Review

Role of hERG potassium channel assays in drug development

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Abbreviations: ECG, electrocardiogram; hERG, human ether-a-go-go related gene; LQTS, long QT syndrome; PPC, population patch clamp

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Numerous structurally and functionally unrelated drugs block the hERG potassium channel. HERG channels are involved in cardiac action potential repolarization, and reduced function of hERG lengthens ventricular action potentials, prolongs the QT interval in an electrocardiogram, and increases the risk for potentially fatal ventricular arrhythmias. In order to reduce the risk of investing resources in a drug candidate that fails preclinical safety studies because of QT prolongation, it is important to screen compounds for activity on hERG channels early in the lead optimization process. A number of hERG assays are available, ranging from high throughput binding assays on stably expressed recombinant channels to very time consuming electrophysiological examinations in cardiac myocytes. Depending on the number of compounds to be tested, binding assays or functional assays measuring membrane potential or Rb+ flux, combined with electrophysiology on a few compounds, can be used to efficiently develop the structure-function relationship of hERG interactions.

Introduction

In drug development, it is advantageous to discover potential safety liabilities associated with a structural series before significant resources have been invested. This process involves early counter screening against related targets, such as a panel of kinases for a program targeting p38 kinase or other potassium channels for a program directed against a member of this class of ion channels. Undesirable activities that are unrelated to the biological target of interest are much more difficult to address at the early stages and are often only discovered during preclinical safety studies, after significant resources have been devoted to the program. One off-target activity that is frequently encountered is block of hERG potassium channels, which has the potential to cause life-threatening arrhythmias. This is a particular troublesome problem due to the fact that block of hERG may be difficult to detect during routine preclinical and clinical safety studies. In this work, we discuss the need for hERG screening and the types of hERG assays that are currently available.

What Is hERG?

Technically, hERG is the name of a human gene and stands for human ether-a-go-go related gene. The name is based on homology to a gene found in Drosophila melanogaster that was named ether-a-go-go (erg) because of the ether-induced leg shaking observed in flies with mutations in this gene. According to the new nomenclature, hERG is KCNH2. The protein encoded by this gene will be referred to as the hERG channel, as is common practice, although the newer nomenclature refers to it as Kv11.1. The hERG channel is a voltage-gated potassium channel and, like other voltage-gated potassium channels, it is highly selective for potassium and is a tetramer formed of four subunits, each containing six transmembrane domains (Fig. 1). Two alternative splice forms, hERG1a and hERG1b, exist, which differ only at the N-terminal domain. The two splice forms can assemble to form functional homo- or hetero-tetramers with somewhat different channel kinetics. Generally, hERG channels are involved in action potential repolarization. In humans, the hERG channel is expressed widely, including in the brain, adrenal gland, thymus, retina, and in cardiac and smooth muscle tissues. In the heart, ERG1 channels appear to be the only members of this potassium channel family, whereas two related channels, ERG2 and ERG3, are expressed in the nervous system. Although in heterologous expression systems, the hERG channel can associate with the β-subunits minK (KCNE1) and MiRP1 (KCNE2), the existence of such complexes in native tissues remains a subject of debate.

HERG channels differ from other voltage-gated potassium channels in the relative kinetics of activation and inactivation. In hERG channels, the development and reversal of inactivation is fast compared to the activation and deactivation steps, leading to a characteristic increase in current upon repolarization as illustrated in Figure 2.

Why and When to Screen on hERG

In the heart, hERG channels are the molecular correlate of the Ikr current which, together with other potassium currents, is involved in action potential repolarization. Reduced function of hERG causes action potential prolongation, which in rare cases can lead to the potentially fatal ventricular tachyarrhythmia Torsades de Pointes. In a body surface electrocardiogram (ECG), ventricular action potential prolongation manifests itself as a prolongation of
hERG assays

the QT interval or the period between the beginning of the QRS complex and end of the T wave (Fig. 3).

A particular form of inherited long QT syndrome (LQTS), LQTS2, has been linked to loss-of-function mutations in hERG. While LQTS2 is typically not associated with any clinical symptoms, except occasional fainting in some patients, it greatly increases an individual's risk for Torsades de Pointes.

In analogy with the inherited syndrome, certain drugs that inhibit hERG channels can cause LQTS (acquired or drug-induced LQTS) and an increased risk of Torsades de Pointes. This was first demonstrated in the late 1980s and 1990s for the histamine H1 receptor antagonist terfenadine (Fig. 4). In 1989, overdoses of terfenadine were shown to prolong the QT interval. Terfenadine use was linked to Torsades de Pointes in 1990, and terfenadine was subsequently shown to inhibit the delayed rectifier potassium current in isolated myocytes, and hERG channels expressed in Xenopus oocytes. The link between QT prolongation and Torsades de Pointes is complex, and not all drugs that prolong the QT interval carry the same risk of arrhythmia. However, QT interval prolongation is the only known surrogate for an increased risk for Torsades de Pointes. Based on this increased risk, a significant number of drugs that prolong the QT interval, ranging from the antihistamines terfenadine and astemizole to the antipsychotic droperidol (Fig. 4), have been withdrawn from the market, while others have received black box warning labels. Although the correlation between block of hERG and Torsades de Pointes is not perfect, the potential for putting patients at risk means that an interaction of a compound with the hERG channel must be taken very seriously. The enormous cost of drug development, coupled with the desire to avoid late-stage compound failures, motivates pharmaceutical companies to test compounds for inhibition of hERG early on in the lead optimization process.

Medium and High Throughput hERG Assays

The ideal hERG assay provides a linear measure of channel activity under physiologically relevant conditions. The closest to this ideal would be voltage clamp recordings in cardiac myocytes using a cardiac action potential waveform as the voltage command. However, such a study is extremely laborious and only amenable to the detailed characterization of very few selected compounds. As previously noted, it is advantageous to screen compounds for hERG activity early on in the lead evaluation and optimization process. However, this approach requires testing of hundreds and potentially thousands of compounds within a single drug discovery program. Although the development of automated electrophysiology technologies has improved the throughput of electrophysiological methods, radioligand binding, fluorescent or ion flux assays may serve as efficient and robust assays for examining the potential hERG liabilities of a large number of compounds.

Electrophysiology. Electrophysiology can provide detailed and quantitative information on the potency and mechanism of hERG block by a test compound. One of the unique advantages of such voltage clamp recordings is the ability to control membrane potential. Since activation and inactivation of hERG is dependent on membrane potential, voltage clamp recordings can differentiate between compounds that preferentially interact with different states of the channel.

Figure 1. Voltage-gated potassium channel family. (A) Diagram showing the predicted topology of an individual potassium channel α and the minK-related β subunit (left), and the arrangement of the pore forming subunits in a potassium channel tetramer (right). (B) Family tree for voltage-gated potassium channels.
Several higher throughput automated electrophysiological instruments have been developed and typically use a planar substrate with holes, illustrated in Figure 5, that replaces the traditional patch pipette.\(^{21,22}\) Cells that seal to the substrate around a hole may be voltage clamped, if the section of plasma membrane covering the hole can be ruptured manually, by applying negative pressure, or perforated using an antibiotic such as amphotericin, allowing electrical access to the cell’s cytoplasm. Currently, the highest throughput devices available are the IonWorks HT and IonWorks Quattro. Both instruments use a disposable 384-well PatchPlate\(^\text{TM}\) and a common ground chamber and achieve increased throughput through synchronous recording from 48 wells combined with simple and versatile software. Typical seal resistances are on the order of 100 mega-Ohms for IonWorks HT and 50 mega-Ohms for IonWorks Quattro. Whole cell access is obtained by perfusion with a membrane-perforating agent, typically amphotericin B. The IonWorks Quattro features a similar hardware and software design but uses an approach called population patch clamp (PPC). PPC involves a modified PatchPlate with 64 holes per well and is especially advantageous when expression levels are not homogeneous from cell to cell. However, recording in PPC mode requires a minimum success rate of sealing to holes and a sufficiently high seal resistance to avoid short circuit currents through holes not occluded by a cell.

Other automated electrophysiology instruments, such as the PatchXpress (Molecular Devices), the QPatch (Sophion Biosciences) and the Patchliner (Nanion Technologies GmbH) also use planar substrates, but record asynchronously from individual wells. Currently, 16-well or 48-well formats are available. High resistance seals and flexible voltage protocols result in recordings that rival traditional manual electrophysiology in quality.

A major limitation of all automated electrophysiology platforms is the high cost of the instruments and consumables. Another concern with regard to the automated platforms is the potential for results that are inconsistent with those from conventional voltage clamp assays. Typically, hERG potencies measured by automated and manual electrophysiology are in good agreement; however, some compounds are known to appear less potent when tested by automated electrophysiology.\(^{23-26}\)

Discrepancies typically arise from hydrophobic compounds being adsorbed to the surface of the plate used to hold the compound or to the patch plate itself. Glass-coated compound plates can help to minimize the nonspecific binding to the compound plate but come at a significant cost. Multiple compound additions can mitigate problems caused by compounds sticking to the patch plate substrate and are common practice.

**Flux assays.** An alternative to either manual or automated electrophysiology is a functional assay that measures ion flux across cell or vesicle membranes. This assay offers higher throughput than the automated voltage clamp protocols and takes advantage of the ability of Rb\(^+\) to permeate through hERG channels.\(^{27}\) Typically, cells are loaded with Rb\(^+\) overnight and hERG-dependent Rb\(^+\) efflux is initiated by an addition of high (50–60 mM) extracellular potassium concentrations to depolarize the cell and open hERG channels. The amount of Rb\(^+\) efflux can be calculated by using \(^{86}\text{Rb}\) as a radioactive tracer or by flame atomic absorption spectrometry (FAAS).\(^{27,28}\) Such flux assays, which can operate in 96- and 384-well formats, afford the testing of several thousand compounds per day. They offer a robust measure of channel activity, but lack voltage control and temporal resolution. In two reports, using either HEK-293 or CHO-K1 cells stably expressing recombinant hERG channels, IC\(_{50}\) values determined in a Rb\(^+\) flux assay were approximately 10-fold higher than those determined by electrophysiology, but the rank order of compounds according to potency was similar for the two assay types.\(^{29,30}\)

**Fluorescence-based assays.** The development of improved fluorescent dyes and plate readers has provided another approach to high throughput screening of ion channel activities. Fluorescent dyes which are sensitive to changes in membrane potential have proved especially useful for studying the pharmacology of many ion channel types.\(^{31,32}\) However, studying hERG by this approach presents a challenge since this channel does not typically control a cell’s resting membrane potential. It has been possible, however, to select HEK-293 and CHO-K1 cell lines stably expressing recombinant hERG channels that give rise to a depolarization signal in response to addition of extracellular potassium (50–60 mM).\(^{29,30}\) At least in the HEK-293 background, this signal is the sum of hERG-dependent and hERG-independent components; a complication that probably contributes to the low signal-to-noise ratio observed in this assay. In both cell lines, IC\(_{50}\) values were significantly right-shifted compared to those determined by electrophysiology, regardless of whether DiBAC\(_4\)(3) (Invitrogen, Carlsbad, CA), FMP or Blue

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**Figure 3.** Electrocardiogram and cardiac action potential trace corresponding to one heartbeat. Adapted with permission from Macmillan Publishers Ltd: Nature Reviews: Drug Discovery (Fermini & Fossa, 2003, Nature Rev Drug Disc 2:439–447), copyright 2003.
Membrane Potential Indicator Dye (both from Molecular Devices, Sunnyvale, CA) was used to monitor membrane potential. The lack of sensitivity of these membrane potential assays resulted in a high incidence of “false negatives”. Moreover, the rank order of compounds by IC50 differed between the membrane potential assay and electrophysiological determinations.

To avoid some of the problems arising from non-hERG potassium currents, it may be possible to take advantage of known activators, such as PD-307243, to open hERG channels under physiological conditions which will yield a hERG-dependent hyperpolarization. One concern associated with this assay format is the potential interference of the channel activator with binding of the test compound.

Yet another approach to hERG fluorescence-based assays involves dyes, such as FluxOR™ (Invitrogen, Carlsbad, CA), that are sensitive to intracellular thallium concentrations, since thallium is known to permeate through hERG channels. Such an assay provides a more direct measurement of hERG activity and may be comparable to Rb+ flux assays, but with the benefit of being amenable to a 1536-well screening format.

In all fluorescent assays there is the potential for interactions between the test compound and the dye that result in quenching of the fluorescent signal, and this may limit the range of compound concentrations that can be tested reliably.

**Binding assays.** Radioligand binding assays have been used extensively to screen for interaction with the hERG channel. These are non-functional assays, usually performed using isolated cell membranes, and they rely on competition or allosteric coupling between the binding sites for the test compound and for the radioligand. Although they do not provide a direct measure of Ik blockade, such binding assays can test 50,000 to 100,000 compounds per day and are relatively inexpensive, which is why they are commonly used in most large pharmaceutical companies. A number of well-characterized radioligands for hERG have been described, including [3H]-dofetilide, [35S]-MK-499, [3H]-astemizole, and [125I]-BeKm-1. In general, binding assays using [3H]-dofetilide, [35S]-MK-499 and [3H]-astemizole (Fig. 4) are very robust and reproducible and IC50 values typically correlate well with those determined in electrophysiology. These three non-peptide radioligands, in analogy with most small molecule hERG blockers, are thought to bind in the large inner cavity of the hERG channel and require channel opening to access their binding sites. In contrast, the scorpion venom peptide BeKm-1 preferentially blocks channels in the closed state and binds to the extracellular S5-pore linker of the channel. The binding site for [125I]-BeKm-1 may be allosterically coupled to the inner cavity, since E-4031, which is believed to bind to the inner cavity, inhibits binding of [125I]-BeKm-1 to recombinant hERG channels expressed in HEK293 cells. Binding assays do not provide detailed information regarding the nature of the interaction of the test compound with hERG, such as the ability to block or activate the channel or a preference for a particular state of the channel. Despite this limitation and the fact that [35S]-MK-499 itself binds with a Kd of < 1 nM but blocks hERG with an IC50 of approximately 30 nM, agreement between compound potencies in [35S]-MK-499 binding and voltage clamp assays is remarkably high (reviewed in ref. 38, personal observation). Radioligand binding assays using isolated membranes differ from whole cell functional assays in that they are amenable to a range of assay conditions that may impact on the ability of test compounds to bind. These include temperature, osmolarity, relative concentration of organic solvent, and K+ concentration. In addition, binding assays typically employ time periods of drug exposure significantly longer than most functional assays, which may allow for a more accurate
determination of IC$_{50}$ values for very potent compounds that require a significant period of time to equilibrate.

**Evaluation of Cardiac Risk**

**Measurements of action potential duration.** As an alternative to assays that utilize recombinant hERG channels expressed in mammalian cell lines, it is possible to examine native hERG channels in myocardial cells from several species. The most common tissue sources are isolated Purkinje fibers from guinea pig, rabbit or dog.\textsuperscript{42-45} The contribution of hERG to repolarization and action potential prolongation in these tissues is similar to the human heart, in contrast to the rat heart, for which hERG does not contribute significantly to repolarization.

Although measurements of action potential repolarization are very labor intensive and can only be used to study a limited number of compounds, they can provide valuable data since they examine effects on native cardiac channels. This is potentially important since the precise molecular composition of the channel that controls the I$_{Kr}$ current remains a matter of debate. Additionally, these assays can detect effects on a number of cardiac channels in addition to hERG.\textsuperscript{43}

**In vivo ECG measurements.** The most reliable measure of cardiac safety is probably afforded by ECG recordings in conscious or anesthetized animals,\textsuperscript{43,46,47} and new drug applications require these data. ECG measurements are only practical for a very small number of select compounds. At the same time, such in vivo studies are the only methods of assessing cardiac safety that take into account the safety liabilities of the test compound and all its metabolites combined. They also allow a direct evaluation of other potential complications, such as plasma protein binding, tissue distribution and poly-pharmacology.

The most commonly used species for these measurements is the dog.\textsuperscript{47} Guinea pigs may also be used but are technically more challenging.\textsuperscript{48} If metabolite profiles are expected to differ between man and dog, ECG measurements can also be made in primates.\textsuperscript{49,50} In addition to the choice of species, the most important choice for ECG measurements is between the use of conscious animals implanted with telemetry devices and anesthetized animals.\textsuperscript{51} Advantages of the anesthetized animals include greater reproducibility of the data and the potential for electrical pacing to study effects of heart rate. Disadvantages include the preclusion of oral dosing and the potential for interference of the anesthetic with normal cardiac function or with metabolism of the test compound.

The effects of a test substance on QT interval can be obscured by changes in heart rate since the QT interval is inversely correlated with heart rate.\textsuperscript{52} Therefore, it is common to report QT intervals corrected for the preceding RR interval (QTC). A number of correction methods exists that attempt to normalize QT intervals to an RR interval of 1000 ms, corresponding to a heart rate of 60 beats per minute.\textsuperscript{52-55}

While ECG measurements directly evaluate the effect of a particular test compound on the QT interval, they may not be the best choice to evaluate the potential off-target activity of a class of compounds on hERG channels since other factors may affect the QT interval and either obscure an effect on I$_{Kr}$ or complicate the interpretation of the results.\textsuperscript{56}

**In Silico Modeling Predictions**

Compounds representing a wide range of chemical structures (Fig. 4) have been shown to block hERG. Alanine-scanning mutagenesis studies on the pore-lining S6 segment and parts of the pore helix identified a number of residues involved in conferring high affinity for MK-499,\textsuperscript{57} including threonine and valine (T623 and V625) residues in the pore and glycine, tyrosine and phenylalanine residues in S6 (G648, Y652, F656). The two aromatic residues in S6, Y652 and F656, also contribute to the block of hERG by terfenadine, cisapride, doxetilide and quinidine.\textsuperscript{57,59} These residues are conserved in ERG and EAG channels, but are not found in other voltage-gated potassium channels. Modeling studies have suggested that F656 may be involved in π-stacking interactions with aromatic rings in hERG ligands, while Y652 may interact with the charged amine that is present in many compounds that bind to this channel.\textsuperscript{60}

A homology model based on the crystal structure of the bacterial potassium channel KcsA suggested that the cavity lined by the membrane-spanning sections of hERG may be larger than the cavities found in most ion channels.\textsuperscript{57} Figure 6 shows a model of hERG with MK-499 docked using the FLOG (flexible ligands oriented on grid) procedure. Together with the interactions afforded by the unique aromatic residues in S6 of hERG, the larger size of the cavity may explain the ability of hERG channels to accommodate binding of a wide variety of structurally distinct compounds. While hERG homology models can help to explain the promiscuity relative to other potassium channels, they are less useful in predicting the ability of test compounds to block hERG.

A different modeling approach involves quantitative structure-activity relationship (QSAR) modeling.\textsuperscript{60} This approach takes advantage of known hERG blockers that are used as a training set to generate a pharmacophore model, which can then be tested against a test set. Several pharmacophore models have been developed and can be used to prioritize compounds for hERG testing. Since these models are generally better at predicting lack of hERG activity, they can be used as a screening tool to reduce the cost of hERG testing in the in vitro assays.
hERG Trafficking

More recently, effects on hERG trafficking have been identified as a mechanism for drug-induced LQTS.61 Drugs that reduce hERG/IKr current may interfere with hERG trafficking, which include the antiparasitic agent mefloquine and the cholesterol-lowering agent probucol.65,66 The mechanisms by which drugs interfere with hERG trafficking are largely unknown. In some cases, interactions with chaperones such as Hsp90 may prevent proper folding of hERG,67 whereas in other cases drugs appear to trap hERG in the endoplasmic reticulum.64 Most in vitro and in vivo assays currently used to detect hERG inhibition and QT prolongation focus on acute effects and are not likely to detect drug effects on channel trafficking. Electrophysiology, Rb+ flux and whole cell binding assays yield signals that are proportional to surface channel expression and could therefore be adapted to detect drug-induced changes in hERG trafficking. In addition, an antibody-based chemiluminescent assay has been developed to specifically monitor effects on hERG cell surface expression.68

hERG Activators

Recently, several hERG activators have been identified.69-71 As expected these compounds shorten the ventricular action potential duration and the QT interval. Several forms of familial short QT syndrome have been identified, one of which has been linked to mutations in hERG.72 Familial short QT syndrome is associated with atrial and ventricular arrhythmias and sudden death, suggesting that activation of hERG carries similar safety liabilities as hERG blockade.

Summary

In order to reduce the risk of a drug candidate failing in preclinical safety studies because of blockade of hERG channels and associated QT prolongation, it is important to screen compounds for activity on hERG channels early in the lead optimization process. However, the earlier in the process hERG screening is to occur, the higher the required assay throughput. Although binding assays do not measure hERG function, they offer a rapid, robust and inexpensive measure of the ability of compounds to interact with the hERG channel. Combined with electrophysiology on a few benchmark compounds to characterize the nature of the compound-channel interaction, binding assays can be used as an efficient means to develop the structure-function relationship of hERG interactions within a structural class of test compounds. This strategy should facilitate the development of new small molecule drugs with improved cardiovascular safety profiles.

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