



ELSEVIER

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Journal of Pharmacological and Toxicological Methods xx (2005) xxx – xxx

**Journal of
Pharmacological
and
Toxicological
Methods**

www.elsevier.com/locate/jpharmtox

Evaluation of functional and binding assays in cells expressing either recombinant or endogenous hERG channel

Steven M. Murphy^a, Marc Palmer^a, Michelle Fontilla Poole^a, Linas Padegimas^a, Karen Hunady^a, Joel Danzig^a, Sikander Gill^b, Rajwant Gill^b, Anthony Ting^a, Bruce Sherf^a, Kurt Brunden^a, Alain Stricker-Krongrad^{a,*}

^a Athersys, Inc., 3201 Carnegie Avenue, Cleveland, OH 44115, United States

^b Aurora Biomed, 1001 East Pender St., Vancouver BC, Canada V6A 1W2

Received 1 September 2005; accepted 18 October 2005

Abstract

Introduction: The hERG (human ether-a-go-go related gene) potassium channel is required for normal cardiac repolarization, is susceptible to inhibition by a wide variety of compounds, and its blockage can lead to cardiac QT interval prolongation and life threatening arrhythmias. The present report examines the ability of hERG binding and functional assays to identify compounds with potential cardiovascular liabilities at the earliest stages of drug discovery. **Methods:** Competitive binding assays were developed using ³H-dofetilide and membranes from HEK293EBNA cells stably expressing recombinant hERG (HEK293-hERG) and IMR-32 cells expressing hERG endogenously. hERG functional assays were also developed using membrane potential indicator dye and rubidium efflux. The ability of these assays to identify compounds with potential adverse cardiac effects was examined using drugs with known cardiac effects ranging from those with no known adverse effects to drugs that were withdrawn from the market due to increased risk of sudden death associated with Torsades de Pointes. **Results:** Binding assays using HEK293-hERG membranes and ³H-dofetilide were robust ($Z=0.69\pm 0.015$, mean \pm S.E.M.), highly reproducible (test–retest slope=1.04, $r^2=0.98$), and correlated well with IC₅₀ values obtained by patch clamp (slope=0.98, $r^2=0.89$). Binding assays using IMR-32 membranes were less sensitive ($Z=0.4\pm 0.03$, mean \pm S.E.M., false negative rate=0.4) but still correlated well with patch clamp data (slope=1.06, $r^2=0.83$). The hERG membrane potential assay could detect potent hERG inhibitors (defined by hERG patch clamp IC₅₀<0.1 μ M) using HEK293-hERG cells, but were prone to generate false-negative results with less potent inhibitors (false negative rate=0.5). Finally, the rubidium efflux assay gave highly reproducible results ($Z=0.80\pm 0.02$, mean \pm S.E.M.) that correlated with patch clamp IC₅₀ values (slope=0.87, $r^2=0.73$). **Discussion:** The hERG binding and rubidium efflux assays are robust, predictive of patch clamp results, and can be used at the earliest stages of drug discovery.

© 2005 Elsevier Inc. All rights reserved.

Keywords: hERG; ³H-dofetilide; Binding assay; Membrane potential dye; QT prolongation; Arrhythmias; Rubidium efflux; Torsades de Pointes

1. Introduction

The identification and elimination of compounds with potentially serious adverse effects early in the drug discovery process holds the promise of significantly reducing the time and cost required to develop new drugs. Despite these potential benefits, early stage safety screening remains limited due to a lack of high-throughput assays that can accurately predict in vivo outcomes. Recent advancements in the molecular characterization of complex biological systems coupled with the

withdrawal of drugs from the market due to life threatening adverse effects has provided the opportunity and sense of urgency required for the development and implementation of highly predictive early stage safety screens.

Increased risk of rare but life threatening cardiac arrhythmias called Torsades de Pointes (TdP) has resulted in market withdrawal or in post-market labeling changes (e.g., black box warnings) for many drugs in the last several years (Fermini & Fossa, 2003). These drugs were designed for a variety of therapeutic indications, yet they all caused cardiac QT interval prolongation. Surprisingly, many of these compounds inhibit a common molecular target, the human ether a-go-go related gene (hERG) product.

* Corresponding author. Tel.: +1 216 431 9900; fax: +1 216 361 9596.

E-mail address: astricker@athersys.com (A. Stricker-Krongrad).

hERG was first identified in a hippocampal cDNA library during the search for the human homolog of the *Drosophila* potassium ion channel ether a-go-go (*eag*) (Warmke & Ganetzky, 1994). hERG shares sequence homology with mouse *eag* over most of the protein including the putative transmembrane regions, pore region, and cyclic nucleotide binding domain, but the level of identity clearly indicates that hERG is a novel related gene rather than homolog of *eag* (Warmke & Ganetzky, 1994). The hERG gene was mapped to chromosome 7 (Warmke & Ganetzky, 1994) where it was found to be linked to the genetic locus for long QT syndrome #2 (LQT2) (Curran et al., 1995).

Long QT syndrome is a group of disorders characterized by abnormally long QT intervals on the EKG and increased risk for cardiac arrhythmias (Keating & Sanguinetti, 2001; Priori & Napolitano, 2004). The genetic form of this syndrome can be caused by mutations in several different genes referred to as LQT1, 2, 3, etc. Many of these genes have now been identified as ion channels or ion channel associated proteins involved in the generation of a normal cardiac action potential (Keating & Sanguinetti, 2001; Priori & Napolitano, 2004). The findings that hERG and LQT2 are genetically linked combined with the observation that multiple different LQT2 families contain mutations in the hERG gene have led to the hypothesis that defects in hERG cause QT prolongation (Curran et al., 1995).

At approximately the same time that mutations in hERG were identified as the cause of LQT2, hERG was expressed in cells and shown to be a voltage-gated potassium channel (Sanguinetti, Jiang, Curran, & Keating, 1995; Trudeau, Warmke, Ganetzky, & Robertson, 1995) whose electrophysiological characteristics were very similar to the rapidly activating delayed rectifier potassium current (I_{kr}) that contributes to the repolarization of cardiac tissue. The ability of class III antiarrhythmia agents, such as dofetilide, to block both potassium conductance by recombinant hERG expressed in various cell types (Kiehn, Lacerda, Wible, & Brown, 1996; Snyders & Chaudhary, 1996; Spector, Curran, Keating, & Sanguinetti, 1996; Trudeau et al., 1995) and the I_{kr} current in cardiomyocytes (Jurkiewicz & Sanguinetti, 1993; Sanguinetti & Jurkiewicz, 1990; Wang, Fermini, & Nattel, 1994; Yang et al., 1994) further supports the hypothesis that hERG is responsible for the I_{kr} current during cardiac repolarization. The expression of hERG in the heart (Curran et al., 1995; Pond et al., 2000; Wymore et al., 1997) is consistent with this hypothesis, but it should be noted that hERG is also expressed in several other tissues including brain, adrenal gland, thymus, and retina (Pond et al., 2000; Saganich, Machado, & Rudy, 2001; Wymore et al., 1997) suggesting that its function extends beyond its role in cardiac repolarization (Schwarz & Bauer, 2004).

Prolongation of the QT interval can, as noted above, be caused by genetic defects in several different ion channels but it can also result from a variety of non-genetic factors such as heart disease, hypokalemia, and pharmacological agents (Roden et al., 1996). Drugs, such as class III antiarrhythmia agents, induce QT prolongation as a means of combating cardiac arrhythmias, whereas non-cardiovascular drugs cause

QT prolongation as an unexpected adverse effect. The discovery that antihistamines, such as terfenadine and astemizole (Roy, Dumaine, & Brown, 1996; Salata et al., 1995; Suessbrich, Waldegger, Lang, & Busch, 1996), and antibiotics such as erythromycin (Daleau, Lessard, Groleau, & Turgeon, 1995) block the hERG channel (I_{kr}) led to the hypothesis that undesired QT prolongation of these and other drugs may result from hERG inhibition. Many drugs that induce unexpected QT prolongation were examined for their ability to inhibit hERG. Surprisingly, structurally diverse drugs designed to target various therapeutic indications were found to block the hERG channel (Fermini & Fossa, 2003; Zolotoy et al., 2003). These findings indicate that blocking the hERG channel is a common cause for QT interval prolongation.

A probable explanation for the susceptibility of hERG to inhibition by diverse small molecules came from structural analyses showing that the hERG channel has an unusually large inner cavity and two aromatic residues that face the core of the channel (Mitcheson & Perry, 2003). It has been proposed that the large inner cavity of the channel may provide the space necessary for compounds to enter the pore and the aromatic amino acids may facilitate the binding of drugs containing aromatic groups and charged amines (Mitcheson & Perry, 2003).

The ability of structurally diverse compounds to block the hERG channel and induce QT prolongation indicates the importance of screening for hERG inhibition during drug development, as recommended by the International Conference on Harmonization (cf. ICHS7B EWG 2002, but also EWG 2004). Possible *in vitro* hERG assays include patch clamp, Fluorometric Imaging Plate Reader (FLIPR) membrane potential method, rubidium efflux, and radioligand binding. Patch clamp is currently considered the gold standard for hERG screening (cf. ICH S7B EWG 2002, however, also EWG 2004), but it has many limitations when it comes to screening a large number of compounds in a short period of time. It is labor intensive, requires a trained electrophysiologist, and is relatively costly. By contrast, the other *in vitro* methods are ideal for rapidly screening large numbers of compounds, but they are less validated. The FLIPR membrane potential method has high throughput capacity but it has been reported to have a high rate of false positives (Tang et al., 2001). The rubidium efflux assay has been reported to correlate with patch clamp results but with a significant rightward shift in IC_{50} values (Rezazadeh, Hesketh, & Fedida, 2004). Radioligand binding assays (e.g. 3H -dofetilide and 3H -astemizole) appear to be an attractive alternative *in vitro* assay for hERG screening (Chiu et al., 2004; Diaz et al., 2004), although some concerns have been raised about their predictive value (Fermini & Fossa, 2003; Netzer, Bischoff, & Ebnet, 2003).

In order to establish a method that can be used in the earliest stages of drug discovery to identify hERG inhibitors and predict the risk that these compounds pose for inducing adverse cardiac effects in man, the present paper compares FLIPR membrane potential dye, rubidium efflux, and 3H -dofetilide binding assays to the patch clamp analysis using compounds with a range of known adverse cardiac effects.

2. Materials and methods

2.1. Compounds

Acetaminophen, bromopride, chloramphenicol, acetylsalicylic acid, imipramine hydrochloride, amitriptyline hydrochloride, quinidine, pimozone, terfenadine, and astemizole were purchased from Sigma-Aldrich (St. Louis, MO). Dofetilide and risperidone were purchased from Apin Chemicals (Abingdon, UK). ^3H -dofetilide (28.1 Ci/mmol) was custom labeled by PerkinElmer (Boston, MA).

2.2. Generation of a hERG expression vector

A full-length hERG cDNA was amplified by polymerase chain reaction (PCR) using gene-specific primers (HE.Fatg 5'caccgatccaccATGCCGGTCCGGAGGGGCCA CGTCG and HE.Rtag 5'atCTAACTGCCCGGGTCCGAGCCGTGTCTGT), PfuTurbo Hotstart DNA polymerase (Invitrogen), PfuTurbo buffer, 1 M GC-Melt (BD Biosciences, Clontech) and cDNA synthesized from human brain RNA (Invitrogen) using Superscript II reverse transcriptase (Invitrogen) per manufacturer's recommendations. PCR was performed in MJR DNA Engine 200 thermocycler (95 °C for 1 min followed by 36 amplification cycles of 95 °C for 10 s, 61 °C for 30 s, and 72 °C for 8 min). The hERG PCR product was digested with *Bam*HI and subcloned into the mammalian expression vector pTgT-Blst (pTgT Blst vector is the pTargetT vector (Promega) modified by replacing the neomycin resistance gene with blasticidin resistance gene) that was digested with *Bam*HI and *Eco*RV. The sequence of hERG in the resulting plasmid (pTgT(Blst)-hERG) matched the published sequence of the human potassium voltage-gated channel, subfamily H (eag-related), member 2 (KCNH2), transcript variant 1, mRNA (Genebank# NM_000238) except for four silent mutations.

2.3. Cell lines

IMR-32 (ATCC# CCL-127), CHP-212 (ATCC# CRL-2273), SK-N-AS (ATCC# CRL-2137), and HEK293EBNA (ATCC# CRL-1573) were grown in MEM (Gibco), MEM/HAMS (1:1 mixture) (Gibco), MEM, and DMEM (Gibco), respectively, plus 10% fetal bovine serum (HyClone) and penicillin/streptomycin/glutamine (Gibco) at 37 °C with 5% CO₂. A CHO cell line stably expressing hERG (Aurora Biomed) was grown in Ham's F-12 (Sigma) with 10% fetal calf serum (Consera International Inc.) and penicillin/streptomycin (Sigma) at 37 °C with 5% CO₂.

A cell line stably expressing hERG (under the control of the CMV promoter) was generated by transfecting HEK293EBNA cells with pTgT(Blst)-hERG using Fugene 6 (Roche) according to manufacturer's instructions. The cells were then plated at low density and grown in the presence of 7 µg/ml Blastacidin (Invivogen) to select for individual cells that integrated pTgT(Blst)-hERG into their genome. Forty-seven clonal HEK293EBNA-hERG cell lines were screened for hERG expression by qPCR (data not shown). Four cell lines

expressing hERG, as determined by qPCR, were examined for hERG expression by ^3H -dofetilide binding (data not shown). The clone with the highest specific binding (95%) was used for further analysis.

2.4. hERG binding assay

Binding assays were performed in triplicate using membranes isolated from either HEK293EBNA-hERG or IMR-32 cells. Briefly, cells were washed in Dulbecco's phosphate buffer saline (Invitrogen), resuspended in hypotonic buffer (10 mM Hepes pH 7.4 and complete EDTA-free protease inhibitors (Roche)), and then lysed with an Omni TH homogenizer. The preparations were clarified by low speed centrifugation (1000×g for 15 min) and the supernatants were subjected to high-speed centrifugation (48,000×g for 20 min) to pellet the plasma membranes. The membranes were resuspended in hypotonic lysis buffer, protein concentrations were determined by Bradford assay (BioRad), and the membranes were stored at –80 °C.

Saturation binding assays were performed by incubating plasma membranes (50 µg/well) in assay buffer (HEK293-hERG assay buffer=50 mM Tris pH 7.4, 10 mM KCl, 1 mM MgCl₂, IMR-32 assay buffer=10 mM Hepes pH 7.4, 5 mM KCl, 5 mM MgCl₂, 130 mM NaCl, 1 mM EGTA, 10 mM glucose, 0.5% powdered milk) with various concentrations of ^3H -dofetilide (1–750 nM for IMR32, 0.78–400 nM for HEK293-hERG) for 90 min at room temperature in the presence (non-specific binding) or absence (total binding) of 50 µM of unlabeled dofetilide. Equilibrium was reached by 30 min and was stable for at least 4 h (data not shown). Membranes were collected by filtration using a Brandel Harvester onto glass fiber filters (Filtermat A, Perkin Elmer) that had been preincubated with 0.15% PEI. The filtermats were washed briefly with ice-cold wash buffer (HEK293-hERG wash buffer=HEK293-hERG assay buffer, IMR-32 wash buffer=25 mM Tris pH 7.4, 130 mM NaCl, 5.5 mM KCl, 0.8 mM MgCl₂, 5 mM glucose, 0.05 mM CaCl₂, 0.01% BSA), dried, and coated with scintillant (MeliLex B/HS, Perkin Elmer). ^3H -dofetilide on the filter was quantified with a Wallac Trilux 1450 MicroBeta scintillation counter (Perkin Elmer). The dissociation constant (K_d) and number of binding sites per milligram of membrane protein (B_{max}) was calculated by non-linear regression using PRISM (Graphpad Software, San Diego, CA).

K_i values were determined for the inhibition of ^3H -dofetilide binding to hERG by incubating 10 nM (HEK293-hERG) or 250 nM (IMR-32) ^3H -dofetilide in assay buffer with 50 µg/well of membrane and varying concentrations of test compound. Curve fitting and K_i calculations were done using the PRISM software (Graphpad Software). Z' values were determined by the method of Zhang, Chung, and Oldenburg (1999).

2.5. hERG membrane potential assay

384-well, black-walled, clear-bottom plates (Corning) were seeded with HEK293EBNA (2×10^4 cells/well), HEK293-hERG (2×10^4 cells/well) or IMR-32 (3×10^4 cells/well). Blue membrane potential indicator dye (Molecular Devices) was

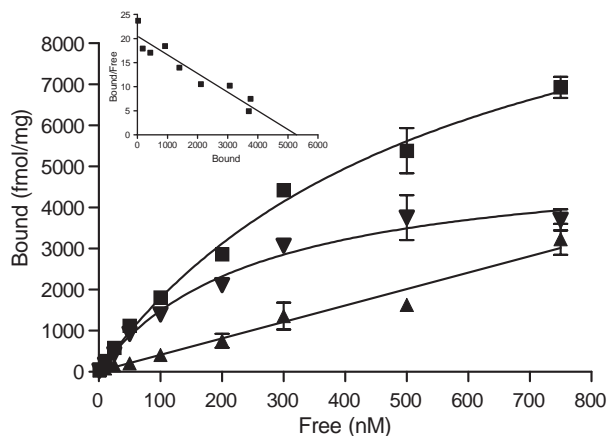


Fig. 1. Saturation binding of ^3H -dofetilide to the hERG channel endogenously expressed in IMR-32 cells. Increasing amounts of ^3H -dofetilide were incubated with cell membranes in the presence (non-specific binding) or absence (total binding) of $50\ \mu\text{M}$ unlabeled dofetilide. Bound ^3H -dofetilide is shown as $\text{fmol } ^3\text{H}$ -dofetilide bound/mg membrane protein. Inset graph represents a Scatchard plot of the specific binding data.

diluted as per the manufacturer's directions and added to the wells along with either test compound or buffer control. The plate was briefly centrifuged and then incubated for 30 min at $37\ ^\circ\text{C}$. The plate was then placed in the FLIPR (Fluorometric Imaging Plate Reader-Molecular Devices) where KCl was added to a final concentration of $60\ \text{mM}$ and fluorescence was recorded for 3 min. Compounds, membrane potential dye, and the cells were dissolved/suspended in Hanks buffered saline plus $10\ \text{mM}$ Hepes pH 7.4.

The depolarization signal observed by the addition of extracellular KCl to HEK293-hERG cells is generated by both hERG channel conductance and non-hERG channel conductance. This conclusion is drawn from the observation that addition of the hERG channel blocker, dofetilide, to HEK293-hERG cells inhibits the depolarization signal by approximately 20%, whereas addition of dofetilide to HEK293EBNA cells does not affect the depolarization signal. The portion of the depolarization signal in HEK293-hERG cells that is contributed by the hERG channel conductance is the depolarization signal observed in the presence of buffer alone minus the depolarization signal observed in the presence of $10\ \mu\text{M}$ dofetilide. To determine the percent inhibition of the hERG dependence conductance, all test compounds were added to the cells in the presence and absence of $10\ \mu\text{M}$ dofetilide. The difference between these two depolarization signals divided by the hERG-dependent depolarization signal (total depolarization observed with buffer alone minus depolarization in the presence of buffer plus $10\ \mu\text{M}$ dofetilide) gives the percent inhibition of the hERG-dependent signal.

2.6. Rubidium efflux assay

CHO-hERG cells were grown to approximately 80–90% confluence in 96-well plates (Falcon). Cells were loaded with rubidium (Rb) by incubating cells in $5.4\ \text{mM}$ RbCl loading buffer (Aurora Biomed) for 1 h at $37\ ^\circ\text{C}$. Extracellular RbCl was removed by washing the cells twice in Rb wash buffer

(isotonic buffer, Aurora Biomed). Test compounds, dissolved in Rb wash buffer, were added to the cells, and incubated for 10 min, then the hERG channels were activated by replacing the test compounds in Rb wash buffer with test compounds in Rb open buffer (Aurora Biomed), which contains $60\ \text{mM}$ KCl. The media was removed after 6 min and then the cells were lysed in 1.5% Triton X-100. The amount of rubidium in the extracellular media and the cytoplasm (cell lysate) was measured using the atomic absorbance spectroscopy-based ICR 8000 (Aurora Biomed). The percent rubidium efflux is the percentage of extracellular rubidium compared to the total rubidium (cytoplasmic plus extracellular). 100% rubidium efflux is the percent rubidium efflux in the buffer control minus the percent rubidium efflux seen in the absence of KCl stimulation.

3. Results

3.1. Cell lines

Development of a hERG binding assay began by identifying a cell line that expresses hERG endogenously and creating a cell line that expresses recombinant hERG. Since previous reports indicated that hERG was expressed in the brain (Pond et al., 2000; Wymore et al., 1997), hERG mRNA levels were examined by qPCR in the neuroblastoma cell lines IMR-32, CHP-212, and SK-N-AS. IMR-32 cells had the highest hERG mRNA level so it was chosen for further analysis (data not shown). A cell line stably expressing recombinant hERG (HEK293-hERG) was generated using HEK293EBNA as the parental cell line.

3.2. Validity of the hERG binding assay

Cell membranes isolated from HEK293EBNA, HEK293-hERG, and IMR-32 cells were examined for their ability to bind to ^3H -dofetilide, which is known to bind to (Finlayson,

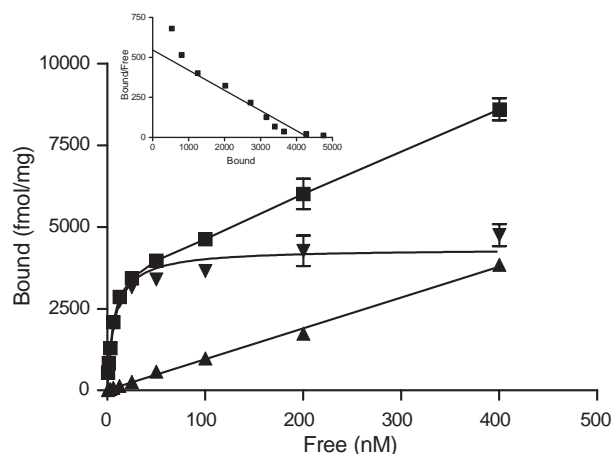


Fig. 2. Saturation binding of ^3H -dofetilide to the hERG channel expressed in HEK293EBNA cells. Increasing amounts of ^3H -dofetilide were incubated with cell membranes in the presence (non-specific binding) or absence (total binding) of $50\ \mu\text{M}$ unlabeled dofetilide. Bound ^3H -dofetilide is shown as $\text{fmol } ^3\text{H}$ -dofetilide bound/mg membrane protein. Inset graph represents a Scatchard plot of the specific binding data.

Turnbull, January, Sharkey, & Kelly, 2001) and inhibit the hERG channel (Kiehn et al., 1996; Snyders & Chaudhary, 1996). Cell membranes from HEK293EBNA displayed no detectable specific ^3H -dofetilide binding (data not shown), whereas membranes from HEK293-hERG and IMR-32 cells displayed specific saturable binding of ^3H -dofetilide. The binding of ^3H -dofetilide to HEK293-hERG and IMR-32 cell membranes resulted in calculated K_d values of 8.2 and 328 nM, respectively, and B_{max} values of 4.4 and 5.5 pmol/mg membrane protein, respectively (Figs. 1 and 2).

In order to determine if the hERG binding assay can identify compounds that inhibit hERG function and potentially cause in vivo cardiovascular side effects, 12 drugs that are presently or were formally on the market were chosen for evaluation in the hERG binding assay. These compounds (Table 1) are grouped into three classes based on adverse cardiac effects in man: compounds in class A have not been reported to inhibit hERG in patch clamp studies or to cause

QT interval prolongation or arrhythmias; class B compounds inhibit hERG in patch clamp studies and cause QT interval prolongation, but have no or only isolated reports of associated Torsades de Pointes; class C compounds inhibit hERG in patch clamp studies, cause QT interval prolongation, and have either been withdrawn from the market due to increased risk of Torsades de Pointes or have measurable incidence of TdP (Fermini & Fossa, 2003; Redfern et al., 2003). The K_i values for the inhibition of ^3H -dofetilide binding to hERG by the compounds in Table 1 were determined using membranes isolated from IMR-32 and HEK293-hERG cells. These K_i values were plotted against published IC_{50} values for the inhibition of hERG function in patch clamp studies (Fig. 3). K_i values for compounds from class A are not included in Fig. 3 because these compounds do not inhibit the binding of ^3H -dofetilide to IMR-32 or to HEK293-hERG cell membranes with K_i values $<20 \mu\text{M}$ (the upper limit of detection in these assays). It should also be

Table 1

| Drug | Target | QT interval prolongation ^{a,b} | Reported Torsades de Pointes ^a | Label warnings/restricted use ^a | Functional assays | | Binding assays | |
|--------------------------|--------------------------|---|---|--|---|--|---|---|
| | | | | | Mammalian patch clamp IC_{50} (μM) | Rb-efflux IC_{50} (μM) | 293-hERG ^3H -dofetilide K_i (μM) | IMR-32 ^3H -dofetilide K_i (μM) |
| <i>Class A</i> | | | | | | | | |
| Acetaminophen | Analgesic | No | None | None | ND | >20 | >20 | >20 |
| Bromopride | Antinausea | No | None | None | ND | >20 | >20 | >20 |
| Chloramphenicol | Antibiotic | No | None | None | ND | >20 | >20 | >20 |
| Aspirin | Antiinflammatory | No | None | None | ND | >20 | >20 | >20 |
| <i>Class B</i> | | | | | | | | |
| Imipramine | Antidepressant | Yes | Isolated incidences | None | 3.4 ^c | 12.22 | 4.6 | >20 |
| Risperidone | Antipsychotic | Yes | Isolated incidences | Label warning | 0.17 ^d | 8.56 | 1.8 | 7.1 |
| Amitriptyline | Antidepressant | Yes | Isolated incidences | Label warning | 10 ^e | 8.35 | 7.2 | >20 |
| <i>Class C</i> | | | | | | | | |
| Pimozide (Orap) | Antipsychotic | Yes | Measurable incidences | ECG before beginning treatment | 0.018 ^c | 0.011 | 0.019 | 5.4 |
| Terfenadine (Seldane) | Antihistamine | Yes | Measurable incidences (1 per 10,000 person years) | Withdrawn from market due to TdP | 0.009 ^f 0.079 ^g 0.016 ^h | 0.345 | 0.13 | 7.3 |
| Astemizole (Hismanol TM) | Antihistamine | Yes | Measurable incidences (8.5 per 10,000 person years) | Withdrawn from market due to TdP | 0.0009 ⁱ 0.0012 ^f 0.0016 ^g | 0.037 | 0.0033 | 0.06 |
| Quinidine (Quinaglute) | Antiarrhythmia class 1a | Yes | Measurable incidences (1–8.8%) | Black box warning | 0.41 ^j 1.0 ⁱ 1.4 ^k | 7.4 | 6 | >20 |
| Dofetilide (Tikosyn) | Antiarrhythmia class III | Yes | Measurable incidences (1–4%) | Black box warning | 0.011 ^l 0.012 ^m | 0.014 | 0.0082 | 0.61 |

^a Redfern et al. (2003).^b Fermini and Fossa (2003).^c Teschemacher, Seward, Hancox, and Witchel (1999).^d Kongsamut et al. (2002).^e Tic, Walker, Valenzuela, Breit, and Campbell (2000).^f Wang et al. (2003).^g Gill et al. (2003).^h Diaz et al. (2004).ⁱ Zhou, Vorperian, Gong, Zhang, and January (1999).^j Paul, Witchel, and Hancox (2002).^k Weerapura et al. (2002).^l Kang et al. (2004).^m Snyders and Chaudhary (1996).

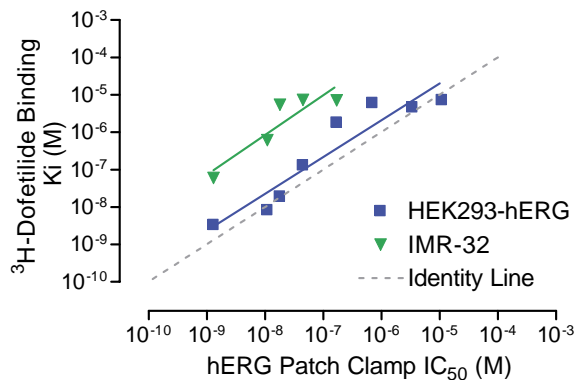


Fig. 3. External validity of the hERG binding assay by comparison with patch clamp. K_i values determined by hERG binding correlate with published IC_{50} values from hERG patch clamp studies. The K_i values for the classes B and C compounds in Table 1 were determined in the hERG binding assay using membranes isolated from either IMR-32 or HEK293-hERG cells (see Materials and methods) and plotted against the published hERG patch clamp IC_{50} values (Table 1). The identity line is shown for reference.

noted that K_i values for three (imipramine, amitriptyline, and quinidine) of the eight classes B and C compounds were not included in the IMR-32 data for Fig. 3 because their K_i values were $>20 \mu\text{M}$. Thus, binding assays using IMR-32 membranes had an estimated false negative rate of 0.4 in these experiments. Linear regression analysis of the data in Fig. 3 gave a slope of 0.98 ($r^2=0.89$) for binding studies using HEK293-hERG membranes and a slope of 1.06 ($r^2=0.83$) for binding studies using IMR-32 membranes. The binding data generated using the HEK293-hERG membranes were right shifted only three-fold (on average) from the identity line. On the other hand, binding data generated using the IMR-32 membranes were rightward shifted 130-fold (on average).

The relationship between the in vitro hERG binding, patch clamp studies, and the in vivo cardiovascular phenotype was examined by plotting the K_i values determined in the HEK293-hERG binding studies and the published IC_{50} values from the patch clamp studies for the compounds in Table 1 against the compounds grouped according to the severity of their in vivo cardiac phenotype (Fig. 4). Although the number of data points is limited, it is clear that the potency of a compound in the hERG binding and patch clamp studies is directly correlated with the severity of the cardiovascular side effects. All compounds in class A had K_i values $>20 \mu\text{M}$. The average K_i values for class B and class C compound were $4.5 \mu\text{M}$ and $1.2 \mu\text{M}$ ($0.04 \mu\text{M}$ without quinidine), respectively.

3.3. Reliability of the hERG binding assay

The robustness and repeatability of the hERG binding assay were examined in two ways. First, the Z' value for this assay has been determined in multiple independent experiments by two different investigators. The Z' value for the hERG binding assay is 0.69 ± 0.015 (mean \pm S.E.M.) when using HEK293-hERG membranes and is 0.40 ± 0.03 (mean \pm S.E.M.) when using IMR-32 membranes. Second, a hERG binding assay test–retest experiment was performed using HEK293-hERG membranes and the compounds in classes B and C. In this experiment, the K_i values for compounds in classes B and C were determined by one investigator and then retested by another investigator. The results of these assays were fit by linear regression (slope = 1.04, $r^2=0.98$) and indicated no significant deviation from the identity line (Fig. 5). Estimation of the test–retest systematic error is 2% ($1 - r^2$). Average intra- and inter-assay variability were 10% and 13%, respectively.

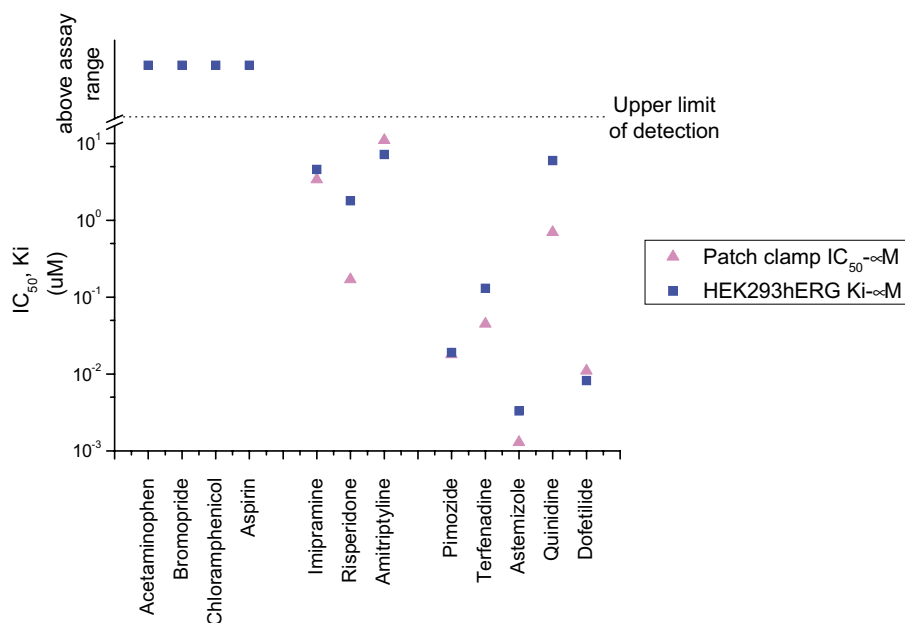


Fig. 4. Correlation of IC_{50} values from hERG patch clamp studies and K_i values from hERG binding to in vivo cardiovascular phenotype. Published IC_{50} values for inhibition of hERG function in patch clamp studies and K_i values for the inhibition of ^3H -dofetilide binding to membranes from HEK293-hERG are plotted above the drugs that were tested. The drugs are grouped into three classes depending on in vivo cardiovascular effects: class A compounds have no known cardiovascular effects, class B compounds cause QT prolongation but do not have arrhythmia warnings, and class C compounds cause QT prolongation and have arrhythmia warnings.

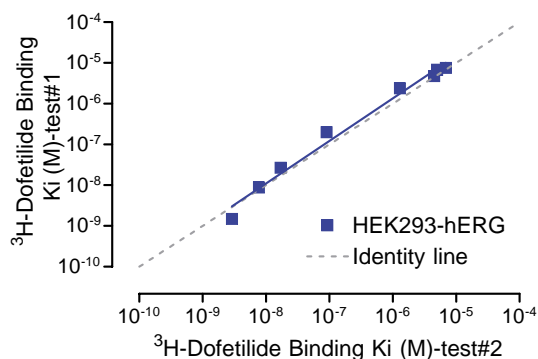


Fig. 5. Repeatability of the hERG binding assay in test–retest assays. The K_i values for the compounds in Table 1 were determined in two independent experiments performed by two different investigators using the HEK293-hERG binding assay (see Materials and methods). The identity line is shown for reference.

3.4. Validity of the membrane potential assay

Two approaches were used to determine if the compounds inhibited the ability of the hERG channel to conduct potassium. The first approach used a membrane potential indicator dye and a Fluorometric Imaging Plate Reader (FLIPR) to indirectly measure potassium conductance. Cells were incubated with a test compound and a membrane potential sensitive dye and then the extracellular potassium concentration was elevated to 60 mM. Cells containing potassium channels depolarize in response to the increase in extracellular KCl, which is reflected by an increase in the fluorescence of the membrane potential dye. Membrane depolarization was recorded in IMR-32, HEK293-hERG, and HEK293EBNA cells indicating the presence of potassium channels in all three cell types. The potassium channels responsible for the depolarization could be at least partially determined by measuring depolarization in the presence and absence of dofetilide, which is known to inhibit the conductance of potassium by the hERG channel. The depolarization of the HEK293EBNA cell line was not inhibited by the presence of 10 μ M dofetilide suggesting that the observed depolarization was not the result of hERG channel potassium conductance. The identity of endogenous potassium channels is unclear but the existence of voltage-gated potassium channels and small inward rectifying currents have been previously reported in HEK293 cells (Yu & Kerchner, 1998). In contrast to HEK293 cells, the depolarization of HEK293-hERG cells was decreased by approximately 20% in the presence of 10 μ M dofetilide suggesting that depolarization in HEK293-hERG cells is the result of conductance by hERG channels in addition to other endogenous ion channels. Higher concentrations of dofetilide did not significantly increase the inhibition of the depolarization in HEK293-hERG cells (data not shown). The level of depolarization of IMR-32 was typically decreased in the presence of 10 μ M dofetilide but this effect was highly variable (data not shown) so studies presented here focus on the HEK293-hERG cell line.

The ability of the compounds in Table 1 to inhibit the dofetilide-sensitive portion of the potassium-stimulated depo-

larization in HEK293-hERG cells was examined using the membrane potential dye assay. As expected, none of the class A compounds significantly inhibited hERG function but unexpectedly none of the class B and only four (dofetilide, terfenadine, astemizole, and pimozone) of the five class C compounds significantly inhibited hERG when tested at 10 μ M (false negative rate=0.5, Fig. 6). In addition to the high false negative rate, this assay also had very low Z' values indicating that it is not a robust assay.

3.5. Validity of the rubidium efflux assay

The compounds in Table 1 were next examined for their ability to inhibit the hERG channel by the rubidium efflux assay. This assay takes advantage of the fact that rubidium is similar in size to potassium, can move through potassium channels, and can be readily measured by atomic absorbance (Terstappen, 1999). Briefly, this assay involves incubating a CHO cell line stably expressing hERG (CHO-hERG) with rubidium to allow rubidium to enter the cells. The cells are then washed to remove the extracellular rubidium, incubated with test compound or buffer control, and depolarized by addition of KCl to the extracellular media. The net movement of rubidium from inside the cell to the extracellular medium is measured by atomic absorbance. The ability of rubidium to move out of the CHO-hERG cell upon KCl-induced depolarization serves as a measure of hERG channel potassium conductance. The IC_{50} values of the compounds in Table 1 determined in the rubidium efflux assay were plotted against the published hERG patch clamp IC_{50} values for these compounds (Fig. 7). Class A compounds were not included in this graph since their IC_{50} values were greater than 20 μ M (the upper limit of detection for the concentrations tested). Linear regression analysis of the data in Fig. 7 gave a slope of 0.87 ($r^2=0.73$) and indicated that the rubidium efflux IC_{50} values were right shifted 10-fold (on average) compared to the identity line. Finally, the reliability of the rubidium efflux assay was examined by determining the Z' value for multiple independent experiments. The average Z'

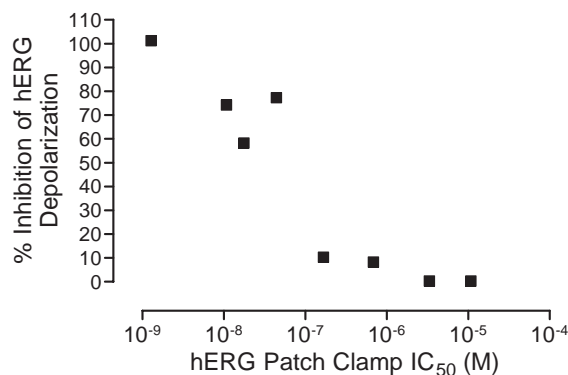


Fig. 6. External validity of the hERG membrane potential assay. HEK293-hERG cells were incubated in the presence or absence of 10 μ M dofetilide and the membrane potential was measured using a membrane potential dye following the addition of 60 mM KCl. The percent inhibition of the hERG-dependent component of the depolarization was calculated (see Materials and methods) and graphed against published IC_{50} values for these compounds in hERG patch clamp studies.

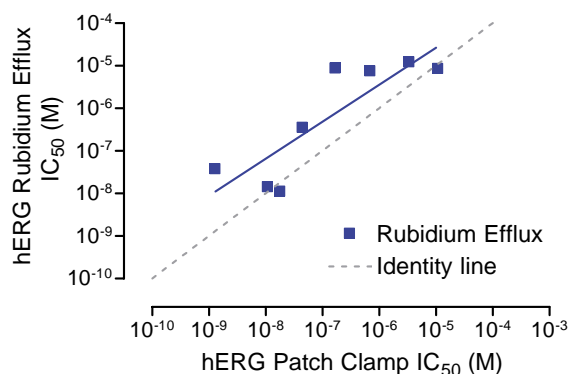


Fig. 7. External validity of the hERG rubidium efflux assay by comparison with patch clamp. IC₅₀ values determined in the hERG rubidium efflux assay correlate with IC₅₀ values determined in hERG patch clamp studies. IC₅₀ values were determined for the compounds in Table 1 in the hERG rubidium efflux assay (see Materials and methods) and plotted against the published IC₅₀ values determined in hERG patch clamp studies. The identity line is shown for reference.

value for the rubidium efflux assay was found to be 0.80 ± 0.02 (mean \pm S.E.M.).

4. Discussion

In the past decade, at least nine drugs targeted against a variety of therapeutic areas have been withdrawn from the market or had their use severely restricted because they caused unexpected QT prolongation and increased risk of sudden death due to Torsades de Pointes (Fermini & Fossa, 2003; Roden, 2004). Although there are multiple potential molecular targets that could be responsible for this effect and the drugs were structurally diverse, most of these drugs were found to block the hERG potassium channel. These findings illustrate the importance of screening compounds for hERG inhibition early in the drug discovery process (ideally at the start of lead optimization) rather than later (preclinical testing).

The assays currently used to determine if a compound can block the hERG channel range from monitoring the QT interval in vivo to screening for hERG blockers in silico (Fenichel et al., 2004). While many of these assays provide valuable information, most are laborious, expensive, and have low throughput capacity. The goal of the present study was to evaluate in vitro assays for their ability to identify compounds that block the hERG channel and thus identify compounds with the potential to cause QT prolongation and associated life threatening arrhythmias. Patch clamp is currently considered the gold standard for analyzing a compound's ability to inhibit hERG and it was therefore used as a comparator. The assays examined in this study were limited to those that provided sufficiently high throughput to allow screening at the earliest stages of drug discovery. The assays fall into two classes: competitive binding assays and functional assays. The competitive binding assay used ³H-dofetilide and membranes isolated from cells expressing either endogenous hERG or recombinant hERG. The hERG functional assays used either a membrane potential sensitive dye or rubidium efflux to examine the ability of a compound to block hERG channel function.

4.1. hERG binding assays

Radioligand binding is a commonly used method to screen for inhibitors of protein function. When the radioligand has a very high affinity for a protein, the assay is highly sensitive and can cover a large range of inhibition values. It is also amenable to different formats (96 and 384 wells) and detection methods (filtration and proximity scintillation). The first hERG binding assays demonstrating that competitive binding could be used to predict functional inhibition of the hERG channel were performed by Chadwick et al. and Rasmussen et al. These two groups demonstrated that IC₅₀ values for several known class III antiarrhythmia agents determined in competitive binding assay with the known I_{kr} (hERG) inhibitor, ³H-dofetilide, and primary myocardial cells from guinea pigs correlated with their ability to inhibit I_{kr} function (Chadwick et al., 1993; Rasmussen, Allen, Blackburn, Butrous, & Dalrymple, 1992). Finlayson et al. (2001) advanced the idea that competitive inhibition of ³H-dofetilide binding to membranes isolated from cells expressing recombinant hERG could be used as means to screen for hERG channel blockers. Recently, K_i values determined using ³H-astemizole and ³H-dofetilide in hERG competitive binding assays have been shown to correlate well with the IC₅₀ values determined with patch clamp (Chiu et al., 2004; Diaz et al., 2004). These results support the concept of using competitive binding as an inexpensive means to identify compounds that inhibit hERG function.

In the present study, hERG binding assays were developed in a neuroblastoma cell line (IMR-32), which expresses hERG endogenously, and in a HEK293EBNA cell line expressing recombinant hERG (HEK293-hERG). Surprisingly, the observed K_d for dofetilide obtained from IMR-32 membranes (328 nM, Fig. 2) was much higher than the K_d reported for either recombinantly expressed hERG [8.2 nM (Fig. 1), 24 nM (Diaz et al., 2004), 59 nM (Finlayson et al., 2001)] or ERG endogenously expressed in guinea pig cardiac myocytes (23 nM, Rasmussen et al., 1992). In addition to the difference in K_d, we also noticed that the binding of ³H-dofetilide to the IMR-32 membranes is affected by the ion concentrations in the binding buffer, whereas the binding to membranes from cells expressing recombinant hERG is relatively unaffected (data not shown). The reason for the differences in the binding of ³H-dofetilide between recombinant and endogenous hERG is unclear. To the best of our knowledge, this is the first time that ³H-dofetilide binding has been characterized in membranes isolated from neuronal cells and it is possible that they express hERG-associated proteins that modulate hERG function. An example of one such protein is MiRP (MinK-related peptide) which has been reported to alter the electrophysiological properties of hERG when it is co-expressed with hERG (Abbott et al., 1999; Weerapura, Nattel, Chartier, Caballero, & Hebert, 2002). Another possible explanation for the differences in binding might be the existence of multiple binding sites, but careful analysis of our saturation binding data with IMR-32 membranes do not provide evidence for the existence of two binding sites (cf. Scatchard inset in Fig. 1).

In order to compare the pharmacological profile of the endogenous hERG with the cloned hERG, we have selected compounds from three classes based on their adverse cardiac effects in man (cf. Table 1): compounds in class A have not been reported to cause QT interval prolongation or arrhythmias; class B compounds cause QT interval prolongation but have no or only isolated reports of associated Torsades de Pointes; class C compounds cause QT interval prolongation and have either been withdrawn from the market due to increased risk of Torsades de Pointes or have measurable incidence of TdP (Fermini & Fossa, 2003; Redfern et al., 2003). The K_i values for the inhibition of ^3H -dofetilide binding to hERG by the compounds in Table 1 were determined using membranes isolated from IMR-32 and HEK293-hERG cells. The rank order of the compounds according to the K_i values determined with IMR-32 cell membranes was similar to the rank order of the compounds according to the K_i values determined with HEK293-hERG. This indicates that the dofetilide binding site in the IMR-32 cells is the hERG channel. There are, however, important differences between the endogenous hERG with the cloned hERG binding. First, in keeping with the lower K_d observed for dofetilide binding, data generated using the IMR-32 membranes were rightward shifted (80-fold on average) when compared to the HEK293-hERG data. Secondly, although the global rank order of the classes were similar (class C < class B < class A) between the IMR-32 and HEK293-hERG assays, in the former assay two compounds in class B (imipramine and amitriptyline) and one compound in class C (quinidine) could not be differentiated from class A compounds and were declared inactive. Although we have used a limited set of drugs, this indicates that the sensitivity of the IMR-32 binding assay is too low and it will be associated with a high rate of false negatives (estimated at 40%).

The ability of the hERG binding assays to identify hERG inhibitors was examined by plotting the K_i values determined in the hERG binding assays against published IC_{50} values for the inhibition of hERG function in patch clamp studies (Fig. 3). The correlation coefficients determined by comparing the hERG patch clamp IC_{50} values for the compounds in Table 1 with the K_i values for these compounds determined in the hERG binding assay using HEK293-hERG and IMR-32 membranes were $r=0.89$ and $r=0.83$, respectively (Fig. 3). These data are in good agreement with the correlation coefficients of $r^2=0.82$ and $r_{\text{sp}}=0.91$ previously reported when the IC_{50} values of various drugs in hERG patch clamp studies were compared with the K_i values determined for these compounds in binding studies using recombinant hERG and ^3H -dofetilide (Diaz et al., 2004) or ^3H -astemizole (Chiu et al., 2004). Moreover, the K_i values generated using the HEK293-hERG membranes were close to the identity line (Fig. 3, Diaz et al., 2004), indicating that the sensitivity of the dofetilide binding assay is comparable to the patch clamp assay. This is in contrast to the data obtained using astemizole, where a 10-fold rightward shift is observed (Chiu et al., 2004), which may indicate a slight loss of sensitivity. However, an accurate comparison can only be made by testing the same set of compounds in both assays.

If an assay is to be used for screening large numbers of compounds, it must be robust and highly reproducible. The quality of the HEK293-hERG binding assay was evaluated by calculating the Z' factor of repeated assays and by performing the test–retest procedure. The hERG competitive binding assay was found to be a robust and highly reproducible assay, with an average Z' of 0.69 ± 0.015 (mean \pm S.E.M.) and a test–retest slope = 1.04 and $r^2=0.98$. These measures indicate that the hERG binding assay would be well suited for high-throughput screening.

Despite the fact that hERG binding assays are robust (Fig. 5) and predictive of adverse cardiac effects (Fig. 4), concerns have been raised about the possibility of false negative results for compounds that inhibit the hERG channel by binding at sites other than the radioligand binding site (Fermini & Fossa, 2003; Netzer et al., 2003). The basic structural organization of the hERG channel and the molecular determinates for high affinity binding by hERG blocking compounds has recently been reviewed (Mitcheson & Perry, 2003). Briefly, the hERG channel is a tetramer with each subunit having six transmembrane segments (S1 to S6) and large intracellular amino- and carboxy-termini. Each subunit has a pore domain S5-P-S6 (P contains the ion selectivity filter), a voltage-sensing domain (S1 to S4), and a cytosolic aperture (S6 overlap). Most drugs bind to the hERG channel in the inner cavity of the channel between the cytosolic aperture and the voltage-sensing domain. Mutagenesis studies have revealed that key amino acids required for high affinity binding are located in the S6 region and are predicted to face the intercavity of the channel (Lees-Miller, Duan, Teng, & Duff, 2000; Mitcheson, Chen, Lin, Culbertson, & Sanguinetti, 2000). In addition, there is converging evidence that the conformational changes associated with the gating of the hERG channel modify drug binding, most probably by changing the availability of the key S6 residues (for a review, see Mitcheson & Perry, 2003). Lastly, it has been demonstrated that most hERG blockers are active when the cytosolic aperture is open (Kiehn et al., 1996; Snyders & Chaudhary, 1996; Spector et al., 1996).

The existence of binding sites outside of the inner cavity is suggested by the observations that macrolide antibiotics such as clarithromycin (Volberg, Koci, Su, Lin, & Zhou, 2002) and peptide toxins such as BeKm-1 (Milnes et al., 2003) do not appear to require channel opening to block the hERG channel. In addition, BeKm-1 was shown to bind to the outer mouth of the channel (Korolkova et al., 2002) with an affinity in the 10 pM range (Angelo et al., 2003) and preferentially block the channel in the closed state (Milnes et al., 2003). Despite the fact that BeKm-1 does not bind to hERG in the inner cavity, the class III antiarrhythmia agent E-4031, which does bind in the inner cavity of the hERG channel (Mitcheson & Perry, 2003), can displace ^{125}I -BeKm-1 with an IC_{50} of 7 nM (Angelo et al., 2003). This is not surprising since it is known that the binding of class III antiarrhythmic methanesulfonanilides to the hERG channel is sensitive to the gating state of the channel (Ficker, Jarolimek, & Brown, 2001; Lees-Miller et al., 2000). Interestingly, we find that ^3H -dofetilide binding is displaced by BeKm-1 with a K_i of 740 nM. These results suggest that, when

compounds block hERG they cause allosteric changes, likely associated with the activation state of the channel, that affect the dofetilide binding site. Thus, these results imply that the ^3H -dofetilide binding assay is not limited to the detection of drugs that bind to the inner cavity of the hERG channel.

4.2. hERG functional assays

As discussed, the hERG binding assay is an excellent predictor of a compound's ability to block the hERG channel and it can be implemented early in drug discovery. The hERG binding assay does not, however, directly measure the effect of compounds on the hERG channel and therefore it is formally possible to miss or underestimate the potency of hERG blocking compounds. hERG functional assays, such as patch clamp, provide valuable information about a compound's ability to block the hERG channel in a living cell but it is typically used relatively late in drug discovery. One of the goals of this work was to identify a robust hERG functional assay that could be used in the early stages of drug discovery in conjunction with the hERG binding assay.

Patch clamp is a highly informative hERG functional assay, but its use is limited by its labor-intensive nature, requirement for specialized equipment, and its relatively high cost. Some of these barriers to early stage testing of compounds by patch clamp may be lowered by the recent introduction of planar patch clamp technology, which holds the promise of dramatically increasing the throughput and decreasing the cost of patch clamp analysis (Guo & Guthrie, 2005; Kiss et al., 2003; Netzer et al., 2003; Wang & Li, 2003; Willumsen et al., 2003). The rubidium efflux assay can also be used to examine the effect of a compound on the hERG channel (Gill et al., 2003; Netzer et al., 2003; Rezazadeh et al., 2004; Tang et al., 2001). This assay takes advantage of the facts that rubidium is similar in size to potassium, can pass through potassium channels, and can be measured by atomic absorbance (Terstappen, 1999). This assay has higher throughput and is less expensive than traditional patch clamp analysis and, therefore, has the potential to be used at an earlier stage of drug discovery. The rubidium efflux assay does, however, still require specialized equipment and personnel. In addition to patch clamp and rubidium efflux assays, which directly measure the ability of hERG to conduct ions, membrane potential indicator dyes can also be used to examine hERG function by measuring changes in membrane potential caused by conductance of ions by the hERG channel (Netzer et al., 2003; Tang et al., 2001). This assay is especially attractive because of its high-throughput capacity and its ability to be performed on equipment that is commonly used in drug discovery (e.g., Fluorescent Light Imaging Plate Reader (FLIPR)).

Rubidium efflux and membrane potential dye assays were performed with 12 compounds shown in Table 1. Published IC_{50} values for the inhibition of hERG by these compounds in hERG patch clamp analysis allowed the comparison of the results from this study to patch clamp analysis. The membrane potential dye was found to give a low signal-to-noise ratio of approximately 1.2 (data not shown). In addition to this narrow

dynamic range, there was considerable variability in this assay, especially with IMR-32 cells. The combination of a narrow dynamic range and high variability caused the Z' values to be very low. It is not unexpected that there would be significant noise in this assay since the assay measures membrane potential and depolarization is required to open the hERG channel. The finding that the membrane potential dye assay gave very low Z' values and a false negative rate of 0.5 (Fig. 6) indicates that this type of assay is not well suited for measuring the ability of compounds to inhibit the hERG channel.

Rubidium efflux is a highly robust assay ($Z'=0.80\pm 0.02$, mean \pm S.E.M.) whose results correlate well with patch clamp analysis (slope=0.87 and $r^2=0.73$, Fig. 7). The finding that IC_{50} values determined using the rubidium efflux assay are right-shifted compared to those determined by patch clamp is consistent with previously reported results (Rezazadeh et al., 2004). Although the right shift in IC_{50} values may lead to an underestimation of a compound's ability to inhibit the hERG ion channel, the rubidium assay gave no false positive or false negative results in this study. The high-throughput capacity, relatively low cost, and robust nature of the rubidium efflux assay indicates that it is a good early stage hERG functional assay.

4.3. Relevance to safety pharmacology

The hERG binding assay provides valuable information during drug discovery but it also has the potential to supplement existing methods used during the preclinical safety evaluation of new chemical entities. The work presented here and elsewhere (Chiu et al., 2004; Diaz et al., 2004; Rezazadeh et al., 2004) provides compelling evidence that the hERG binding, rubidium efflux, and patch clamp assays have similar predictive value for in vivo cardiac outcomes. Rubidium efflux and patch clamp are both functional assays and are therefore prone to generate false positive/negative results for similar types of molecules. In contrast, the hERG binding assay is not a functional assay so similar molecules would not be expected to give false positive/negative results in the hERG binding and patch clamp assays. Compounds that inhibit hERG in one but not the other type of assay could be singled out for more in-depth analysis to determine if they are true hERG inhibitors. Thus, performing the hERG binding assay in addition to patch clamp analysis would potentially improve the predictability of in vivo outcomes compared to performing either assay alone.

4.4. Relevance to clinical pharmacology

We have thus far described hERG binding and functional assays and their use as in vitro safety screens for drug discovery and development. However, questions regarding the interpretation of the results remain. First, what degree of selectivity between the target and hERG should be considered acceptable? Second, what impact do metabolic and pharmacokinetic considerations have on the potential for a compound to induce QT prolongation? Third, how does hERG affinity in

vitro relate to the QT prolongation effects observed in clinical studies?

Although we used a limited sample of drugs, considerations about the degree of selectivity needed can be made from examining Table 1. Among the three compounds in class B, there is general agreement about the identity of the molecular targets of risperidone, an antipsychotic, and imipramine, and an antidepressant. The targets for risperidone are thought to be the dopamine D2 and the 5HT2a receptors, for which it has an affinity of 2.7 and 1.1 nM, respectively (Kongsamut, Kang, Chen, Roehr, & Rampe, 2002; Schotte et al., 1996). The targets for imipramine are the 5-HT and norepinephrine transporters for which it has an affinity of 1.3 and 20 nM, respectively (Owens, Morgan, Plott, & Nemeroff, 1997). The ratio of hERG K_i to target K_i is 947 and 438 for risperidone and imipramine, respectively. The ratio of rubidium efflux IC_{50} to target K_i is 4505 and 1164 for risperidone and imipramine, respectively. Among the five compounds in class C, the non-antiarrhythmia drugs are pimozone, which is an antipsychotic, and terfenadine and astemizole, which are antihistaminergic. The target for pimozone is the D2 receptor, for which it has an affinity of 12 nM (Kongsamut et al., 2002; Schotte et al., 1996). The target for terfenadine and astemizole is the histamine H1 receptor, for which they have an affinity of 7.1 and 8.3 nM, respectively (Gillard, Van Der Perren, Moguilevsky, Massingham, & Chatelain, 2002; Ter Laak, Donne-Op den Kelder, Bast, & Timmerman, 1993). The ratio of hERG K_i to target K_i is 1.6, 18,

and 0.4 for pimozone, terfenadine, and astemizole, respectively and the ratio of rubidium efflux IC_{50} to target K_i is 0.9, 49, and 4.5, respectively. These data suggest that QT prolongation and TdP are likely when the hERG K_i and the target K_i are separated by less than 20-fold and rubidium efflux IC_{50} and the target K_i are separated by less than 50-fold. On the other hand, they also suggest that a separation of more than 3 log units between the hERG K_i and target K_i , and a separation of more than 3.5 log units between the rubidium IC_{50} and target K_i may be necessary to minimize the risk of inducing QT interval prolongation (Table 2). In addition to the degree of selectivity in vitro, metabolic and pharmacokinetic considerations will also determine the potential for a drug to induce QT prolongation. Three main considerations are plasma protein binding, the maximum plasma levels achieved when a drug is administered and tissue distribution. Recent reports have indicated that the ratio of hERG patch clamp IC_{50} to the maximum free (unbound) effective therapeutic plasma concentration (ETPC- $C_{unbound}$) corresponded well to the observed changes in QT (Kongsamut et al., 2002; Redfern et al., 2003). In the present report, the ratio between the hERG K_i and the ETPC $_{unbound}$ (Redfern et al., 2003) ranged from 42 to 1000 for class B compounds and from 1.9 to 44 for class C compounds, in agreement with the report from Redfern et al. The ratio between rubidium IC_{50} and the ETPC $_{unbound}$ ranged from 111 to 4756 for class B compounds and from 2.3 to 142 for class C compounds. Therefore, these data suggest a minimal

Table 2

| Drug | Target K_i (μ M) ^a | ETPC- unbound (μ M) ^b | Functional assays | | | | Binding assays | | | |
|-----------------------------|---|---|--|-----------------------------------|---|------------------------------------|---|---|-----------------------------------|---|
| | | | Mammalian patch clamp IC_{50} (μ M) | Rb-efflux IC_{50} (μ M) | Rb IC_{50} / Target K_i ratio | Rb IC_{50} / ETPC- unbound | 293-hERG ³ H-dofetilide K_i (μ M) | Binding K_i /target K_i ratio | Binding K_i /ETPC unbound | IMR-32 ³ H-dofetilide K_i (μ M) |
| <i>Class A</i> | | | | | | | | | | |
| Acetaminophen | | | ND | >20 | | | >20 | | | >20 |
| Bromopride | | | ND | >20 | | | >20 | | | >20 |
| Chloramphenicol | | | ND | >20 | | | >20 | | | >20 |
| Aspirin | | | ND | >20 | | | >20 | | | >20 |
| <i>Class B</i> | | | | | | | | | | |
| Imipramine | 0.0013 0.020 | 0.11 | 3.4 | 12.22 | 1164 | 111 | 4.6 | 438 | 42 | >20 |
| Risperidone | 0.0027 0.0011 | 0.0018 | 0.17 | 8.56 | 4505 | 4756 | 1.8 | 947 | 1000 | 7.1 |
| Amitriptyline | | 0.041 | 10 | 8.35 | | 204 | 7.2 | | 176 | >20 |
| <i>Class C</i> | | | | | | | | | | |
| Pimozone (Orap) | 0.012 | 0.00043 | 0.018 | 0.011 | 0.9 | 26 | 0.019 | 1.6 | 44 | 5.4 |
| Terfenadine (Seldane) | 0.0071 | 0.004 (with P450 blocker) | 0.009 0.079 0.016 | 0.345 | 49 | 86 | 0.13 | 18 | 32 | 7.3 |
| Astemizole (Histanol TM) | 0.0083 | 0.00026 | 0.0009 0.0012 0.0016 | 0.037 | 4.5 | 142 | 0.0033 | 0.4 | 12.7 | 0.06 |
| Quinidine (Quinaglute) | | 3.2 | 0.41 1.0 1.4 | 7.4 | | 2.3 | 6 | | 1.9 | >20 |
| Dofetilide (Tikosyn) | | 0.002 | 0.011 0.012 | 0.014 | | 7 | 0.0082 | | 4.1 | 0.61 |

^a See text for references.

^b Redfern et al. (2003).

safety margin of 1000-fold between hERG binding K_i and $ETPC_{unbound}$ and 5000-fold between rubidium IC_{50} and $ETPC_{unbound}$. These data are in good agreement with the in vitro selectivity needed as described above and thereby illustrate the importance of knowing the percentage of compound bound to plasma proteins when considering safety margins in vivo. Even if the ratio of target K_i to hERG K_i /rubidium IC_{50} is reasonably large, the data in Table 1 suggests that the absolute hERG affinity of a compound is an important consideration when determining if a compound should move forward during early stages of drug discovery. Factors such as tissue distribution may give different exposures of a compound to hERG and the intended target, thereby reducing the predictability of a ratio of K_i values for hERG and the intended target. Thus, the absolute hERG affinity may be an important consideration during the very earliest stages of drug discovery. The average K_i values for class B and class C compound were 4.5 μ M and 1.2 μ M (0.04 μ M without quinidine), respectively. Therefore, these data suggest that, when the hERG binding potency is less than 5 μ M, there is a risk of inducing QT prolongation, which will become more significant when the potency is less than 1 μ M. Similar analysis with the rubidium efflux assay suggests that, when the IC_{50} is less than 10 μ M, there is a risk of inducing QT prolongation, which will become more significant when the potency is less than 1 μ M. This information will be important early in discovery for guiding SAR or choosing between multiple possible lead series.

4.5. General conclusion

The hERG binding assay has been shown to be highly robust and predictive of in vivo cardiac liabilities. In addition, it can be performed in a high-throughput format, it is inexpensive, and does not require dedicated equipment or highly specialized personnel. Thus, it can be used in the earliest stages of drug discovery to identify compounds that either should be eliminated or require SAR to decrease the hERG liability. The rubidium efflux assay is a highly robust functional assay that is predictive of in vivo cardiac liabilities. In addition, it has the throughput capacity and cost that allow it to be used much earlier in drug discovery than other functional assays like patch clamp. Although these assays will never replace the need to perform more laborious functional assays, they will allow for the elimination of problematic compounds earlier in the drug discovery process and therefore lead to lower failure rates in more complex assays thereby saving time and money.

References

- Abbott, G. W., Sesti, F., Splawski, I., Buck, M. E., Lehmann, M. H., Timothy, K. W., et al. (1999). MiRP1 forms IKr potassium channels with HERG and is associated with cardiac arrhythmia. *Cell*, *97*, 175–187.
- Angelo, K., Korolkova, Y. V., Grunnet, M., Grishin, E. V., Pluzhnikov, K. A., Klaerke, D. A., et al. (2003). A radiolabeled peptide ligand of the hERG channel, [125I]-BeKm-1. *Pflugers Archiv*, *447*, 55–63.
- Chadwick, C. C., Ezrin, A. M., O'Connor, B., Volberg, W. A., Smith, D. I., Wedge, K. J., et al. (1993). Identification of a specific radioligand for the cardiac rapidly activating delayed rectifier K⁺ channel. *Circulation Research*, *72*, 707–714.
- Chiu, P. J., Marcoe, K. F., Bounds, S. E., Lin, C. H., Feng, J. J., Lin, A., et al. (2004). Validation of a [3H]astemizole binding assay in HEK293 cells expressing HERG K⁺ channels. *Journal of Pharmacological Science*, *95*, 311–319.
- Curran, M. E., Splawski, I., Timothy, K. W., Vincent, G. M., Green, E. D., & Keating, M. T. (1995). A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. *Cell*, *80*, 795–803.
- Daleau, P., Lessard, E., Groleau, M. F., & Turgeon, J. (1995). Erythromycin blocks the rapid component of the delayed rectifier potassium current and lengthens repolarization of guinea pig ventricular myocytes. *Circulation*, *91*, 3010–3016.
- Diaz, G. J., Daniell, K., Leitza, S. T., Martin, R. L., Su, Z., McDermott, J. S., et al. (2004). The [3H]dofetilide binding assay is a predictive screening tool for hERG blockade and proarrhythmia: Comparison of intact cell and membrane preparations and effects of altering [K⁺]_o. *Journal of Pharmacological and Toxicological Methods*, *50*, 187–199.
- Fenichel, R. R., Malik, M., Antzelevitch, C., Sanguinetti, M., Roden, D. M., Priori, S. G., et al. (2004). Drug-induced Torsades de Pointes and implications for drug development. *Journal of Cardiovascular Electrophysiology*, *15*, 475–495.
- Fermini, B., & Fossa, A. A. (2003). The impact of drug-induced QT interval prolongation on drug discovery and development. *Nature Review Drug Discovery*, *2*, 439–447.
- Ficker, E., Jarolimek, W., & Brown, A. M. (2001). Molecular determinants of inactivation and dofetilide block in ether a-go-go (EAG) channels and EAG-related K(+) channels. *Molecular Pharmacology*, *60*, 1343–1348.
- Finlayson, K., Turnbull, L., January, C. T., Sharkey, J., & Kelly, J. S. (2001). [3H]Dofetilide binding to HERG transfected membranes: A potential high throughput preclinical screen. *European Journal of Pharmacology*, *430*, 147–148.
- Gill, S., Gill, R., Lee, S. S., Hesketh, J. C., Fedida, D., Rezazadeh, S., et al. (2003). Flux assays in high throughput screening of ion channels in drug discovery. *Assay Drug Development Technology*, *1*, 709–717.
- Gillard, M., Van Der Perren, C., Moguilevsky, N., Massingham, R., & Chatelain, P. (2002). Binding characteristics of cetirizine and levocetirizine to human H(1) histamine receptors: Contribution of Lys(191) and Thr(194). *Molecular Pharmacology*, *61*, 391–399.
- Guo, L., & Guthrie, H. (2005). Automated electrophysiology in the preclinical evaluation of drugs for potential QT prolongation. *Journal of Pharmacological and Toxicological Methods*, *52*, 123–135.
- Jurkiewicz, N. K., & Sanguinetti, M. C. (1993). Rate-dependent prolongation of cardiac action potentials by a methanesulfonanilide class III antiarrhythmic agent. Specific block of rapidly activating delayed rectifier K⁺ current by dofetilide. *Circulation Research*, *72*, 75–83.
- Kang, J., Chen, X. L., Wang, H., Ji, J., Reynolds, W., Lim, S., et al. (2004). Cardiac ion channel effects of tolerodine. *Journal of Pharmacology and Experimental Therapeutics*, *308*, 935–940.
- Keating, M. T., & Sanguinetti, M. C. (2001). Molecular and cellular mechanisms of cardiac arrhythmias. *Cell*, *104*, 569–580.
- Kiehn, J., Lacerda, A. E., Wible, B., & Brown, A. M. (1996). Molecular physiology and pharmacology of HERG. Single-channel currents and block by dofetilide. *Circulation*, *94*, 2572–2579.
- Kiss, L., Bennett, P. B., Uebele, V. N., Koblan, K. S., Kane, S. A., Neagle, B., et al. (2003). High throughput ion-channel pharmacology: Planar-array-based voltage clamp. *Assay Drug Development Technology*, *1*, 127–135.
- Kongsamut, S., Kang, J., Chen, X. L., Roehr, J., & Rampe, D. (2002). A comparison of the receptor binding and HERG channel affinities for a series of antipsychotic drugs. *European Journal of Pharmacology*, *450*, 37–41.
- Korolkova, Y. V., Bocharov, E. V., Angelo, K., Maslennikov, I. V., Grinenko, O. V., Lipkin, A. V., et al. (2002). New binding site on common molecular scaffold provides HERG channel specificity of scorpion toxin BeKm-1. *Journal of Biological Chemistry*, *277*, 43104–43109.
- Lees-Miller, J. P., Duan, Y., Teng, G. Q., & Duff, H. J. (2000). Molecular determinant of high-affinity dofetilide binding to HERG1 expressed in *Xenopus* oocytes: Involvement of S6 sites. *Molecular Pharmacology*, *57*, 367–374.

- Milnes, J. T., Dempsey, C. E., Ridley, J. M., Crociani, O., Arcangeli, A., Hancox, J. C., et al. (2003). Preferential closed channel blockade of HERG potassium currents by chemically synthesised BeKm-1 scorpion toxin. *FEBS Letters*, *547*, 20–26.
- Mitcheson, J. S., Chen, J., Lin, M., Culberson, C., & Sanguinetti, M. C. (2000). A structural basis for drug-induced long QT syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 12329–12333.
- Mitcheson, J. S., & Perry, M. D. (2003). Molecular determinants of high-affinity drug binding to HERG channels. *Current Opinion in Drug Discovery & Development*, *6*, 667–674.
- Netzer, R., Bischoff, U., & Ebneith, A. (2003). HTS techniques to investigate the potential effects of compounds on cardiac ion channels at early-stages of drug discovery. *Current Opinion in Drug Discovery & Development*, *6*, 462–469.
- Owens, M. J., Morgan, W. N., Plott, S. J., & Nemeroff, C. B. (1997). Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *Journal of Pharmacology and Experimental Therapeutics*, *283*, 1305–1322.
- Paul, A. A., Witchel, H. J., & Hancox, J. C. (2002). Inhibition of the current of heterologously expressed HERG potassium channels by flecainide and comparison with quinidine, propafenone and lignocaine. *British Journal of Pharmacology*, *136*, 717–729.
- Pond, A. L., Scheve, B. K., Benedict, A. T., Petrecca, K., VanWagoner, D. R., Shrier, A., et al. (2000). Expression of distinct ERG proteins in rat, mouse, and human heart. Relation to functional I(Kr) channels. *Journal of Biological Chemistry*, *275*, 5997–6006.
- Priori, S. G., & Napolitano, C. (2004). Genetics of cardiac arrhythmias and sudden cardiac death. *Annals of the New York Academy of Sciences*, *1015*, 96–110.
- Rasmussen, H. S., Allen, M. J., Blackburn, K. J., Butrous, G. S., & Dalrymple, H. W. (1992). Dofetilide, a novel class III antiarrhythmic agent. *Journal of Cardiovascular Pharmacology*, *20*(Suppl 2), S96–S105.
- Redfern, W. S., Carlsson, L., Davis, A. S., Lynch, W. G., MacKenzie, I., Palethorpe, S., et al. (2003). Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and Torsades de Pointes for a broad range of drugs: Evidence for a provisional safety margin in drug development. *Cardiovascular Research*, *58*, 32–45.
- Rezazadeh, S., Hesketh, J. C., & Fedida, D. (2004). Rb⁺ flux through hERG channels affects the potency of channel blocking drugs: Correlation with data obtained using a high-throughput Rb⁺ efflux assay. *Journal of Biomolecular Screening*, *9*, 588–597.
- Roden, D. M. (2004). Drug-induced prolongation of the QT interval. *New England Journal of Medicine*, *350*, 1013–1022.
- Roden, D. M., Lazzara, R., Rosen, M., Schwartz, P. J., Towbin, J., & Vincent, G. M. (1996). Multiple mechanisms in the long-QT syndrome. Current knowledge, gaps, and future directions. The SADS Foundation Task Force on LQTS. *Circulation*, *94*, 1996–2012.
- Roy, M., Dumaine, R., & Brown, A. M. (1996). HERG, a primary human ventricular target of the nonsedating antihistamine terfenadine. *Circulation*, *94*, 817–823.
- Saganich, M. J., Machado, E., & Rudy, B. (2001). Differential expression of genes encoding subthreshold-operating voltage-gated K⁺ channels in brain. *Journal of Neuroscience*, *21*, 4609–4624.
- Salata, J. J., Jurkiewicz, N. K., Wallace, A. A., Stupinski III, R. F., Guinasso Jr., P. J., & Lynch Jr., J. J. (1995). Cardiac electrophysiological actions of the histamine H1-receptor antagonists astemizole and terfenadine compared with chlorpheniramine and pyrilamine. *Circular Research*, *76*, 110–119.
- Sanguinetti, M. C., Jiang, C., Curran, M. E., & Keating, M. T. (1995). A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr potassium channel. *Cell*, *81*, 299–307.
- Sanguinetti, M. C., & Jurkiewicz, N. K. (1990). Two components of cardiac delayed rectifier K⁺ current. Differential sensitivity to block by class III antiarrhythmic agents. *Journal of General Physiology*, *96*, 195–215.
- Schotte, A., Janssen, P. F., Gommeren, W., Luyten, W. H., Van Gompel, P., Lesage, A. S., et al. (1996). Risperidone compared with new and reference antipsychotic drugs: In vitro and in vivo receptor binding. *Psychopharmacology (Berl)*, *124*, 57–73.
- Schwarz, J. R., & Bauer, C. K. (2004). Functions of erg K⁺ channels in excitable cells. *Journal of Cellular and Molecular Medicine*, *8*, 22–30.
- Snyders, D. J., & Chaudhary, A. (1996). High affinity open channel block by dofetilide of HERG expressed in a human cell line. *Molecular Pharmacology*, *49*, 949–955.
- Spector, P. S., Curran, M. E., Keating, M. T., & Sanguinetti, M. C. (1996). Class III antiarrhythmic drugs block HERG, a human cardiac delayed rectifier K⁺ channel. Open-channel block by methanesulfonanilides. *Circular Research*, *78*, 499–503.
- Suessbrich, H., Waldeger, S., Lang, F., & Busch, A. E. (1996). Blockade of HERG channels expressed in *Xenopus* oocytes by the histamine receptor antagonists terfenadine and astemizole. *FEBS Letters*, *385*, 77–80.
- Tang, W., Kang, J., Wu, X., Rampe, D., Wang, L., Shen, H., et al. (2001). Development and evaluation of high throughput functional assay methods for HERG potassium channel. *Journal of Biomolecular Screening*, *6*, 325–331.
- Ter Laak, A. M., Donne-Op den Kelder, G. M., Bast, A., & Timmerman, H. (1993). Is there a difference in the affinity of histamine H1 receptor antagonists for CNS and peripheral receptors? An in vitro study. *European Journal of Pharmacology*, *232*, 199–205.
- Terstappen, G. C. (1999). Functional analysis of native and recombinant ion channels using a high-capacity nonradioactive rubidium efflux assay. *Analytical Biochemistry*, *272*, 149–155.
- Teschmacher, A. G., Seward, E. P., Hancox, J. C., & Witchel, H. J. (1999). Inhibition of the current of heterologously expressed HERG potassium channels by imipramine and amitriptyline. *British Journal of Pharmacology*, *128*, 479–485.
- Tie, H., Walker, B. D., Valenzuela, S. M., Breit, S. N., & Campbell, T. J. (2000). The heart of psychotropic drug therapy. *Lancet*, *355*, 1825.
- Trudeau, M. C., Warmke, J. W., Ganetzky, B., & Robertson, G. A. (1995). HERG, a human inward rectifier in the voltage-gated potassium channel family. *Science*, *269*, 92–95.
- Volberg, W. A., Koci, B. J., Su, W., Lin, J., & Zhou, J. (2002). Blockade of human cardiac potassium channel human ether-a-go-go-related gene (HERG) by macrolide antibiotics. *Journal of Pharmacology and Experimental Therapeutics*, *302*, 320–327.
- Wang, J., Della Penna, K., Wang, H., Karczewski, J., Connolly, T. M., Koblan, K. S., et al. (2003). Functional and pharmacological properties of canine ERG potassium channels. *American Journal of Physiology. Heart and Circulatory Physiology*, *284*, H256–H267.
- Wang, Z., Fermi, B., & Nattel, S. (1994). Rapid and slow components of delayed rectifier current in human atrial myocytes. *Cardiovascular Research*, *28*, 1540–1546.
- Wang, X., & Li, M. (2003). Automated electrophysiology: High throughput of art. *Assay Drug Development Technology*, *1*, 695–708.
- Warmke, J. W., & Ganetzky, B. (1994). A family of potassium channel genes related to eag in *Drosophila* and mammals. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 3438–3442.
- Weerapura, M., Nattel, S., Chartier, D., Caballero, R., & Hebert, T. E. (2002). A comparison of currents carried by HERG, with and without coexpression of MiRP1, and the native rapid delayed rectifier current. Is MiRP1 the missing link? *Journal of Physiology*, *540*, 15–27.
- Willumsen, N. J., Bech, M., Olesen, S. P., Jensen, B. S., Korsgaard, M. P., & Christophersen, P. (2003). High throughput electrophysiology: New perspectives for ion channel drug discovery. *Receptors and Channels*, *9*, 3–12.
- Wymore, R. S., Gintant, G. A., Wymore, R. T., Dixon, J. E., McKinnon, D., & Cohen, I. S. (1997). Tissue and species distribution of mRNA for the IKr-like K⁺ channel, erg. *Circular Research*, *80*, 261–268.
- Yang, T., Wathen, M. S., Felipe, A., Tamkun, M. M., Snyders, D. J., & Roden, D. M. (1994). K⁺ currents and K⁺ channel mRNA in cultured atrial cardiac myocytes (AT-1 cells). *Circular Research*, *75*, 870–878.

- Yu, S. P., & Kerchner, G. A. (1998). Endogenous voltage-gated potassium channels in human embryonic kidney (HEK293) cells. *Journal of Neuroscience Research*, *52*, 612–617.
- Zhang, J. H., Chung, T. D., & Oldenburg, K. R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *Journal of Biomolecular Screening*, *4*, 67–73.
- Zhou, Z., Vorperian, V. R., Gong, Q., Zhang, S., & January, C. T. (1999). Block of HERG potassium channels by the antihistamine astemizole and its metabolites desmethylastemizole and norastemizole. *Journal of Cardiovascular Electrophysiology*, *10*, 836–843.
- Zolotoy, A. B., Plouvier, B. P., Beatch, G. B., Hayes, E. S., Wall, R. A., & Walker, M. J. (2003). Physicochemical determinants for drug induced blockade of HERG potassium channels: Effect of charge and charge shielding. *Current Medicinal Chemistry Cardiovascular Hematology Agents*, *1*, 225–241.