findings suggest that a drug that reduces outward (repolarizing) I_{Kr} might still be sufficiently safe with regard to proarrhythmic risk owing to the mitigating effects of reduced inward (depolarizing) current such as I_{CaL} and I_{NaLate}. From a cardiac perspective it is possible that drugs that affect multiple cardiac currents (including I_{Kr}) but marginally affect delayed repolarization (owing to multiple channel blockade) pose minimal proarrhythmic liabilities compared to drugs that exert selective blockade of I_{Kr} at therapeutic exposures.

As repolarization on a cellular level represents an integrated response of multiple cardiac currents, pumps and exchangers, so the QT interval represents the integrated response of temporal and spatial differences in repolarization within and across the ventricles as filtered and recorded from the body surface[38]. The relationship between changes in the QT interval (prolongation as well as morphological changes) and electrophysiological effects across the ventricular wall are complex and poorly understood, and simpler ECG recordings are not necessarily informative about spatial and temporal effects on repolarization that are linked to proarrhythmic liabilities[39]. Thus, QT prolongation is a poor representation of changes in repolarization at the cellular or regional levels. This fact was recently

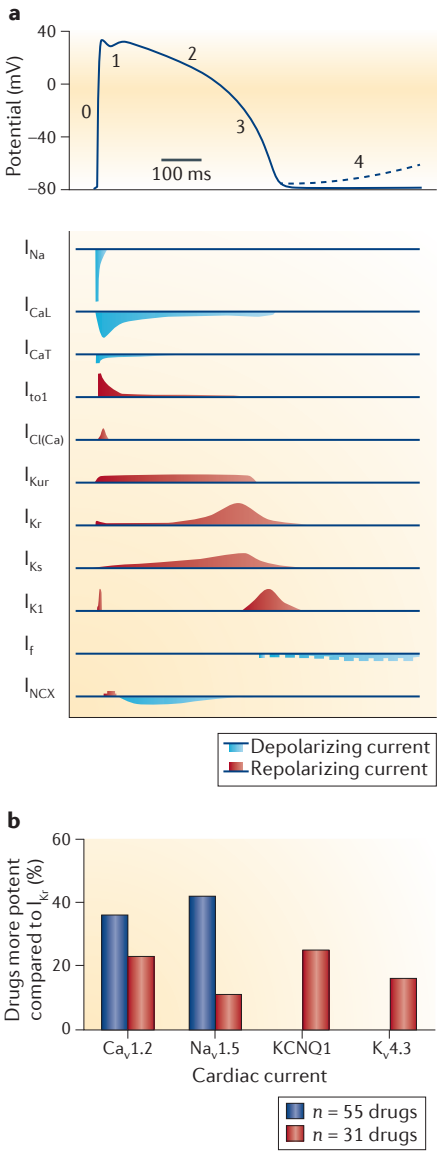

Figure 2 | **Ionic basis for ventricular repolarization and drug-induced proarrhythmia.** **a** | Multiple ionic currents define the ventricular action potential. Voltage-dependent and time-dependent inward (depolarizing) and outward (repolarizing) currents are displayed. The outward repolarizing current, I_{Kr} (also known as the $K_v11.1$ current, conducted by hERG channels), is only one of multiple overlapping currents that define repolarization. Numbers indicate the phases of the action potential: rapid depolarization (phase 0), early repolarization (phase 1), plateau (phase 2), rapid repolarization (phase 3) and resting membrane potential (or phase 4 depolarization). **b** | Multi-channel blockade of cardiac currents by marketed drugs. The graph compares half-maximal inhibitory concentration (IC_{50}) values for the blockade of I_{Kr} with values for the blockade of two depolarizing currents (I_{CaL} through $Ca_v1.2$ channels and I_{Na} through $Na_v1.5$ channels) and two repolarizing currents (I_{Ks} through KCNQ1 channels and I_{to} through $K_v4.3$ channels). Two published datasets were used: a 55-drug set[27] (blue bars), which characterized blockade of I_{CaL} and I_{Na}, and a 31-drug dataset[30] (red bars), which characterized blockade of I_{CaL}, I_{Na}, I_{Ks} and I_{to}. Eleven compounds were common between the two datasets. Both datasets also characterized potency of I_{Kr} blockade, used heterologously expressed channels and used the same automated patch platform at the respective test sites to compare blockade potency (thus minimizing variability). The bars represent the percentage of drugs that blocked each current more potently than I_{Kr} for each drug collection. The results show that a single-current 'I_{Kr}-centric' approach neglects to inform on the lack of selectivity of many drugs, which results in blockade of multiple prominent cardiac currents. Figure part **a** is reproduced with permission from REF. 101, Frontiers (http://dx.doi.org/10.3389/fphys.2012.00346).

highlighted in a technically challenging noninvasive ECG imaging study in subjects with long QT, which recorded 256 body surface ECGs (along with a thoracic CT scan gated to the R–R interval) to construct maps of epicardial repolarization dispersion[40]. The results showed that subjects with long QT had epicardial regions with steep repolarization dispersion caused by heterogeneities of action potential duration, a proarrhythmic substrate that was not detected with the surface ECG. The steep repolarization gradients did not correlate with QTc values from 12-lead ECG recordings, highlighting a limitation of resolution of standard ECG monitoring to assess proarrhythmic liabilities.

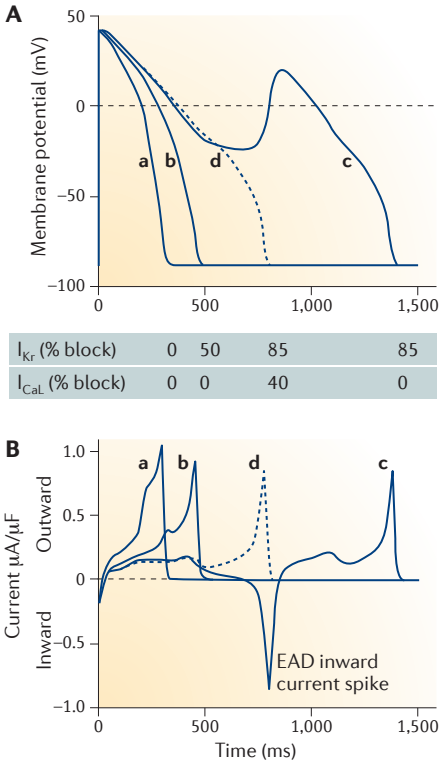

Figure 3 | **Role of delayed repolarization and multi-channel blockade in defining pro-arrhythmic risk.** Representative *in silico* reconstructions show action potentials (part **A**) and corresponding net ionic current (part **B**) in the presence of different levels of blockade of hERG current (I_{Kr}) and L-type calcium current (I_{CaL}). Under drug-free conditions (0% blockade of I_{Kr} and I_{CaL}), repolarization proceeds normally (shortest action potential (part **Aa**) with net outward current throughout repolarization (part **Ba**). A 50% blockade of outward repolarizing I_{Kr} prolongs the action potential duration from 306 to 477 milliseconds (part **Ab**, part **Bb**), and, further, an 85% I_{Kr} blockade elicits an early afterdepolarization (EAD) before full repolarization, which is associated with a prolonged action potential duration of 1,466 milliseconds (part **Ac**). The inward current responsible for the EAD is evident in the lower panel (part **Bc**). Concomitant 40% blockade of the inward (depolarizing) I_{CaL} in the setting of sustained 85% I_{Kr} blockade partially reverses delayed repolarization (part **Bc** and part **Ad**), shortening the action potential duration from 1,380 to 782 milliseconds, and abolishes the net inward current spike responsible for initiating the EAD (part **Bd**). The simulation is based on the O'Hara-Rudy model[44] with slow stimulation (15 beats per minute), a recognized risk factor for torsades de pointes.

I_{Kr} assay performance characteristics in relation to proarrhythmia and clinical QTc prolongation. Redfern and colleagues[41] compared potency of I_{Kr} blockade (within the context of clinical exposures) with subjective clinical experiences related to QT-interval prolongation and TdP. Based on a five-category ranking of proarrhythmic risk, the authors suggested that most drugs with half-maximal inhibitory concentration (IC_{50}) values for I_{Kr} blockade that are comparable to the highest free-drug plasma concentrations observed clinically were associated with TdP and that a provisional safety margin of 30 (defined as IC_{50}/C_{max}, where C_{max} is the maximum free plasma concentration a drug achieves clinically) was generally predictive of cardiac safety (although a higher margin of 100 was suggested in the case of less serious disease indications). Wallis[42] compared the potency of I_{Kr} blockade (based on a 10% inhibition of current) with clinical QTc prolongation (powered to detect 7–10 millisecond QTc prolongation) using a 19-compound dataset (of which 11 prolonged QTc clinically). When assessed at twofold clinical exposure, the I_{Kr} assay was shown to be 82% sensitive (in simplest terms, the proportion of true positives correctly identified by the assay) and 75% specific (the proportion of true negatives correctly identified by the assay).

Sensitivity and specificity both describe the concordance between test assay results based on a clinical 'gold standard', but it can be argued that the ability of a preclinical test to predict QT prolongation is more appropriately described by reverse conditional probabilities (known as positive and negative predictive values) that describe the probabilities of clinical effects given assay results. However, positive and negative predictive values depend on the prevalence of the disease or condition in the study sample and typically cannot be generalized beyond a study. Furthermore, highly sensitive tests may actually have low predictive values in study populations with a low prevalence of the disease or condition. By contrast, likelihood ratios (LRs) summarize sensitivity and specificity in a form that is useful for defining the utility of an assay for increasing certainty about a diagnosis — thus representing a meaningful measure of test accuracy — and they have the advantage of being less dependent on disease prevalence[43], a particularly important benefit for low incidence events such as TdP. In clinical decision making, a positive LR (+LR) value of 1 indicates no influence on the risk of disease, values of 2–5 indicate a small increase in probability and values of 10 or greater indicate a large and often conclusive increase in the likelihood of disease. The +LR value calculated for I_{Kr} assay results from the Wallis study[42] was 3.3, consistent with a minimal-to-moderate influence on decision making.

A later retrospective study[26] evaluated the performance of an I_{Kr} assay in predicting QT prolongation by comparing potency of I_{Kr} blockade (in accordance with S7B) to results from strictly controlled clinical TQT studies (in accordance with E14) of a 39-drug dataset submitted for regulatory approval. Based on IC_{50} values for I_{Kr} blockade (relative to unbound clinical exposures) and a mean QTc prolongation time of 5 milliseconds defining a positive TQT study, the I_{Kr} assay provided only moderate overall performance (sensitivity and specificity values of 64% and 88%, respectively, and a positive likelihood ratio of 3.5, based on an optimal safety margin of 45). This study demonstrates the overall minimal performance of an I_{Kr} assay alone to reliably predict QT prolongation (itself a surrogate marker of proarrhythmia) and highlights the need to consider a more comprehensive view of drug effects on cardiac currents to better assess proarrhythmia.

Limitations of animal models for predicting delayed repolarization and proarrhythmic risk in humans. The need for proarrhythmia evaluations based on human models is emphasized by species differences in ventricular repolarization and responses to drugs. For example, the rat is an inappropriate species for modelling human repolarization as it possesses a minimal I_{Kr} (instead relying on a transient outward current (I_{to}) through channels composed of $K_v4.3$ subunits. Another example is provided by the guinea pig, which relies on two plateau-repolarizing currents (I_{Kr} and I_{Ks}) to modulate ventricular repolarization, whereas humans, dogs and rabbits rely

primarily on I_{Kr} (REFS 44,45). Differences in drug responses across species have also been reported: manifestation of EADs in response to dofetilide and quinidine were readily observed in rabbit Purkinje fibres but not in those of guinea pigs, dogs, pigs, goats, or sheep under identical conditions *in vitro*[46]. In a recent study, prominent differences in the ability of combined pharmacological blockade of the inward rectifier current (I_{K1}) and either I_{Kr} or I_{Ks} to elicit TdP across both rabbits and dogs were attributed to a stronger repolarization reserve in dogs[45]. Various *in vitro* proarrhythmia models have been used to screen for proarrhythmic risk, most notably the rabbit ventricular wedge[47–49] and the rabbit Langendorff heart preparations[50–52]. Although such empirical *in vitro* models are useful in defining mechanisms of proarrhythmia, the technical expertise necessary to maintain these models and the electrophysiological differences (both between non-human species and between non-human species and humans) make the assessment of proarrhythmic risk difficult.

Differences in the ionic current densities of canine and human ventricle tissues have been documented[53]; although I_{Kr} density and kinetics are similar in both species, in humans, I_{CaL} and I_{to} are approximately 30% larger and 29% smaller, respectively. In addition, the ability to resist changes in repolarization (repolarization reserve) is less in humans because of reduced outward current contributions from the potassium currents I_{K1} and I_{Ks}. These later results are consistent with the increased sensitivity of humans (versus dogs) to drug-induced QT prolongation with sotalol, cisapride and moxifloxacin[54].

Towards a mechanistic approach to assess proarrhythmic risk using human cellular proarrhythmia assays. Knowledge of the mechanisms responsible for TdP provides the opportunity to develop a more comprehensive approach for assessing proarrhythmic risk. Briefly, impaired and delayed cellular repolarization can, through various mechanisms, promote net inward current during the action potential plateau that gives rise to EADs (FIG. 3). These emergent cellular effects are not predicted based on effects on individual cardiac currents. When coupled temporally and spatially with a vulnerable electrophysiological substrate that includes heterogeneous repolarization, EADs may give rise to early ventricular excitation. Dispersion of repolarization across the ventricles (which is often exaggerated under pathological conditions with remodelled or hypertrophied myocardium) may also promote EADs and is recognized as a substrate vulnerable to unstable re-entrant excitation supporting TdP. Multiple studies have identified EADs as the likely triggering event for TdP[4,55–58]. QT prolongation alone does not necessarily lead to TdP in the absence of EADs.

Determination of the exact conditions necessary for the initiation of TdP requires sophisticated 3D computer modelling, which is not routinely available (and is not characterized in detail here; see REFS 4,59–61). Although a 3D heart model may be informative about proarrhythmic mechanisms, it is difficult to define parameters for these models from the available data. This is not the case with cellular membrane models, which can be fully parameterized from readily available data. Reconstructions of cellular proarrhythmic activity based on integrated effects on multiple cardiac currents (delayed repolarization, repolarization instability and EADs) can be assessed with precision using appropriate *in silico* reconstructions[62–64].

The discussions above highlight key aspects of our understanding of TdP, an event no longer viewed as an idiosyncratic response to a seemingly diverse array of drugs. The evolution of a defined mechanistic understanding of the initiation of TdP provides the basis for a more comprehensive mechanistic preclinical assessment of proarrhythmic risk. Such an approach is less reliant on surrogate markers (such as QT prolongation) and irrational disqualification of preclinical drug candidates based on one-dimensional assessments of delayed repolarization. With this as the background, a meeting was held at the US Food and Drug Administration (FDA) in the summer of 2013 to discuss a new paradigm for cardiac safety evaluations based on *in vitro* human models and mechanistic understandings of TdP proarrhythmia. The evolving paradigm — CiPA — represents a novel preclinical approach focused on the evaluation of TdP risk by the pharmaceutical industry, regulators and academic investigators[65,66].

The CiPA initiative

The three main preclinical elements of this proposed paradigm are: first, *in vitro* evaluation of drug effects on multiple individual currents using human ion channels in heterologous expression systems; second, integration of this information into an *in silico* computational reconstruction of a human ventricular myocyte to assess proarrhythmic liability; and third, confirmation of effects on ionic currents using *in vitro* electrophysiological responses from hSC-CMs (FIG. 4). A clinical element that includes the assessment of ECGs from Phase I studies represents a fourth component of the CiPA. This component remains an essential part of a drug's safety evaluation and functions to confirm the absence of unanticipated electrophysiological effects (for example, from a metabolite) as well as detect other forms of untoward electrophysiological effects (for example, AV-nodal blockade). The three preclinical components of the CiPA, along with the challenges associated with each and progress in their development, are discussed below.

Component 1: drug effects on human cardiac ion channels in vitro. Although there are multiple ion channels, transporters and exchangers in the human ventricular myocyte, the Ion Channel Working Group (ICWG, under the auspices of the Safety Pharmacology Society) of the CiPA initiative has proposed assessing drug effects on 7 ionic currents chosen based on their prominent role in defining ventricular repolarization and experiences from the pharmaceutical and safety pharmacology communities. These currents are: I_{CaL} (through channels formed of $Ca_v1.2$ subunits), the fast sodium current (I_{NaFast}) and I_{NaLate} (both $Na_v1.5$) for inward depolarizing currents; and I_{Kr} ($K_v11.1$), I_{Ks} ($K_v7.1$), I_{to} ($K_v4.2$) and I_{K1} ($K_{ir}2.1$, $K_{ir}2.2$, $K_{ir}2.3$ and $K_{ir}2.4$) for outward repolarizing currents. The use of automated patch-clamp platforms is proposed to characterize the effects of drugs on multiple human currents (expressed individually in heterologous expression systems, such as human embryonic kidney cells or Chinese hamster ovary cells). This strategy will allow for earlier and more-comprehensive ion channel screening across multiple currents, which is not realistically possible using manual patch-clamp techniques[67].

Challenges with component 1. Recent studies have emphasized the importance of time- and voltage-dependent drug–channel interactions to characterize drug blockade of I_{Kr} beyond simple IC_{50} values (for an example see REF. 68). Indeed, differences in channel gating between normal versus mutated hERG channels[69], as well as differences in drug-binding kinetics and mode of action (trapped or unblocked) to

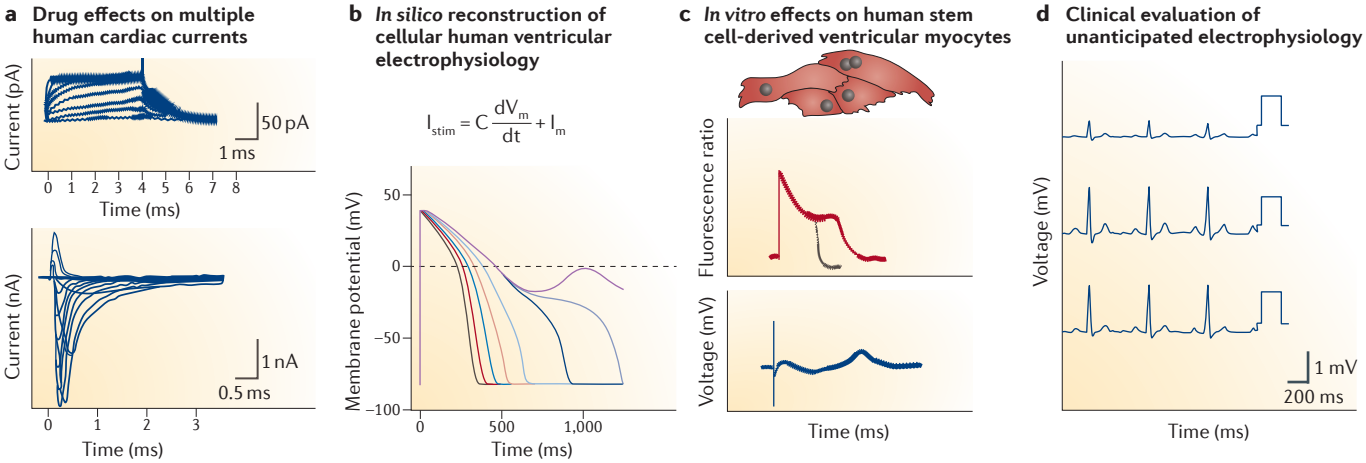

Figure 4 | **Elements of the Comprehensive *in vitro* Proarrhythmia Assay. a** | Drug effects on multiple currents provide input for *in silico* reconstructions of cellular ventricular proarrhythmia, including delayed repolarization and early after-depolarization (EAD). Two representative currents — K_v11.1 current (also known as hERG current or I_{Kr}; top panel) and Ca_v1.2 (I_{CaL}; bottom panel) — are shown. **b** | *In silico* reconstructions of ventricular myocytes based on the O'Hara-Rudy model[44] with progressive I_{Kr} blockade leading to an EAD. **c** | The effects of drugs on ionic currents are confirmed *in vitro* with human stem cell-derived ventricular cardiomyocytes, using electrophysiological recordings of field potentials from microelectrode arrays or action potential configurations from voltage-sensitive dyes. The graphs show delayed repolarization resulting from I_{Kr} blockade, which was recorded from human stem cell-derived cardiomyocytes using voltage-sensitive dyes (upper traces) and extracellular field potentials (lower traces). Note the presence of an EAD clearly identified using voltage-sensing optical (VSO) techniques. **d** | Early clinical electrocardiogram (ECG) evaluations are used to evaluate unanticipated electrophysiological effects. Figure part **a** is adapted with permission from REF. 118, American Physiological Society. The upper trace in figure part **c** is courtesy of Clyde Biosciences, UK. The lower trace in figure part **c** is courtesy of Axion Biosystems, USA.

wild-type hERG channels, have a crucial role in affecting a drugs' ability to delay repolarization[70]. Such factors need to be considered when designing standardized patch-clamp protocols that more fully characterize drug effects on currents. Such details (especially for the crucial I_{Kr}) are essential to adequately inform *in silico* reconstructions of ventricular electrical activity. Towards this goal, the ICWG (with input from the *In Silico* Working Group (ISWG)) is developing standardized voltage-clamp protocols to characterize the dynamics of I_{Kr} blockade, and they are presently testing the utility of these protocols to best parameterize the *in silico* reconstruction model. These protocols will first be tested using manual patch-clamp techniques, with data to be subsequently compared to results from automated patch platforms. Efforts are also ongoing to standardize the voltage-clamp protocols for the remaining six ionic currents.

An additional challenge for this workgroup is to define standardized experimental conditions and operating specifications for automated planar patch-clamp platforms[71,72]. Standardization is essential for benchmarking variability within and across laboratories, a key challenge for implementation of the CiPA paradigm. Best practices need to be defined that consider the potential loss of compounds studied owing to compound handling and perfusion systems[73]. The effects of temperature (room temperature versus 37°C)[74,75], buffer systems (serum versus serum-free solutions), and plasma protein binding[76] on data interpretation also need to be addressed when translating drug effects to *in silico* reconstructions. Study designs are currently being considered to address these practical aspects.

Component 2:* in silico *reconstruction of human ventricular repolarization changes. Several factors make it difficult, by inspection, to directly translate drug effects on individual cardiac currents to their effect on either delayed repolarization or a propensity for proarrhythmia. These factors are the integrated nature of repolarization, the dynamic time- and voltage-dependent interplay of drug-induced current blockade, the consequent effects on ionic gradients and the emergent nature of repolarization abnormalities such as EADs. For these reasons, *in silico* reconstructions of ventricular electrical activity are essential to understand proarrhythmic effects. Robust cellular models such as those described by ten Tusscher *et al.*[77] and O'Hara *et al.*[78] represent reasonable initial approaches, and the latter is based on extensive data derived from human ventricles. Although whole-heart models also exist and give useful insight into how arrhythmias arise[79], they are not necessary for understanding proarrhythmia on a cellular level and they impose technical challenges that are more appropriate for specialized centres of modelling excellence to tackle[80].

Challenges with component 2. The ability of *in silico* reconstructions to predict proarrhythmia relies on the validity of the human myocyte model, the quality of voltage-clamp data and appropriate definition of proarrhythmic end points[81]. Discrepancies between experimental and simulated electrophysiological responses could be attributed to parameter fitting that is not exclusively based on scarce available experimental human ventricular data. Although different models demonstrate different strengths and limitations (when compared to cellular and clinical data) and may prove instructive[82–84], one based closely on extensive human data would probably best serve the present objectives. The best cell models represent the best selection of ionic current models. Thus, it was considered reasonable to start with the collection of human currents from the O'Hara and Rudy model[78], with the possibility of systematically incorporating additional features within each ionic current to account for cell-specific electrophysiological dynamics[85] and drug effects (including use dependence). Furthermore,

although it is best to understand the limitations of each model based on the most valuable output parameters, the complexity introduced by considering multiple models probably provides little further advantage for assessing proarrhythmic risks in normal populations. In the future, the inclusion of known proarrhythmic risk factors (for example, hypokalaemia, bradycardia and irregular rhythms) shown to reduce repolarization reserve may be helpful to further refine proarrhythmic risks of vulnerable populations.

It is also essential to determine the optimum number of currents to consider, based on the roles of various currents in proarrhythmia and the off-target effects of compounds; this will affect the quality of the predictions as well as the time and cost for compound screening. Using *in silico* reconstructions of rabbit ventricular myocytes, Beattie *et al.*[86] demonstrated that blockade of I_{Kr}, I_{CaL} and I_{NaFast} provided better predictions of results obtained with the *in vitro* rabbit left ventricular wedge proarrhythmia model than did blockade of I_{Kr} alone. Reconstructions of human ventricular action potential prolongation with these three currents also provides improved prediction of the risk of TdP compared to I_{Kr} blockade alone[87], consistent with conclusions derived for predictions of TdP using these three currents and logistic regression models[27]. A sensitivity analysis of preclinical biomarkers of proarrhythmic risk to changes in the conductance and kinetics of select ionic currents that affect repolarization (I_{CaL}, I_{Kr}, I_{Ks} and I_{K1}, as well as the sodium-potassium pump current (I_{NaK}) and the sodium-calcium exchange current (I_{NaCa})) was performed using the ten Tusscher and Panfilov human ventricular model[88]. The findings demonstrated that changes in action potential duration at 90% repolarization (APD90) were particularly sensitive to the inactivation kinetics of I_{Ks} and I_{CaL} and the conductances of I_{CaL}, I_{Ks} and I_{Kr}, whereas triangulation was shown to be most dependent on I_{K1}, and to a lesser extent on I_{Kr}.

A less appreciated factor to consider is the extent to which concentration-dependent kinetics of blockade may influence proarrhythmic risk. The use of voltage-clamp protocols to characterize differences in the time-dependent blockade of I_{Kr} and the incorporation of a Markov model of hERG gating into a dog epicardial action potential model provided a better prediction of the action-potential-prolonging effects of three drugs that were assessed for concentration-dependence and modulation using pacing frequency[89]. A more detailed kinetic model of I_{NaLate} was recently shown to better define the effects of ranolazine on repolarization[90]. Ongoing studies by the ISWG under the auspices of the FDA have demonstrated improvements on current methods, which were provided by incorporating more-complex models of I_{Kr} blockade (based on Markov models) for defining cellular proarrhythmic risk, thus moving beyond traditional conductance reduction models and simpler IC_{50} values to characterize I_{Kr} blockade.

Reconstructions of human cellular ventricular repolarization delays or instability provide an integrated proarrhythmia risk assessment. This effort would include assessing the concentration–response relationship for delayed repolarization or other torsadogenic indicators (including the propensity to elicit EAD-triggered activity that gives rise to TdP[91]) in relation to clinical exposures. Recent experimental and modelling studies highlight the complex interactions that contribute to the initiation and perpetuation of EADs, and such studies emphasize the involvement of I_{CaL} reactivation and the dynamic interplay of I_{NaLate}, late potassium currents, I_{NaCa} and intracellular calcium cycling[92–94]. Additional proarrhythmic biomarkers under consideration include changes in membrane resistance, triangulation of the shape of the action potential plateau and final repolarization phases, changes in refractoriness (an integrated response reflecting the time courses of repolarization and recovery of cardiac excitability) and the dispersion of repolarization across different cell types. Beat-to-beat variability of repolarization (BVR, a recognized marker of TdP in proarrhythmia models) could also be considered as a proarrhythmic marker when using stochastic models of ion channels and transporters[95] (rather than non-deterministic models cited above), as could heterogeneity of delayed repolarization (a recognized substrate for TdP proarrhythmia) across epicardial, midmyocardial and endocardial ventricular layers that define repolarization gradients (and the QT interval).

It will be necessary to calibrate the *in silico* model for sensitivity using appropriate positive controls based on clinical experience[96,87]. Within the CiPA, the Clinical Translation Working Group, under the auspices of the Cardiac Safety Research Consortium, has selected a set of 28 drugs ranked as high, intermediate or low proarrhythmic risk based on clinical experiences (TABLE 1). As an initial test of the *in silico* model, the ISWG plans on evaluating the effects of a subset of 12 compounds (representing high-, intermediate- and low-risk categories) using ionic current data generated by the ICWG using overexpressing heterologous expression systems.

Component 3: confirmation using hSC-CMs. Under ideal circumstances, the *in vitro* assessment of a drug's proarrhythmic risk would use adult human ventricular tissue or myocytes. However, such preparations are not readily available for the routine evaluation of even small numbers of drug candidates. Fortunately, hSC-CMs hold great promise in regards to preclinical cardiac safety (as well as efficacy) testing[97,98–101].

Table 1 | **Set of 28 drugs ranked according to high, intermediate and low proarrhythmic risk**

CiPA high risk	Redfern category*	CiPA intermediate risk	Redfern category	CiPA low or no risk	Redfern category
Azimilide	1	Astemizole	2	Diltiazem	5
Bepridil	3	Chlorpromazine	–	Loratadine	5
Dofetilide	1	Cisapride	2	Metoprolol	–
Ibutilide	1	Clarithromycin	4	Mexiletine	–
Quinidine	1	Clozapine	–	Nifedipine	4
Vandetanib	–	Domperidone	4	Nitrendipine	5
Methadone	–	Droperidol	–	Ranolazine	–
D,L-sotalol	1	Terfenadine	2	Tamoxifen	5
		Pimozide	3	Verapamil	5
		Risperidone	5		
		Ondansetron	–		

CiPA, Comprehensive *in vitro* Proarrhythmia Assay. *See REF. 41 for details.

The present role of hSC-CMs in the CiPA is to confirm drug effects identified in ionic current studies. Any additional, novel electrophysiological effects not anticipated from simpler isolated current measures with heterologous expression systems can be readily detected using hSC-CMs. Such drug effects could include effects mediated by cellular metabolism, oxidative stress or altered calcium-handling[8,102–104]. Additional examples could include modulation of I_{Kr} through intracellular second messengers[105–107] and augmentation of I_{NaLate} through the phosphoinositide 3-kinase pathway[108,109]. Discrepancies found between ionic current studies (or *in silico* reconstructions) and hSC-CM effects would subsequently be investigated to improve future risk assessments. Effects of longer-term, chronic drug exposure on hSC-CMs — related to channel expression, metabolic modulation or structural changes (for example, owing to off-target effects on kinases) — can also be detected using hSC-CM studies.

Various approaches have been used to evaluate electrophysiological markers of proarrhythmia with hSC-CMs. These approaches include transmembrane action potentials, microelectrode array (MEA) recordings, impedance measurements, calcium transients and voltage-sensitive dyes[110,111] (FIG. 5). Although all these approaches reflect aspects of integrated cellular responses, those describing the effects of transmembrane potentials are most easily comparable with *in silico* reconstructions of transmembrane electrical activity and ECG effects. Typical end points for MEA studies include field potential duration and incidence of EADs (direct measures of altered repolarization), beat frequency (known to modulate repolarization independently of effects on repolarizing currents), amplitude of initial depolarizing spike (a measure of sodium current) and conduction patterns (a general measure of experimental conditions). Analogous measures are provided by voltage-sensing optical (VSO) approaches, which show changes in the duration as well as the time course of repolarization, thus providing measures of action potential triangulation (a linearization of repolarization that is recognized as a hallmark of proarrhythmia). Although it is possible to measure action potentials (and isolated currents) directly from hSC-CMs[112], such efforts are presently technically difficult using automated patch-clamp platforms and are not readily available for widespread use. Furthermore, most currents of particular interest (such as I_{Kr}) are quite small and difficult to measure from hSC-CMs (as is the case with human adult ventricular myocytes).

Experimental work continues to define the roles, variability of responses and utility of hSC-CMs as a readout of integrated cellular electrophysiological responses. In late 2014, the CiPA Myocyte working group (MWG, under the auspices of the Health and Environmental Sciences institute (HESI)) conducted a pilot study across 12 volunteer sites to evaluate and compare the electrophysiological effects of eight blinded compounds on repolarization. This study tested the effects of compounds on four different hSC-CM types using MEA and VSO approaches (voltage-sensing dyes and integrated voltage-sensing membrane proteins). Although analysis is ongoing, preliminary results suggest reasonable concordance of changes in repolarization among the hSC-CM types (attesting to the reproducibility of MEA and VSO assessments), with some differences in sensitivity noted across myocyte preparations. Any differences in myocyte sensitivities can easily be calibrated and controlled by the concomitant evaluation of known standards run alongside test compounds on each plate, taking advantage of the higher-throughput multi-well formats of MEA or VSO approaches to test for a consensus of positive controls with each assay.

Parallel to the work described above, a Japanese consortium recently reported on a prospective comparison study using the MEA approach, a hSC-CM line and the I_{Kr} blocking agent E-4031 across three 'trained' independent Japanese research laboratories (REF. 113). Their results demonstrated minimal inter-facility differences in the ability to detect delayed repolarization. Recently expanded as the Japan iPS Cardiac Safety Assessment (JiCSA), this consortium is presently conducting a multi-site study comparing the electrophysiological effects of 60 drugs on hSC-CMs using the MEA platform. Recognizing their common goals, the HESI MWG and the JiCSA have recently formed a joint group to share results and plan future complementary hSC-CM studies in support of the CiPA initiative.

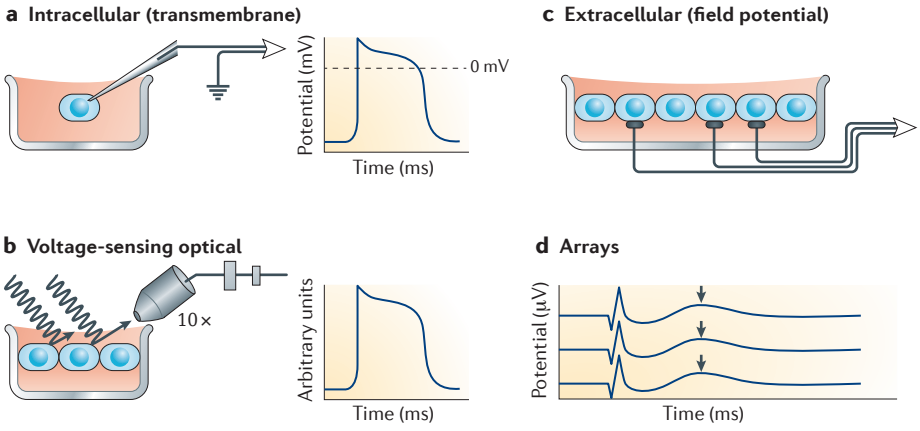

Figure 5 | **Electrophysiological approaches for evaluating proarrhythmia with human stem cell-derived cardiomyocytes. a** | Intracellular transmembrane potentials that are recorded using microelectrodes provide the most detailed characterization of electrophysiological effects on cellular repolarization. Such studies are slow, technically demanding and not easily adapted to higher-throughput techniques. **b** | Voltage-sensitive dyes provide optical signals of transmembrane potential based on changes in fluorescence[170,171]. Although the absolute membrane potential is not directly measured, changes in the action potential duration and the configuration of repolarization (including triangulation of the plateau, a recognized risk factor for early after-depolarizations (EADs)) are readily obtained from multiple myocytes with minimal cellular disruption and reasonable throughput. **c** | Microelectrode array (MEA) recordings provide localized extracellular field potentials from electrodes located on the chamber floor. **d** | Changes in the field potential duration (FPD) reflecting changes in cellular repolarization are most reliably measured from the peak of the repolarization wave (indicated by arrows, just before full repolarization)[172,173] and are easily translated to clinical repolarization effects. Repolarization instability and EAD activity can also be recorded with this technique.

Challenges for component 3. As with adult ventricular myocytes, it is recognized that repolarization of hSC-CMs is affected by heart rate, and that drug effects on spontaneous rhythm (rate or rhythmicity) may confound the interpretation of FPD changes. Electrical or optogenetic pacing of

hSC-CMs at slow rates is not feasible, with some preparations displaying rapid intrinsic beating, which also limits the ability to characterize reverse use-dependent effects on repolarization (the greater prolongation of repolarization at slower (rather than rapid) stimulation rates, a recognized hallmark of proarrhythmic risk linked to EAD generation). Further efforts to develop platforms that include optional pacing protocols and myocytes with minimal intrinsic beat frequencies should alleviate this limitation.

Commercially available hSC-CM preparations represent a mix of atrial, nodal and ventricular cells (the latter being the predominant phenotype from most commercial cell sources). It will be challenging to standardize protocols across all hSC-CMs for either present or future preparations given the differences in the distribution of electrophysiological phenotypes in cells from different vendors; different plating, maturation and experimental conditions suggested for different cell sources; and the different requirements imposed by the different electrophysiological approaches. Thus, it is more amenable to consider standardizing assay calibration efforts to confirm their sensitivity and the functional roles of key ion channels (for example, those carrying I_{Kr}, I_{CaL}, I_{Ks} and I_{NaLate}). This can be accomplished by defining concentration–response curves for specific current blockers on each high-throughput assay plate. Such data will define assay sensitivity (providing a basis for comparing drug effects across multiple cell types, laboratories and platforms) and provide a benchmark for future myocyte preparations (for example, 3D structures or myocyte–fibroblast co-cultures).

No standardized guidelines currently exist for the evaluation of hSC-CM phenotypes or functionality that may affect drug responses[114]. Recent papers have proposed a multi-parametric quality-assessment rubric to assess the ventricular myocyte phenotype[115–117]. Although too extensive for routine measurements (including genetic, structural, electrophysiological and contractile measurements), this does provide a guide for general further considerations.

Although most currents found in adult ventricular myocytes are also present in hSC-CMs[118,119], the electrophysiological phenotype of hSC-CMs is generally characterized as being immature, as reflected in a less-negative resting membrane potential, reduced upstroke velocities and spontaneous automaticity[120,121]. This electrophysiological phenotype is due (at least in part) to reduced I_{K1} density (the current responsible for the resting membrane potential of adult myocytes) as well as greater density of pacemaker current I_F. Despite these limitations, hSC-CMs consistently demonstrate delayed repolarization when challenged with QT-prolonging drugs[122–126]. For example, Harris and colleagues[125] demonstrated relevant pharmacological responses and excellent correlations to functional cardiac electrophysiological studies based on ten compounds evaluated using hSC-CMs and an MEA platform. A subsequent study correctly identified more than 20 drugs with single and multiple ion channel pharmacology[127].

Although single MEA parameters (such as field potential duration) may be more easily compared with more standard preclinical models (for example, QT prolongation), a multi-parametric approach considering complex patterns of responses to drugs may prove valuable to realize the full breadth of cardiotoxicity. Techniques such as hierarchical clustering and parallel coordinate data visualization (as used in microarray gene expression) have been applied to inform on mechanisms and rank ordering of novel compounds with known clinical risk.

Recent ongoing efforts to direct maturation of hSC-CMs involve electrical stimulation, co-culture with fibroblasts, and 3D tissue engineering[128,129]. A recent study demonstrated that prolonged periods of culture (up to 120 days) of hSC-CMs derived from either embryonic or induced stem cells resulted in the maturation of their structural and contractile properties towards a more adult-like phenotype[130], with increased cell size and anisotropy, greater myofibril density and alignment, an increase in multi-nucleated cardiomyocytes, doubling in shortening magnitude, hyperpolarized diastolic potentials, faster upstroke velocities and reduced beat rate. With important advances towards the faithful recapitulation of a mature electrophysiological (and contractile) phenotype[131], we anticipate that hSC-CMs will have a more prominent role in the direct evaluation of proarrhythmic risk, with ionic current and reconstruction studies subsequently relegated to clarifying and confirming myocyte study results.

As stable hSC-CMs can be maintained in culture, it is possible to evaluate the longer-term electrophysiological effects of compounds that require hours to days of exposure. A classic example would be the ability of drugs to reduce I_{Kr} by reducing *KCNH2* transcription or translation of *KCNH2* mRNA (a process that is termed trafficking, an example of which is provided by the antiprotozoal drug pentamidine)[132,133]. Longer-term studies also provide the opportunity for the *in vitro* evaluation of chronic drug effects. This may be particularly important with oncological therapeutics, for which debilitating cardiotoxic effects often occur later in the course of therapy (and may be detected only after expensive and longer-term non-clinical toxicology studies with non-human models). With hSC-CMs, it is also be possible to assess safety (and efficacy) end points specific to monogenic cardiac diseases that are related to cardiac rhythm abnormalities (such as congenital long QT syndromes) using patient-derived cardiomyocytes[134,135].

Additional practical considerations regarding the CiPA. The CiPA represents an evolving proposal that will substantially affect the workflow for definitive preclinical proarrhythmia assessment. At its simplest levels, more-comprehensive channel-screening efforts could be implemented early in drug discovery using automated planar patch-clamp techniques, with the resulting data used to feed early *in silico* reconstructions (the latter possibly at centralized facilities or cloud based, providing standardized software and results). It is encouraging that elements of the CiPA are already being used in early drug discovery (for example, increasing the number of ionic currents being interrogated and screening for integrated responses with human-derived cardiomyocytes) as well as guiding early medicinal chemistry efforts and refining early hazard identification. At a minimum, such efforts should reduce the automatic exclusion of I_{Kr} -blocking drugs from early drug discovery. It is anticipated that the commercial costs for such services would be substantially reduced as these efforts are implemented.

It is essential to correctly identify clinically positive and negative torsadogenic compounds to include in validation and test sets; within the CiPA, this task was undertaken by a group of academics, clinicians and regulators who are familiar with clinical QT studies, mechanisms of proarrhythmia, drug safety assessments and regulatory submissions. Under the auspices of the Cardiac Safety Research Consortium, the Clinical Translation Working Group has selected 28 compounds for evaluation

in the CiPA based on the absence of active metabolites, good solubility and defined cellular electrophysiological effects. Based on clinically demonstrated torsadogenic risk, proarrhythmic incidence, published reports, the FDA Adverse Event Reporting System (AERS) database and expert opinion, these compounds were ranked into three categories of TdP risk: high, intermediate and low or no risk. An initial set of 12 compounds, divided equally between the three proarrhythmia risk categories, will be used as a test set for the initial *in silico* reconstructions and hSC-CM calibration studies. With the expectation of reasonable concordance established between the preclinical and clinical rankings, the remaining drugs will be prospectively tested as a validation exercise.

In mid-2015, official discussions commenced with international regulatory authorities regarding expectations of the CiPA initiative, its role in future regulatory guidelines and validation efforts. Such dialogue is essential to support the replacement of costly TQT studies, as drugs demonstrating low proarrhythmic risk based on the mechanistic-based CiPA approach, despite some degree of QTc prolongation, could proceed with uncomplicated clinical monitoring for QT prolongation during development. Although timelines for regulatory implementation of the CiPA depend on such discussions with regulators, a review of the progress made by the various workgroups is planned for early 2016. Key differences between the present ICH S7B and E14 approaches and the evolving CiPA paradigm are depicted in TABLE 2.

Contractile and structural cardiotoxicity

Effects on cellular cardiac contractility: potential role of hSC-CMs. Drug effects on cardiac contractility *in vivo* (defined most broadly as the ability to maintain appropriate cardiac output given cardiovascular haemodynamics) represent the complex interplay of direct drug effects on the myocyte as well as extrinsic factors (for example, resulting from changes in heart rate, preload, afterload and autonomic tone). However, such extrinsic effects do not negate the importance of the ability to directly influence sarcomere shortening or relaxation in response to varying calcium levels, as modulated by multiple interrelated signalling cascades. The ability to detect potential inotropic liabilities at an early stage is useful in guiding subsequent *in vivo* preclinical investigations, compound selection and first-in-human studies.

Differences in cardiac contractility between species are well known and would be expected to support the wide range of heart rates and haemodynamic requirements that occur across species. For example, whereas calcium reuptake by the sarcoplasmic reticulum calcium pump is largely responsible for relaxation in the adult mouse myocyte, this mechanism accounts for approximately 65% of calcium reuptake in normal human adult myocytes (with the remainder accounted for by sodium-calcium exchange and the sarcolemmal calcium pump[136–138]). Given such differences between species, human-derived cardiomyocytes have a potential advantage over myocytes from other species in assessing drug effects on contractility (assessed based on the ability of the drug to shorten or generate tension) at the cellular level for acute or longer-term studies.

Owing to the complexity involved in producing cardiac contractions, it is desirable to evaluate a drug's inotropic and lusitropic effects with phenotypic assays. Although it may be possible to assess the effects of drugs on some specific regulatory mechanisms that are involved in contraction or relaxation (for example, calcium-induced calcium release, or calcium reuptake into the sarcoplasmic reticulum), such studies do not provide an integrated perspective of a drug's overall effect on inotropy (just as evaluating blockade of I_{Kr} fails to provide a comprehensive proarrhythmic risk assessment). In contrast to the analogy with I_{Kr} and proarrhythmia, no single predominant cellular mechanism is recognized as affecting cardiac contractile performance.

A detailed review is outside the scope of this article, but numerous reports indicate the relative heterogeneity and immaturity of the contractile machinery of some hSC-CMs compared to adult ventricular myocytes — with myofibrillar disorganization, lack of T tubules and heterogenous distribution of ryanodine receptors[139–141], reduced twitch amplitude compared to native tissues[142], and leaky sarcoplasmic reticulum without a mature terminating mechanism for the calcium transient[131,143–147]. Although adult ventricular myocytes demonstrate a positive force–frequency relationship (increasing force with faster stimulation rates) and post-rest potentiation of contraction, some hSC-CMs demonstrate

Table 2 | **Key differences between the ICH S7B and E14 approaches and the evolving CiPA paradigm**

	ICH S7B	ICH E14	CiPA
Goals	Non-clinical studies of the potential of compounds to delay ventricular repolarization	Clinical studies of the potential of compounds to delay ventricular repolarization	Non-clinical mechanistic studies to determine the proarrhythmic risk of compounds, the results of which are confirmed with clinical studies
Focus	I_{Kr} and *in vivo* QTc; supplemental studies possible	QTc prolongation and clinical TQT studies	Multiple human ionic currents informing *in silico* reconstructions of human myocyte electrophysiology to determine TdP risk: electrophysiological confirmation from human stem cell-derived cardiomyocytes and clinical ECGs
Evidence of risk	Risk assessment of delayed repolarization or QT interval prolongation translated from models to humans	QTc prolongation: studies give a dichotomous output based on exclusion of a 10-millisecond clinical effect	Proarrhythmic risk: studies provide a mechanism-based assessment calibrated against clinical comparators ranked according to a consensus of clinical experience
Standardization	None	Moxifloxacin as a positive control arm	Standardized ion channel protocols; a moderated, publicly available *in silico* model; a standardized, calibrated core myocyte protocol and a standardized Phase I ECG protocol
Validation	None	None	Planned, but fundamentally limited by the inability to categorize known drugs for TdP risk independent of the context of their use

CiPA, Comprehensive *in vitro* Proarrhythmia Assay; ECG, electrocardiogram; ICH, International Conference on Harmonization; TdP, torsades de pointes; TQT, thorough QT, QTc, QT interval corrected for changes in heart rate.

a negative force–frequency relationship and absent or weak post-rest potentiation[148,149]. Encouragingly, more-mature calcium handling could be induced in hSC-CMs by culturing them for a longer time[130], using electrical stimulation and/or 3D cultures[150], or patterning them onto microgrooved culture substrates[151], providing promise for near-term enhancements. As discussed with respect to electrophysiological characteristics, it is expected that evolving engineered hSC-CM preparations[152] will better recapitulate mature inotropic mechanisms, thus allowing for a more robust evaluation of direct inotropic effects of drugs in the future.

Some studies have demonstrated the utility of hSC-CMs for detecting positive (for example, isoprenaline) and negative inotropic agents[116], whereas others have demonstrated the immaturity of the sarcoplasmic reticulum and β-adrenergic response[153,154]. Further work is necessary to evaluate the baseline characteristics of the effects of drugs on contractility, their variability across hSC-CM preparations and their reproducibility, in order to define the utility of hSC-CMs for detecting positive and negative inotropic effects consistent (in direction and amplitude) with clinical responses.

Drug effects on structural cardiotoxicity: potential role of hSC-CMs. The growing list of cardiac toxicities associated with established anticancer drugs (for example, anthracyclines and trastuzumab) and more-recently introduced classes of tyrosine kinase inhibitors poses a challenge for the development of novel anticancer agents[10,155,156], and these toxicities could also be of concern in other therapeutic areas. In the past, the ability to detect structural cardiotoxicity (defined here as morphological damage or loss of viability) using *in vitro* approaches has been hampered by the inability of non-human cardiac models to recapitulate the mechanisms involved in human toxicity, the stability of *in vitro* models to evaluate longer-term effects (measured in days rather than hours) and the inability to simultaneously assess a broad range of toxicity end points (thus providing a more comprehensive assessment).

The advent of high-content screening (merging automated, high-throughput screening techniques with cellular image-based microscopic assessments involving spatial, temporal and spectral resolution with multiple probes) has enabled the multi-parametric, quantitative assessment of cellular toxicities for cardiac as well as other tissues[157–160]. Studies using hSC-CMs and high-content screening provide the ability to detect cardiotoxicity early in drug discovery, before using traditional, histologically based (and costly) assessments. These later studies require substantially larger compound amounts, larger animals and extended dosing periods (days to weeks), and such studies are not readily amenable to early compound-selection efforts.

A recent example of using high-content screening to assess structural cardiotoxicity in hSC-CMs compared responses between rat myoblastic H9c2 cells (derived from embryonic rat hearts) and embryonic hSC-CMs to clinical structural cardiotoxicants[161]. Cellular response profiles characterizing toxicity following 3-day exposures were evaluated using multiple end points (including ATP depletion to assess cell viability, live-cell fluorescent imaging of mitochondrial membrane potential, endoplasmic reticulum integrity, calcium mobilization and membrane permeability). Mitochondrial and calcium mobilization were the predominant effects for detection of structural cardiotoxicity. Embryonic hSC-CMs offered somewhat improved detection of structural toxicity, detecting 25 of the 34 structural cardiotoxicants at or below C_{max} concentrations compared with 18 compounds in H9c2 cells. In another example, Mioulane and colleagues[162] described a high-content imaging approach to detect cardiomyocyte apoptosis and necrosis using an automated microscopy platform based on monitoring nuclear remodelling, mitochondrial status and membrane potential. They noted that the susceptibility of hSC-CMs to chelerythrine-stimulated apoptosis varied with time after differentiation, but that hSC-CMs were generally more resistant to apoptosis than rat neonatal ventricular myocytes. The authors further commented that such studies, although not fully able to replace *in vivo* studies, would allow for a more targeted assessment of human-based cardiotoxicity (despite challenges posed by the immature characteristics of hSC-CMs) in early drug discovery, with confidence increasing as their predictive value was confirmed.

Several multi-parametric imaging studies have investigated the effects of tyrosine kinase inhibitors on hSC-CMs. Doherty *et al.*[163] found that three tyrosine kinase inhibitors linked with clinical cardiotoxicity — crizotinib, sunitinib and nilotinib — showed unique toxicity profiles, reflecting multiple off-target mechanisms (including increased ROS production, caspase activation, cholesterol accumulation and decreased myocyte viability) that may contribute to overall cardiotoxicity. By contrast, minor changes were observed with erlotinib, an oncology drug not associated with cardiotoxicity. Another imaging study, which examined the relationship between hESC-CM bioenergetics and the toxicity of tyrosine kinase inhibitors[164], revealed sensitization to mitochondrial toxicity in cells utilizing oxidative phosphorylation compared to cells relying on glycolysis for ATP production. This sensitization was exaggerated with the HER2 kinase inhibitor mubritinib, which was non-toxic in glycolytic cells but affected mitochondrial integrity and toxicity in myocytes with altered bioenergetics. Complementary high-content profiling and hierarchical clustering of cell-surface nutrient transporter expression data indicated the involvement of bioenergetic modulation of kinase inhibitor toxicity in the altered myocytes. These results demonstrate the importance of considering metabolic conditions when designing and translating *in vitro* studies, as well as the utility of multi-parametric analysis techniques.

Collectively, the studies cited above demonstrate the utility of hSC-CMs and image-based high-content screening to distinguish subtle mechanistic differences among drugs based on drug- and class-specific patterns of responses. The wealth of data generated from such studies poses challenges for analysis and storage. A recent review of high-content screening studies revealed that the majority of high-content screens use only a few image-based features (for example, disregarding the distribution of features among each cell population). This oversimplification of informational content was posited to result from more-traditional data analysis processes; problems associated with larger, multi-parametric data sets; and a lack of familiarity of assay statistics[165]. Defining cardiotoxicity-based high-content screening is somewhat analogous to defining proarrhythmia based on effects on multiple cardiac currents. In the electrophysiological example, a single ionic current (I_{Kr}) is now recognized as an oversimplified representation of proarrhythmia, and drug effects on multiple human channels in the CiPA paradigm provide multi-parametric data for analysis, which lead to integrated electrophysiological responses and better predictivity guided by *in silico* reconstructions. In the cardiotoxicity example, the need exists to more fully utilize the multi-parametric data generated from high-content screens, using more-comprehensive integrated assessments (guided, at present, by phenotypic responses)

that will lead to integrated cellular responses and better predictivity. Although both examples should benefit from the use of human cells and tissues, the major distinction between these two examples is the lesser understanding of inputs into the more complex interrelated mechanisms responsible for structural and contractile cardiotoxicity.

Assay validation: overall considerations

In general, when mechanistic insights into safety liabilities are lacking, assays are developed that rely on complex interactions to probe adverse effects (for example, heart failure). With such assays, validation consists of testing the effects of numerous compounds and defining the relationship between the preclinical effects or biomarkers and the clinical observation (for example, a study ranking the proarrhythmic effects of 55 drugs on the spontaneous beat rate of hSC-CMs[166]). Using such an approach, one might expect diminished sensitivity or specificity with compounds different to those within the validation dataset. Importantly, with an understanding of the response profiles of mechanisms associated with safety liabilities, a larger validation dataset is not needed. Instead, the larger dataset is supplanted by demonstration of the presence (and sensitivity) of the mechanism and an understanding of assay results and limitations in the larger context of the biological (or pathological) system.

Conclusions

We are in the midst of an evolving paradigm shift for preclinical cardiac safety assessments. This shift is founded on a growing knowledge of the basic cellular mechanisms underlying electrophysiological, contractile and structural cardiac toxicity; the availability of human channels and hSC-CMs as the most relevant *in vitro* test systems; the availability of automated, high-throughput and high-content screening platforms for evaluating electrophysiological and multi-parametric subcellular and cellular responses; and *in silico* models that adequately describe and integrate complex cellular electrophysiological responses. These mechanistic-based approaches could replace the more traditional 'black box' approaches that rely on characterization of the effects of a larger number of compounds on poorly characterized animal models to define assay performance.

Recent opinions regarding declining pharmaceutical productivity have highlighted the potential contribution of excessive focus on target-centric approaches to the observed decline, and, conversely, the potential for phenotypic screening strategies to help to reverse it[167–169]. Similarly, we argue that focused, 'target-based', reductionist approaches for early cardiac safety screening (for example, studying hERG alone for TdP proarrhythmia) hinder drug discovery efforts and result in the premature termination of drug candidates, and we also assert that a mechanistic-based systems approach using human-derived preparations provides a more comprehensive and specific evaluation of cardiac proarrhythmia liabilities. Although structural cardiotoxicities are less well understood, multi-parametric phenotypic patterns (or signatures) obtained with human-derived cardiomyocytes will provide valuable insights into cardiotoxic liabilities, leading to a better understanding of novel mechanistic pathways to be avoided in early drug discovery.

Despite the many practical challenges remaining for the human-based cellular assessment of cardiotoxicity, continuing to rely on more-precise measures of imperfect *in vitro* surrogate markers and simpler (and poorly understood) metrics of cardiotoxicity with non-human tissues will not lead to more efficient development of safer drug candidates. The proposed human-based integrated *in vitro* approaches discussed herein will guide and complement preclinical *in vivo* cardiac safety (including traditional telemetry ECG evaluations) and toxicity studies, and these approaches provide a model for future efforts aimed at assessing toxicities in other organs. The growing use (and refinement) of hSC-CMs will provide a unifying bridge for the efficient evaluation of proarrhythmic, contractile and structural cardiotoxicity. Lessons learned from years of efforts avoiding proarrhythmia linked to delayed repolarization (once considered an idiosyncratic response) at all costs have provided a new paradigm for rational and more efficient *in vitro* preclinical safety assessments.

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Competing interests statement
The authors declare competing interests: see Web version for details.