Cardiotoxicity screening: a review of rapid-throughput in vitro approaches

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Abstract Cardiac toxicity represents one of the leading causes of drug failure along different stages of drug development. Multiple very successful pharmaceuticals had to be pulled from the market or labeled with strict usage warnings due to adverse cardiac effects. In order to protect clinical trial participants and patients, the International Conference on Harmonization published guidelines to recommend that all new drugs to be tested preclinically for hERG ($K_v11.1$) channel sensitivity before submitting for regulatory reviews. However, extensive studies have demonstrated that measurement of hERG activity has limitations due to the multiple molecular targets of drug compound through which it may mitigate or abolish a potential arrhythmia, and therefore, a model measuring multiple ion channel effects is likely to be more predictive. Several phenotypic rapid-throughput methods have been developed to predict the potential cardiac toxic compounds in the early stages of drug development using embryonic stem cells- or

human induced pluripotent stem cell-derived cardiomyocytes. These rapid-throughput methods include microelectrode array-based field potential assay, impedance-based or Ca^{2+} dynamics-based cardiomyocytes contractility assays. This review aims to discuss advantages and limitations of these phenotypic assays for cardiac toxicity assessment.

Keywords Cardiotoxicity · Rapid throughput · hERG · Automated patch clamp · Microelectrode array · Fluorescence plate reader

Abbreviations

AP	Action potential
APC	Automated patch clamping
ESC	Embryonic stem cell
FPD	Field potential duration
FLIPR	Fluorescent imaging plate reader
iPSC	Induced pluripotent stem cell
IC ₅₀	Half-maximal inhibitory concentration
K _v	Voltage-gated potassium channels
LQTS	Long-QT syndrome
MEA	Microelectrode array
Na _v	Voltage-gated sodium channels
RTCA	The real-time cell analyzer
TdP	Torsade de pointes

Introduction

Cardiotoxicity is a leading cause for drug failure

Cardiotoxicity is one of the leading causes for failure, during drug development as well as after a compound has been approved for therapy. Between 1990 and 2013, a total of 81 drugs had to be taken off of the market in the USA, Europe,

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and Asia, due to treatment-emergent effects, with 16 of the compounds producing cardiac arrhythmias. Moreover, since 1997, cardiotoxicity-related withdrawal has dramatically increased from 5.1 to 33.3 % (Table 1—MacDonald and Robertson 2009; Qureshi et al. 2011). This includes compounds seeking to rectify cardiovascular problems such as the antiarrhythmic agent encainide (Apostolakis et al. 2013) or the hypertensive mibefradil (Huang et al. 2015), but also drugs with entirely different indication, for example the antihistamine terfenadine (Braam and Mumery 2010; Hondeghem et al. 2011; Sirenko et al. 2014) or the gastroprokinetic cisapride (Sirenko et al. 2014; Wilhelms et al. 2012). In some cases, the drugs were not withdrawn, but amended with cautionary notes informing

about possible adverse cardiac effects, as was the case with the antidiabetic rosiglitazone, where a meta-analysis had revealed increased incidence of myocardial infarction and death (Sager et al. 2015). While still available in the US market, rosiglitazone is now under strict scrutiny, in that it can only be prescribed by certified doctors and purchased through special pharmacies, which led to a dramatic drop in prescriptions in 2012, amounting to only ~0.4 % of what had been seen in 2006.

Early-stage drug discovery commonly uses mechanism-based, high-throughput screening, which allows candidate identification from compound libraries comprising >100,000 different molecules. Stringent toxicity analyses follow, but 20–50 % of all advanced candidates have to be

Table 1 Drugs withdrawn due to arrhythmia (1990–2010)

Drug name	Usage	Year withdrawn	Remarks	References
Encainide (Enkaid)	Antiarrhythmia	1991 (US, UK)	QT prolongation ^a	Echt et al. (1991)
Terodiline (Micturin)	Antispasm	1991 (Germany, UK)	QT prolongation, torsade de pointes (TdP) ^b	Thomas et al. (1995)
Dexfenfluramine (Redux)	Antiobesity	1997 (US, EU, UK)	QT prolongation, tachycardia, arrhythmia	Weissman et al. (1998)
Terfenadine (Seldane/Tri-ludan)	Antihistamine	1997 (US, France)	QT prolongation, ventricular tachycardia	Roy et al. (1996)
Mibefradil (Posicor)	Antihypertension	1998 (US, EU)	QT prolongation, fatal arrhythmia	Bezprozvanny and Tsien (1995)
Sertindole (Serdolect/Ser-lect)	Antipsychotic	1998 (EU)	QT prolongation, risk of TdP	Karamatskos et al. (2012)
Astemizole (Hismanal)	Antihistamine	1999 (US)	QT prolongation, risk of TdP	Zhou et al. (1999)
Grepafloxacin (Raxar)	Antibacterial	1999 (Worldwide)	QT prolongation	Roy et al. (1996)
Cisapride (Propulsid)	Gastroprokinetic agent	2000 (US)	QT prolongation, risk of TdP	Durdagi et al. (2014)
Sparfloxacin (Spacin/Zagam)	Antibacterial	2001 (US)	QT prolongation and photo-toxicity	Agarwal et al. (2014)
Levomethadylacetate (OrLAAM)	Treatment of opioid dependence	2003 (US)	QT prolongation, cardiac arrest, risk of TdP	Hamilton et al. (2013)
Thioridazine (Mellaril/Melleril)	Antipsychotic	2005 (Germany, UK)	QT prolongation	Fenton et al. (2007), Fornaro et al. (2002), Keks et al. (1994)
Clobutinol (Clobutinol)	Cough suppressant	2007 (Germany)	Ventricular arrhythmia, QT prolongation	Bellocq et al. (2004)
Propoxyphene (Darvocet/Darvon)	Analgesic	2010 (Worldwide)	QT prolongation, elevated risk of MI and stroke	Becquemont et al. (2014), Butola and Rajagopal (2015)
Rosiglitazone (Avandia)	Antidiabetic drug	2010 (EU)	Cardiac arrhythmias, risk of MI and death	Chen et al. (2012), Nissen and Wolski (2007)
Sibutramine (Reductil/Meridia)	Anorexiant	2010 (Worldwide)	QT prolongation, risk of MI and stroke	Abe et al. (2015), Hassan and El-Gharib (2015), Hayes et al. (2015), Khorassani et al. (2015)

MI myocardial infarction

^a Refers to an abnormally long-QT interval in electrocardiographic analyses, indicating abnormalities in ventricular

^b Ventricular tachycardia associated with QT prolongation with characteristic waxing and waning of the QRS amplitude de-/repolarization

abandoned due to unwanted effects, even in the late stages of drug development (Kola and Landis 2004). Only 1 in 5000–10,000 screened chemicals reaches the market, with an associated cost of \$1–2 billion for development and a 12- to 15-year commitment (Boverhof and Zacharewski 2006). The associated economic risk can be significantly reduced if compounds with therapeutic but toxic properties are recognized early. Since cardiotoxicity is the leading cause for drug failure, emphasizing screens for cardiac side effects is especially helpful (Brana and Tabernero 2010; Doherty et al. 2015; Du et al. 2011; Stoelzle et al. 2011; Su et al. 2011).

The role of *hERG* in cardiotoxicity screening

Cardiac contraction is initiated by the cardiac action potential (AP), which involves the harmonious cooperation of several distinct ion channels (Fig. 1). As in neurons, voltage-gated sodium (Na_v) channels stand at the beginning of all activity: they allow for influx of Na^+ ions (I_{Na}), which is responsible for the initial, rapid depolarization (phase 0). The change in the membrane potential opens voltage-gated potassium channels carrying a transient outward current (I_{to}) that begins to repolarize the cardiomyocyte (phase 1). However, opening of L-type Ca^{2+} channels (I_{Ca}) and rapid as well as slow delayed rectifier K^+ channels (I_{Kr} , I_{Ks}) temporarily plateau repolarization (phase 2), which is only overcome once additional rectifier K^+ channels open (rapid delayed, slow delayed, and inward, mediating I_{Kr} , I_{Ks} , and I_{K1} , respectively) that fully repolarize the cell (phase 3—Abriel et al. 2015). The *human Ether-à-go-go-related gene* (*hERG*, official gene name *KCNH2*, which encodes the protein $\text{K}_{v11.1}$) mediates the repolarizing I_{Kr} current in the cardiac AP during phase 2 and phase 3. Loss of *hERG* channel function by pharmacological inhibition or genetic mutation has been shown to delay the AP repolarization and widen the ventricular AP, which is the underlying physiological abnormality in long-QT syndrome (Fig. 1a), a disorder associated with syncope, seizures, and even sudden death (Curran et al. 1995; Danker and Moller 2014; Priest et al. 2008; Wible et al. 2005). A great number of previously withdrawn drugs (Table 1) have been found to induce arrhythmia through *hERG* channel blockade. Interestingly, many of these are small molecules carrying hydrophobic rings and a hydrogen bond acceptor that are critical for interaction with *hERG* (Villoutreix and Taboureau 2015). Ligand-based *hERG* channel modeling furthermore revealed three major factors that elevate a compound's likelihood of blocking *hERG*, which include (1) a positively charged nitrogen atom, (2) high lipophilicity, and (3) the absence of negatively charged oxygen atom (Doddareddy et al. 2010; Villoutreix and Taboureau 2015). These interaction characteristics are tightly connected to

Fig. 1 Schematic alignment of a whole-heart electrocardiogram (ECG) and action potential of a cardiac myocyte. **a** Schematic presentation of a normal ECG trace. Ventricular myocyte depolarization correlates with the start of the QRS complex, whereas repolarization is reflected by the T wave (Bridal et al. 2010). The QT interval (healthy: 350–440 ms) refers to the time from the beginning of the QRS complex to the end of the T wave. Genetic predisposition or drug administration can delay the repolarization phase of the action potential, which reflects as prolongation of the QT interval to unhealthy levels (commonly > 500 ms), hence the name “long-QT syndrome”. **b** Schematic presentation of an action potential of a cardiac myocyte. The rapid entry of Na^+ through voltage-gated sodium channels (I_{Na}) is responsible for rapid, Phase 0, depolarization. The opening of transient potassium channels (I_{to}) mediates rapid, phase 1, repolarization, which is followed by the opening of L-type calcium channels (I_{Ca}) and slow delayed rectifier potassium channels (I_{Ks}) that are responsible for subsequent action potential plateau (phase 2). The opening of the potassium channels, including those mediating the rapid delayed rectifier current (I_{Kr}), slow delayed rectifier current (I_{Ks}), and the inward rectifier K^+ current (I_{K1}), ultimately repolarizes the cardiomyocyte during phase 3. The final phase of the action potential signals a return of the membrane potential to its baseline value near -90 mV (phase 4)

structure: *hERG* channels are homotetramers of four identical α -subunits, each comprising six α -helical transmembrane segments (S1–S6). Voltage sensitivity is mediated by the voltage sensor domain (S1–S4), more specifically positively charged lysine and arginine in S4 that move in response to the membrane potential change. Segments S5

and S6 form the pore that allows K^+ ions cross to lipid bilayer. A lengthy S5-P linker that contains an amphipathic helix (the turret helix) and a selectivity filter loop is also present in these segments. Both the N-terminus and the C-terminus locate to the intracellular side (Stansfeld et al. 2007). Unlike most other K_v channels, hERG channels lack two kinking proline residues that restrict the channel's aqueous volume. The consequently larger hERG channel cavity is more accommodating, allowing comparatively large drugs to enter and interfere with K^+ conduction (Mitcheson 2003; Recanatini et al. 2005). In addition, unlike most voltage-gated K_v channels where the inner helices are lined by aliphatic isoleucine or valine residues, hERG channels have two aromatic residues (Tyr652 and Phe656 in the S6 domain) which are important for the binding of cisapride (Chen et al. 2002; Mitcheson et al. 2000), terfenadine (Mitcheson et al. 2000), quinidine (Lees-Miller et al. 2000; Sanchez-Chapula et al. 2003), and chloroquine (Sanchez-Chapula et al. 2002). The sensitivity for blocking hERG channel activity resulting from Phe656 binding was linearly correlated with the hydrophobicity of the structure (Fernandez et al. 2004). In addition to Tyr652 and Phe656, a number of other amino acid residues, such as Ile647 in the S6 segment (quinidine, E-4031 and terfenadine—Ishii et al. 2001) as well as Ser620 (dofetilide—Ficker et al. 1998), Thr623, Ser624, and Val625 in the P helix (vesnarinone—Kamiya et al. 2001), have been identified as critical for drug binding. Rather than directly inhibiting hERG, some drugs were also found to alter hERG trafficking (Dennis et al. 2012; Villoutreix and Taboureau 2015). The combination of broadly distributed binding sites and the susceptibility to trafficking modulation explains why many structurally diverse small molecules can affect the hERG channel activity and trigger long-QT syndrome.

The challenge in screening for adverse cardiac effects is that the most informative screening methods use animal or ex vivo modeling, which is distinctly low throughput and costly, and it requires large compound quantities. It is, therefore, desirable to identify approaches that combine strong prediction power for cardiotoxicity with experimental convenience. To that end, hERG screening offers itself, owing to the channel's tight connection to pharmacologically induced long-QT syndrome and *torsades de pointes* (or simply, “torsade”) (Curran et al. 1995; Danker and Moller 2014; Priest et al. 2008; Wible et al. 2005). Based on the 2002 recommendation by the International Conference on Harmonization (Danker and Moller 2014; Doherty et al. 2015), hERG testing has been adopted for all new drugs prior to regulatory review (Dorn et al. 2005; Giacomini et al. 2015; Guthrie et al. 2005; Huang et al. 2010), which must include direct (i.e., current modulation) as well as indirect (e.g., trafficking) effects on hERG channel function (Wible et al. 2005). To that end, it is useful to

first employ in silico analyses that may help identify possible problem molecules; several Web sites offer these services, including Pred-hERG (<http://labmol.farmaciaci.ufg.br/predherg>), Tox-Comp.net (<http://www.toxcomp.net/>), and ACD-/I-Lab (<https://ilab.acdlabs.com/iLab2/index.php>) (Villoutreix and Taboureau 2015). In addition, chemogenomics databases such as ChEMBL (Bento et al. 2014), PubChem (Wang et al. 2014), and ChemProt (Kim Kjaerulff et al. 2013) can help narrow the compound candidate list based on chemical similarity to known hERG interacting agents. There are also target prediction Web services such as the SEA search tool (Keiser et al. 2007), PASS (Filz et al. 2008), Swiss Target Prediction (Gfeller et al. 2014), HitPick (Liu et al. 2013), SuperPred (Nickel et al. 2014), and admetSAR (Cheng et al. 2012; Villoutreix and Taboureau 2015). There is, hence, an abundance of resources that can first-level filtering for hERG channel interaction.

Of course, while hERG screening is an efficient way of identifying potential for cardiac adverse effects, it is not wholly predictive. One preclinical analysis found that only 55 % of confirmed hERG inhibitors ($IC_{20} \leq 3 \mu M$) prolonged the cardiac action potential by more than 10 % (Lu et al. 2008). Similarly, lack of hERG activity is no certain indicator of cardiac tolerability. The α -1 blocker alfuzosin, for example, has little effect on hERG, but it delays cardiac repolarization through increased sodium channel activity, which results in long-QT syndrome (Navarrete et al. 2013). The need for more reliable, rapid-throughput prediction of cardiotoxicity has prompted the development of several new in vitro technologies to help detect adverse cardiac effects in the early stages of the drug development through the use of automated planar patch clamping, microelectrode arrays (MEA), real-time cell analyzers (RTCAs), and fluorescence-based screens (Benay et al. 2015; Elkins et al. 2013; Numann and Negulescu 2001; Su et al. 2011). In this review, we summarize these new methods for cardiotoxicity testing and discuss their advantages and disadvantages.

Non-hERG drug targets in cardiac electrophysiology

Cardiotoxicity is frequently related to abnormal excitation of the heart, which, viewed broadly, is the result of neuronal input, cardiac conduction (involving the sinoatrial/atrioventricular nodes and the bundle branches), and cardiomyocyte contraction. As a self-excitatory organ with autonomous rhythmic activity, the heart does not rely on external input to maintain its basic functionality, which may explain why agents affecting neuronal input and cardiac conduction are less likely to precipitate clinical issues. Significant problems arise, however, when (left) ventricular contraction is compromised, as this has direct impact on systemic blood delivery (Cardinale et al. 2013; Keating and Sanguinetti 2001; Zaniboni et al. 2000).

Under certain circumstances, cardiac excitation can become abnormal, which can lead to arrhythmias. Regardless of whether the heart beat is abnormally slow or fast (i.e., bradycardia and tachycardia, respectively), syncope and death are possible outcomes (Baudenbacher et al. 2008; Huikuri et al. 2001, 2009; Keating and Sanguinetti 2001). Because the ventricles provide the mechanics that move the blood through the system, ventricular arrhythmias are clinically particularly problematic, examples being the aforementioned torsade and ventricular fibrillation (Keating and Sanguinetti 2001; Maeno et al. 2009; Simpson et al. 2009). Epidemiological studies have revealed that gain or loss of function of different ion channels can alter cardiac action potential properties and contribute to arrhythmia susceptibility (Beattie et al. 2013; Burashnikov and Antzelevitch 2003; Cheng et al. 2003; Frea et al. 2015; Kurtzswald-Josefson et al. 2014; London et al. 2007; McNair et al. 2004; Medeiros-Domingo et al. 2007; Nabel 2003; Pfeufer et al. 2005; Schmitt et al. 2014; Splawski et al. 2004; Steffensen et al. 2015; Tester et al. 2005). Heritable dysfunction of the voltage-gated sodium (Na_v) channel $\text{Na}_v1.5$ (gene name: *SCN5A*), for example, has been associated with long-QT (LQT3) and Brugada syndrome (Giudicessi and Ackerman 2013; Miller et al. 2014). Voltage-gated potassium (K_v) channels are also involved; loss of K_v channel conductance results in action potential prolongation and long-QT syndrome, while K_v channel gain-of-function gives rise to an abbreviated cardiac action potential and short-QT syndrome (Schmitt et al. 2014), and modulation of aforementioned hERG channels, of course, is a primary cause for cardiac drug complications. As carriers of I_{Kr} , which mediates cardiomyocyte repolarization (Priest et al. 2008), the hERG channel, mores specifically its block, has strong ties to long-QT syndrome, ventricular arrhythmia, and sudden cardiac death (Sanguinetti and Tristani-Firouzi 2006). In addition to Na_v and K_v channels, gain-of-function mutations on $\text{Ca}_v1.2$ (calcium channel subtype 1.2) have been found to produce Timothy syndrome (Splawski et al. 2004, 2005). RyR2 point mutation-related aberrant diastolic Ca^{2+} leak from sarcoplasmic reticulum contributes to the formation of delayed after-depolarization, leading to heart failure and catecholaminergic polymorphic ventricular tachycardia (Blayney and Lai 2009). Clearly, ion channels of the heart's electrophysiological apparatus constitute potential targets in cardiotoxicity, which provides the basis for many in vitro methods attempting to predict cardiac drug toxicity.

Rapid-throughput cardiotoxicity screening methods

Fluorescence-based hERG assays

The hERG channel carries the rapid delayed rectifier K^+ current, which is a common player in abnormalities of

the QT interval and in ventricular arrhythmia (Sanguinetti and Tristani-Firouzi 2006). As previously discussed, drug-induced inhibition of hERG has good prediction value for cardiotoxicity. Several assays take advantage of this correlation, providing indirect information about hERG targeting through radioligand binding (Cheng et al. 2003) and Rb^+ efflux (Ding et al. 2006), or by directly assessing a substance's impact on hERG current using automated patch clamping (Bridgland-Taylor et al. 2006). More recently, another in vitro rapid-throughput method was added to this list, which exploits the hERG channel thallium (Tl^+) conductance (Xia et al. 2011). Cells are loaded with a Tl^+ -sensitive dye. Upon hERG channel opening, Tl^+ permeates down its concentration gradient into the cell, where it interacts with the dye to generate a fluorescent signal. A reduction in Tl^+ fluorescence suggests hERG blockade (Huang et al. 2010; Xia et al. 2011). Because the same assay is amenable to other K^+ channels, it has been successfully used to identify modulators of $\text{K}_v7.2$, $\text{K}_v7.3$, Kir2.1 (Beacham et al. 2010), $\text{K}_v7.4$ (Li et al. 2011), and Ca^{2+} -activated K^+ channels (Jorgensen et al. 2013). The major advantage of this approach is its experimental convenience, permitting use of 96-, 384-, and even 1536-well formats, as well as its relatively low running cost.

To validate whether Tl^+ influx is a reliable assay for predicting hERG channel blockade, experiments were set up, side-by-side, to compare each assay's sensitivity for hERG modulation, including [^3H]-dofetilide binding, Tl^+ influx using FLIPR™ technology, and automated patch clamping (Huang et al. 2010). The study showed that, on average, the IC_{50} values generated from automated patch clamping were markedly lower, reaching only 1/5_{th} and 1/17_{th} of the values produced with Tl^+ influx and [^3H]-dofetilide binding, respectively (Huang et al. 2010; Priest et al. 2008). Some of the tested compounds (e.g., ouabain, phenytoin, venlafaxine, and amantadine) produced false-negative data in Tl^+ influx assay, while false-positive data were absent (Huang et al. 2010). Data from Tl^+ influx, therefore, must be interpreted with caution. The assay has its advantages, in that it is cost-effective and rapid, generating up to 50,000 data points per day (where one data point informs on the effect of one compound at a single concentration; more data points per day, therefore, equal higher throughput). However, it cannot reliably predict hERG inhibition, especially for those compounds at low potency, which limits its utility to the elimination of compounds with relatively strong hERG inhibition during the early stages of drug development.

Another option to assess hERG modulation is the deployment of voltage-sensitive, fluorescent dyes. The underlying principle is that K^+ channel opening can lead to hyperpolarization. Potassium channel inhibition, conversely, pushes the cell in the depolarizing direction; all of

which can be visualized with voltage-sensitive dyes such as the FLIPR membrane potential dye (FMP). Cells stably transfected with hERG and loaded with FMP showed that this technology requires compound concentrations 5 times higher than the detection limit established in automated patch clamping, which translates into a significant risk for false negatives (~12 % in this study—Dorn et al. 2005). The advantages and disadvantages of fluorescent dye technology, therefore, are similar to what has been discussed above for Ti^+ influx analyses: low cost point and rapid data generation (again up to 50,000 data points/day), albeit with the limitation that only comparatively potent hERG inhibitors are detected. Because of its screening power and its well-defined shortcomings, voltage-sensitive fluorescent dye assays have been adopted as a standard in the *Comprehensive In Vitro Proarrhythmia Assay* (CIPA) pilot study organized by the ILSI *Health and Environmental Sciences Institute* (Sager et al. 2014). It should be emphasized that Ti^+ permeability and membrane potential changes are highly unspecific changes that must be assessed under heterologous expression (e.g., in HEK or CHO cells) to ensure that the observed effect is not mediated through a non-hERG target (Cao et al. 2014). This brings with it the concerns common for analyses employing heterologous

expression. The biophysical properties of hERG behavior in these systems are highly similar, but not identical, to what is seen endogenously. Deactivation of hERG channels, for example, is significantly faster when they are assessed in their natural cardiomyocyte environment (Sanguinetti and Tristani-Firouzi 2006), which may further impact the prediction reliability of these assays (Table 2).

Automated patch clamping

While hERG channel blockade obviously plays a pivotal role in unwanted cardiac events, various other channels contribute to the cardiac action potential, and their modulation is similarly prone to trigger long-QT syndrome (Beattie et al. 2013; Liu and Trudeau 2015; Medeiros-Domingo et al. 2007; Moric-Janiszewska et al. 2004; Schmitt et al. 2014; Zhang et al. 2012). The gold standard in measuring the compound's effect on ion channel behavior is manual patch clamping (Hamill et al. 1981). This technique, while extremely sensitive, is also immensely time-consuming, labor-intensive, and technically challenging, which limits its usefulness for rapid-throughput cardiotoxicity screening. The last decade has seen a proliferation of platforms that automate patch clamping, and the technology has made

Table 2 Technical summary of assays

Assay type ^a	Platform	Cell type	Well format	Sampling rate	Consumable cost ^b	Subacute measurement
<i>Target-based assay (hERG)</i>						
Ti^+ influx ^c	FLIPR	Stable hERG lines	96, 384, 1536	8 Hz	Clear-bottom, black well 96 or 384-well plate (~\$5/plate)	No
hERG channel current ^d	APC	Stable hERG lines	96, 384	10 kHz	PatchPlate (~\$400/plate)	No
<i>Phenotypic assays</i>						
Action potential ^e	APC	ESC-CM, iPSC-CM	96, 384	10 kHz	PatchPlate (~\$400/plate)	No
Field potential (FP) ^f	MEA	ESC-CM, iPSC-CM	12, 48	50 kHz	MEA plate (~\$500/plate)	Yes
Impedance ^g	RTCA	ESC-CM, iPSC-CM	96	80 Hz	E-plate (~\$500/plate)	Yes
Ca^{2+} dynamics ^h	FLIPR	iPSC-CM	96, 384, 1536	8 Hz	Clear-bottom, black well 96 or 384-plate (~\$5/plate)	No

^a Cardiomyocytes-based, unless indicated otherwise (e.g., stably expressing lines using HEK or CHO cells)

^b Bulk ordering may reduce the running cost of different systems. Abbreviations as defined in the text

^c Notes: Predicts LQT based on hERG channel inhibition with 78 % accuracy. Less sensitive than APC (Bridal et al. 2010; Huang et al. 2010; Xia et al. 2011)

^d Notes: Predicts LQT based on hERG channel inhibition with 73 % accuracy (Beacham et al. 2010; Danker and Moller 2014; Jorgensen et al. 2013; Li et al. 2011; Ly et al. 2007; Wible et al. 2005; Zeng et al. 2008)

^e Notes: Recorded in current-clamp mode. Altered APD from multiple mechanisms (Becker et al. 2013; Ma et al. 2011)

^f Notes: Predicts LQT with 100 % accuracy based on FPD prolongation; Noninvasive, medium throughput (Asakura et al. 2015; Clements and Thomas 2014; Fendyur and Spira 2012; Harris et al. 2013; Navarrete et al. 2013; Pradhapan et al. 2013)

^g Notes: Predicts cardiotoxicity with 84 % accuracy based on beating irregularities. Noninvasive, medium throughput (Doherty et al. 2015; Guo et al. 2011; Himmel 2013; Nguemo et al. 2012; Pan et al. 2015; Wang et al. 2013; Xi et al. 2011)

^h Notes: Predicts cardiotoxicity with 96 % accuracy based on the alternation of Ca^{2+} dynamics. Dye loading may affect the electrophysiological properties of the cardiomyocytes (Sirenko et al. 2014)

impressive advances since its introduction. So far, however, the hands of a skilled experimenter still give the conventional approach a decisive edge over automated patch clamping, which provides unparalleled sensitivity and data reliability. Yet, industrial as well as academic laboratories have recognized the value of automated patch clamping which drives the development over ever-more sophisticated, rapid-throughput systems that through the use of automated cell handling and drug dispensing can now generate up to 10,000 data points per day (Dunlop et al. 2008; Elkins et al. 2013; Farre and Fertig 2014; Farre et al. 2009; Stoelzle et al. 2011). Essential for the successful deployment of these machines is their ability to establish good cell–electrode contact, because ionic current behave much like water: It will find the path of lowest resistance. Consequently, if the connection between the pipette mouth and the cell is loose, the measured signal will be a mixture of current going through the cell’s ion channels and current escaping between the pipette mouth and the cell membrane (“leak current”). Since ion channels commonly provide comparatively high-resistance pathways, leak current can make up the majority of the measured signal. The goal is, therefore, to provide the best possible seal between the pipette mouth and the cell membrane. To afford high-quality measurements, the resistance of this seal normally lies at or above 1 gigaohm ($G\Omega$), which explains the origin of all-decisive “gigaseal” in electrophysiology. In conventional patch clamping, gigaseals are the result of careful pipette fabrication, highly trained micromanipulation, pressure changes, and voltage adjustments. In automated patch clamping, the borosilicate pipette electrode has been replaced by a planar electrode arrangement where dissociated cells are dispensed into a small chamber with a single hole at the bottom. The hole is the equivalent of the pipette’s mouth; it can be occluded by a cell covering it. The system then sucks the cell into a high-resistance position to rupture its cell membrane at the hole interface, effectively establishing a bottom-up connection to the cell. The chamber is, hence, the extracellular side (comparable to the bath in conventional patch clamping), while the circuitry under the chamber’s hole is the intracellular side (i.e., the “pipette” with attached amplifier in conventional patch clamp terms). Providing high-quality gigaseals with planar electrodes employs diverse approaches which have been reviewed in elsewhere (Dunlop et al. 2008; Farre and Fertig 2012; Farre et al. 2007).

Automated patch clamping has been used to establish the potencies of a variety of ion channel ligands using heterologously expressed ion channels in mammalian cells. These ion channels include voltage-gated K^+ channels, such as hERG (Huang et al. 2010; Stoelzle et al. 2011; Tang et al. 2001), hKCNQ1/minK (Cao et al. 2010), voltage-gated Na^+ channels (Cao et al. 2010; Castle et al.

2009; Stoelzle et al. 2011), and voltage-gated Ca^{2+} channels (Cao et al. 2010). In many, but not all cases, the concentration-dependent inhibition values matched what had been established by conventional patch clamping. For some hKCNQ1/minK channel blockers, however, the suggested potencies were 25–40 times lower than indicated by the IC_{50} values from conventional patch clamping data, demonstrating that automated patch clamping can have its fallacies (Cao et al. 2010). Commonly, failure of automated patch clamping is related to the biophysical requirements of the experiment. The conventional approach has a skilled experimenter who can intervene if the recording conditions change. Automated patch clamping accomplishes this only with difficulty. In many cases, certain parameters are measured at the beginning and assumed to be constant throughout the experiment. This, however, is rarely the case. The seal may deteriorate (thus elevating the leak current component), or the cell may reseal and decrease the diameter of the hole connecting the amplifier with the cell’s interior. The latter defines the so-called *access resistance* (R_a), which is a functional bottleneck that limits the system’s ability to mediate rapid and potentially large current flow during voltage steps. For channels with fast gating kinetics, low R_a is vital to enable a rapid clamp to a new potential. This is especially true for Na_v channels whose gating kinetics terminate within 3 ms, making fast clamping speed, and, by extension, very low R_a , a prerequisite for good voltage control. Successful measurements of Na_v currents are technically most demanding, but various Na_v channels ($Na_v1.3$, $Na_v1.5$, $Na_v1.7$, etc.) have been clamped and pharmacologically screened using automated patch clamping systems. The data suggest that the biophysical parameters (i.e., IV relationship, steady-state inactivation, etc.) as well as the IC_{50} values seem to be consistent with what has been seen with conventional measurements (Cao et al. 2010; Castle et al. 2009). Moreover, automated patch clamp technology advances rapidly, now even accommodating measurements on use-dependent sodium channel blockade (e.g., tetracaine on $Na_v1.5$), which suggests that current experimental limitations may be overcome soon, providing a highly sensitive, rapid-throughput screening platform for cardiotoxicity studies (Stoelzle et al. 2011).

Of course, cardiotoxicity predictions based on automated patch clamp data can also be misleading, owing to the analytical environment. Heterologous expression experiments, with their approach of reflecting drug actions on an isolated protein, carry the potential that natural, more complex drug relationships are missed. For example, a drug could bind to multiple targets, which may potentiate or mitigate the predicted arrhythmia (Moller 2010; Witchel et al. 2003a, b). Verapamil, for instance, is a potent hERG channel blocker, but it is not torsadogenic because of its concomitant action on L-type Ca^{2+} channels (Lacerda et al.

2008; Navarrete et al. 2013). In these cases, reliance on heterologous automated patch clamp data would unnecessarily exclude an otherwise promising drug candidate. Compared to FLIPR-based hERG channel screening, which predicts long-QT syndrome propensity with 77.7 % accuracy, automated patch clamping does not necessarily perform better (Beattie et al. 2013). It should also be kept in mind that, while systems show an almost exponential increase in the well format (8, 16, 64, 384 wells), the success rate approximates 60–80 % for highly trained personnel; for users with less experience, success of <50 % is not uncommon. In most cases, failure is attributed cell quality, where “quality” is determined by cell membrane properties, dissociation, and the percentage of cells expressing the desired channel (Guthrie et al. 2005). In addition, the running cost for automated patch clamping is commonly high due to sophisticated plates, such that any one experiment may cost hundreds of dollars. This, however, may be more than off-set by the versatility of automated patch clamping: Aside from measuring heterologously generated currents (Bruggemann et al. 2006; Farre et al. 2007, 2009), these systems can also record from cell with endogenous currents, for example cardiomyocytes derived from embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC) (Becker et al. 2013; Ma et al. 2011). This has the decisive advantage that the entire complement of cardiac cofactors and channels are present. Furthermore, cardiomyocytes derived from iPSCs or ESCs express cardiac-specific genes and proteins, allowing them to assume atrial, nodal, and ventricular cardiac action potential behavior within the physiological range (Haythornthwaite et al. 2012; Ma et al. 2011). Automated patch clamping with such setups, especially in the current-clamp mode, can be highly predictive of a drug’s potential to affect cardiac excitation. Veratridine, for example, has been examined in this fashion, and its sodium channel activation has been shown to prolong the action potential duration (Chevalier et al. 2014). Nifedipine and lidocaine, examined under current clamp, similarly have been found to modulate various parameters, including the cardiac action potential amplitude/duration (Stoelzle et al. 2011).

Stem cell-derived cardiomyocytes nicely reproduce human physiological conditions, but data produced with these systems still requires validation with large sets of positive and negative reference compounds, because the correlation with adult cardiac physiology, and, by extension, its pharmacological sensitivity is, at this time, not very well understood. The problem is that iPSC- and ESC-derived cardiomyocytes are likely in a neonatal state, making the type and/or expression level of ion channels uniquely distinct from that of adult cells (Binah et al. 2007; Xi et al. 2011). Also, consideration must be taken that automated patch clamping requires cellular dissociation, which

disrupts intercellular networks. It is unclear at this point, if human ESC-/iPSC-derived cardiomyocytes altered their ionic currents with the loss of network connections in suspension (Kujala et al. 2012; Lahti et al. 2012).

Microelectrode array field potential measurements

The microelectrode array (MEA) is a medium-throughput assay designed to measure field potentials from electrically active cellular networks. It has been used on different types of cardiomyocytes, primary, as well as those derived from ESCs, and iPSCs, to predict QT prolongation and torsadogenicity (Fendyur and Spira 2012; Guo et al. 2011; Harris et al. 2013). The field potential duration (FPD) has been shown to correlate with action potential duration when both are recorded simultaneously (Fendyur and Spira 2012; Halbach et al. 2003). More importantly, QT correlation appears to be in place as well, as established by in vivo ECG data (Guo et al. 2011; Harris et al. 2013). An MEA system combines a cell culture plate with an embedded array of high-impedance or low-impedance electrodes, an amplifier, a digitizer sampling at >50 kHz, and associated analysis software (Meyer et al. 2004; Scheel et al. 2014). Using this setup, FDP modulation of ESC-derived cardiomyocytes has been examined under exposure to different reference compounds (Braam and Mummery 2010). Lidocaine, a sodium channel blocker, terminated spikes in the 30–100 μM concentration range, which is consistent with published IC_{50} values. Nifedipine, a Ca^{2+} channel blocker, shortened FPD, but increased spike frequency, albeit with no arrhythmic behavior. The hERG channel blocker E-4031 induced sustained FDP prolongation at 30 nM (Braam and Mummery 2010; Navarrete et al. 2013). Most informative were the effects of nine compounds with established torsadogenicity and QT prolongation (alfuzosin, cisapride, sparfloxacin, terfenadine, domperidone, sertindole, quinidine, sotalol, and amiodarone). Three independent studies confirmed their FDP prolonging characteristic; no false negatives were observed (Braam and Mummery 2010; Meyer et al. 2004). Interestingly, FPD prolongation appears to have superior cardiac event prediction value, since hESC-derived cardiomyocytes flag no adverse drug effects with verapamil and ketoconazole, which have been demonstrated to be hERG channel inhibitors (Braam and Mummery 2010; Redfern et al. 2003). These findings suggest that electrophysiological properties and drug responses of human ESC-derived cardiomyocytes match clinical observations on QT prolongation/shortening and arrhythmogenicity. More importantly, when the drug profiling is extended from the single parametric FDP approach to consider multiple field potential characteristics (e.g., spike amplitude, spike frequency, etc.), the assay’s sensitivity for cardiac risk is further enhanced (Clements and Thomas 2014).

The great advantage of the MEA platform is that it affords the opportunity to conduct experiments with intact cellular networks. Also, because the cells are continuously cultured and maintained, it is possible to conduct true time response measurements, which provide insight into subacute drug responses. Of course, due to its tissue complexity, the method is relatively low throughput, currently topping out at the 48-well format, although 96-well systems are available (Scheel et al. 2014). Another drawback is the comparatively high cost of the MEA plates (~\$500/plate), although wider adoption of this technology may soon make these types of platforms more economical. It should be emphasized that MEA recordings produce enormous amounts of data that can obscure the important from the irrelevant. This, however, is not different from many other modern research technologies (e.g., next-generation sequencing), and it can be assumed that the analysis burden will soon become more manageable. First, semiautomatic data analysis tools have already become available (Pradhapan et al. 2013). Even with this analysis obstacle still in place, MEA allows for high-resolution, noninvasive electrophysiological recordings on cardiomyocytes with a high correlation between FDP prolongation and clinical long-QT syndrome, which makes it a remarkably powerful cardiotoxicity prediction tool (Braam and Mummery 2010).

Real-time cell analysis

Real-time cell analysis (RTCA) is a proprietary, impedance-based technology that can monitor the cardiomyocyte contraction–relaxation cycle at millisecond resolution in 96-well format without the need for labeling (Xi et al. 2011). A variety of reference cardiac modulators have been tested using RTCA, in an effort to determine their influence on cardiomyocyte contractility (Xi et al. 2011). In setups using murine ESCs, tetrodotoxin, a sodium channel blocker, decreased the beating rate, whereas isradipine, a voltage-gated Ca^{2+} channel inhibitor, decreased spike amplitude and frequency; similar analyses showed that (S)-(-)-Bay K 8644 depressed the beating rate (Abassi et al. 2012; Xi et al. 2011). Four drugs withdrawn from the market due to increased torsade incidence, astemizole, cisapride, droperide, and sertindole, all produced beating irregularities consistent with those observed for the hERG blocker E4031. However, another hERG channel blocker, terfenadine, failed to produce any effect (Abassi et al. 2012). These data suggest that different mechanisms are in place when it comes to disrupting the mechanical activity of cardiomyocytes, and multiple beating parameters such as rate, intensity, and duration can be used to assess contraction abnormalities (Himmel 2013). One study examining the effects of 49 positive and negative inotropes as well as non-actives using human iPSC-derived cardiomyocytes

showed that non-active controls were identified as such with 74 % prediction reliability. Seven out of nine positive inotropes were correctly identified as active (78 % reliability), while 20 out of 21 negative inotropes produced the expected outcome, indicating 95 % reliability (Scott et al. 2014). This shows that RTCA-based assaying has fairly good prediction value based on *European Centre for the Validation of Alternative Methods* criteria (Genschow et al. 2002).

Like MEA, RTCA is noninvasive, permitting recordings from cultures following subacute compound exposure (Pan et al. 2013). This is of particular relevance for molecules binding to non-ion channel targets, whose effects may take longer to manifest, making it impossible to capture them with assays emphasizing hERG activity or action potential parameters (Himmel 2013). The chemotherapeutic agent doxorubicin, for example, has been shown to induce arrhythmia as well as cardiotoxicity by interfering with mitochondrial function. It was RTCA that enabled the detection of the delayed decrease in the cardiomyocyte beating frequency (Abassi et al. 2012). Despite these promising results, the predictive or even translational value of RTCA is considered limited, because its validity is not yet firmly established (Himmel 2013). Also challenging is the cost of the RTCA consumables, in particular for the plates, which makes RTCA medium throughput. Nevertheless, this technology, coupled with stem cell-derived cardiomyocytes, has the potential for primary, secondary, and safety pharmacology (Harding et al. 2007; Mandenius et al. 2011; Pouton and Haynes 2007).

Measurement of intracellular Ca^{2+} dynamics

The mechanical beating process of a syncytial bed of cardiomyocytes is highly synchronous, and throughout the cardiac cycle, it correlates with the dynamic changes in intracellular Ca^{2+} (Sirenko et al. 2014). Visualization of intracellular Ca^{2+} dynamics, therefore, provides insight into cardiomyocyte mechanics (Sirenko et al. 2014). The FLIPR platform, initially developed to monitor Ca^{2+} changes associated with G-protein coupled receptor activation (Niedernberg et al. 2003), has been repurposed toward primary cultured neuronal networks (Cao et al. 2012, 2014) and cardiomyocytes, where it can provide real-time (8 Hz) fluorescence data for 96-, 384-, and even 1536-well plates (Sirenko et al. 2014). With a throughput of 50,000 data points per day (Dorn et al. 2005), FLIPR-based Ca^{2+} imaging is highly efficient, which has prompted the development of follow-up, improved technology. Various positive and negative reference compounds targeting distinct ion channels have been tested with the FLIPR platform, which confirmed this method's capability of capturing the cardiomyocytes Ca^{2+} oscillations to make measurements

on fluorescence peak width, peak spacing, peak rise, and peak decay times. When tested with hERG channel inhibitors cisapride, terfenadine, astemizole, and pimozide, all compounds characteristically delayed Ca^{2+} baseline recovery (Sirenko et al. 2014). Sodium channel blockers lidocaine and tetrodotoxin produced slowed beating frequency and various other irregularities in a concentration-dependent fashion with potencies similar to what has been established for their actions on sodium channels (Sirenko et al. 2014). Drugs with positive chronotropic effects can be readily identified, and various ion channel modulators induce the expected inhibition of contractile activity (Sirenko et al. 2014). The FLIPR system is also suitable for capturing subacute responses in Ca^{2+} dynamics following compound exposure, as demonstrated by delayed cardiotoxic effects 24 h after administration of pentamidine and quinidine (Sirenko et al. 2014). These findings are encouraging, but whether or not the measured Ca^{2+} dynamics can sufficiently parallel critical clinical events such as long-QT syndrome or torsade remains to be established. There is, however, good concordance with clinical data for known chronotropes and/or inotropes. Coupled with an immensely high-throughput capability and comparatively low cost, FLIPR Ca^{2+} imaging will likely find widespread utility for preclinical cardiotoxicity testing. It is also experimentally convenient, requiring only standard black well, clear-bottom 96- or 386-well plate, which is much more economical than the high-tech plates required for MEA and RTCA analyses. Of course, at 8 Hz, the temporal resolution of FLIPR is relative low, which limits its detail on Ca^{2+} dynamics, especially when the beating rate is stimulated (Cao et al. 2015). As in other systems where indicators must be loaded, caution is necessary when it comes to interpreting the data, as the presence of the labeling chemicals may affect the electrophysiological properties of the cells (Fendyur and Spira 2012; Peters et al. 2015).

Conclusion

Cardiotoxicity is one of the leading causes for discontinuation at different phases of drug development. Various screening technologies seek to predict the same based on hERG activity, yet inhibition of hERG current alone cannot possibly encompass the diverse set of mechanisms behind drug-induced cardiac arrhythmias. Promising in this regard are phenotypic measurements of more native cellular systems examining action potential or field potential duration, contractility, and Ca^{2+} dynamics in human ESC- and iPSC-derived cardiomyocytes. These assays, however, are comparatively time-consuming, which is in conflict with screening economy, suggesting that simpler and more

convenient approaches such a FLIPR-based hERG TI^+ influx and Ca^{2+} dye technology ought to be used in the earliest stages. Compounds displaying a safe profile in the latter two assays could then be subjected to MEA testing, while automated patch clamping ought to be employed to study associated mechanisms of action.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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