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Abstract

Introduction: The Comprehensive *in vitro* Proarrhythmia Assay (C*i*PA) is a nonclinical Safety Pharmacology paradigm for discovering electrophysiological mechanisms that are likely to confer proarrhythmic liability to drug candidates intended for human use.

Topics covered: Key talks delivered at the 'C*i*PA on my mind' session, held during the 2015 Annual Meeting of the Safety Pharmacology Society (SPS), are summarized. Issues and potential solutions relating to crucial constituents [e.g., biological materials (ion channels and pluripotent stem cell-derived cardiomyocytes), study platforms, drug solutions, data analysis, etc.] of C*i*PA core assays are critically examined.

Discussion: In order to advance the C*i*PA paradigm from the current testing and validation stages to a research and regulatory drug development strategy, systematic guidance by C*i*PA stakeholders is necessary to expedite solutions to pending and newly arising issues. Once a study protocol is proved to yield robust and reproducible results within and across laboratories, it can be implemented as qualified regulatory procedure.

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Keywords

Comprehensive *in vitro* proarrhythmia assay (C*i*PA)

Field potential data sampling and analysis

Induced pluripotent cell cardiomyocytes (*h*iPSC-CMs)

Multi electrode array (MEA)

Patch clamp technologies

Pending issues and solution

Validation

Voltage sensitive dye (VSD)

Abbreviations: AP, action potential; ADP_{90}, action potential duration at 90% repolarization; Ca_V, voltage dependent calcium channel; CDI, Cellular Dynamics International; C*i*PA, comprehensive *in vitro* proarrhythmia assay; CROs, contract research organizations; CSA, consortium for safety assessment; DMSO: dimethyl sulfoxide; FP, field potential; FPD, field potential duration; FPDcB, FPD corrected according to the Bazett formula; FPDcF, FPD corrected according to the Fridericia formula; GEVI: genetically-encoded voltage indicators; GLP: good laboratory practices; HESI, Health and Environmental Sciences Institute; HPF, high pass filter; *h*iPSC-CMs, human induced pluripotent stem cell cardiomyocytes; HTS, high throughput screening; ICH, International Conference on Harmonization; I_{CaL}, L-type (long-lasting voltage-gated) depolarizing calcium current; I_{Kr}, rapidly activating delayed rectifier potassium current; I_{hERG}, human ether-a-go-go-related gene potassium current; I_{Ks}, slowly activating delayed rectifier potassium current; I_{K1}, inward rectifier potassium current; I_{to}, transient outward potassium current; I_{NaFast}, fast depolarizing sodium current; I_{NaLate}, late depolarizing sodium current; ICWG, ion channel working group; ILSI, International Life Sciences Institute; *hiPSC*-CMs,: human induced pluripotent stem cell derived cardiomyocytes; ISI, interspike interval; ISWG, *in silico* working group; LQTS, long QT syndrome; JiCSA, Japan iPS Cardiac Safety Assessment consortium; JSPS, Japan Safety Pharmacology Society; LQT, long QT interval syndrome; LPF, low pass filter; MEA, multi-electrode arrays; MWG, myocyte working group; Na_V, voltage dependent sodium channel QMS, Quality Management System; QTc, QT interval corrected according to the Fridericia algorithm; SD: Standard deviation; SEM: standard error of the mean; SPS, Safety Pharmacology Society; TdP, Torsade de Pointes arrhythmia; TQT, thorough QT study, V_{max}, maximal velocity of depolarization; VSD, Voltage-sensitive dyes; VSO, Voltage-sensitive optical devices; WG, working group.

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1. Introduction

C*i*PA is a novel Safety Pharmacology paradigm undergoing systematic evaluation for fitness to discover candidate drugs with the potential to trigger ventricular arrhythmic events in humans. If these evaluation efforts succeed, C*i*PA will become a Safety Pharmacology screening tool for drug research and development purposes (Cavero & Holzgrefe, 2015).

The C*i*PA paradigm has been designed to provide an accurate and comprehensive assessment of the cardiac ventricular electrophysiological properties of candidate drugs for identifying mechanisms that may mediate life-threatening ventricular proarrhythmic events. This preclinical approach can be considered as an extension of the currently applied ICH S7B guideline strategy (ICH, 2005a) which is designed to detect whether a candidate drug adversely affects the physiological function of the cardiac channel encoded by the ether-à-go-go related gene (*h*ERG) which conducts the delayed rectifier K^+ current (I_{Kr}). This concern arises primarily from clinical findings that drug-induced *h*ERG inhibition can provoke a particular type of polymorphic ventricular arrhythmia called torsade de pointes (TdP). This tachyarrhythmia, at times, culminates in irreversible ventricular fibrillation. However, within the large number of drugs demonstrating potent I_{Kr} channel blocking activity in the *in vitro* patch clamp assay, some are free of proarrhythmic effects in integrated nonclinical assays, as well as in man (Kannankeril, Roden, & Darbar, 2010; Vandenberg, Perry, Perrin, Mann, Ke, & Hill, 2012).

C*i*PA is an initiative sponsored by a multi-partner international consortium which includes the FDA, HESI, CSRC, SPS, Japan National Institute of Health Sciences, Health Canada, European Medicines Agency, Pharmaceutical and Japan Medical Devices Agency, Japan iPS Cardiac Safety Assessment Group, academic electrophysiologists, *in silico* modelers, pharmaceutical industry associations, contract research organizations (CROs), stem cell manufacturers, and companies producing hardware and software for Safety Pharmacology research.

The C*i*PA core components are:

1) *In vitro* patch clamp assays in stably expressed recombinant human ion channels. The aim of these studies is to evaluate the effects of candidate drugs on key depolarizing and repolarizing ion currents participating in the formation of human ventricular action potential (AP).
2) An *in silico* AP assay which is performed to verify whether the results obtained in the previous investigations engender either phenotypic indicators signaling proarrhythmic liability on the human ventricular AP [e.g., prolongation of AP duration (e.g;, ADP_{90}), EADs (Gintant, 2008)] evidenced on the AP profile generated by a mathematical model of the human ventricular myocyte AP (O'Hara, Virag, Varro, & Rudy, 2011) or mechanism-related proarrhythmic metrics (section 2.3) allowing the latter model to classify each drug candidate as no/low, intermediate or high proarrhythmic risk agents.
3) An *in vitro* assay designed to investigate the electrophysiological effects of candidate drugs in ventricular cardiomyocytes derived from human induced pluripotent stem cells (*h*iPSC-CMs). The aim is to confirm or cast doubt on the *in silico* predictions and to broaden the cardiac safety

assessment of the candidate drug to include additional proarrhythmic mechanisms not discoverable by the ion channel assay investigation or *in silico* analysis.

The CiPA initiative encompasses also the E14 guideline (ICH, 2005b) since it has the potential to de-emphasize the clinical thorough QT/QTc study (TQT) and replace it with an intensive Phase ECG investigation (Cavero, Holzgrefe, & Clements, 2016).

The first part of this article provides extended summaries of key presentations on the C*i*PA paradigm given at the 'C*i*PA on my mind session' which was part of the scientific program of the 15th Annual Meeting of the Safety Pharmacology Society (SPS) held in Prague in October 2015.

The second part of this report threads through issues concerning biological material, experimental platforms, measured parameters, and data analysis approaches that need to be resolved to qualify each C*i*PA core assay for Safety Pharmacology research and regulatory purposes. While available solutions for these issues are mentioned in this report, it will be the role of the C*i*PA Steering Committee and the various C*i*PA Working Groups and Teams to recommend the best possible solutions for bench stakeholders.

2. Key presentations from 'C*i*PA on your mind' session held at the Annual Meeting of the Safety Pharmacological Society

2.1. CiPA introduction. Ongoing activities (CiPA Steering Team) and updates. Dr. G. Gintant, Abbvie,North Chicago, IL, USA

The 2015 C*i*PA *h*iPSC-CM pilot study program was designed to test whether a set of reference drugs yielded reproducible effects on field potentials (FP) measured with multi-electrode arrays (MEA) and action potentials (AP) obtained by using voltage-sensitive optical dye (VSD) (Cavero & Holzgrefe, 2014). The knowledge acquired from these investigations will provide indications for optimizing experimental protocols with regard to the selection of the most informative parameters to measure, sampling times during the execution of a protocol, analytical approaches, and data reporting formats.

In September 2015, the FDA Cardiac Safety Committee (CSC), through an FDA Broad Agency Announcement (BAA) awarded a grant to HESI for 'Validating human stem cell cardiomyocyte technology for better predictive assessment of drug-induced cardiac toxicity' (HESI, 2014). This endowment will be used to finance studies to characterize the electrophysiological properties of *h*iPSC-CMs in studies using MEA and VSD platforms.

HESI funding will also support a pilot Phase I study (to be completed by the end of 2016) of 12 reference drugs selected by the C*i*PA Clinical Subteam) which includes compounds with no/low (diltiazem, mexiletine, ranolazine and verapamil), intermediate (chlorpromazine, cisapride, terfenadine, and ondansetron), and high proarrhythmic risk (quinidine, bepridil, dofetilide, and sotalol). These drugs were selected from a larger set of 28 drugs [Table 1 in (Gintant, Sager, & Stockbridge, 2016)] which the C*i*PA Clinical Translation Working Group proposed for achieving the validation of the components of the C*i*PA paradigm (Cavero & Holzgrefe, 2015). The drugs within

this set have different physico-chemical properties and cover a wide spectrum of electrophysiological endpoints (in particular, the degree of torsadogenic risk, multichannel blockade, and variable degrees of *h*ERG channel blockade).

The results obtained from ongoing studies will be used to test whether the C*i*PA *in vitro* ion channel assays provide appropriate markers of risk (metrics) for training the *in silico* AP model to recognize and assign an appropriate level of proarrhythmic risk to each of the 12 initial reference drugs. Phase II studies will investigate the entire collection of 28 drugs.

The C*i*PA Steering Committee has accepted a proposal from the Japan iPS Cardiac Safety Assessment (JiCSA) group to sponsor and coordinate, within Japan, an experimental study of 60 compounds with varying degrees of proarrhythmic potential for testing and evaluating the C*i*PA *h*iPSC-CM assays.

The C*i*PA Steering Committee and the ICH S7B and E14 Working Groups have initiated discussions to define the possible place of the C*i*PA paradigm within the upcoming revisions of these regulatory documents. For instance, the E14 Q&As (R3), released by the ICH in December 2015 (ICH, 2015) opens the avenue to perform intensive ECG studies, in replacement of the thorough QT/QTc study, conducted within Phase I safety clinical investigations. The latter trials, if are designed in accordance with the rich cardiac electrophysiological knowledge generated by the C*i*PA assays, can be expected to accelerate and improve the human assessment of the proarrhythmic safety profile of candidate drugs (Cavero, et al., 2016).

2.2. Ion channel project update. Dr. Bernard Fermini Global Safety Pharmacology, Pfizer, Groton, CT, USA and Dr. Najah Abi Gerges, AnaBios Corporation, San Diego, CA, USA,

The Ion Channel Working Group (ICWG), under the auspices of the Safety Pharmacology Society, was charged with the following tasks:

1) Selection of cardiac ventricular ion channels requiring experimental scrutiny because drug-induced perturbations of their physiological function have been found to mediate proarrhythmic events in patients (Kannankeril, et al., 2010; Vandenberg, et al., 2012);
2) Establishing best practice protocols for manual and automated patch-clamp studies to determine whether candidate drugs disrupt human ventricular ion channel functions;
3) Performing pilot studies to identify those ion channel parameters that can best predict a proarrhythmic liability of candidate drugs (Fermini, Hancox, Abi-Gerges, Bridgland-Taylor, Chaudhary, Colatsky, et al., 2016).

For the first core assay of the C*i*PA paradigm, the ICWG selected three depolarizing [I_{NaFast} ($Na_V1.5$ peak current), I_{NaLate} ($Na_V1.5$ late current), I_{Ca} ($Ca_v1.2$),] and three repolarizing [(I_{to} ($K_V4.3$), I_{Kr} (*h*ERG), and I_{Ks} (K_VLQT1/KCNE1)] cardiac ion channels and I_{K1} (Kir2.1) channel which can behave as a depolarizing or repolarizing current according to the phase of the cardiac cycle to which it contributes. This selection was made by taking into consideration the key role played by the channel in

shaping the ventricular depolarization and repolarization phases of the ventricular AP profile. A definitive triage will be made according to experimental data collected by ICWG and the *in silico* working groups (ISWG) and by taking into consideration of the results of an SPS inquiry concerning these channels most often investigated by the pharmaceutical industry for assessing the cardiac safety of candidate drugs which are I_{NaFast}, I_{CaL}, I_{Kr}, and I_{Ks}.

For each of the seven ion channels selected by the ICWG, two sets of experimental protocols have been prepared:

1) Physiological protocols. These are designed to mimic the behaviors of an ion channel during the cardiac action potential by employing AP-like current stimuli to activate channels for generating IC_{50} values as well as data on voltage-, rate- and use-dependence, and channel activation, inactivation, and deactivation rates. The physiological protocol for I_{Kr} has been tested in manual patch clamp platforms and the results of the study have been recently published (Crumb, Vicente, Johannesen, & Strauss, 2016). The data obtained from the latter (pilot) and the ongoing post-pilot (investigating the 12 reference drugs listed in section 2.1) studies will be used by the *in silico* working Group (ISWG) to train the O'Hara-Rudy model (O'Hara, et al., 2011) to assign the correct proarrhythmic liability (no/low, intermediate, and high).
2) Dynamic block protocols (Milnes, Witchel, Leaney, Leishman, & Hancox, 2010) are designed to generate parameters (e.g., drug channel affinity (IC_{50}), kinetics, and state-dependence of the drug-channel interaction) of potential interest for the *in silico* assay. Pilot I_{Kr} studies on dofetilide, cisapride, and verapamil, applying this protocol have already been carried out at room and physiological (37°C) temperatures by using manual patch clamp setups. Additional, ongoing patch clamp pilot studies on the effects of other reference *h*ERG blocking drugs according to a dynamic protocol are expected to be completed within 2Q 2016. It is of interest to note that results reported by (Milnes, et al., 2010) indicate a marked ability of cisapride, but not dofetilide, to interact with the *h*ERG channel on a dynamic pulse-by-pulse basis. These authors note that that if a candidate drug were to undergo voltage-dependent cycles of dissociation and reassociation, its effects might be overestimated by *h*ERG-blocking assays using certain stimulus frequencies and voltage protocols. For these drugs (e.g., cisapride, droperidol, haloperidol, amiodarone) the time period between resting and gated (open/inactivated) states should be well-controlled during concentration-response studies and this may be achieved by using continuous voltage clamp applying a sustained-depolarization [Fig. 6 in (Milnes, et al., 2010)].

   The ability of cisapride to rapidly associate and dissociate from the *h*ERG channel during the AP cycle may account for the large variability of its potency as blocker of the *h*ERG channel reported in the literature [Table 2 in (Milnes, et al., 2010)]. In contrast, dofetilide is a slow *h*ERG blocker which becomes and remains associated to the *h*ERG channel throughout the AP cycle. Hence, patch clamp studies applying dynamic block protocols may provide useful

information on the development of channel blockade produced by a drug candidate and may account for possible variation in a drug IC_{50} determined by using different channel activation voltage protocols, and possibly, at different study temperatures.

The protocols tested in pilot and post-pilot studies are consensus protocols. However, the protocols, that will be officially proposed by the ICWG for the C*i*PA ion channel assays, will be best practice in nature since they will incorporate the knowledge acquired during the testing and validation studies.

All manual patch protocols, starting from those on *h*ERG channels, will be adapted for high throughput screening (HTS) platform application and optimized to yield data of the same high quality as those obtained or obtainable with the manual patch clamp technique.

2.3. In silico modeling: update and next steps. Dr. Sara Dutta, Division of Applied Regulatory Science OCP/OTS/CDER/FDA, Silver Spring, MD, USA

The C*i*PA ISWG has been entrusted with selecting, training, and making publicly available a human (adult) ventricular AP computational model that has the power to detect the proarrhythmic potential of candidate drugs from patch clamp results generated in the C*i*PA cardiac ion channel assays.

The O'Hara-Rudy model (O'Hara, et al., 2011) was selected as a starting point for *in silico* simulations by a large panel of leading cardiac modeling experts because it is the cardiac cell electrophysiological model most tightly linked to human ventricular cell data obtained from over 100 undiseased human hearts. This choice is further supported by the following desirable features characterizing the model:

1) Simplicity of use;
2) Transparency of assumptions;
3) Direct relationship to experimentally derived and verifiable datasets;
4) Freedom from intellectual property protection;
5) Implementation as a community resource that will not require specialized hardware or software to be used.

The chief challenge for *in silico* programming scientists is to provide a model that can predict the clinical proarrhythmic risk of candidate drugs from *in vitro* ion channel results generated in patch clamp studies. In order to attain this goal, the ICWG should find reasonable solutions to the following issues:

1) Identification of key ion channels and parameters for accurate *in silico* prediction of the proarrhythmic risk of a drug candidate;
2) Selection of the most appropriate experimental protocol for generating, in the envisaged assay system (manual or automatic patch clamp), the most robust ion channel results for *in silico* purposes;
3) Definition of quality control criteria for data input into the computational model (Elkins, Davies, Brough, Gavaghan, Cui, Abi-Gerges, et al., 2013).

Issues relating to the use of the O'Hara-Rudy model (O'Hara, et al., 2011), which may still require attention, include:

1) Optimization of the basic model performance;
2) Identification of the best method to represent drug-channel interactions;
3) Enabling the ready use of patch clamp data as a model input;
4) Definition of the underlying principles to evaluate the fitness-for-purpose of simulation protocols.

Of particular importance, the *in silico* assay-generated information on the clinical proarrhythmic risk of a candidate drug should:

1) Be mechanism-based (e.g., either producing phenotypic manifestations on the reconstructed AP such as EADs, or linked to one or more functional parameters, such as the value of the net repolarization current or amount of current necessary to trigger EADs);
2) Enable the rank categorization of a candidate drug into no/low, intermediate, and high proarrhythmic risk in relation to reference drugs assigned to these three classes by the C*i*PA Clinical Translational Working Group (Cavero & Holzgrefe, 2015);
3) Be usable for regulatory requirements.

In order to be compatible with the O'Hara-Rudy model (O'Hara, et al., 2011), patch clamp data should be obtained in ion channel preparations that generate data qualitatively and quantitatively similar to those measured in native adult cardiomyocytes since this model has been built according to electrophysiological data generated in preparations from human adult hearts.

Possible issues concerning datasets obtained in patch clamp studies are:

1) Certain cloned ion channels used for patch clamp studies may be expressed without their native complementary channel subunits that may play a critical role for physiological ion channel kinetics;
2) High throughput screening (HTS) patch clamp platforms may use bath solutions that differ from the native environment within which human cardiac ion channels function;
3) Currently available automated patch clamp platforms do not routinely provide the possibility of performing studies at physiological temperature, a feature necessary to observe rapid drug-induced channel blockade. This may explain why the IC_{50} values of certain drugs differ substantially from those obtained at room temperature. For example, this is the case for erythromycin and d,l-sotalol which exhibited an IC_{50} against I_{Kr} of 1410 and 810 µM, respectively, at 24 °C and 199 and 278 µM at 37°C (Kirsch, Trepakova, Brimecombe, Sidach, Erickson, Kochan, et al., 2004). In order to overcome this potential issue, the ICWG is generating data with the 12 reference compound set (2.1) at 24 and 37°C in order to support an ISWG project aimed at developing a computational routine that can use 24°C data to predict the behavior of a drug at 37°C. However, most of I_{Kr} blockers studied to date appears to be characterized by IC_{50} values that are often virtually the same when determined at either 24° C or 37° C (Li, Dutta, Sheng, Tran, Wu, & Colatsky, 2016). However, it should be noted that for

certain drugs block kinetics (and, therefore, risk prediction) may be vastly different at room and physiologic temperatures.

A candidate proarrhythmic metric under evaluation is the value of the residual net repolarizing current ($I_{net} = I_{Kr} + I_{Ks} + I_{NaLate} + I_{CaL}$ which can be derived from the effects of a candidate drug on the repolarization phase under the 'worse case' EAD scenario which can be achieved by applying a current patch clamp step protocol. When I_{net} crosses 0 during repolarization, EADs are likely to occur [details in (Starmer, 2005)].

Another proarrhythmic metric under evaluation obtainable from patch clamp studies is I_{bias} (μA/μF) current which is the threshold current necessary to trigger EADs in the absence and presence of a candidate drug. For instance, a drug concentration producing a 50% block of the *h*ERG channel reduced the control value of I_{bias} (0.734) by 50% (0.375) (Gray & Huelsing, 2008).

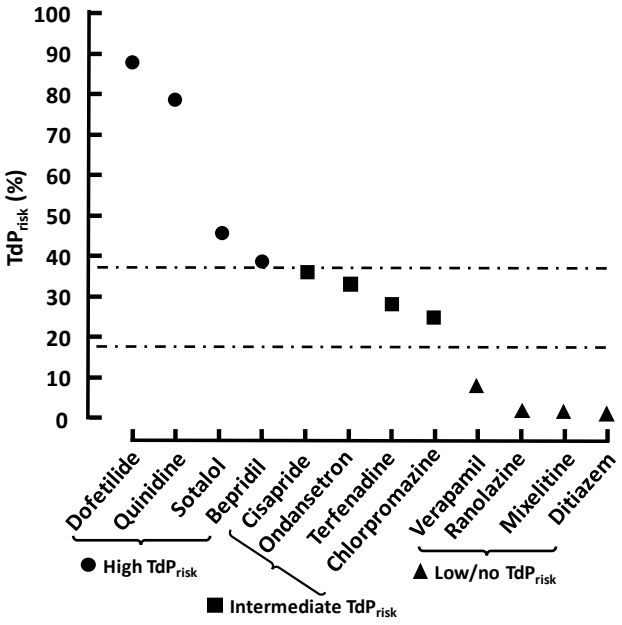

Fig. 1. Proarrhythmic metric (TdP_{risk}) derived using three variables (see details in the text), namely, the AUC under I_{Kr}, I_{CaL} and I_{NaLate} current contours generated in AP patch clamp studies under vehicle (control) and drug exposure (at human concentrations) conditions. Figure redrawn from a slide presented by Dr. Sara Dutta at the 2015 Annual Meeting of the Safety Pharmacological Society. The results of this figure should be considered exploratory in nature, since the data used to build it were not obtained using C*i*PA ion channel protocols and do not take into consideration *h*ERG blocking kinetics. The authors of this work are in the process of further refining this metric and evaluating its performance using CiPA ion channel protocols and training compounds (personal communication)

A new and potentially interesting proarrhythmic metric referred as a 'TdP_{risk}' (Fig 1) is firstly provided by calculating the area under the time-current profile (AUC) of three key ventricular AP currents (I_{Kr}, $I_{CaL,}$ and I_{NaLate}) determined by the application of an AP patch clamp protocol. Then, the ratios of the AUCs calculated during control condition and AUC under drug (at clinical concentrations) exposure were computed. Model inputs included experimental manual patch clamp IC_{50} data obtained at 37°C, amount of block for each channel produced by drug exposures achieved in patients, and current conductance scaled accordingly by the computational model (Crumb, et al., 2016). The TdP_{risk} metric is obtained in the following manner: $TdP_{risk} = a(AUC_{ICaL})_{Control}/(AUC_{ICaL})_{Drug} + b(AUC_{INaLate})_{Control}/(AUC_{INaLate})_{Drug} + c$ $c(AUC_{IKr})_{Control}/(AUC_{IKr})_{Drug} + d$. The parameters a, b, c, and d are calculated using the proportional odds model (Brant, 1990). The input of the TdP_{risk} metric into the O'Hara-Rudy model provided a correct classification (Fig. 1) of the clinical proarrhythmic risk of the reference training set of 12 drugs.

The ability of the *in silico* assay to link accurately ion channel pharmacology to clinical risk prediction requires ion channel datasets characterized by minimal variability and high reproducibility within and across laboratories and platforms.

The *in silico* C*i*PA shared community resource for public access will be accompanied by ISWG-prepared documentation concerning:

1) Quality of patch clamp data necessary to obtain reliable information from the AP model;
2) Boundaries (in terms of compound actions) within which the model predictions can be considered reliable;
3) Model assumptions;
4) A description of how data should be input into the model to perform the computational analysis.

Envisaged future development of the *in silico* model includes:

1) Improvement of basic performance by refining the contribution of certain ionic currents, namely I_{K1} and I_{NaLate};
2) Use of drug-channel interaction data obtained from the dynamic block protocol for generating reliable proarrhythmic predictions for drugs specifically blocking I_{Kr};
3) Enabling the use of patch clamp data obtained at 24 °C to satisfy the model requirement for 37°C;
4) Inclusion of disease state models by introducing the potential pathological influences of key cardiovascular changes due for example to bradycardia, hypokalemia, co-medications, and certain inherited LQT syndromes;
5) Influence of data obtained by applying different patch protocols (current/voltage clamp) on simulation outputs;
6) Additional considerations for use of *in silico* models as discussed in a recently published review article (Davies, Wang, Mirams, Caruso, Noble, Walz, et al., 2016).

2.4. Current situation and future plan of Japan iPS Cardiac Safety Assessment (JiCSA) consortium. Yuko Sekino, National Institute of Health Sciences, Tokyo, Japan

The JiCSA (Jicsa, 2016) is a joint consortium (funded by the Japan Ministry of Health, Labour and Welfare) under the leadership of the Japan National Institute of Sciences, the Japanese Pharmacology Society, pharmaceutical companies, CROs, academia, and research institute representatives.

JiCSA established an active collaboration with the C*i*PA Steering Committee for participating in the testing and validation of core C*i*PA assays.

The key objectives of the JiCSA are:

1) Standardization and validation of Safety Pharmacology methods for identifying drugs which have the potential of inducing proarrhythmic events;
2) Validation of the *h*iPSC-CM assay for regulatory purposes.

In order to ensure the reproducibility of results of *h*iPSC-CMs electrophysiological investigations, the JiCSA is funding and conducting a study in four independent laboratories. The protocol of this

study calls for the use of the same *h*iPSC-CM line (CDI-iCells Cardiomyocytes) and the same experimental platform (MED64) for determining the effects of candidate drugs. In particular, to constrain sources of potential assay variability, technicians received special training concerning all experimental protocol provisions such as *h*iPSC-CM culture density, period to wait for the experimental procedure following cell seeding, drug sampling times for data points following drug exposure, temperature within the experimental well, and instrumentation calibration settings [e.g., high pass (HPF) and low pass (LPF) filters fixed at 0.1 Hz and 5 kHz, respectively]. A pilot study carried out according to these experimental conditions investigated five concentrations of reference *h*ERG blockers added in a cumulative manner (observing 20 min intervals between 2 contiguous concentrations) to six independent experimental wells seeded with *h*iPSC-CMs. For statistical analysis, parameters derived from the field potential (FP) signals included interspike interval (ISI) in ms, field potential duration (FPD) in ms, early afterdepolarizations (EAD) incidence, and cell monolayer beating arrest. FPD values were corrected by using Fridericia [(FPDcF= FPD / (ISI / 1000)$^{1/3}$] or Bazett (FPDcB = FPD / (inter-spike interval / 1000)$^{1/2}$]) formula (Nakamura, et al., 2014; Asakura et al, 2015).Steady state FP signals (golden waves) within the exposure time selected for measuring ISI and FPD were characterized by an FPA ≥200 μV, an FPDcF ≥340 ms, and a second peak amplitude following the FP spike ≥ 15 μV. This study provided evidence that FPD was of greater amplitude at 0.1 Hz than with a 1.0 Hz high pass filter. The use of 1 Hz high pass filter (HPF) reduced FPD prolongation and obscured EAD detection produced by *h*ERG blockers. The application of the above protocol by four geographically separated research sites to the study of E-4031 yielded the following results: a 3 nM concentration produced a 10% increase in FPDcF in all laboratories, 10 nM produced EADs in 2/6 preparations in 2 laboratories and in 5/6 preparations in the remaining laboratories. However, all four laboratories observed EADs in the 6 CM preparations after 10 min exposure at 30 nM.

The JiCSA has asked its NIHS member to discuss with the Medical Devices Agency (PMDA) and the MHL, the regulatory acceptance of electrophysiology datasets produced by Japan Pharma in studies using *h*iPSC-CMs. At the level of the ICH organization and international regulatory institutions, JiCSA consortium envisages a gateway role for the acceptance of the C*i*PA assays for drug development and regulatory purposes. Finally, it plans to relay all data generated by the studies carried out under its sponsorship to the Pharmacological Evaluation Institute of Japan (PEIJ), an institution supported by a grant from the Japanese government for the development of widely applicable data validation platforms.

2.5. Current situation and future plans in Japanese activity from the Kirishima meeting. Dr Atsushi Sugiyama, Department of Pharmacology, Faculty of Medicine, Toho University, Ota-ku, Tokyo, Japan

In January 2014, representatives of the Japanese health regulatory authority, pharmaceutical industry, and academia met in Kirishima (Japan) to discuss recent scientific advances and technologies for assessing the cardiac safety of candidate drugs (stem cell, computational, clinical, and integrated cardiac safety pharmacology-based methods). The meeting produced a consensus report (Sekino, 2014) in which it is stressed, *inter alia*, the desire to involve Japanese scientists in the evaluation of the C*i*PA paradigm. Additionally, the meeting participants considered that the C*i*PA strategy should be enriched of an *in vivo* nonclinical assay as currently recommended by the ICH S7B guideline (ICH, 2005b) and an early clinical ECG investigation to replace the ICH E14 (ICH, 2005b) TQT study (Cavero & Holzgrefe, 2014; Sugiyama, 2014) for ensuring the cardiac electrophysiological safety of candidate drugs for human use. At this meeting, the following dedicated working groups were designated: *Stem Cell Pharmacology*, *In Silico Pharmacology, Clinical Pharmacology, and Integrated Cardiac Safety Pharmacology*. The common mission of these groups is to contribute via appropriate initiatives to the determination of the human safety of candidate drugs selected for clinical investigation.

The specific assignments of each group are briefly indicated here below.

1) The *Stem Cell Pharmacology Group* has already established formal collaboration with the J*i*CSA for the development and standardization of experimental protocols to predict adverse drug reactions in nonclinical electrophysiological studies using *h*iPSC-CMs (see section 2.4).
2) The *In Silico Pharmacology Group* in charge with thee activity on the development of a novel three dimension (3D), multi-scale, and multi-physics computational heart model (UT Heart) to correctly predict the proarrhythmic potential of candidate drugs from a 12-lead human ECG generated from nonclinical electrophysiological data obtained in automated patch clamp studies. In pilot studies, the UT Heart model satisfactorily predicted the expected human ECG effects of E-4031 and verapamil.
3) The *Clinical Pharmacology Group* is tasked with the elaboration of Phase I ECG protocols that incorporate C*i*PA generated knowledge for the rapid, early clinical assessment of the proarrhythmic risk of candidate drugs in volunteers.
4) The *Integrated Safety Pharmacology Group* is in charge of the integration of C*i*PA generated datasets into the nonclinical integrated risk assessment scheme recommended by the ICH S7B (ICH, 2005b). Additionally, it evaluates the predictability of candidate drug clinical proarrhythmic liability from the integrated analysis of nonclinical *in vitro* and *in vivo* data in conjunction with human ECG datasets obtained during Phase I investigations.

3. The C*i*PA core assays: potential issues and possible solutions

Safety Pharmacology studies should be designed, executed, and analyzed according to best scientific principles which entails, whenever possible, the adoption and scrupulous application of best practice

protocols in the execution of experimental procedures, particularly if these are carried out for regulatory purposes.

The following sections examine issues requiring resolution to render C*i*PA core assays fit for the discovery of proarrhythmic mechanisms of candidate drugs.

3.1. Ion channel assays

The objective of the C*i*PA ion channel assays is to determine whether candidate drugs disrupt the physiological function of any ion channels playing a fundamental role in the generation of a key electrical current contributing to the generation of the human ventricular AP.

3.1.1 Biological material for the CiPA ion channel assay

The core structural component of any cardiac ion channel is the pore-forming α-subunit protein which functions as pathway for the entry into, and the exit of ions (Na^+, K^+ and Ca^{2+}) from, the CM. This protein is often complemented by one or more accessory proteins (subunit β, γ, δ) which modulate the pore activity.

Ion channel functions can be easily explored in channel-proteins heterologously expressed in host cells. Whereas this expression process generally succeeds in the permanent expression of the ion conducting α-subunit protein, it does not routinely result in the co-expression of the multiple proteins needed to fully recapitulate the functions of the native channel.

For the C*i*PA ion channel assay, the cloned cardiac ion channels should ideally possess the protein (primary, secondary, and tertiary) structure of native channels present on ventricular myocytes of healthy adult individuals, be stably expressed and functional within the host cells, and conduct a current density sufficiently large to allow its accurate measure in automated patch clamp platforms.

3.1.2 Study of I_{CaL} in heterologously expressed Ca^{2+} channels

While various heterologous cell lines express I_{Kr}, I_{Ks}, and I_{NaFast} ($Na_v1.5$) populations which conduct currents of elevated amplitude (≥ nA/cell), this is not generally the case for I_{CaL} ($Ca_v1.2$) (Lory, Varadi, Slish, Varadi, & Schwartz, 1993; Nishimura, Takeshima, Hofmann, Flockerzi, & Imoto, 1993). An additional experimental issue with heterologously expressed I_{CaL} is that, with dialysis of the intracellular constituents in the whole-cell recording setup, I_{CaL} amplitude rapidly declines, making it difficult to accurately determine the time-course of the electrophysiological effects of candidate drugs (Bean, 1984). However, $Ca_V1.2$ screening conditions (peak current amplitude maximization and rundown attenuation) have recently been optimized for an automated planar patch clamp platform (PatchXpress® 7000A) in an HEK-293 cell line stably expressing a human L-type cardiac Ca^{2+} channel assembly of α1C, α2δ, and β2a proteins along with the $K_{ir}2.3$ inward rectifier K^+ channel. The concurrent expression of $K_{ir}2.3$ is a crucial strategy, adopted for maintaining host cell resting potential below the threshold resting current activating $Ca_V1.2$. Indeed, this feature facilitates an effective culture of the HEK-293 cell line without the need of using Ca^{2+} channel blockers to prevent the excessive influx of extracellular Ca^{2+} that is detrimental to cell health and growth. The selected

automated patch platform routinely achieved GΩ seals and could record relatively small current amplitudes. Additionally, the assay conditions were optimized to yield IC_{50} values that were similar to those obtained in manual patch clamp studies for six established Ca^{2+} antagonists [diltiazem $IC_{50:}$ 44 (automated patch champ) and 36 (manual patch clamp) µM, gallopamil: 17 and 1.1 µM, isradipine 2.2 and 8 nM, nifedipine 22 and 16 nM, nimodipine 0.11 and 0.7 µM; verapamil 15 and 44 µM] and a Ca^{2+} agonist [S(-)-Bayk8644: 3.1 and 8.2 nM)] [Table 1 in (Balasubramanian, Imredy, Kim, Penniman, Lagrutta, & Salata, 2009)].

It should also be recalled that published potency (IC_{50}) values for verapamil as a blocker of $Ca_v1.2$ are rather heterogeneous. These differences are likely to depend not only from the voltage used to activate the channel and the presence or absence of subunits in the cloned $Ca_v1.2$, but also from the ion species (Ca^{2+} or Ba^{2+}) used in the extracellular solution as charge carrier, and other experimental conditions such as study temperature (Dilmac, Hilliard, & Hockerman, 2004).

Recently, a new $Ca_V1.2$ cell line by NMITT (Nmitt Pharmaservices, 2016) has been claimed to stably express all three subunits (α1c/β2/α2δ) present in the native $Ca_V1.2$ channel, have high success rates and reliable performance in automated patch clamp assays, afford rundown-free recordings, easy maintenance in cell culture, and possibility of custom adjustment of expression levels.

These developments mandate that the cell line for the C*i*PA Ca^{2+} channel assay should be selected after appropriate pharmacological characterization in automated patch clamp platforms according to the ICWG protocols.

3.1.3 Recording the small I_{NaLate}

I_{NaLate} is a small fraction of the classic "peak" I_{Na} current carried by $Na_V1.5$ which is generated by voltage-dependent Na^+ channels entering in burst-gating mode (repeated openings and closures) during myocyte depolarization. Genetic mutations causing defective I_{Na} channel inactivation, myocardial ischemia, and drug treatments can upregulate I_{NaLate} and expose patients to arrhythmic risk (Chevalier, Amuzescu, Gawali, Todt, Knott, Scheel, et al., 2014). Drugs like ranolazine, which is a blocker of this current, can reduce or prevent the proarrhythmic propensity inherent to *h*ERG channel blockade (Gupta, Khera, Kolte, Aronow, & Iwai, 2015). In contrast, drugs enhancing I_{NaLate} can favor the development of arrhythmic events. This underscores the importance of the inclusion of I_{NaLate} in the CiPA ion channel panel.

Because of the small amplitude of I_{NaLate}, agents which impair sodium channel inactivation such as veratridine or ATX-II, are routinely employed to overcome the small amplitude of this current in order to facilitate a more accurate characterization of candidate drug effects on this current. Nevertheless, it should be noted that it remains to be demonstrated that toxin-enhanced currents fully replicate native I_{NaLate}.

A recent report describes the successful use of the CytoPatch™ automated patch-clamp platform and a HEK293 cell line stably expressing human $Na_V1.5$ to determine I_{NaLate} (Chevalier, et al., 2014). The

baseline amplitude of this current averaged -10.4 ± 2.2 pA (n=18) during the last 100 ms of 300 ms depolarizing pulses to -10 mV from an initial holding potential of -100 mV, delivered at 0.33 Hz. Ranolazine (30 μM) reduced this current by 58.4 ± 3.5% (N=18) after a 3 min incubation. In contrast, in cells exposed for 5 min to 1 μM of veratridine I_{NaLate} was increased by 269.1 ± 16.1 % (n=28) from a baseline value of -12.4 ± 1.9 pA.

3.1.4 Studying I_{Ks} in automated patch clamp platforms

Under baseline conditions, the repolarizing current I_{Ks} contributes minimally to the repolarization phase of the cardiomyocyte AP which is primarily mediated by I_{Kr}. However, I_{Ks} exerts a major and vital function during sympathetic tone elevation leading to enhanced cardiac chronotropic and inotropic activity. Indeed, in these physiological settings, the increase in I_{Ks} limits the degree of QT interval prolongation that would result from the augmented $Ca_V1.2$ currents in the absence of the I_{Ks} intervention. Therefore, I_{Ks} functions as cardiac repolarization reserve antiarrhythmic mechanism precluding excessive, potentially dangerous repolarization lengthening (Jost, Papp, & Varro, 2007).

Mutations in the gene encoding *h*KCNQ1 are responsible for the most common form of congenital long QT (LQT) syndrome (characterized by loss of channel function and referred to as LQT1 syndrome) which predisposes affected patients to excessive QT interval prolongation during surges in adrenergic stimulation (e.g., occurring during intensive exercise and emotions) that can trigger lethal TdP events. Indeed, in these patients, I_{Ks} fails to increase as a counterbalance to the enhanced depolarization mediated by β-adrenergic stimulation to the heart (Jost, et al., 2007).

HEK-293 cells transfected with the *h*KCNQ1 and *h*KCNE1 genes stably express I_{Ks} channels with voltage- and temperature-dependent gating characteristics, pharmacological responses, and β-adrenergic modulation, as exhibited by I_{Ks} in native human CMs (Wu, Naiki, Ding, Ohno, Kato, Zang, et al., 2014).

ICWG protocols should be tailored, tested, and validated for the ability to study I_{Ks} expressed in suitable cell models in automated patch clamp platforms that allow the adoption of desired pulsing rates.

3.1.5 Points to consider for the selection of automated patch clamp equipment and studies

Automated patch clamp platforms for C*i*PA ion channel assays should ideally:

1) Provide easily obtained knowledge-rich proarrhythmic metrics for the *in silico* assay;
2) Offer the possibility of studying candidate drugs at 37°C and at desired pacing rates;
3) Allow sampling of well solutions for the determination of drug candidate concentrations;
4) Allow superfusion of well preparations to uniformly expose CMs to desired concentrations;
5) Offer the possibility of rapidly evaluating possible rundown phenomena;
6) Ensure satisfactory reproducibility of results within and across platforms/cell lines/laboratories;
7) Provide easily programmable features for standardizing dataset output.

3.1.6 Validation of critical components of automated patch clamp platforms

Available automated patch clamp plates used for cell growth and experimental procedures require validation for fitness-for-purpose. Dr. Fermini, during his presentation at the 2015 SPS meeting (section 2.1), mentioned that the IC_{50} of cisapride varied from 28 to 62 nM when determined in two different types of plates, despite the application of the same assay protocol by the same technician. Hence, each key component of the automated patch clamp platform requires careful evaluation for data reproducibility and reliability.

3.1.7 Solubility of test articles for automated patch clamp

Solubility of test articles may pose problems specific to HTS patch clamp platforms. Indeed, current HTS plates are generally passive microfluidic (low volume, small size) systems (Wikipedia, 2016) that are characterized by high shear stress affecting the conformation of patched cells and delaying homogenous solubility of the tested compounds within the experimental well solution (Cioffi, Moretti, Manbachi, Chung, Khademhosseini, & Dubini, 2010; Kraly, Holcomb, Guan, & Henry, 2009). The latter issue is routinely addressed by formulating poorly soluble compounds with substances [e.g., dimethyl sulfoxide (DMSO)] that render test articles more readily bioavailable to the patched cells. However, to be successful this strategy should be accompanied by means to rapidly achieve the homogenous mixing of small quantities of drugs or solvent containing solutions (see section 3.3.5). Finally, to ensure that homogenous mixing is achieved, the platform should allow sampling of well solutions for the determination of test substance concentration to correctly assess the potency of the candidate drug under evaluation.

3.1.8 Data management

Patch clamp dataset analysis should be standardized in terms of models used for IC_{50} (IC_x and Hill coefficient of the concentration-response curve) calculations, minimal number of assays/data points to obtain meaningful, robust data, and correction methods for possible rundown phenomena.

3.1.9 Expectations from ion channel patch clamp generated data

Ion channel patch clamp datasets generated by the ICWG-organized pilot studies with a set of 12 reference drugs will be relayed to the ISWG to train the O'Hara-Rudy model (O'Hara, et al., 2011) to identify key parameters (metrics) that have the ability to cause modifications in the computationally derived AP signaling proarrhythmic potential. From these liability fingerprints, the computational model, once appropriately trained and calibrated for fitness-for-purpose, will be able toautomatically attribute a low/no, intermediate, or high proarrhythmic risk label to any candidate drug.

3.2. In silico assay

Potential issues and solutions pertaining to C*i*PA *in silico* assay were discussed by Dr. Sara Dutta (section 2.3). In addition to these, the ISWG should consider developing simulations to assess the potential proarrhythmic danger of candidate drugs which block the I_{Ks} channel (Towart, Linders, Hermans, Rohrbacher, Van Der Linde, Ercken, et al., 2009), given the role of this channel in the cardiac repolarization process particularly during cardiac activation (Liu, Du, & Li, 2012).

3.3. hiPSC-CM assay

The objective of the C*i*PA *h*iPSC-CM assay is not only to verify the proarrhythmic predictions obtained from the *in silico* simulations but also to discover proarrhythmic mechanism(s) that the ion channel and *in silico* assays did not, or were not powered (e.g., disruption of cardiac channel expression and trafficking) to discover.

3.3.1 Qualification of hiPSC-CMs for CiPA purpose

Commercially available *h*iPSC-CM cell lines exhibit different mechanical, electrophysiological, and pharmacological properties (Ji, Kang, & Rampe, 2014; Millard, Chvatal, & Ross, 2015). Hence, in order to be adopted for the C*i*PA assay, they need to be carefully characterized for fitness-for-purpose, i.e. for their ability to generate the sought knowledge in a reproducible manner within and across laboratories. Issues deserving targeted attention are:

1) Definition of requisite features of *h*iPSC-CM cell lines to be adopted for the C*i*PA paradigm;
2) C*i*PA-specific procedures concerning cell seeding and growth in HTS plate wells;
3) Desirable properties of cardiac ion channel expressed by *h*iPSC-CMs for the C*i*PA studies;
4) Drug testing solution preparation;
5) Homogeneous mixing of µL quantities of solutions containing testing agents within the experimental well and precautions to ensure that drug candidates are not adsorbed to platform wares;
6) Adoption, if necessary for experimental purposes, of experimental platforms offering pacing capability;
7) Study design;
8) Data point sampling timing;
9) Dataset analysis approaches.

3.3.2 hiPSC-CM cell line features

The phenotypic structure of current CMs is structurally and functionally characteristic of the embryonic and/or fetal development stage as documented by electrophysiological, calcium handling, and metabolic signatures exhibited by these cell preparations. Indeed, in contrast to native adult CMs, *h*iPSC-CMs have small cellular size, lack T-tubule structures and well-formed sarcomeres, and possess multiple nuclei, poor overall calcium storage and handling capability, and relatively small populations of mitochondria. In addition, their metabolic processes depend entirely upon glycolytic activity, and have intrinsic contractile automaticity (Ivashchenko, Pipes, Lozinskaya, Lin, Xiaoping, Needle, et al., 2013; Keung, Boheler, & Li, 2014; Robertson, Tran, & George, 2013; Roden & Hong, 2013). The lack of working load for *h*iPSC-CMs in culture conditions is responsible for the absence of the development of a cellular sarcomeric organization during the maturation process which, in turn, results in a poorly organized syncytium lacking adult phenotypic characteristics.

Intensive research efforts are ongoing for obtaining *h*iPSC-CMs exhibiting adult phenotypic features. Currently, any small progress toward the latter goal is obtained by *h*iPSC-CM production process changes which, in certain cases, are accompanied by changes in the pharmacological properties of *h*iPSC-. If the latter occurs, it infringes a central tenet of safety disciplines which stipulates that the biological material used for a safety test, particularly whenever performed for regulatory purposes, should, over time, provide reliable and reproducible datasets within and across laboratories. In order to fulfil this requirement, the production process of any *h*iPSC-CM line, if adopted for C*i*PA safety investigations, should be constrained to preserve the properties exhibited during the qualification process. To this end, it is incumbent on the manufacturers of *h*iPSC-CMs to ensure the consistency of cell products from batch to batch within the requirements of a Quality Management System (QMS) certified according to the International Organization for Standardization (ISO) principles. This entails, *inter alia*, that for experimental replication purposes, the manufacturer complies with ISO standards and maintains in its archive system (and, if requested, provides) documentation concerning the entire manufacturing process of any *h*iPSC- CM batch (World Health Organization, 2011). As a corollary to these basic principles, on material delivery the manufacturer has to inform investigators of any change in the production process of the *h*iPSC-CMs since this may require additional pharmacological validation assaysto assess whether the modified biological material responds to reference drugs in the same manner as established during the C*i*PA validation exercise.

3.3.3 Cell seeding and growth into HTP plate wells

The growth of *h*iPSC-CMs seeded into experimental wells uses solutions generally supplied by cell manufacturers. For proprietary reasons, the composition of these solutions is not always detailed on the container labels. It is, however, a safety assay requirement that the composition of any solutions used during the experimental procedure [for biological material preparation (monolayer differentiation) or assay execution] should be part of the experimental protocol. For example, if such culture or assay solutions contain proteins of animal or human origin (serum), they may modify candidate drug availability to the biophase sites.

Whenever possible, the use of protein-free differentiation and recording media should be considered, particularly if these media work well for the selected hiPSC-CM line. For example, commercial media such as mTeSR (Stemcell™ Technologies, 2016) and Essential 8^{TM} (Thermofisher Scientific, 2016), are claimed to increase reproducibility of *h*iPSC-CM culture and differentiation by eliminating the use of serum, serum replacements, or conditioned media. Relative to recombinant growth factors, chemically synthesized small molecules incorporated into protein-free media reduce variability and cost and enhance the robustness of the *h*iPSC-CM assays. Additionally, these substrates stabilize certain properties of the cell culture systems and improve *h*iPSC-CM maturation processes when complemented with soluble cues of 3D design (e.g. substrate physical form optimizing cell-to-cell contacts) that may facilitate *h*iPSC- CM electrophysiological maturation (Denning, Borgdorff,

Crutchley, Firth, George, Kalra, et al., 2015; Patel, Celiz, Rajamohan, Anderson, Langer, Davies, et al., 2015).

Several *h*iPSC-CM manufacturers (e.g., Axiogenesis™, Pluriomics™) have developed proprietary protein-free media for their cell lines. End-users have also adopted personalized recipes for protein-free media used in their experimental procedures [e.g., for CDI *h*iPSC-CMs (Harris, 2015). As such, the C*i*PA MWG should consider recommending the use of protein-free media for C*i*PA *h*iPSC-CM assays if the use of these solutions provides datasets of enhanced value compared to traditional, protein-containing media.

3.3.4 Measuring platforms

Platforms currently available for HTP determination of the electrical activity of *h*iPSC-CMs use MEA or VSD technologies (Cavero & Holzgrefe, 2015).

MEA is a multi-well plate technology for label-free, real-time electrophysiological characterization of cardiomyocyte drug responses by recording extracellular field potentials (FP) from cell monolayers or clusters. Each well bottom is fitted with multiple recording electrodes [e.g., Maestro MEA system has a 16 electrode grid (Axion Biosystems, 2016b)].

MEA technology can be used to determine effects (concentration-response curves, time-course over a prolonged period) of test articles on FP. Endpoints from FP recordings include amplitude (FPA in µV), duration (FPD in ms), beat period (BP in s) from which BR (contractions per min = 60/BP] can be calculated. Arrhythmic events are quantified by the number of experimental preparations showing rhythm irregularities. Classifications of these irregularities have been proposed according to specific parameters (Gilchrist, Lewis, Gay, Sellgren, & Grego, 2015).

Due to the variability of the FP shape, a critical issue concerns the selection of the control FP wave(s) to enable clear measurements of FP parameters (in particular FPD). The changes produced by a candidate drug on the baseline FP are used to determine proarrhythmic safety or liability of a candidate drug. To this end, four approaches appear applicable:

1) Golden electrode. The underlying rationale is that monolayers of *h*iPSC-CMs are typically linked in an electrically coupled syncytium beating as a whole unit. As a result, the characteristics of the culture recorded at a given electrode could be considered to represent the activity detectable by any other electrodes within the well. Therefore, data points from a single electrode (i.e., the 'golden electrode') exhibiting an excellent FP wave may be presumed to be representative of the entire preparation. A requirement for this approach is that the same electrode should be used for sampling baseline and drug or vehicle exposure data [Fig. 7 in (Clements, 2016)].
2) Well-wide mean. This refers to the mean response across all well electrodes. The morphology of the FP waves from the entire set of MEA electrodes is generally rather heterogeneous. In particular, some electrodes can display prominent repolarization waves and others none at all. This can result in the misidentification of a weak repolarization wave, in particular, by automatic detection software with possible inaccuracy in FPD measurements [Fig. 7 in (Clements, 2016)].

The Cardiac Data Aggregation Tool within the AxIS software (Axion Biosystems, 2016a) allows setting user-specific boundaries for improving the accuracy of well-wide statistics.

3) Golden FP wave. This is defined by the following criteria: FPA ≥200 μV, FPDcF ≥340 ms, and a second wave following the FP spike of ≥ 15 μV amplitude. This approach is used by Japanese investigators, as described in section 2.4. A requirement for this approach, as noted for the Golden electrode, is that the same electrode should be used for sampling data under baseline and drug or vehicle exposure conditions.
4) Golden class. This method is proposed by NOTOCORD Systems through their NOTOCORD-Field Potential Screener (fps) (Notocord Systems, 2016). For each electrode, it clusters, FP waveforms recorded from all electrodes within a well according to morphology to ensure coherent measurements made over short sampling periods throughout the whole experimental time. Waveforms within each cluster (or class) are then averaged to improve the signal/noise ratio. The user has the possibility to select his quality control criteria for the first analysis period (used then throughout the experiment) for BR, acceptable maximal variability, cluster number, and beat number. These criteria allow the automatic selection of all electrodes that can be analyzed. Then, on each analyzable electrode, the software uses the most populated clusters for the calculation of the desired biomarkers (e.g., FPA, FPD, BP)

The variability in the FP shape of the preparation generally recorded from the electrodes within the experimental well may have multiple origins, some of which are preparation specific (e.g., position of the electrode within the well in relation to the pacemaker driving the preparation, ground position, cell organization within the sheet, and differences in resistance and capacitance between myocytes and electrodes).

The most relevant issues with MEA technology requiring appropriate resolution include:

1) Determination of the time necessary to achieve homogenous mixing of the test article solution with the drug-free solution within the well. This may be a function of the solubilizing medium (e.g., use or not of DMSO) and amounts of drug-containing solution selected to replace the drug-free solution;
2) Validation of electronic amplifier circuits and all connected hardware (e.g. electrode forms and position in relation to grounds) necessary to measure FP waveforms;
3) Use of a low frequency (0.1 Hz) digital filter for FP measurements in order to avoid information loss on recorded FP waveforms (Asakura, Hayashi, Ojima, Taniguchi, Miyamoto, Nakamori, et al., 2015);
4) Identification of recording electrodes within the well for capturing FP waves clearly exhibiting the parameters to be measured;
5) Selection of the most pertinent FP endpoint parameters for further analysis;
6) Detection and analysis of certain proarrhythmic events (EADs, beat-to-beat variability).

Voltage-sensitive dyes (VSD) and genetically-encoded voltage indicators [GEV (Q-State Biosciences, 2016)], together referred to as voltage sensitive optical probes (VSOs) can be used to measure the integrated AP generated by multicellular *h*iPSC-CM monolayers (Zhang, Gintant, & Pierson, 2014). Parameters that are generally calculated from these AP are BR, depolarization V_{max}, APD_{30}, APD_{50}, APD_{90}, AP triangulation (APD_{90}-APD_{30}) and number of proarrhythmic events such as early after depolarizations (EADs). AP shapes recorded with VSDs and the associated AP metric values (e.g., ADP_{90}) may be specific to each *h*iPSC-CM line.

3.3.5 There are several theoretical limitations to VSD and GEVI technology which have been succinctly summarized by Leyton-Mange and colleagues (Leyton-Mange et al., 2014): 1) Fluorescence signals report relative, not absolute, membrane potentials; 2) The slow temporal response of these VSOs results in loss of high frequency elements and leads to small but systematic overestimation of APD, although, VSD ANEPPS dyes have a faster temporal response to GEVIs (e.g. ArcLight). However, for the moment, this slow temporal response is not a critical issue since currently available hiPSC-CM preparations exhibit low Vmax values than primary CMs (Robertson et al., 2013); 3) Phototoxicity associated with VSOs can limit the duration of experimental recordings. Comparatively, GEVIs have been reported to have a lower phototoxicity than VSDs (ArcLight vs. di-4-ANEPPS and di-8-ANEPPS); 4) Additionally, a possible interaction of VSOs with the candidate drug should also be taken into consideration when using these technologies (Hardy et al., 2009).Experimental platform offering the possibility of pacing the preparations

Platforms offering the possibility of pacing *h*iPSC-CM sheets [e.g., (Axion Biosystems, 2016b; Multichannel Systems, 2016)] should be selected to determine the electrophysiological effects of drugs blocking I_{Ks}. Indeed, the activation of this channel occurs at high driving rates (Braam, Tertoolen, Casini, Matsa, Lu, Teisman, et al., 2013; Liu, et al., 2012). As such, the determination of the effects of a candidate drug at low and high pacing rates can provide key information on the potential proarrhythmic liability of a candidate drug. It should be noted that for spontaneously beating *h*iPSC-CM sheets, low pacing refers to a threshold rate just above the baseline rate (e.g., for CDI-iCell *h*iPSC-CM spontaneously contracting at ~60-times/min, stimuli delivered each 900-800 ms will raise the rate by 10-20 contractions). The baseline heart rate of *h*iPSC-CM sheets depends not only on the individual cell line under study but also on experimental protocol provisions (e.g., presence or not of glucose in the recording well solution).

The 48-well MEA optical stimulation system Lumos® is claimed to provide artifact-free pacing (Axion Biosystems, 2016c). In this regard, it should be mentioned that other optical electrophysiology platforms for driving and recording the activity of *h*iPSC-CMs are now available and are based on proprietary ultrasensitive optogenetic reporters and actuators that convert APs into visible fluorescence flashes easily detectable by microscopy. These methodologies can also be used to deliver excitatory photo impulses to drive the preparation at a desired frequency (Q-State Biosciences, 2016).

3.3.6 Properties of cardiac ion channels expressed by hiPSC-CM in growth wells

The populations of cells constituting *h*iPSC-CM sheets are likely to be heterogeneous in nature (Herron, et al., 2012) as the channel properties of each cell type depends on the progression from the initial embryonic to the successive fetal and final adult status. For example, the maximum diastolic potential (MDP) of commercially available *h*iPSC-CMs is relatively low or depolarized [e.g., more than -75 mV (Ma, Guo, Fiene, Anson, Thomson, Kamp, et al., 2011; Peng, Lacerda, Kirsch, Brown, & Bruening-Wright, 2010)]. This accounts for the sustained conducting (open) state exhibited by Na^+ channels in commercially available *h*iPSC-CMs, in contrast to the closed or inactivated state displayed by these channels in healthy native resting CMs. These electrophysiological properties, characteristic of immature *h*iPSC-CMs, remain virtually unchanged even after maintaining CMs in established culture systems for prolonged periods. It is possible that the composition of culture media favors the persistence of the immature phenotype observed in currently available *h*iPSC-CM lines [Fig. 1 in (Drawnel, Boccardo, Prummer, Delobel, Graff, Weber, et al., 2014)]. Additionally, the structure of ion channels and the functional role of associated regulatory sub-units in *h*iPSC-CM lines remain to be determined. In order to shed light on ion channel expression issues, the new CRISPR Genome Editing technology could be used since it offers tools for easy site-specific DNA deletions, insertions, inversions, and replacements. These genomic manipulations would help researchers to understand the gene function in various cellular contexts, to explore the mode of gene regulation at endogenous loci, and, most importantly, to model human disease conditions in in vitro cellular and *in vivo* systems (Shui, Hernandez Matias, Guo, & Peng, 2016) since patients harboring genetic disease are the most vulnerable human subjects to drug-induced proarrhythmic events.

A further issue with spontaneously beating *h*iPSC-CM sheets is whether either multiple synchronized or master pacemakers are responsible for the innate automaticity of pulsating monolayer syncytia. This information would be extremely useful for improving the analysis of FP waveforms, the shape of which varies with the contractile state of the preparation. A potential solution for this issue could be the adoption of standardized pacing protocols for *h*iPSC-CM assays. Alternatively, preparations exhibiting continuously changing sites of contraction initiation could be excluded from analysis. The acquisition software that accompanies the Maestro 48-/96-well MEA system allows the identification of the well site where the contractile wave starts (Axion Biosystems, 2016a).

The relative contribution of individual ion channels to FP shaping is poorly understood. The current knowledge on this matter is primarily provided by pharmacological maneuvers using channel blockers which are presumed to bind selectively and specifically to the channel under investigation (Ma, et al., 2011). However, the relative specificity and selectivity of these agents are properties which have not been established in *h*iPSC-CM preparations, but rather in conventional *in vitro/in vivo* preparations fully expressing functional and structurally organized human and non-human ion channels that have been studied under well-defined experimental conditions. Hence, the selectivity and efficacy of reference pharmacological agents remain to be established in immature *h*iPSC-CMs.

It should also be noted that no information is available on whether epi-, mid-, and /or endo-myocardial cells are present and, if so, which role(s) they play, in *h*iPSC-CM monolayer syncytia. Since these cell types mediate repolarization dispersion, this crucial mechanism responsible for triggering arrhythmia in the integrated human heart (Burton & Cobbe, 2001) cannot be currently evaluated in *h*iPSC-CM monolayer preparations.

Finally, the genetic background of available *h*iPSC-CM lines is generally not provided by the cell suppliers. Yet, this information could offer critical insights on CM line-specific characteristics.

3.3.7 Diffusion and time necessary for homogeneous mixing of candidate drug solutions within the experimental well

When the effects of candidate drugs on the electrical activity of *h*iPSC-CM are determined in 48/384 well platforms (e.g., study volume in each well of a 48 well plate is 450 µL per well), specific attention should be given to several potential issues.

Tight and homogeneous adherence of *h*iPSC-CM monolayers to the well floor should be ensured to obtain reliable FP measurements. Although this issue is now of reduced concern if fibronectin- or Kapton-coated wells are used, deficient adherence can cause trapping of part of the bath solution between the non-adhering cells and the well bottom. This phenomenon can prevent or delay mixing of the trapped solution with the drug-containing solution or expose non-adherent cell surfaces to the candidate drug. The non-adherence can be verified by using, for example, light green SF yellowish dye. When a drop of this dye is added to the well solution, the colored fluid surrounds the adherent cell monolayer but, in the presence of non-adherence, it rapidly penetrates between non-adherent cells and the well floor. The time to obtain a homogenous mixing of this dye with the well solution (300 µL) was found to be 10-15 min. This observation demonstrates that for small volume wells, the time for obtaining homogenous mixing between drug-free and drug-containing solutions (the density of which may differ from that of the control medium) should be experimentally assessed. The latter remark highlights a possible issue that may derive from the amount of a DMSO-solubilized drug that is selected for replacing the drug-free well solution. For example, if the desired final concentration of a drug solubilized in 0.3% DMSO within a 300 µL well is 300 pM (300 pM/mL) and this has to be achieved by replacing 1/30 of the total well solution (i.e., 10 µL), then the added 10 µL solution should contain 9000 pM of the candidate drug.

However, to achieve the same final concentration (300 pM) by replacing 1/2 (i.e. 150 µL) of the total well drug-free solution, the added 150 µL of a drug stock solution should contain 600 pM of the candidate drug. The 10 µL solution of 9000 pM of drug in 0.3% DMSO has a higher density than the 150 µL solution of 600 pM. Under the static microfluidic mixing conditions of currently used multi-well plates (Cioffi, et al., 2010; Kraly, et al., 2009), the small quantity of solution with the higher drug concentration reaches the cell monolayer attached to the floor of the well before becoming uniformly mixed with the drug-free solution. This may explain the results depicted in Fig.2 which were obtained in experiments conducted at 37°C by replacing 1/2 and 1/30 of the drug-free well medium with matched volumes of drug solubilized in an appropriate quantity of DMSO to achieve the same final well drug concentration containing 0.3% DMSO (v/v). Fig. 2 reports FP tracings recorded from 2 sets of 2 contiguous wells located in the same experimental plate containing cell sheets grown from the same *h*iPSC-CM batch and subjected to the same experimental procedure. Although the preparations were theoretically exposed to the same final concentration (300 pM) of a proprietary *h*ERG blocker by replacing 1/30 and 1/2 of the well drug-free solution, the observed effects were dramatically different. Indeed, almost full inhibition of the FP (persisting for the 30-min of the experimental procedure) was produced by the addition of 10 µL of a 9000 pM drug solution, while no effect was produced by 150 µL of a 0.6 nM drug solution. DMSO (0.3%) used to solubilize the drug cannot be considered responsible for the effects produced by the 10 µL solution containing the candidate drug since the matched 10 µL control solution containing only DMSO did not affect FP responses.

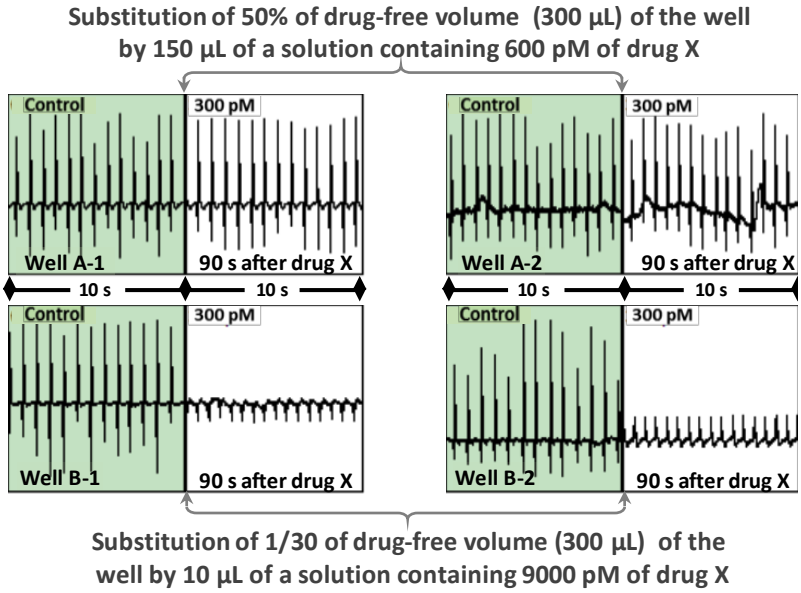

Fig 2. Effects of final drug concentration (300 pM) in 300 µL well achieved by replacing 150 µL (well A-1 and A-2) or 10 µL (B-1 and B-2) drug-containing solutions on the FP in hiPSC-CMs. Control responses were obtained by adding the same volumes of solution for achieving a 0.3% DMSO content in the final bath volume.

This problem could be avoided if the platform allowed the continuous superfusion of the preparation with solutions containing the desired final concentration of the drug. However, this would increase background noise and require greater system complexity and far larger amounts of test article. In view of these observations, it would appear that the largest possible volume not affecting the contractile activity of the preparation should be replaced to achieve the desired final concentration.

Additionally, if the responses to small volume containing high test article concentrations appear exaggerated, the possible influence of the density of the added solution in the observed response should questioned and verified by appropriately designed experimental procedures that avoid the use of very small quantities of drug-containing solutions which are generally used for the construction of concentration-response relationships in small volume wells.

3.3.8 Study design

The study protocol for the C*i*PA *h*iPSC-CM assay should incorporate the knowledge provided by the *in silico* assay. Since the final effects of the candidate drugs may change with length of the exposure time, time point measurements for short duration assays should be carefully standardized to allow comparisons across assays and laboratories. If warranted by the candidate drug therapeutic mechanism (e.g., biopharmaceutical anticancer agents), the effects of the candidate drug on ion channel expression and trafficking mechanisms should be assessed, if possible, during a prolonged (>8h) exposure of *h*iPSC-CMs to the test article (Guo, Coyle, Abrams, Kemper, Chiao, & Kolaja, 2013; Himmel, 2013). For this type of assay (but ideally also for short duration assays), recording and maintenance media should be identical in nature, notably in terms of protein content to avoid potential undesirable drug binding and pH effects, which can affect drug solubility and receptor availability.

3.3.9 Sampling frequency and timing

Data sampling times and frequency should be selected to provide the best evidence possible in favor of, or against, proarrhythmic liability of a candidate drug. In particular, it is crucial to generate data at steady-state conditions, as this is a fundamental pharmacological tenet for calculating IC_{50} values correctly. However, if arrhythmic events (EADs) occur at steady state, FPD measurements should be performed before their manifestation, or disregarded, since arrhythmia is a more meaningful proarrhythmic signal than FPD prolongation. Whereas EADs occurrence is a major indicator of a proarrhythmic potential of a candidate drug, the absence of EADs cannot be taken as an indication of the absence of proarrhythmic risk. The analysis of FP and AP datasets can make use of conventional or data-tailored methods, as discussed below.

3.3.9.1 Conventional dataset analysis

FP (FPA, FPD, BP or BR) and AP (ADP_{50}, ADP_{90}) parameters from a meaningful number of preparations are conventionally reported as means plus/minus associated errors [(Standard deviation (SD) or standard error of the mean (SEM)]. Additionally, if concentration-response datasets are available, the concentration inhibiting the response by 50% (IC_{50}) should be calculated together with associated parameters such as the slope and the maximum of the concentration-response curve. The logistic model to be used for such calculations should be recommended by a C*i*PA expert team in order to minimize possible data analysis variability due to use of different models and fitting options (e.g. methods to weight data for optimized fitting).

The experimental protocol should clearly define the FP or AP parameters to be selected to support proarrhythmic liability conclusions for the candidate drug and the threshold changes (e.g., FDP increase >20%) or X number of EADs within a given time period) for assuming that the observed effects are of biological significance.

3.3.9.2 Application of QT correction algorithm to FPD

An issue requiring a specific comment concerns the standardization of the FPD to a reference-beating rate to remove the possible influence of heart rate differences across preparations and *h*iPSC-CM lines. Many C*i*PA investigators standardize FPD values as if it were an ECG QT interval by considering that its duration is inversely related to the contracting rate of the preparation. Indeed, ECG QT intervals are generally corrected according to Bazett or Fridericia formulas (QTc) to obtain values for a reference heart rate of 60 beats/min for inter-subject (or inter preparation) comparisons (Davey, 2002). Several investigators apply this method to FPD analysis (Asakura,et al., 2015; Harris, Aylott, Cui, Louttit, Mcmahon, & Sridhar, 2013; Nakamura, et al., 2014; Navarrete, Liang, Lan, Sanchez-Freire, Simmons, Gong, et al., 2013). However, they do not provide experimental evidence to justify the application of either the Bazett or Fridericia algorithm to FPD values generated in electrophysiological assays with *h*iPSC-CMs (Cavero & Holzgrefe, 2015). For the moment, a less than optimal solution to this issue could be the reporting of uncorrected and corrected FPD values (Clements, Millar, Williams, & Kalinka, 2015; Clements & Thomas, 2014) to enable comparison with publications which exclusively report corrected FPD data. Recently, electrical pacing has been used to demonstrate that the optimal FPD correction factor for CDI hiPSC-CMs is intermediate of Bazett's and Fridericia's formulae (Axion Biosystems, 2016b).

3.3.9.3 In silico approaches to FP analysis

Computational *in silico* models of cardiac electrophysiology are now available and routinely used for reconstructing the AP and determining the contribution of key ion channels to the AP formation (Corrias, Giles, & Rodriguez, 2011). However, although FP data acquired by MEA technologies have not yet received extensive attention for simulation purposes, such efforts are ongoing as indicated by posters presented at the 2014 (Zitoun, Raphel, Boulakia, & Gerbeau, 2015) and 2015 (Bowler, Harris, Gavaghan, & Mirams, 2016) Annual Meetings of the Safety Pharmacology Society.

Examination of the relationships linking FP electrical signals to AP profiles is possible by using mathematical models incorporating partial differential equations (bidomain equations) for adequately describing the time- and space-dependence of intra- and extra-cellular electrical potential diffusion within the *h*iPSC-CM monolayers in experimental wells. By making the reasonable assumption that the FP is the electrical phenotypical manifestation of extra-cellular potentials, bidomain equations can convert FPs into APs and vice versa. This computational approach can be designed to integrate information concerning MEA well geometry and size, electrode surface areas, and positions within the well, etc. The model also needs to include values for the extra-cellular potential, or its derivatives, at

the boundary of the computational domain. These "boundary conditions" can be established, for example, on the observation that FP vanishes on that portion of the well connected to the ground wire, or that extracellular currents normally vanish on any insulated portion of the boundary.

Bidomain equations are necessarily based on an appropriate cell model. This *in silico* approach should be viewed, therefore, as an extension of, rather than an alternative to, the *in silico* model used to describe APs on their own. In other words, the FP model can provide reliable information only if it is based on a reliable cell models. Recently, a computation model for the electrophysiology behavior of *h*iPSC-CM line has been published (Paci, Hyttinen, Aalto-Setala, & Severi, 2013), but the fact that various commercially available *h*iPSC-CM lines exhibit different mechanical, electrophysiological, and pharmacological properties, implies that FP models should be developed by taking into consideration the electrophysiological features of the *h*iPSC-CM line of interest.

Bidomain equations used to model FPs recorded with MEA technology are constructed assuming a homogenization process which assumes that the system generating FPs is explored at a scale

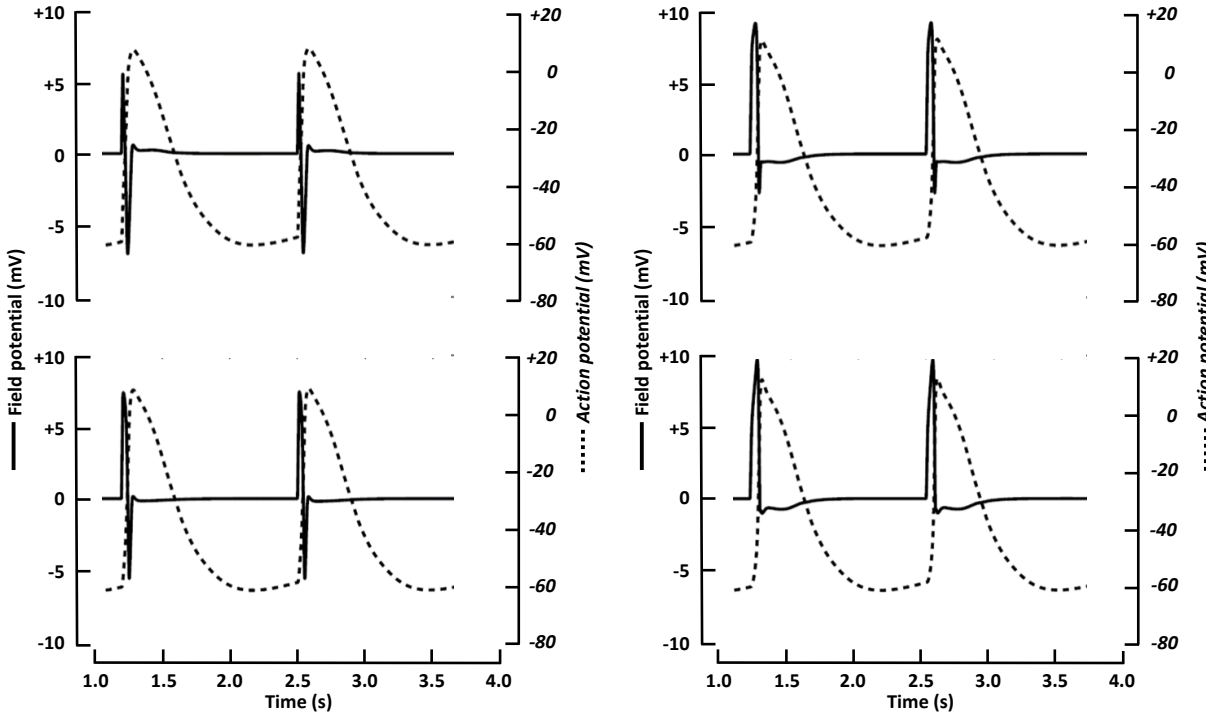

Fig. 3. Conversion of 4 *in silico* FPs [obtained by means of a computational model developed from electrophysiological data obtained in *h*iPSC-CMs (Paci, et al., 2013)] into AP profiles (Zitoun, et al., 2015). Note the qualitative agreement between simulated and experimentally obtained FPs in *in vitro h*iPSC-CMs assays. The *in silico* FPs, despite being obtained from homogenous cell sets, exhibit shape differences. The geometry used for this simulation corresponds to a 60-6 well MEA by Multi Channel Systems (Multichannel Systems, 2016).

substantially larger than the scale of the cells generating these signals. However, rejecting the latter assumption would require resolving the spatial scale of each single cell of the biological preparation, an approach which dramatically increases the complexity of the computational approach.

In spite of the aforementioned issues, promising results have been recently obtained with the bidomain incorporating an electrode model (Zitoun, et al., 2015) based on the model of *h*iPSC-CM.

The *in silico* FPs obtained within this framework (Fig 3) appear to be in good qualitative agreement with the FPs recorded from *in vitro* experiments (Zitoun, et al., 2015). Additionally, such a model can be used to scrutinize various aspects of the FP which are difficult to clarify in wet assays. For example, inter-electrode variability in FP forms, routinely observed in biological assays and generally attributed to the phenotypic expression of the different cell types constituting the *h*iPSC-CM monolayer, is reproducible in computer simulations using monolayers of perfectly homogeneous cells. Interestingly, there are configurations where the differences in FP shapes, relayed by spatially separated electrodes, do not translate in notable AP shape differences.

In silico models of FP can also be used to further our understanding of the effects of ion channel blocking drugs on the shapes of FPs obtained in *h*iPSC-CM experiments.

The *in silico* analysis of the FP based on a cell model is referred to as a forward (or direct) problem analysis whereas the inverse problem refers to the determination of cell AP properties derived from biologically measured FPs. For example, the application of inverse problem solutions could be exploited to automatically identify the ion channel species affected by a drug from the experimental FP data and underlying cell model. However, the results of the *in silico* FP models cannot outperform the *in silico* AP model upon which they are based. Hence, if the effects of a channel blockade are difficult to interpret on the simulated AP, the simulated FP would be unable to provide additional information. Ongoing computational efforts are expected to generate additional tools for rendering FP measurements generated by MEA platforms as informative as possible, particularly in the determination of the effects of candidate drugs on ion channel functions.

4. Discussion

The first part of this report reviews presentations delivered at the 'C*i*PA on my mind' session held at the 2015 Annual Meeting of the Safety Pharmacological Society. The presentations commented on accomplished, ongoing, and future activities of selected C*i*PA working groups for the qualification of the C*i*PA paradigm.

The second part of the report analyses a series of key issues requiring expedited solutions for the completion of initial testing and successive validation activities of C*i*PA core assays. Although it proposes possible solutions to certain issues it will be the role of C*i*PA Steering Committee and C*i*PA specialized working Groups to provide documented guidance on how these issues should be handled, particularly by bench investigators. In the absence of such an official intervention, substantial delays in the implementation of the C*i*PA as a valuable Safety Pharmacology strategy are likely to occur.

4.1 *Considerations on the 'CiPA on my mind' session held at the 2015 Annual Meeting of SPS*

The presentations on C*i*PA at the 2015 SPS Annual Meeting delivered limited new information in relation to the Dec 2014 C*i*PA Update Workshop (Cavero & Holzgrefe, 2015).

The Leaders of the Ion Channel Working Group (ICWG) informed the audience that the drafting of a physiological and dynamic block protocols for each of the seven cardiac ion channels selected as potential key investigational targets for the C*i*PA paradigm had been completed. Additionally, the testing of these protocols concerning the *h*ERG channel had been successfully achieved in manual patch clamp platforms. However, the screening technology for C*i*PA ion channel assays will be the automated patch clamp platform [which lacks the experimental flexibility of the manual patch clamp approach (Danker & Moller, 2014)]. The adaptation of the ICWG *h*ERG ion channel protocols to automated patch platforms remains to be done. If the protocols for the remaining six channels of interest have to be tested manually and then adapted to automated patch clamp platforms, an updated plan for expediting this process should be devised and implemented.

The substantial resources spent so far by the ICWG for optimizing *h*ERG channel protocols are justified by the recognized crucial role played by this channel in drug-induced lethal arrhythmia (Kannankeril, et al., 2010; Vandenberg, et al., 2012). Nevertheless, the protocol optimization process for automated patch clamp platforms concerning other cardiac ion channels should be accelerated.

The ongoing and future activities for the C*i*PA *in silico* assay were detailed at the 'C*i*PA on my mind' session. In particular, Dr. Sara Dutta emphasized that the successful training of the O'Hara-Rudy model with the set of twelve reference proarrhythmic drugs relies entirely on the quality and reliability of experimental data generated by the ICWG studies. It was also stressed that the input metrics should be quantitative in nature, drug mechanism-linked, and ultimately allow the classification of candidate drugs into one of three categories of risk (no/low, intermediate or high) as established by the C*i*PA Clinical Translation Group (Cavero & Holzgrefe, 2015). Metrics with these features will have to be obtained by using best practice protocols in optimized ion channel assays carried out desirably in automated patch clamp platforms.

Special mention deserves the novel TdP_{risk} metric since it succeeded in correctly classifying each of 12 established proarrhythmic drugs (Fig. 1) although it should be noted that the generation of the data necessary to calculate it may not be easily accomplished in automated patch platforms and will be expensive, particularly if this has to be done in manual patch clamp studies as was the case for the data presented in Fig. 1 (Crumb, et al., 2016). However, if such a novel metric provides robust and reliable information on the intrinsic proarrhythmic risk of a very promising drug candidate, it may be sought whenever a questionable safety signal is suggested by the overall C*i*PA studies. It is also important to keep in mind that the data presented in Fig. 1 are not the only possible ones for the correct classification of the 12 drug data set since various avenues are still being investigated, including a dynamic *h*ERG model and an improved representation of I_{NaLate}.

Drs. Sekino and Sugiyama (sections 2.4 and 2.5) discussed the contribution of Japanese organizations to the international C*i*PA validation efforts, including a 60 drug study to be performed in 2016. The primary goal of this study, in order to adopt appropriate correction measures, is the discovery of potential causes for the high inter- and among-laboratory variability currently

characterizing C*i*PA *h*iPSC-CM assays. To this end, the adopted protocol stipulates the application of strictly standardized conditions for which the technical teams of four geographically separated laboratories received specific training. However, certain protocol provisions may merit reconsideration in the light of issues detailed in section 3.2 of this report which deals with various potential concerns around biological material, experimental platforms, cell growth and testing solutions, small volumes of drug solutions added to the experimental well for generating concentration-response curves, and data analysis still requiring optimization. Additionally, the JiCSA consortium study protocol, despite the remarkable efforts to pinpoint causes to be corrected to constrain bench-related data variability (Kitaguchi, Moriyama, Taniguchi, Ojima, Ando, Uda, et al., 2016), may not have given sufficient weight to concurrent causes which are independent of the experimental laboratory setup. These include, for example, the manufacturing processes of the *h*iPSC-CM batches, and seeding, growing, and testing solutions. Indeed, if these experimental solutions contain animal or human sera, even strict control of the manufacturing procedure of these materials may not be sufficient to prevent possible between-manufacturer differences in the amounts of proteins (in particular growth factors) that can cause changes in the pharmacological properties of each newly produced batch of material. If this were the case, part of the knowledge provided by the Japanese efforts to standardize the C*i*PA *h*iPSC-CM assay would not be entirely translatable to novel batches of biological material or solutions required for seeding and growing cells or testing drugs. Hence, such commendable standardization activity to reduce dataset variability may risk introducing systematic errors in the experimental protocol assumed to be optimized. y. Therefore, it is essential that any standardization procedures for *in vitro* C*i*PA assays adopt experimental conditions that take into account, and attempt to correct, any possible intra and extra laboratory sources of experimental data variability. For example, the use of seeding, growing, and testing solutions used for CiPA *h*iPSC-CM assay containing uncharacterized factors (e.g., human or animal sera) should be circumvented, whenever possible, and replaced by well-defined, currently available, chemically homogeneous media (Denning, et al., 2015; Patel, et al., 2015). Indeed, the ongoing rapid improvements of experimental tools could quickly render obsolete prior systematic validation of the C*i*PA *h*iPSC-CM assay.

4.2 *Considerations for core CiPA assay components needing targeted attention*

Cardiac toxicity represents a leading cause of drug attrition along the nonclinical and clinical drug development paths (Ferri, Siegl, Corsini, Herrmann, Lerman, & Benghozi, 2013). Extensive ongoing international efforts are directed at validating the C*i*PA paradigm as a nonclinical drug safety strategy for the identification of candidate drugs with proarrhythmic potential. However, any strategy engineered to attain this objective is contingent, *inter alia*, on rigorous characterization of each crucial component of the experimental model. The core of this report highlights various pending issues for the *in vitro* C*i*PA assays that will require focused attention by the C*i*PA Steering Committee, C*i*PA Working Groups, and bench investigators striving to achieve the qualification of the C*i*PA paradigm.

4.2.1. In vitro ion channel assay

The mission of the ICWG can be considered successful only when it delivers optimized and validated automated patch clamp platform protocols providing, for each key cardiac current, easily generated metrics that, when fed into the *in silico* model (O'Hara, et al., 2011), can predict with confidence the degree of proarrhythmic danger of a candidate drug.

It is noteworthy that the adaptation of optimized manual patch protocols for I_{NaLate}, I_{CaL} and I_{Ks} to automated patch clamp platforms may pose specific problems if HTP patch clamp protocols have to be of simple execution, reasonably priced, and, at the same time, generate robust and reliable proarrhythmic metrics.

4.2.2. In vitro hiPSC-CM assay

The optimization of the *h*iPSC-CM assay appears to be particularly challenging due to thepending issues concerning biological material, and numerous assay variables that need to be locked down.

As emphasized earlier, all commercially available *h*iPSC-CM lines have not yet been scrupulously characterized as far as structural, electrophysiological, and pharmacological properties are concerned in relation to native human adult left ventricular CMs. These potential hurdles could be circumvented by clearly defining the advantages and limitations of any cell line selected for the C*i*PA *h*iPSC-CM assay and by interpreting the results obtained in this assay in the light of published evidence concerning 'abnormal' responses produced by certain drug classes in this biological material (Jonsson, Vos, Mirams, Duker, Sartipy, De Boer, et al., 2012; Kang, Chen, Ji, Lei, & Rampe, 2012). If warranted by the experimental results, the need of complementary assays to address unclear results should be considered and implemented.

A further issue with *h*iPSC-CMs concerns the selection of cell lines that are guaranteed by the producer to preserve constant pharmacological properties from batch to batch. This requires that the production process be strictly controlled to prevent any drift in the *h*iPSC-CM maturation status (currently a major preoccupation of manufacturers) for the C*i*PA CM assays. However, the latter limitation may be overcome if the improved *h*iPSC-CM cell lines are subjected to a novel standardized pharmacological validation. The C*i*PA Myocyte Working Group (MWG) is about to propose the use of a set of positive and negative reference drugs as a validation procedure for improved cell line.

In section *3.3*, additional issues with the *h*iPSC-CMs were discussed. In particular, experimental evidence has been presented (Fig. 2) indicating that the volume of the drug-containing solutions used to replace the drug-free well solution can markedly affect experimental results. Indeed, while 10 µL of a DMSO solution containing a large concentration of a *h*ERG blocking drug stopped FP generation, a volume of 150 µL giving the same final bath concentration of test article and DMSO had no effect. It should be emphasized that if small volumes containing large concentrations of a drug that binds irreversibly to key ion channel receptor sites are used to construct concentration-response curves in small volume wells, the drug dilution process will be unlikely to result in a rapid homogenous equilibrium between the bath solution drug levels and the drug receptor biophase. This is a

fundamental issue to be addressed for the construction of reliable and scientifically sound concentration-response curves. As illustrated in Fig. 2, failing to satisfy this requirement may result in classifying a drug substantially more potent than it is in reality. Another issue on test article evaluation concerns any drug candidate with reduced solubility in the testing solution. This also may lead to faulty estimations of drug potency.

4.2.3. In silico analysis of FP data generated in the hiPSC-CM assay

The analysis of experimental FP is often performed employing questionable criteria. For instance, certain investigators (Asakura, et al., 2015; Nakamura, et al., 2014) have overcome the differences in FP shapes measured from the various electrodes present within the experimental well by selecting 'good looking' FP shapes (e.g., golden FP) satisfying pre-established criteria for the calculation of parameters (e.g., FPA, FPD) necessary for the determination of the proarrhythmic potential of candidate drugs. This does not appear to be a robust scientific approach. Similarly, the FPD is often taken to reflect the QT interval of the ECG and thus, on this assumption, standardized at a reference beating rate [e.g., 60 contractions per minute as done for human ECG) to remove the influences of variable beating rates across preparations and cell lines (Asakura, et al., 2015; Harris, et al., 2013; Nakamura, et al., 2014; Navarrete, et al., 2013)]. However, this approach should be justified by experimental data since the spontaneous contractile rate of *h*iPSC-CMs is not only determined by the pacemaker driving the preparation but also by the FPD (it is recalled that beating rate is inversely proportional to FPD: the higher is the rate and the shorter is FPD and vice versa). Algorithms used for the rate-correction of the QT-interval of the ECG are not powered to handle the contribution of the repolarisation phase to the beating rate.

In silico simulations of FP can provide insights difficult to obtain in biological assays or traditional analytical approaches. For these purposes, the use of systems of differential equations necessary to model the time and spatial nature of FP signals provides a straightforward relationship between FP signals and AP profiles which can be readily used for the derivation of proarrhythmic-relevant parameters (e.g. APD_{50} and APD_{90}, triangulation). Simulation experiments do not support assumptions based on visual observation concerning the variation of FP shapes recorded with MEA technology (see Fig. 3.). Indeed, they indicate that the variability of the FP shapes recorded from different electrodes within the experimental well are not necessarily the reflection of the activity of different cell types entering into the formation of the *h*iPSC-CM monolayer. It is more likely that FP shape variability is due to the position of the electrodes within the experimental well in relation to the pacemaker cells driving the monolayer contractions, or to ground electrodes. Indeed, simulation conditions using homogeneous cell features within the monolayer show that the shapes of FPs recorded from spatially separated electrodes are not identical (Fig. 3). This supports the usefulness of simulation studies to test hypotheses and to generate insights on certain *in vitro* observations whereupon hypothesis may be based.

The use of simulation studies to facilitate the solution of biological problems is an opportunity offered by the current availability of potent computational tools. In line with this tenet, the C*i*PA paradigm has also incorporated such an approach for predicting the proarrhythmic risk of candidate drugs from *in vitro* ion channel datasets. The introduction of *in silico* simulations using FP and AP data generated in *h*iPSC-CM studies could be a logical complement to the C*i*PA simulation assay. Indeed, such a novel tool could be used to predict proarrhythmic risks and the possible mechanisms mediating such risks. Additionally, the results of this analysis could be compared to information provided by the computational reconstruction of the AP by using channel assay generated metrics. Since the *h*iPSC-CMs are, for the moment, an integrated humanoid experimental material, computational analysis of results obtained from its experimental use may have a greater human translational power than those from the cloned ion channel assays.

The above reflections might be considered too optimistic in view of the fact that the proposed *in silico* analysis of FP is based on a cell line-specific model of *h*iPSC-CMs (Paci, et al., 2013) and thus it may not be applicable to FP measures made from other cell lines. This judicious objection can be partly addressed by noting that any *in silico* model is built on data which do not incorporate the universal complexity of the biological system modelled. This is also the case for the O'Hara-Rudy model (O'Hara, et al., 2011) adopted for a core assay within the C*i*PA paradigm. Nevertheless, the proposed *in silico* simulations are likely to provide knowledge common to multiple *h*iPSC-CM cell lines. Moreover, future development of cell line-specific mathematical models (Bowler, et al., 2016)will help to further refine these models (Johnstone, Chang, Bardenet, De Boer, Gavaghan, Pathmanathan, et al., 2015).

4.3 *Good laboratory practices (GLP) for CiPA assays*

An issue mentioned in a previous report (Cavero & Holzgrefe, 2015) concerns the requirement of GLP application to any CiPA assays. The established policy for safety studies carried out for regulatory purposes is that they should be run in the observance of GLP principles. Therefore, it is a decision of regulatory authorities to wave such a requirement if this cannot be scrupulously applied to a given assay. Nonetheless, if this were this case, the sponsor would, in our opinion, be expected to justify the non-application of GLP and provide the best possible documentation concerning the possibility to accurately replicate an assay provided to regulatory authorities in support of the proarrhythmic safety of a candidate drug.

Since discussions are ongoing on the possible incorporation of all or part of the C*i*PA paradigm into the upcoming revision of the ICH S7B guideline, the issue of GLP for CiPA assays is likely to be matter of reflection at the level of the ICH organization.

4.4 *Discrepancies between the in silico and the hiPSC-CM assays*

If the results from the *in silico* model and the *in vitro h*iPSC-CM assay are in agreement, then a decision on successive development steps may be more straightforward. However, if the conclusions

derived from the two assays diverge, how can C*i*PA investigators clarify the discrepancy? This may be the case when the *in silico* (or the *h*iPSC-CM) assay results portend proarrhythmic liability but the *h*iPSC-CM assay (or the *in silico* assay) provide contradictory evidence. In this case, the application of the *in vivo* strategy recommended by the S7B (ICH, 2005a) could be invoked in an attempt to resolve the discrepancy. To this end, the C*i*PA Steering Committee should envisage complementing the current version of the C*i*PA *in vitro* and *in silico* strategy with a follow-up nonclinical *in vivo* assay as proposed by the S7B guideline (ICH, 2005a). Alternatively, data generated by using human primary cardiac tissues (Page et al., 2016) may be sought to provide a more relevant answer when the C*i*PA data package does not provide satisfactory proarrhythmic safety evidence concerning a therapeutically promising drug candidate.

4.5 *General conclusions*

Significant scientific advances often require the adoption of cutting-edge technologies, which initially do not operate at their intrinsic potential, but with time and hard work, are likely to fully satisfy envisaged expectations. This consideration applies to C*i*PA, an innovative Safety Pharmacology paradigm which is grounded on teachings from recent scientific advances and development of novel technologies. The approach adopted to enter C*i*PA into the Safety Pharmacology laboratory armamentarium diverges from the strategy followed by the ICH organization which previously recommended the blind adoption of harmonized strategies prepared by Expert Working groups (Cavero & Crumb, 2005). The latter consensus documents generally proposed the use of established experimental tools without subjecting them to a systematic validation for the envisaged purpose because they had been traditionally applied, but often not for specific Safety Pharmacology purposes. In this regard, the decision taken by the C*i*PA Steering Committee to qualify the C*i*PA safety tool prior to the official adoption should be considered revolutionary in nature. It should not be surprising that the path to achieve such a qualification goal has encountered and will encounter difficulties requiring solutions and reflection as they arise. This is also because the Safety Pharmacology community has no previous experience on how to accomplish a qualification process in a structured manner. If the establishment of C*i*PA, as a qualified Safety Pharmacology drug development strategy succeeds, it may become a reference to be followed by regulatory organizations, even for ICH guidelines calling for the use of traditional assays. It is recalled that the costs, supported by the pharmaceutical industry (and ultimately by patients), to optimize the S7B (ICH, 2005a) and E14 (ICH, 2005b) guideline assays have been unnecessarily excessive and required many years of trials and failures post official implementation to achieve the current status. As such, future ICH guidelines should be subjected to a formal validation process prior to their official adoption. The adoption of this proposal requires industrial and governmental support and resources as well as the nomination, within the ICH, of a Committee in charge of the qualification process for future experimental strategy proposed by any ICH Expert Working Group. Qualification is not a consensus between ICH partners concerning a strategy, but rather a process generating scientific evidence and

objective proofs that the proposed experimental approach is scientifically valid, robust and fit for the envisaged purpose.

Declaration of interest

The authors of this report state that the content of this manuscript exclusively reflects their personal opinion and that no financial assistance was provided to them for the conception and preparation of the manuscript.

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