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Evaluation of in vitro models of stem cell-derived cardiomyocytes to screen for potential cardiotoxicity of chemicals

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ABSTRACT

Cardiotoxicity is an important toxicological endpoint for chemical and drug safety assessment. The present study aims to evaluate two stemcell-based in vitro models for cardiotoxicity screening of chemicals. Eleven model compounds were used to evaluate responses of mouse embryonic stem cell-derived cardiomyocytes (mESC-CMs) using beating arrest as a readout and the analysis of electrophysiological parameters measured with a multi-electrode array (MEA) platform of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). Results revealed that the hiPSC-CM MEA assay responded to all compounds. The mESC-CM beating arrest assay was not responsive to potassium channel blockers and showed a lower sensitivity to sodium channel blockers and Na^+/K^+ ATPase inhibitors compared to the hiPSC-CM MEA assay. Calcium channel blockers and a β-adrenergic receptor agonist showed comparable potencies in both models. The in vitro response concentrations from hiPSC-CMs were highly concordant with human effective serum concentrations of potassium and sodium channel blockers. It is concluded that both in vitro models enable the cardiotoxicity screening with different applicability domains. The mESC-CM beating arrest assay may be used as a first step in a tiered approach while the hiPSC-CM MEA assay may be the best starting point for quantitative in vitro to in vivo extrapolations.

1. Introduction

Cardiotoxicity is considered as an important endpoint in the safety testing of chemicals and drugs. Many promising drug candidates are discontinued during the development because of undesired cardiotoxic effects. In addition, there is an increasing need for the evaluation of food-borne constituents like alkaloids and environmental pollutants that are associated with potential cardiotoxicity (Ainerua et al., 2020; Kratz et al., 2017; Pang et al., 2019; Stevens and Baker, 2009). For these reasons the development of new approaches that can quickly and reliably identify and characterize the cardiotoxicity of chemicals would be of a great value. Traditional laboratory animal studies are gradually considered as an inappropriate approach for cardiac safety assessment due to the fact that animal studies are costly, labour intensive and considered unethical (Pang et al., 2019). These considerations promote the development of new technologies where in vitro assays play an important role in characterizing the toxicity of chemicals (Bernauer et al., 2005). The present study aims to evaluate the potential applicability domain of two stem cell-based in vitro models to rapidly screen for the potential cardiotoxicity of chemicals.

Normal cardiac functioning requires cellular ion homeostasis in cardiomyocytes that is maintained by the concerted action of membrane ion channels and ion transporters (Priest and McDermott, 2015; Schwinger et al., 2003). Brief controlled changes in ionic homeostasis lead to changing inward and outward ion fluxes, generating action potentials that ultimately result in the contraction of cardiomyocytes

Abbreviations: AIC, Akaike's Information Criterion; AMP_{10}, concentration causing 10% reduction in sodium spike amplitude; BPM, beats per minute; BMC, benchmark concentration; BMD, benchmark dose; BR_{10}, concentration causing 10% increase in beating rate; DMSO, dimethyl sulfoxide; ECG, electrocardiogram; f_u, unbound fraction; FPD, field potential duration; FPDc, corrected field potential duration; $FPDc_{10}$, concentration inducing 10% prolongation of FPDc; -$FPDc_{10}$, concentration shortening FPDc by 10%; $hECG_{10}$, human plasma concentrations corresponding to 10% change in the ECG; hERG, human ether-a-go-go related gene; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; MEA, multi-electrode array; mESC-CMs, mouse embryonic stem cell-derived cardiomyocytes

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(Rougier and Abriel, 2016; Huang, 2016; Jeevaratnam et al., 2018). Sodium (Na^+) channels are the key drivers for inducing the depolarization of the cell membrane (DeMarco and Clancy, 2016) and calcium channels contribute to maintaining the plateau phase of action potentials (Bers and Perez-Reyes, 1999). Various types of potassium (K^+) channels are involved in different phases of repolarization (Priest and McDermott, 2015). In addition to ion channels, several enzymes and transporters such as Na^+ /K^+ ATPase also play critical roles in maintaining the ion homeostasis. Chemical-induced cardiotoxicity is often caused by the off-target interactions with these ion channels and transporters, resulting in aberrant electrophysiological function of cardiomyocytes (Priest and McDermott, 2015). Chemicals induce various types of adverse cardiac events, depending on the affected ion channels or transporters.

Up to date, several in vitro methods are being explored to screen for cardiotoxicity. These models range from reductionistic single ion channel binding studies to technological advanced patch clamp techniques that are essential for mechanistic studies. A conventional assay is to measure the inhibitory effect of compounds on individual ion channels. For this, transfected cell lines are used that allow a highly sensitive detection of binding to the target ion channel (Clements and Thomas, 2014). However, this approach fails to address the effects induced by drugs targeting multiple channels (Rehnelt et al., 2017), while also extrapolation to the in vivo situation from transfected cell lines may be dfficult given the differences in expression levels. Models that use the patch clamp technique are considered as the gold standard for detecting cardiotoxicity since it can accurately measure relevant electrophysiological parameters including single ion currents, action potential duration and peak amplitude (Rehnelt et al., 2017). Yet, the patch clamp technique is labour intensive, and the stability of the system is limited due to damage of the cell membrane (Laurila et al., 2016; Tertoolen et al., 2018). Recently, fluorescent imaging techniques have been applied to screen for potential cardiotoxicity of chemicals. For this, voltage-sensitive dyes are used to measure parameters which are comparable to those targeted by the patch clamp technique but without invasive measurement (Laurila et al., 2016). However, this approach is limited by the potential cytotoxicity of these dyes (Chang and Mummery, 2018).

In the past decade, stem cell-derived cardiomyocytes have been integrated as in vitro models in preclinical safety assessments (Pouton and Haynes, 2007; Denning and Anderson, 2008; Kettenhofen and Bohlen, 2008; Freund and Mummery, 2009). Stem cell-derived cardiomyocytes have first been obtained from mouse embryonic stem cells (Wobus et al., 1991; Maltsev et al., 1994). Mouse embryonic stem cell derived cardiomyocytes (mESC-CMs) express the major cardiac contractile proteins, ion channels and receptors (Abassi et al., 2012; Himmel, 2013), which allow them to serve as comprehensive models to detect the cardiotoxic effect of compounds which target multiple mechanisms. Functional beating cardiomyocytes are obtained easily from mouse embryonic stem cells by spontaneous differentiation, without the need of specific growth factors (Seiler and Spielmann, 2011; Kamelia et al., 2017). Moreover, Nicolas et al. (2015) reported that mESC-CMs can successfully detect in vitro cardiotoxicity of various ion channel blockers, by determining chemical-induced concentration-dependent cardiac beating arrest. This provides a robust and easy to use platform for the detection of cardiotoxicity. Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs) have shown their potential as the in vitro model for cardiotoxicity testing (Freund and Mummery, 2009). HiPSCs do not spontaneously differentiate into functional cardiomyocytes, but require more elaborate culturing techniques, including the application of growth factors in the medium (Lewandowski et al., 2017; Sala et al., 2017). HiPSC-CMs express the major cardiac ion channels, receptors, transporters and electrophysiological responses, known to be present in human cardiomyocytes (Ma et al., 2011; Karakikes et al., 2015; Chang and Mummery, 2018; Pourrier and Fedida, 2020). These hiPSC-CMs have often been applied in combination with the multi-electrode array (MEA) technique, which has proven to be a medium throughput and non-invasive approach for the detection of cardiotoxicity (Harris et al., 2013; Li et al., 2016; Nozaki et al., 2016; Kitaguchi et al., 2017; Ando et al., 2017). By measuring extracellular field potential for monolayers of cardiomyocytes grown on the chip, the MEA technique can characterize several electrophysiological parameters which specifically correspond to the specific phases of the in vivo electrocardiogram (ECG) and can thus be used to correlate the in vitro functional measurements to human in vivo clinical data (Halbach et al., 2003; Sala et al., 2017).

This study aimed to identify the applicability domain of two stem cell-based assays to screen for the potential cardiotoxicity of chemicals. For this we used the mESC-CMs with a simple readout (beating arrest) as a relatively high throughput and low-cost assay and compared it with the lower throughput and high-cost hiPSC-CM MEA assay. Eleven compounds with known mode-of-action of cardiac effects that target potassium channels, calcium channels, sodium channels, Na^+/K^+ ATPase and β-adrenergic receptor were tested in both models. The effect concentrations were compared to reported serum concentrations related to in vivo cardiotoxicity obtained from human studies.

2. Materials and methods

2.1. Chemical

Dofetilide (product #PZ0016, ≥98%), amiodarone hydrochloride (product #A8423, ≥98%), sematilide monohydrochloride monohydrate (product #S0323, ≥98%), moxifloxacin hydrochloride (product #SML1581, ≥98%), mexiletine hydrochloride (product #M2727, ≥98%), flecainide acetate salt (product #F6777, ≥98%), verapamil hydrochloride (product #V4629, ≥99%), nifedipine (product #N7634, ≥98%), digoxin (product #D6003, ≥95%), ouabain octahydrate (product #O3125, ≥97%) and isoproterenol hydrochloride (product #1351005, ≥98%) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Dimethyl sulfoxide (DMSO, > 99.7%) was obtained from Merck (Schiphol-Rijk, the Netherlands). All stock solutions and dilutions of test compounds were prepared in DMSO.

2.2. In vitro cardiotoxicity in the mESC-CM beating arrest assay

In the mESC-CM beating arrest assay the in vitro cardiotoxicity was characterised by quantifying the effect of test compounds on the beating of cardiomyocytes formed from the pluripotent mouse embryonic stem cell line D3 (ATCC, Wesel, Germany). The cells were cultured in HyClone AdvanceSTEM™ Low Osmo Dulbecco's Modified Eagle Medium (DMEM, Fischer Scientific, Landsmeer, the Netherlands) supplemented with 20% heat inactivated fetal bovine serum (FBS, ATCC, Manassas, USA), 50 U/ml penicillin (Invitrogen, the Netherlands), 50 μg/ml streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen). The cells were cultured at 37 °C with 5% CO_2 in a humidified atmosphere and subcultured three times per week. Non-enzymatic cell dissociation solution (Sigma-Aldrich) was used to detach cells and 1,000 U/ml murine leukemia inhibiting factor (mLIF, Sigma-Aldrich) was added to prevent spontaneous differentiation. Cells were grown in 25 cm^2 flask (Corning, Amsterdam, the Netherland) precoated with 0.1% m/v gelatine (Sigma-Aldrich).

To obtain beating cardiomyocytes, the differentiation process of cells was performed according to previously published protocols (Nicolas et al., 2015; Kamelia et al., 2017) with minor modifications. On day 0, 20 μl cell droplets containing 3.75×10^4 cells/ml were hung on the lid of 96-well plates (Greiner BioOne, Alphen a/d Rijn, the Netherlands). Phosphate buffered saline (PBS, Invitrogen) was added to all wells of the 96-well plate to provide humidity and prevent evaporation of the hanging drops. After 3 days incubation at 37 °C and 5% CO_2, the embryonic bodies formed were transferred to a 60 × 15 mm bacteriological petri dish (Greiner Bio-One) containing 5 ml medium

and incubated for 3 days. On day 5, embryonic bodies were transferred to 48-well plates (Greiner Bio-One) (one embryonic body/well). The 48-well plates were incubated at 37 °C and 5% CO_2 for another 5 days and the cardiomyocytes started beating from day 10 onwards. On day 11, contracting cardiomyocytes were treated with compounds to detect the cardiotoxicity. For each concentration of test compounds, ten wells containing beating cardiomyocytes (10 beating embryonic bodies; 1/well) were exposed and the number of wells containing beating embryonic bodies after one-hour incubation with test compound was counted by visual inspection under the microscope. After this visual inspection all wells were washed with fresh medium and incubated for one hour in medium without added test compounds to determine the recovery of beating in the cardiomyocytes. 0.25% DMSO was used as solvent control.

2.3. In vitro cardiotoxicity in the hiPSC-CM MEA assay

The MEA technology of Multi Channel System (MCS GmbH, Ruetlingen, Germany) was used to assess the field potentials generated by hiPSC-CMs (Pluricyte® Cardiomyocytes) obtained from Ncardia (Leiden, the Netherlands). The cells were prepared according to the manufacturer's protocol. Briefly, cells were thawed in the incubator at 37 °C for exactly 4 min and gently transferred to a 50 ml tube. The vial was rinsed with 1 ml serum free Pluricyte® Cardiomyocyte Medium (Ncardia) added drop-wise to the tube containing the cardiomyocytes. Then an additional 5 ml medium were added drop-wise to the tube. 20 µl of the homogenous cell suspension thus obtained were taken for manual cell counting using a Buerker-Tuerk Counting Chamber (Marienfeld Superior GmbH & Co. KG, Lauda-Königshofen, Germany). At the same time cells were centrifuged at 300*g* for 3 min. Then the supernatant was removed and medium was drop-wisely added to reach the aimed concentration of cells in the suspension (2×10^4 cells/2 µl). Cells were placed on the 6-well MEA chips (60-6well MEA200/30iR-Ti-tcr) from the Multi Channel System (MCS GmbH) at the concentration of 2×10^4 cells/2 µl/well. Each well was precoated with fibronectin (Sigma-Aldrich) before seeding. MEA chips were incubated at 37 °C with 5% CO_2 and refreshed with medium every 2 days.

Electrically coupled monolayers of hiPSC-CMs with spontaneous beating behaviour can be obtained 7–8 days post-seeding. MEA chips containing the hiPSC-CMs were placed on the headstage of a MEA2100-System (MCS GmbH) for signal selection. Only the wells with a signal showing clearly visible depolarization and repolarization peaks were selected for further assessment (Sala et al., 2017). As indicated in Fig. 1, a typical extracellular field potential waveform consists of a rapid upstroke corresponding to depolarization, a slow wave/plateau and a repolarization peak. Prior to the measurement, MEA chips containing the cells were equilibrated for at least 20 min in the chamber of the MEA system which provided a stable atmosphere at 37 °C with 5% CO_2. Then, cells were exposed to increasing concentrations of the model compounds in a cumulative manner as follows: after an equilibration period, DMSO (0.2%) was added into the well by replacing half of original medium to reach a final concentration of 0.1% DMSO. Then test compound was cumulatively added to the well with increasing concentrations in the same way. Including the baseline condition (0.1% DMSO), seven concentrations of each compound were tested. Test compounds were diluted from stock solutions into medium to reach the aimed final concentrations. The final concentration of DMSO in exposure medium was kept at 0.1%. At each concentration of test compounds, the extracellular field potential was recorded for 1 min after 10 min exposure. Data were collected using Cardio 2D software (MCS GmbH) with a sample frequency of 10 kHZ and a 0.1–3.5 kHz band-pass filter.

2.4. Data analysis

In the mESC-CM beating arrest assay, the cardiotoxicity of model compounds was presented as the percentage of wells containing beating cells compared to the solvent control condition for which the response was set at 100%. The concentration-response curves for amiodarone, sematilide, verapamil, digoxin and ouabain were obtained from our previous study (Nicolas et al., 2015). Concentrations with a recovery less than 50% (1 µM verapamil, 1 µM nifedipine and 600 µM ouabain, data not shown) were excluded from concentration-response curves obtained from the mESC-CM beating arrest assay to minimize the risk that arrest was elicited by general cytotoxicity instead of the reversible interaction with cardiac ion channels. Data represent the mean of at least three independent experiments.

MEA data were analysed using Multiwell-Analyzer software Version 1.5.1.0 (MCS GmbH). Only the electrodes showing a stable field potential trace (Fig. 1) were selected for analysis. The following parameters were measured as the average of at least 30 beats from one-minute recording of each concentration of the compounds (Fig. 1): sodium spike amplitude (defined as the absolute amplitude of the depolarization peak), field potential duration (FPD, defined as the duration between the beginning of the sodium spike and the repolarizing peak) and RR-interval (the duration between two depolarization peaks). To correct the effect of beat rate on FPD, the clinically used Fridericia 's formula was applied (Vandenberk et al., 2016), which is commonly used in cardiotoxicity-related studies (Ando et al., 2017; Kitaguchi et al., 2017):

$$FPDc = \frac{FPD}{\sqrt[3]{RR\ interval}} \quad (1)$$

In this formula the FPD and RR-interval were expressed in seconds. Beat per minute (BPM) was derived from RR-intervals, being the duration between two depolarization peaks:

$$BPM = \frac{60}{RR\ interval} \quad (2)$$

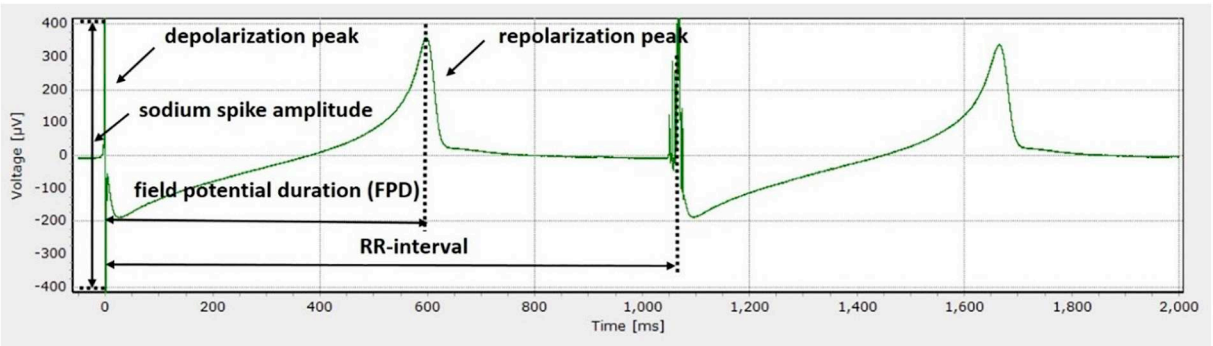

Fig. 1. Typical waveforms of the extracellular field potential signal generated by human cardiomyocytes derived from human induced pluripotent stem cells cultured in 6-well MEA-chips.

Table 1
Summary of in vitro and in vivo effect data of model compounds used in the present study.

Compounds	Main mode of action	mESC-CM beating arrest assay		hiPSC-CM MEA assay					f_u in human plasma	Human clinical data		
		BMC_{10}	95% CI	endpoint	BMC_{10}	95% CI	arrhythmia-like waveform	beating cessation		Clinical effect on human ECG	$hECG_{10}$ [unbound]	95% CI [unbound]
Dofetilide	(h)ERG potassium channel blocker	–		$FPDc_{10}$	0.86 nM	0.39–1.5 nM	3 nM: 1/4 wells; 10 nM: 2/4 wells	10 nM:2/4 wells 30 nM:4/4 wells	0.35 [a]	prolonged QTc interval	5.3 [1.9] nM [h, o] [1.3] nM [a]	4.4–7.3 [1.5–2.6] nM [1.2–1.4] nM
Amiodarone	(h)ERG potassium channel blocker	–		$FPDc_{10}$	1.6 μM	0.13–9.2 μM	> 30 μM	3 μM: 1/6 wells 10 μM: 2/6 wells 30 μM: 3/6 wells	0.0002 [a]	prolonged QTc interval	164.8 [0.033] nM [i, o]	67.1–313 [0.013–0.063] nM
Sematilide	(h)ERG potassium channel blocker	–		$FPDc_{10}$	0.69 μM	0.36–1.1 μM	1 μM: 1/4 wells 3 μM: 1/4 wells	3 μM: 1/4 wells 10 μM: 4/4 wells	0.96 [b]	prolonged QTc interval	[3.6] μM [a, p]	not available
Moxifloxacin	(h)ERG potassium channel blocker	–		$FPDc_{10}$	6.5 μM	3.1–11.3 μM	30 μM: 1/3 wells 100 μM: 3/3 wells	> 100 μM	0.5 [c]	prolonged QTc interval	63.8 [31.9] μM [j, o]	44.2–152.0 [22.1–76.0] μM
Mexiletine	Na^+ channel blocker	85.4 μM	70.2–122.0 μM	AMP_{10}	0.89 μM	0.45–1.70 μM	> 30 μM	10 μM: 1/4 wells; 30 μM: 3/3 wells	0.37 [d]	QRS change	[1.63] μM (120 bpm) [k]	[1.6–1.7] μM
Flecainide	Na^+ channel blocker	13.4 μM	10.6–18.5 μM	AMP_{10}	0.12 μM	0.045–0.26 μM	> 3 μM	0.3 μM: 1/7 wells 1 μM: 2/7 wells 3 μM: 6/7 wells	0.39 [d]	QRS change	[0.22] μM (70 bpm) [l] [0.36] μM [m] [0.29] μM [n]	[0.22–0.23] μM [0.3–0.4] μM [0.28–0.32] μM
Verapamil	Ca^{2+} channel blocker	68.9 nM	46.4–80.1 nM	$-FPDc_{10}$	4.4 nM	0.52–15.3 nM	> 3 μM	1 μM: 1/4 wells 3 μM: 4/4 wells	0.1 [a]	shortened QTc interval/prolonged PR interval	not available	
Nifedipine	Ca^{2+} channel blocker	5.9 nM	4.3–7.7 nM	$-FPDc_{10}$	13.4 nM	10.1–25.5 nM	> 3 μM	> 3 μM	0.04 [a]	shorten QTc interval/ prolong PR interval	not available	
Digoxin	Na^+/K^+-ATPase inhibitor	–		$-FPDc_{10}$	0.24 μM	0.19–0.31 μM	> 3 μM	1 μM: 1/4 wells 3 μM: 4/4 wells	0.75 [e]	shorten QTc interval	not available	
Ouabain	Na^+/K^+-ATPase inhibitor	170.5 μM	160.0–189.0 μM	$-FPDc_{10}$	0.14 μM	0.047–0.25 μM	> 1 μM	1 μM: 3/3 wells	0.92 [f]	shorten QTc interval	not available	
Isoproterenol	adrenergic receptor agonist	2.3 nM	1.6–3.3 nM	BR_{10}	5.0 nM	0.19–34.0 nM	> 30 μM	> 30 μM	0.12–0.14 [g]	increase heart beating rate	not available	

FPDc, corrected field potential duration; $FPDc_{10}$, concentration inducing 10% prolongation on FPDc; AMP_{10}, concentration reducing 10% sodium spike amplitude; $-FPDc_{10}$, concentration shortening 10% of FPDc; BR_{10}, concentration increasing 10% beating rate; NA, not available. [a] Redfern et al., 2003; [b] Hinderling et al., 1993; [c] Kramer et al., 2013; [d] Lombardo et al., 2002; [e] Baggoi and Davis, 1973; [f] Kramer et al., 1970; [g] Kelly and McDevitt, 1978; [h] Allen et al., 2000; [i] Debbas et al., 1983; [j] Démolis et al., 2000; [k] Sadanaga et al., 1993; [l] Heath et al., 2011; [m] Tennezé et al., 2002; [n] Shimizu et al., 2000; [o] calculated based on f_u from literature; [p] induce a 10%–20% change in QTc.

The RR-interval was expressed in seconds. Concentrations that induced arrhythmia-like changes in the waveform and/or beating arrests were excluded from analysis of these parameters since the FPD, RR-interval and sodium spike could not be determined (Kitaguchi et al., 2016; Zwartsen et al., 2019). Data were collected from at least three independent experiments (3–7 wells, 11–37 electrodes). Results are expressed as relative percentage compared to the results obtained for the baseline control (0.1% DMSO). The response of baseline control was set at 100%. The target ion channels or receptors and relevant endpoints of the compounds are summarized in Table 1.

The benchmark dose (BMD) approach was applied on the in vitro concentration-response curves obtained from both assays to derive the benchmark concentrations. A 10% change in the readouts (beating arrest for mESC-CMs and electrical activity for hiPSC-CMs) was used as the benchmark response to calculate the benchmark concentration (BMC_{10}) for cardiotoxicity with lower-upper 95% confidence interval. As the model compounds target different ion channels or receptors, and thus cause different electrophysiological effects in the hiPSC-CM MEA assay, BMC_{10} values were expressed in a mode-of-action specific way (see Results).

BMD analysis was performed using the European Food Safety Authority (EFSA) web-tool[2] for BMD analysis based on the R-package PROAST version 66.40 developed by the Dutch National Institute for Public Health and the Environment (RIVM). Model selection and model fitting was performed according to the flow-chart described in the manual provided by EFSA[1]. Briefly, the quantal data obtained from the mESC-CM beating arrest assay were fitted using the available quantal models including (Log)-logistic, (Log)-probit, Weibull, Gamma, two-stage, Exponential and Hill model. The continuous data from the hiPSC-CM MEA assay were fitted to a set of models including Exponential, Hill, Inverse Exponential model and Log-Normal Family. Analysis was performed according to the flow-chart described in the manual[1]. All fitted models excluding FULL and NULL were used for model averaging described in Wheeler and Bailer (2007) where a weighted average model was constructed to estimate model averaged confidence intervals using bootstrap sampling. Weighting was based the model's Akaike's Information Criterion (AIC) values where models with lower AIC values count with larger weight. The final BMC confidence intervals from model averaging were based on 200 bootstrap data sets. The final BMC_{10} values were obtained by averaging the model-specific BMC estimates by the following equation as described by Buckland et al. (1997), Bailer et al. (2005) and Wheeler and Bailer (2007):

$$\widehat{BMC} = \sum_{k=1}^{K} BMC_k * \omega_k \quad (3)$$

where BMC_k is estimated based on the accepted model k and ω_k represents the corresponding weight for the model k. Detailed information on the BMD analysis of in vitro data can be found in the supplementary materials (Table S1-S17).

The concentration response curves obtained from both in vitro assays were plotted with Graph Pad Prism 5.0 (GraphPad Software Inc., San Diego, USA). Each data point is presented as the mean value ± standard error of the mean (SEM). Statistical significance was analysed by one-way ANOVA followed by post Dunnett test. Values of $p < .05$ were regarded as statistically significant. Statistical analysis was performed by Graph Pad Prism 5.0 (GraphPad Software Inc.).

2.5. Comparison of in vitro and in vivo human cardiotoxicity

To further evaluate the sensitivity of the hiPSC-CM MEA assay the in vitro response concentrations were compared with reported internal effect concentrations related to human clinical ECG data. For this, the in vitro BMC_{10} concentrations were compared with unbound human plasma concentrations corresponding to 10% change on ECG ($hECG_{10}$). In vivo human data are especially available for the endpoint of the prolongation of the QT interval defined as the prolonged duration between the beginning of ventricular repolarization (QRS complex) and the end of depolarization (T wave) in the ECG, and the change of the QRS complex. Potassium channel blockers increased the in vitro FPDc in the hiPSC-CM MEA assay which can be seen as the surrogate for the QT interval in the ECG (Halbach et al., 2003; Zwartsen et al., 2019). The effect of sodium channel blockers on the sodium spike amplitude in the hiPSC-CM MEA assay was correlated to the change of the QRS complex in the human ECG.

Effective concentrations derived from the hiPSC-CM MEA assay are considered as unbound concentrations due to usage of serum free medium in this assay (Harris et al., 2013). Human ECG data were obtained from published literature (Table 1) where the concentration-response curves were extracted from graphs using GetData Graph Digitizer 2.26[3] to calculate the $hECG_{10}$. A zero-effect was included in the dataset, assuming a no effect at a zero compound concentration in serum (in vivo). Obtained $hECG_{10}$ values were derived from the ECGs using BMD analysis as described for the in vitro data for continuous data. Detailed information on the BMD analysis of in vivo data can be found in the supplementary materials (Table S18-S25). The unbound $hECG_{10}$ values were directly taken from literature when reported or were calculated by multiplying $hECG_{10}$ values with unbound fraction (f_u). The fractions unbound were taken from literature (see Table 1).

3. Results

3.1. Screening for cardiotoxic effects using the mESC-CM beating arrest assay

Dofetilide, amiodarone and sematilide are class III antiarrhythmic agents which inhibit the repaid delayed rectifying potassium current through the (human) Ether-a-go-go Related Gene (ERG) potassium channel. Moxifloxacin is known as an antibiotic but blocks the (h)ERG potassium channel as side effect. These four (h)ERG potassium channel blockers did not significantly inhibit the beating of mESC-CMs within the tested concentration ranges (data are shown in Fig. S1A-D in the supplementary data). Fig. 2A-B show that the sodium channel blockers, mexiletine and flecainide inhibited the beating of the mESC-CMs in a concentration-dependent manner and induced maximum inhibition at 1000 μM and 300 μM, respectively. This resulted in a BMC_{10} value of 85.4 μM for mexiletine and 13.4 μM for flecainide (Table 1). The calcium channel blockers verapamil and nifedipine significantly inhibited the beating of mESC-CMs from 0.1 μM and 0.01 μM onwards (Fig. 2C-D). The BMC_{10} value derived from the mESC-CM beating arrest assay for verapamil was 68.9 nM while nifedipine was more potent with a BMC_{10} of 5.9 nM (Table 1). Digoxin and ouabain are cardiac glycosides that disturb the intracellular Na^+ and K^+ ion balance by inhibiting the Na^+/K^+ ATPase on the membrane of cardiomyocytes (Guo et al., 2013). As depicted in Fig. 2E, no inhibitory effect of digoxin on mESC-CMs was found within the tested concentration range. However, ouabain significantly inhibited the beating of mESC-CMs with a BMC_{10} of 170.5 μM (Fig. 2F, Table 1). The β-adrenergic receptor agonist isoproterenol that is used as an antiarrhythmic drug. Fig. 2G shows that isoproterenol inhibited the beating of cardiomyocytes in a concentration-dependent manner with a BMC_{10} of 2.3 nM .

3.2. Screening for cardiotoxic effects using the hiPSC-CM MEA assay

The (h)ERG potassium channel blockers dofetilide, amiodarone, sematilide and moxifloxacin significantly prolonged the FPDc in a

[2] EFSA Statistical Models-BMD. [Online]. Available at: https://shiny-efsa.openanalytics.eu/app/bmd [Accessed August 1, 2019]

[3] Available at: http://getdata-graph-digitizer.com[Accessed May 30, 2019]

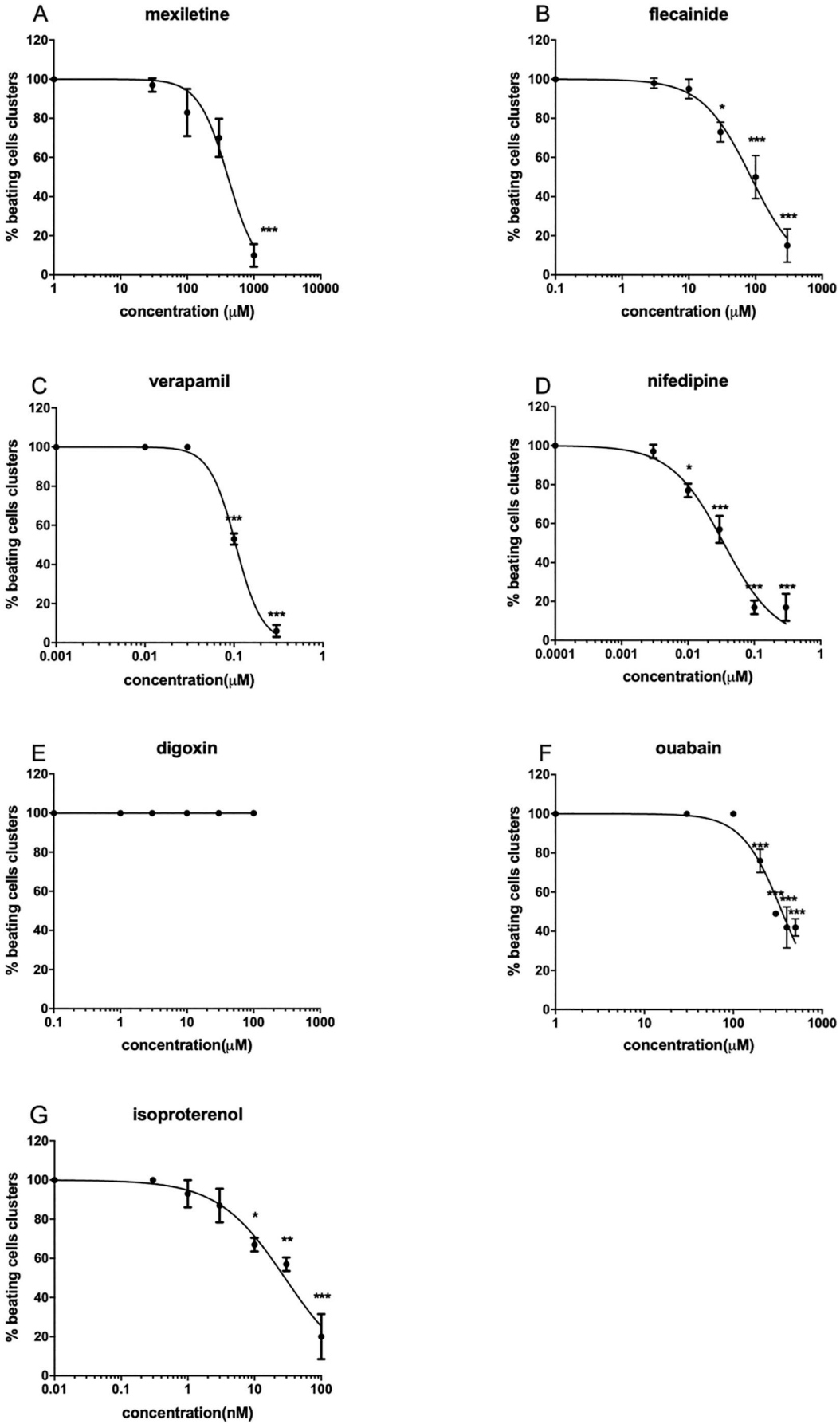

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Fig. 2. Concentration-response curves for cardiotoxicity in mESC-CMs of the sodium channel blockers mexiletine (A) and flecainide (B), the calcium channel blockers verapamil (C) and nifedipine (D), the Na^+/K^+ ATPase inhibitors digoxin (E) and ouabain (F), and the β-adrenergic receptor agonist isoproterenol (G). The response of the solvent control (DMSO) was set at 100%. mESC-CMs data represent the mean of at least three independent experiments. Each data point represents the mean ± SEM. Statistically significant changes compared to the solvent control are marked with * with $p < .05$: *, $p < .01$: ** and $p < .001$: ***.

concentration-dependent manner and induced 10% prolongation of the FPDc ($FPDc_{10}$) at 0.86 nM, 1.6 μM, 0.69 μM and 6.5 μM, respectively (Fig. 3A-D, Table 1). The results presented in Table 1 also reveal that both arrhythmia-like waveforms and the cessation of beating were observed upon treatment of the hiPSC-CM with dofetilide (at 3 nM and 10 nM, respectively) and sematilide (at 1 μM and 3 μM, respectively). Amiodarone caused beating arrest at the highest test concentration of 30 μM without inducing arrhythmia-like waveforms, while moxifloxacin induced arrhythmia-like waveforms from 30 μM onwards but did not induce beating cessation within the tested concentration range (Table 1).

Two sodium channel blockers mexiletine and flecainide induced a 10% reduction of the amplitude (AMP_{10}) at 0.89 μM for mexiletine and 0.12 μM for flecainide (Fig. 3E-F, Table 1). Neither mexiletine nor flecainide induced arrhythmia-like waveforms within the tested concentration ranges while the cessation of beating was observed in most wells at the highest concentrations of mexiletine and flecainide (Table 1).

The calcium channel blockers, verapamil and nifedipine shortened the FPDc in a concentration-dependent manner (Fig. 3G-H). A 10% shortening of the FPDc (-$FPDc_{10}$) was observed at a concentration of 4.4 nM for verapamil, and of 13.4 nM for nifedipine (Table 1). Verapamil did not induce arrhythmia-like waveforms up to 3 μM where complete cessation occurred (Table 1). In contrast, nifedipine was not associated with the arrhythmia or beating arrest within the tested concentration range (up to 3 μM).

Fig. 3I and J show that the Na^+/K^+ ATPase inhibitors digoxin and ouabain significantly shortened the FPDc in the hiPSC-CM MEA assay with the maximum reduction occurring at comparable concentrations of 1 μM and 0.3 μM, respectively. Arrhythmia-like waveforms were not observed during the exposure to digoxin and ouabain while complete beating cessation occurred at the highest tested concentrations of both compounds (Table 1). The $FPDc_{10}$ was 0.24 μM for digoxin and 0.14 μM for ouabain (Table 1).

The antiarrhythmic drug isoproterenol increased the beating rate in a concentration-dependent manner with the concentration causing 10% increase in beating rate (BR_{10}) amounting to 5.0 nM (Fig. 3K and Table 1). No arrhythmia-like waveforms or beating arrest of hiPSC-CMs was observed up to the highest isoproterenol concentration tested (30 μM).

3.3. Comparison of in vitro and in vivo human cardiotoxicity

Based on the obtained results, the hiPSC-CM MEA assay shows a higher sensitivity and broader compound coverage than the mESC-CM beating arrest assay. Therefore, we next evaluated whether the hiPSC-CM MEA assay provides adequate data to predict human in vivo responses, by comparing the in vitro $FPDc_{10}$ and AMP_{10} values for the compounds that induced concentration-dependent changes in these parameters in the hiPSC-CMs, with the unbound $hECG_{10}$ derived from the related change of waveforms in the ECG from clinical studies (Fig. 4). All available human ECG data are reported in the supplementary file (Table S26).

Table 1 and Fig. 4 illustrate the comparison for the four (h)ERG potassium channel blockers between their in vitro $FPDc_{10}$ values and the in vivo unbound hEC_{10} values, being the unbound plasma concentrations that would prolong the QTc interval by 10%. The $FPDc_{10}$ of dofetilide derived from the MEA assay was comparable with its reported unbound $hECG_{10}$ showing 1.5- to 2.2-fold differences and the $FPDc_{10}$ of sematilide was 5-fold lower than the $hECG_{10}$ value derived from the in vivo data (Table 1). For moxifloxacin, the unbound $hECG_{10}$ (31.9 μM) was 5-fold higher than its $FPDc_{10}$ (Table 1), also indicating limited in vitro-in vivo differences. In contrast, the in vitro data for amiodarone were far out of range, resulting in an $FPDc_{10}$ value (1.6 μM) that was five orders of magnitude higher than the unbound $hECG_{10}$ (0.033 nM).

The in vitro-in vivo comparison for the sodium channel blockers mexiletine and flecainide is also shown in Fig. 4 and Table 1. The comparison reveals that the AMP_{10} values obtained in the hiPSC-CM MEA assay were comparable to the unbound $hECG_{10}$ of mexiletine (1.6 μM) (1.8-fold difference) and the range of the unbound $hECG_{10}$ values reported for flecainide (0.22–0.36 μM) derived from the clinical studies (1.8- to 3- fold difference), indicating an adequate match between in vitro and in vivo human effect concentrations.

For the calcium channel blockers verapamil and nifedipine, the Na^+/K^+ ATPase inhibitors digoxin and ouabain and the adrenergic receptor antagonist isoproterenol, no adequate human data were available for a comparison between the in vitro data and the in vivo situation.

4. Discussion

The present study aimed to evaluate use of the mESC-CM beating arrest assay and of the hiPSC-CM MEA assay to screen for the potential cardiotoxicity of chemicals. To evaluate these two models, the effects of eleven model compounds were quantified in both in vitro assays. The in vitro effect concentrations of the hiPSC-CM MEA assay were compared with reported internal effect concentrations related to human clinical ECG data. Based on the obtained results it was concluded that the hiPSC-CM MEA assay is the most versatile assay as it is responsive to all evaluated compounds with a higher sensitivity for (h)ERG potassium and sodium channel blockers and Na^+/K^+ ATPase inhibitors, with the mESC-CM beating arrest assay being not responsive to (h)ERG potassium channel blockers and to one of the Na^+/K^+ ATPase inhibitors. Furthermore, two calcium channel blockers and isoproterenol showed comparable potencies in the two assays. The in vitro effective concentrations obtained from the hiPSC-CM MEA assay correlated well with available in vivo effective concentrations related to human ECG data for (h)ERG potassium and sodium channel blockers.

Given that mESC-CMs are easy to obtain without ethical problems and the mESC-CM beating arrest assay is cost-friendly and easy, requiring less operator skills, the use of the mESC-CM beating arrest assay could be considered as a good first-choice candidate for cardiotoxicity screening. However, from the results obtained in the mESC-CMs, none of the (h)ERG potassium channel blockers induced beating cessation of mESC-CMs within the tested concentration ranges. Corroborating this, similar results (no inhibitory effect) have been found in other studies in which dofetilide and E4031, a typical (h)ERG potassium channel blocker, were both unable to induce the cessation of beating in mESC-CMs (Jonsson et al., 2011; Abassi et al., 2012; Himmel, 2013). No literature data are available on the sodium channel blockers mexiletine and flecainide on mESC-CMs to benchmark our observations. Thus, no comparison could be made. The sodium channel blocker diphenhydramine was reported to induce cessation of beating in mESC-CMs (Nicolas et al., 2015), which is in line with our results showing that sodium channel blockers are active in the mESC-CM beating arrest assay. The calcium channel blockers verapamil and nifedipine showed concentration-dependent inhibition of beating, which corroborates results from the study of Himmel (2013) where both compounds induced beating cessation. Digoxin did not induce a response in the mESC-CMs

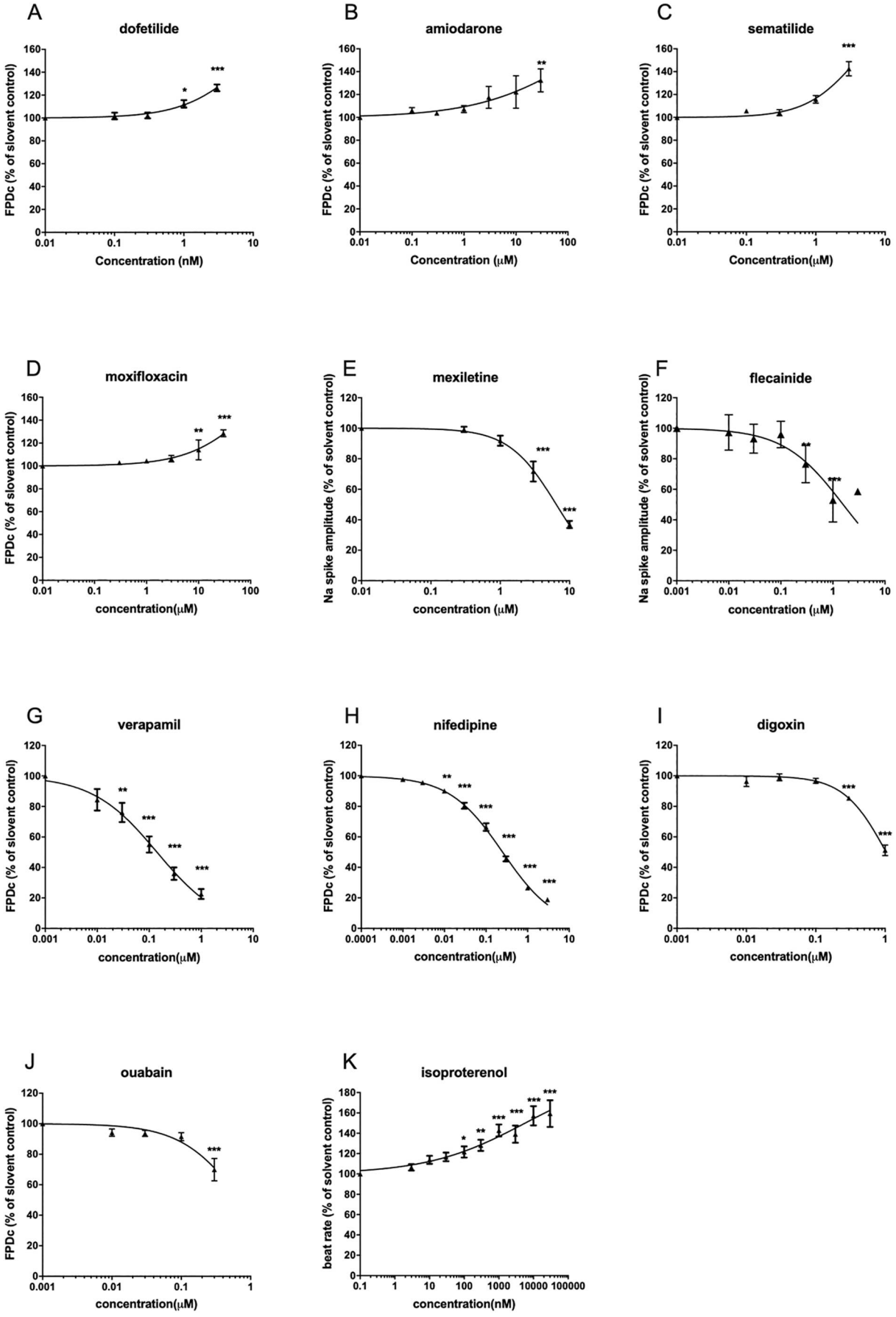

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Fig. 3. Concentration-response curves for cardiotoxicity in hiPSC-CMs of the (h)ERG potassium channel blockers dofetilide (A), amiodarone (B), sematilide (C), and moxifloxacin (D), the sodium channel blockers mexiletine (E), and flecainide (F), the calcium channel blockers verapamil (G) and nifedipine (H), the Na^+/K^+ ATPase inhibitors digoxin (I) and ouabain (J), and the β-adrenergic receptor agonist isoproterenol (K). The response of the solvent control (DMSO) was set at 100%. hiPSC-CMs data represent the mean of at least three independent experiments with minimum of eleven electrodes. Each data point represents the mean ± SEM (3 μM flecainide is an exception as hiPSC-CMs stopped beating with the exposure of 3 μM flecainide and detectable Na spike amplitude was obtained from 1 out of 7 wells). Statistically significant changes compared to the solvent control are marked with * with $p < .05$: *, $p < .01$: ** and $p < .001$: ***.

while ouabain inhibited the beating of mESC-CMs at relatively high concentrations (> 100 μM), which is in line with the study of Himmel (2013) where ouabain failed to induce beating arrest in mESC-CMs at concentration from 0.03 to 3 μM. Isoproterenol was used as a model compound for β-adrenergic receptor agonists and regulates the cardiac pacemaker action potentials by activating hyperpolarization activated pacemaker channels, further resulting in an increased beating rate (Bers, 2002; Nozaki et al., 2017). An inhibitory effect of isoproterenol on mESC-CMs was noted in the present study at the concentration of 1 nM and higher. However, Ikeuchi et al. (2015) did not observe the beating cessation in mESC-CMs up to 1 μM. A possible reason for the inconsistency between the studies could be related to the various types of cardiac cells in embryonic bodies, resulting in different expression patterns of hyperpolarization activated pacemaker channels. It is reported that these channels are highly expressed in the sinoatrial node cells but low in normal atrial and ventricular cardiomyocytes (Baruscotti et al., 2010; Sartiani et al., 2011).

The hiPSC-CM MEA assay provides insight into the real-time electrophysiological response of compounds in hiPSC-CMs (Li et al., 2016). Clearly it is a sensitive and frequently used platform that allows a detection of cardiotoxicity (see supplementary Table S27; Harris et al., 2013; Nozaki et al., 2016; Nozaki et al., 2017; Kitaguchi et al., 2017; Ando et al., 2017). Most studies have focused on the compounds that target the (h)ERG potassium channels and the current study provides a more comprehensive evaluation of compounds that target other main ion channels and receptors. Our MEA data indicate that all model compounds induced concentration-dependent effects on hiPSC-CMs with the BMC_{10} values being in accordance with published MEA data showing 1.2- to 5.7-fold differences. In addition, we report concentrations that induce arrhythmia-like waveforms and beating cessation in the same range as obtained from the literature with a maximum 3-fold difference (see references in Table S27). Such differences are within the range of inter-laboratory variability of 1.8- to 20-fold reported by Kitaguchi et al. (2016), Nozaki et al. (2016) and Tamargo et al. (2004).

Both the mESC-CM beating arrest assay and the hiPSC-CM MEA assay can be considered to be functional models to detect cardiotoxicity. However, differences in sensitivity are observed between the two models. The mESC-CM beating arrest assay was not responsive to (h)ERG potassium channel blockers and the Na^+/K^+ ATPase inhibitor digoxin. The hiPSC-CM MEA assay appeared able to detect the effects of all model compounds. Compared with BMC_{10} values from the mESC-CMs, the BMC_{10} obtained from hiPSC-CMs were almost two orders of magnitude lower for the sodium channel blockers and three orders of magnitude lower for the Na^+/K^+ ATPase inhibitor ouabain. Two calcium channel blockers and isoproterenol showed comparable potencies in the two assays.

Given the differences obtained between the two assays it is of interest to consider that there are several factors that could explain the distinct sensitivity of the mESC-CM beating arrest assay and the hiPSC-CM MEA assay. Although hiPSC-CMs and mESC-CMs express the typical cardiac channels, the expression level and function of these channels are known to be species dependent (Maltsev et al., 1994; Nerbonne, 2004; Jonsson et al., 2011). In the case of potassium channels, the repaid and slow delayed rectifier potassium currents are two predominant currents involved in action potential repolarization in human ventricular cardiomyocytes (Li et al., 1996), while in mouse cardiomyocytes the other three subtypes of delayed rectifier currents (the fast activating and slowly inactivating and steady state current) mainly regulate the repolarization (Xu et al., 1999; Zhou et al., 2003). Thus, all tested (h)ERG potassium channel blockers are only partly involved in

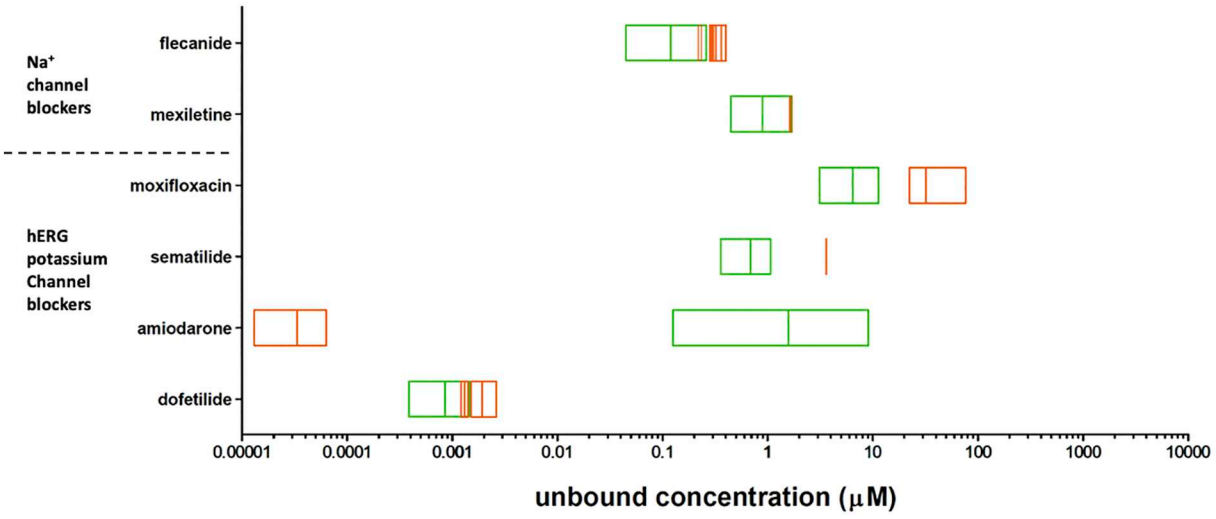

Fig. 4. Comparison between in vitro $FPDc_{10}$ and AMP_{10} values obtained in the hiPSC-CM MEA assay and in vivo unbound plasma $hECG_{10}$ values. The comparison between in vitro and in vivo data was made for four (h)ERG potassium channel blockers (dofetilide, amiodarone, sematilide and moxifloxacin) and two sodium channel blockers (mexiletine and flecainide). Green boxes represent the range between the lower and upper bound of $FPDc_{10}$ and AMP_{10} from the hiPSC-CM MEA assay, giving the obtained $FPDc_{10}$ and AMP_{10} values as the vertical line in between. Orange boxes represent the range between the lower and upper bound of unbound $hECG_{10}$ derived from human ECG data available in the literature (Table 1), giving the obtained unbound $hECG_{10}$ values as the vertical line in between. $FPDc_{10}$ and AMP_{10} derived from the hiPSC-CM MEA assay are considered equal to unbound concentrations due to the usage of serum free medium. The unbound $hECG_{10}$ values were determined from concentrations from studies that reported the unbound concentrations in serum or calculated by multiplying the $hECG_{10}$ determined from concentrations from studies that reported serum concentrations by the f_u in human plasma. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the repolarization of the action potential in mouse cardiomyocytes, providing a possible explanation for the lower sensitivity of mESC-CMs towards the cardiotoxicity of compounds acting as potassium channel blockers. Furthermore, the maturity of ion channels may also contribute to the differences in sensitivity between the two assays. It has been shown that sodium channels are well-developed at intermediate stage of post-differentiation (15 days after culturing) (Maltsev et al., 1994). While the exposure was performed earlier in our experiments (on day 11), which may have added to the lower sensitivity because of potentially sodium channels present in the mESC-CMs.

In addition, the differentiation level of stem cells appears to be different in the two in vitro models. The hiPSC-CMs protocol results in high purity ventricular cardiomyocytes while a combination of diverse cell types with less than 5% cardiomyocytes are present in the mESC-CMs (Kolossov et al., 2005). This can explain the less extended applicability domain of the mESC-CM beating arrest assay compared to the hiPSC-CM MEA assay. Such variation in the type of cells present upon differentiation of the stem cells could influence the diffusion of compounds to their targets in the cell models as compounds were supposed to have a better diffusion in the monolayer of hiPSC-CMs (Harris et al., 2013), which may result in an apparent lower sensitivity of the mESC-CM beating arrest assay. To add, the lower sensitivity of the mESC-CM beating arrest assay may be in part related to the serum that is present in the exposure medium of the mESC-CMs but not in the hiPSC-CMs medium, potentially reducing the fraction unbound of test compounds. However, given the f_u values for binding of test compounds to serum protein (Table 1) this could not fully explain the orders of magnitude difference in sensitivity observed. Lastly, the differences in sensitivity could be due to the endpoint that is used in the mESC-CMs. Beating arrest can be regarded as a late cardiac event that follows the initial early markers of cardiotoxicity detected by the MEA-related endpoints. For mESC-CMs inactive compounds, including the (h)ERG potassium channel blockers and Na^+/K^+ ATPase inhibitors, alteration of contraction frequency might reflect their potential effects better than beating cessation (Himmel, 2013; Ikeuchi et al., 2015). However, scoring contraction frequency instead of beating arrest as endpoint studied will clearly make the assay labour intensive, thereby removing one of its advantages as a simple and cheap assay. Considering the sensitivity and practical characteriscs, both in vitro models can be used for screening cardiotoxicity. The mESC-CM beating arrest assay could be used as a first step in a tiered approach as a first screen for cardiotoxicity. Negative responding chemicals can be further evaluated in the hiPSC-CM MEA assay as a second tier to exclude cardiotoxicity for humans. Furthermore, positive chemicals in the mESC-CM beating arrest assay, can be further tested in the hiPSC-CM MEA assay to provide relevant human mechanistic data.

Finally, the hiPSC-CM MEA assay showed a high sensitivity to the effects of the chemicals. Therefore, the obtained effective concentrations were compared to internal effect concentrations related to human clinical ECG data. Given that mice show differences in the response doses, duration and certain morphology features of action potentials compared to humans (Danik et al., 2002; Edvardsson et al., 1984; Huang, 2016; Kaese and Verheule, 2012), such a direct comparison was not made for the murine data. Both the in vitro $FPDc_{10}$ and AMP_{10} values derived from the hiPSC-CM MEA assay matched well with the corresponding unbound $hECG_{10}$ values derived from human ECG data. Remarkably, the $hECG_{10}$ of amiodarone was five orders of magnitude lower than the in vitro $FPDc_{10}$. The extent to what this discrepancy is related to its high lipophilicity that results in high levels of protein binding in different matrices with f_u values amounting to values as low as 0.0002 (Redfern et al., 2003; Ando et al., 2017), and/or to other reasons underlying the discrepancy between the in vitro and in vivo situation for amiodarone remain open for further studies. Combining the in vitro cardiotoxicity data with so-called physiologically based kinetic modelling will facilitate incorporation of such factors in making ultimate quantitative in vitro to in vivo extrapolations based predictions.

The present study evaluated the sensitivity of mouse (mESC-CMs) and human (hiPSC-CMs) stem cell-derived in vitro models to screen for the potential cardiotoxicity of chemicals. The hiPSC-CM MEA assay showed a higher sensitivity for (h)ERG channel potassium and sodium channel blockers and Na^+/K^+ ATPase inhibitors while the mESC-CM beating arrest assay appeared to be not responsive to (h)ERG potassium channel blockers and the Na^+/K^+ ATPase inhibitor digoxin. The two models showed comparable sensitivity to calcium channel blockers and a β-adrenergic receptor agonist. Comparison of in vitro responses with available human clinical data revealed that effect concentrations obtained in the hiPSC-CM MEA assay were highly concordant with reported human in vivo effective concentrations of potassium and sodium channel blockers. In conclusion, both in vitro models can be considered as functional models to detect cardiotoxicity with different applicability domains. Given its ease of handling the mESC-CM beating arrest assay may be used as a first step in a tiered approach to screen the cardiotoxicity. While negative compounds could be further tested in the hiPSC-CM MEA assay as a second tier to quantify the cardiotoxicity of compounds and reflect human in vivo cardiotoxicity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2020.104891.

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