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Evaluation of the Rubidium Efflux Assay for Preclinical Identification of hERG Blockade

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Abstract: Inhibition of the delayed-rectifier potassium channel current, human *ether-a-go-go* (hERG), by pharmaceutical agents can lead to acquired long QT syndrome and the generation of potentially lethal arrhythmias and sudden death. There remains an unmet need for higher-throughput assays to screen compounds in preclinical development for the potential to block hERG and cause QT prolongation. We evaluated the rubidium efflux assay for its ability to determine block of the hERG potassium channel. hERG-transfected human embryonic kidney-293 cells were cultured on 96-well assay plates and loaded with rubidium ion by incubating in media in which potassium was replaced by 5.4 m*M* Rb^+. Cells were exposed to test compounds and then depolarized with a K^+ channel opening buffer containing 50 m*M* K^+. The supernatant was removed, and cells were lysed using 0.1% Triton X-100. Concentration–response curves were generated for test agents by determining the Rb^+ efflux using a flame atomic absorption spectrometer. Multiple trials with cisapride yielded 50% inhibitory concentration values between 308.1 ± 11 n*M* to 456.3 ± 24 n*M* for inhibition of Rb^+ efflux and a *Z* factor of 0.80 ± 0.07 ($n = 5$ plates, 12 wells per plate). The values for inhibition of the hERG channel exhibited a rightward shift in potency as compared to those measured using electrophysiological techniques. In addition, we evaluated 19 blinded compounds at 10 μM in the Rb^+ efflux assay, and compared results to those using patch clamp electrophysiology and the dofetilide displacement binding assay. The dofetilide displacement binding assay yielded a good correlation with electrophysiological measurements of hERG block. The rubidium efflux assay lacked sensitivity to consistently identify significant channel blockade. In conclusion, the rubidium efflux assay provides a higher-throughput means to identify potent hERG channel blocking agents, but lacks the sensitivity required to accurately determine the potency of blockade.

Introduction

RECENT ADVANCES in the structure of potassium channels and their interactions with different compounds has furthered our understanding of drug-induced or acquired long QT syndrome.[1–7] At the cellular level, inhibition of the hERG-related potassium channel leads to a prolongation of the cardiac action potential or the QT interval, and can lead to re-entrant arrhythmias and tachycardia.[8] Prolonged instances of arrhythmia can lead to sudden death caused by induction of *torsades de pointes* with subsequent degeneration into cardiac fibrillation. Moreover, modulation of potassium channel currents has historically been the therapeutic target for anti-arrhythmic and antifibrillatory drugs.[9] Paradoxically, current drugs that are often prescribed to treat the onset of ventricular fibrillation and arrhythmia are often, by nature, proarrhythmogenic.[10]

Recently, several Food and Drug Administration-approved medications not intended for use as anti-arrhythmics have been retracted from commercial sales because of their affinity for the hERG channel and their propensity to cause acquired long QT syndrome.[11,12] One of these drugs, cisapride, was commonly prescribed to enhance gastric motility. Although this drug accounted for

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; HEK, human embryonic kidney; hERG, human *ether-a-go-go*; IC_{50}, 50% inhibitiory concentration.

several millions of dollars in sales, its inhibitory effects on hERG current and subsequent induction of *torsades de pointes* led to its removal from the market.[13–15]

The potential of compounds from different pharmaceutical classes to cause alterations in the cardiac action potential is an important consideration when devising screening strategies. From the perspective of cost alone, instances such as the retraction of cisapride further reinforce the utilization of preclinical testing to determine whether a new pharmaceutical agent will have contraindications such as QT prolongation.

Historically, the "gold standard" for measurement and screening of ion channel modulators has been electrophysiology. Although this method produces data of high fidelity, the procedure is labor intensive and cost prohibitive. Medicinal chemists produce thousands of variants of each novel chemical structure in the hopes to find a few with a high degree of efficacy for the intended use, which makes screening each one of these compounds virtually impossible using conventional patch clamp techniques. With the advent of robotics and microfluidics, the enormous volume of compounds developed can be screened using the proper HTS. Automation of the patch clamp electrophysiology technique is ideally the most favorable approach. Swanson *et al.*[16] have recently evaluated one of the more common HTS electrophysiology platforms, and found shifts in the potency of compounds up to 200-fold compared to manual patch clamp electrophysiology—averaging upwards of a fivefold shift for their study. In addition, the current automated patch clamp platforms have a relatively high initial cost as well as a higher per data point cost as compared to most HTS-type assays. Alternatively, fluorescence-based techniques, competitive displacement assays, and ionic flux assays have been developed to meet this challenge of higher throughput while retaining a lower cost.

In this study, we aim to highlight the rubidium efflux assay, which allows for the functional measurement of HERG inhibition by different compounds. Several reference agents were tested to compare compound affinity as measured by electrophysiology and radioactive dofetilide displacement. Finally, compounds were used in a blinded comparison between the dofetilide displacement assay and rubidium efflux assay. Our results suggest a possible role for the rubidium efflux assay to test compounds within a series for relative potency against the hERG channel; however, this platform does not prove sensitive enough to make comparisons between multiple families of compounds because of the unpredictably large rightward shift in potency.

Materials and Methods

Cell culture and isolation

HEK-293 cells (Wisconsin Alumni Research Foundation, Madison, WI), which stably express the hERG potassium channel, were incubated at 37°C in 5% CO_2 in Minimum Essential Medium (Invitrogen Corp., Carlsbad, CA) containing the following: 10% fetal bovine serum (Harlan Bioscience, Indianapolis, IN), 1× penicillin-streptomycin, 1× sodium pyruvate, 1× non-essential amino acids (Invitrogen), and 400 μg/ml geneticin (Mediatech Inc., Herndon, VA). Cells were washed with phosphate-buffered saline and trypsinized with 0.25% trypsin/EDTA (Invitrogen). After isolation, cells were plated at a density of 60,000 cells per well on poly-D-lysine-coated 96-well assay plates (Becton Dickinson, Billerica, MA). Assay plates were incubated for 24 h in supplemented media to allow cells to adhere and become 90% confluent.

Rb^+ efflux assay

After a 24-h incubation, assay plates were washed three times with phosphate-buffered saline to remove remaining media. Cells were then incubated (37°C in 5% CO_2) with Rb-loading buffer containing the following: 150 m*M* NaCl, 2 m*M* $CaCl_2$, 0.8 m*M* NaH_2PO_4, 1 m*M* $MgCl_2$, 5 m*M* glucose, 25 m*M* HEPES, and 5.4 m*M* RbCl. Meanwhile, test compounds were prepared in 100% DMSO and diluted to a 10× final concentration in the Rb-loading buffer. After a 3-h incubation, each well was spiked to give a 1× concentration of test compound and incubated for an additional 30 min. Wells were then washed three times with phosphate-buffered saline to remove excess Rb loading buffer. To activate the opening of hERG channels, cells were stimulated with buffer (37°C) containing 50 m*M* KCl instead of RbCl, for an additional 10 min. The channel opening buffer also contained 1× test compound to avoid washout.

Finally, 200 μl of supernatant was collected in a separate assay plate, while cells were lysed with an equal volume of 0.1% Triton. The ICR-8000 flame atomic absorption spectrometer (Aurora Biomed Inc., Vancouver, BC, Canada) was used to generate a standard curve for Rb^+ concentration, and 100-μl quantities of the supernatant and lysate from each experiment were run separately through the spectrometer to determine fractional Rb^+ efflux. Rb^+ efflux was used to quantify channel activity and was represented as the ratio of the Rb^+ content of the supernatant with respect to the total Rb^+ in each well. Data were then normalized to account for variation in total Rb^+ efflux between multiple experiments using the following equation:

$$\text{Normalized efflux} = ([Rb^+]_{frac} - [Rb^+]_{bkgd})/([Rb^+]_{max} - [Rb^+]_{bkgd})$$

where $[Rb^+]_{frac}$ = fractional efflux, $[Rb^+]_{bkgd}$ = unstimulated efflux, and $[Rb^+]_{max}$ = maximum efflux, respectively.

Patch clamp electrophysiology

HEK-293 cells stably expressing hERG were maintained in our tissue culture facility. On the day of the experiment the cells were lifted from the bottom of the culture flask using 0.05% trypsin-EDTA and stored at room temperature in standard Dulbecco's Modified Eagle Medium culture medium with HEPES (to maintain the pH at 7.4) until use.

The standard external bath solution used to record hERG currents had the following ionic composition: 137 m*M* NaCl, 4 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES, pH 7.4 with NaOH. The standard internal pipette solution was composed of: 130 m*M* KCl, 5 m*M* MgATP, 1.0 m*M* $MgCl_2$, 10 m*M* HEPES, and 5 m*M* EGTA, pH 7.2 with KOH. All experiments were performed at 35 ± 1°C, and bath temperature was maintained by a temperature controller system (Cell MicroControls, Norfolk, VA). All drugs were dissolved in DMSO at 10 m*M* and added directly into the bath solution to obtain the final desired concentration. Final DMSO concentration was <0.3%.

Currents were measured using the whole-cell configuration of the patch clamp technique. The hERG currents were elicited by 1-s voltage pulses to +20 mV (0.25 Hz) from a holding potential of −80 mV followed by a repolarizing ramp (0.5 mV/ms) back to −80 mV. Recordings were started approximately 5–10 min after membrane rupture to allow for cell dialysis with the pipette solution. Compounds were perfused in the bath after steady-state control conditions were observed for at least 3–5 min. The effects of the compounds were studied on the peak current measured at approximately −40 mV. The rate of current rundown over the course of the experiment was assumed to be equal to the slope of the regression of current against time during the linear portion of the control period. Steady-state block was determined as the difference between the amplitude of the current measured in the presence of the drug and the corresponding predicted control value based on the rundown rate:

$$\% \text{ Inhibition} = [(I_{\text{Control}} - I_{\text{Drug}})/I_{\text{Control}}] \times 100$$

Data are expressed as mean ± SEM.

Dofetilide displacement binding assay

Membrane aliquots from hERG-transfected HEK-293 cells were thawed, homogenized, and diluted in 50 m*M* Tris-HCl, pH 7.4 at 4°C, with 1 m*M* $MgCl_2$ and 10 m*M* KCl. Test compounds were serially diluted from DMSO stocks into 50 m*M* Tris-HCl assay buffer, pH 7.4, at room temperature with 1 m*M* $MgCl_2$ and 10 m*M* KCl using a PerkinElmer (Boston, MA) MultiProbe II HT EX automation system. The binding assay consisted of test compounds at appropriate dilutions (1% DMSO final concentration), 5 n*M* [^{3}H]dofetilide (specific activity, 84 Ci/mmol; Amersham Biosciences, Piscataway, NJ), and hERG-transfected HEK-293 membranes at 0.24 mg/ml. Following a 90-min incubation at room temperature, separation occurred by filtration through PerkinElmer UniFilter-96 GF/B polyethyleneimine-coated filter plates using a Packard Filtermate (PerkinElmer). After filters were air-dried, 25 μl of Microscint 20 was added, and filter plates read on a Packard TopCount (PerkinElmer). Nonspecific binding was determined using unlabeled E-4031 at 10 μ*M*. From the seven-point dose–response curves generated, an IC_{50} was determined for each drug and converted to K_i using the Cheng-Prussoff equation.

Materials

Reagents were purchased from Sigma Aldrich (St. Louis, MO), unless stated otherwise.

Statistics

All data are given as mean ± SEM, unless stated otherwise. IC_{50} relationships were determined using Origin version 5.0 software (Originlab, Northampton, MA), with a Boltzmann fit normalized to maximum and minimum efflux.

Results

hERG activation

Our initial experiments compared the efflux of Rb^+ from wild-type versus hERG-expressing HEK-293 cells.

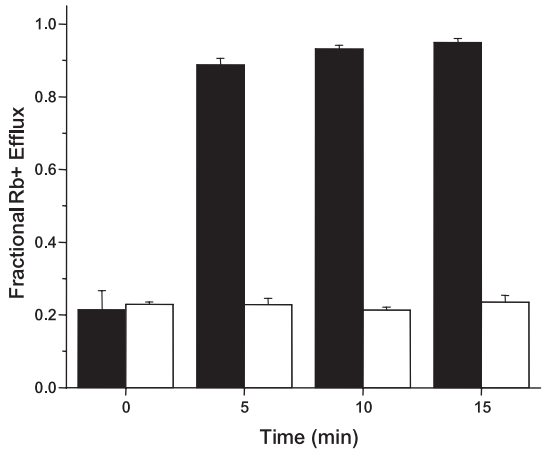

FIG. 1. Fractional rubidium efflux measured in wild-type human embryonic kidney (HEK)-293 cells (white columns), compared to human *ether-a-go-go* (hERG)-expressing HEK-293 cells (black columns). Each group was treated with high K^+ (50 m*M*)-containing depolarizing buffer to elicit HERG channel opening. Different durations of depolarization were tested to determine maximum possible efflux and optimal conditions for subsequent experiments. Data are expressed as mean ± SEM (n = 6 wells for each group per time point).

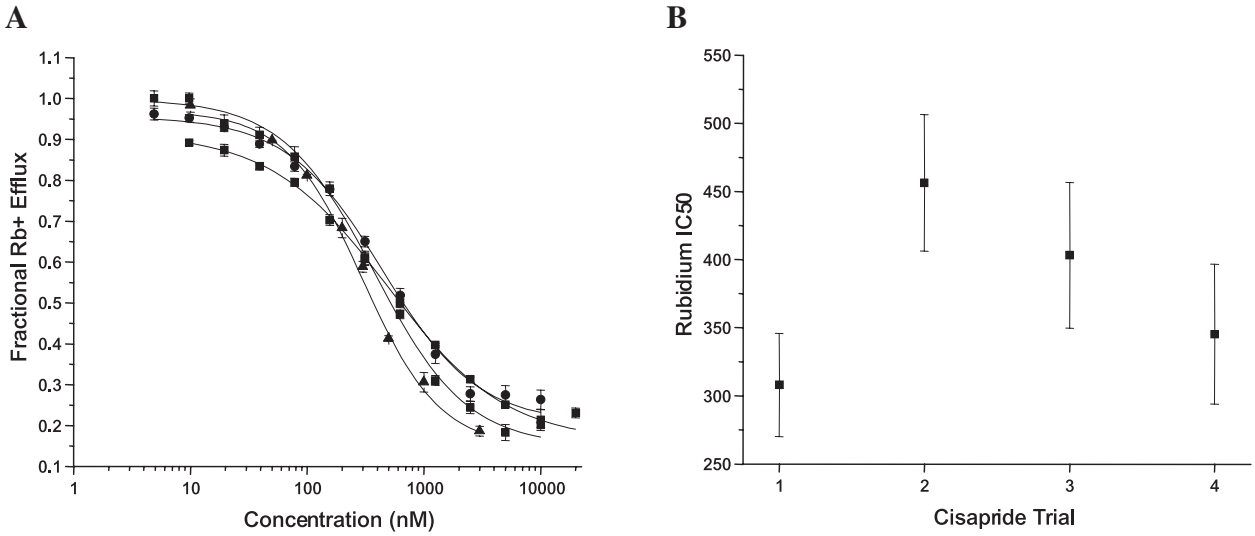

FIG. 2. (**A**) Multiple concentration–response curves generated for the potent human *ether-a-go-go* blocker, cisapride. Fractional rubidium efflux was normalized using the equation given in Materials and Methods to allow for multiple assay comparison. The average 50% inhibitory concentration (IC_{50}) value for the four trials was 397.8 ± 37.3 n*M*. Data are expressed as mean ± SEM ($n = 7$ wells per concentration for each trial). (**B**) Average IC_{50} values for the four individual cisapride trials, with confidence intervals ± 1 SD.

These experiments also determined the maximum possible efflux from hERG-expressing cells after varying durations of exposure to depolarizing buffer. Wild-type HEK-293 cells load with Rb^+ similar to hERG-expressing cells, but do not efflux rubidium upon depolarization (Fig. 1). However, a small amount of Rb^+ (23.8 ± 1.6%) does passively diffuse from the cells. Interestingly, the amount of passive efflux is not significantly different in unstimulated HEK-293 cells expressing the hERG channel (21.4 ± 5.2%). Depolarization of these cells was accomplished with physiological buffer containing 50 m*M* K^+, as previously reported.[17,18] Upon replacement of extracellular buffer with the high K^+ buffer, a maximum of 88.7 ± 1.7% efflux was recorded in hERG-expressing cells after 5 min of depolarization. Subsequent experiments were carried out with depolarization times of 10 min, which was found to be the optimal duration of chemical depolarization using 50 m*M* K^+, allowing for maximum Rb^+ efflux.

Repeatability

To evaluate the dynamic range of the rubidium efflux assay, a *Z* factor was calculated using control data obtained from multiple plates.[19] According to Zhang *et al.*,[19] a high-throughput assay with a *Z* factor value greater than 0.5 can be categorized as an "excellent assay." The observed *Z* factor of 0.80 ± 0.07 ($n = 5$ plates, 12 wells per plate) is indicative of a high-quality assay.

In order to test the reproducibility of the assay, multiple experiments were carried out using the standard reference agent, cisapride. Ninety-six-well assay plates with serial dilutions of cisapride were prepared while cells were incubating separately in load buffer for 3 h. Measured IC_{50} values for cisapride ranged from 308.1 ± 11 n*M* to 456.3 ± 24 n*M* (Fig. 2A). Furthermore, three out of four of the IC_{50} values determined for cisapride fall within 1 SD of each other (Fig. 2B).

Reference compounds

Concentration–response curves were generated for several reference agents known to inhibit the hERG potassium channel. These compounds are representative of different pharmaceutical classes, including anti-arrhythmics, antipsychotics, and antihistamines, as well as others. Figure 3 depicts the concentration–response curves obtained for each of these compounds. Table 1 lists the rank order of IC_{50} values for each compound as generated by the rubidium efflux assay, in comparison to reported values for two other widely used preclinical screens—conventional patch clamp electrophysiology and the radioactive dofetilide displacement binding assay.

Unknown compounds

As with any preclinical screening tool, the goal is to provide accurate liability/potency information for compounds in development. We sought to test several internal compounds of different pharmaceutical classes, in a blinded study. Nineteen compounds in various stages of development were chosen for analysis by the rubidium efflux assay. Each compound was tested us-

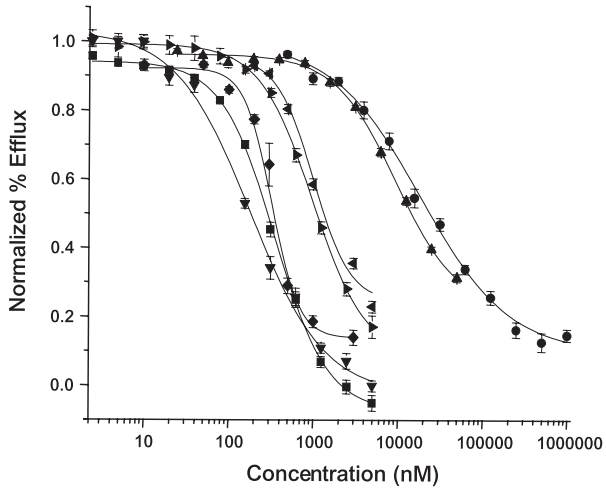

FIG. 3. Concentration–response curves generated for reference agents of various pharmaceutical classes with known human *ether-a-go-go* blocking properties: astemizole (■), quinidine (●), risperidone (▲) E-4031 (▼), sotalol (◆), terfenadine (◀), and pimozide (▶). Data are expressed as mean ± SEM ($n = 7$ wells per concentration of drug).

ing a conventional strategy, *i.e.*, at a fixed concentration of 10 μM, in 0.1% DMSO (final concentration). The data generated by the rubidium efflux assay (Fig. 4) were then compared to historical data from two other screening modalities—the dofetilide displacement binding assay as well as patch clamp electrophysiology (Fig. 4). The rubidium efflux assay was observed to lack the sensitivity necessary to rank order the unknown compounds (Fig. 4A). In fact, only four out of 19 compounds tested positive for hERG blockade at 10 μM. Conversely, retrospective comparison of the dofetilide displacement binding assay versus patch clamp electrophysiology (Fig. 4B) provides a coefficient of determination (R^2) of 0.90.

Solvents and cytotoxicity

Several different solvents can be used to solubilize compounds. DMSO and ethanol, two commonly used solvents, have the potential to cause cytoxicity at elevated concentrations. To avoid the confounding effects of these solvents, each was tested to determine the maximum allowable concentration in compound formulation. In Fig. 5, the basal/unstimulated and active rubidium efflux values were measured after exposure to increasing concentrations of DMSO. HEK-hERG cells seem to tolerate concentrations of DMSO up to 1.25% in buffer, while higher concentrations tend to cause erratic responses in the measured efflux. The total rubidium from each well of the assay plate was measured (Fig. 5) to interpret these results, and results suggested that rubidium was being lost at DMSO concentrations greater than 1.25%. These data provide evidence that DMSO can be cytotoxic at high concentrations, causing unexpected rubidium "efflux," representative of membrane dissolution and passive rubidium leak.

Similar experiments were performed to determine the effects of ethanol on hERG current and cytotoxicity. In this case, increasing concentrations of ethanol caused a decrease in rubidium efflux and in channel function (Fig. 6). Retrospective evaluation of the rubidium content in each well (Fig. 6) shows that in fact, at concentrations greater than 1.25% ethanol, there is an abrupt loss of rubidium from the cells. The apparent decrease in hERG function can thus be explained as an artifact of rubidium loss due to the direct effects of ethanol on the cell.

Finally, an unexpected result was observed while generating the concentration–response curve for the neuroleptic compound, thioridazine. Although increasing concentrations of thioridazine resulted in the expected inhibition of hERG efflux, concentrations greater than 12.5 μM thioridazine resulted in an increase in channel function (Fig. 7). Further analysis of the total rubidium found in each well (Fig. 7) suggests that at concentrations higher than 6.25 μM, there is an abrupt loss of rubidium. This observation suggests that thioridazine becomes cytotoxic to the HEK-293 cells at high concentrations, resulting in loss of rubidium from the cell.

TABLE 1. RANK ORDER OF REFERENCE COMPOUNDS WITH KNOWN INHIBITORY EFFECTS ON HUMAN *ETHER-A-GO-GO* CURRENT

Compound	*Electrophysiology IC_{50}*	*Dofetilide displacement* K_i	*Rubidium efflux IC_{50}*
Astemizole	1 nM[17,20]	1 nM	515.42 nM
E-4031	10 nM[21]	6.9 nM	150.3 nM
Cisapride	23 nM[13]	36 nM	308.1 nM
Terfenadine	56 nM[17]	49 nM	1 μM
Risperidone	167 nM[22]	839 nM	9.45 μM
Quinidine	1 μM[23]	2.1 μM	18.49 μM
Sotalol	320 μM[24]	>20 μM	324.4 μM

IC_{50}, 50% inhibitory concentration.

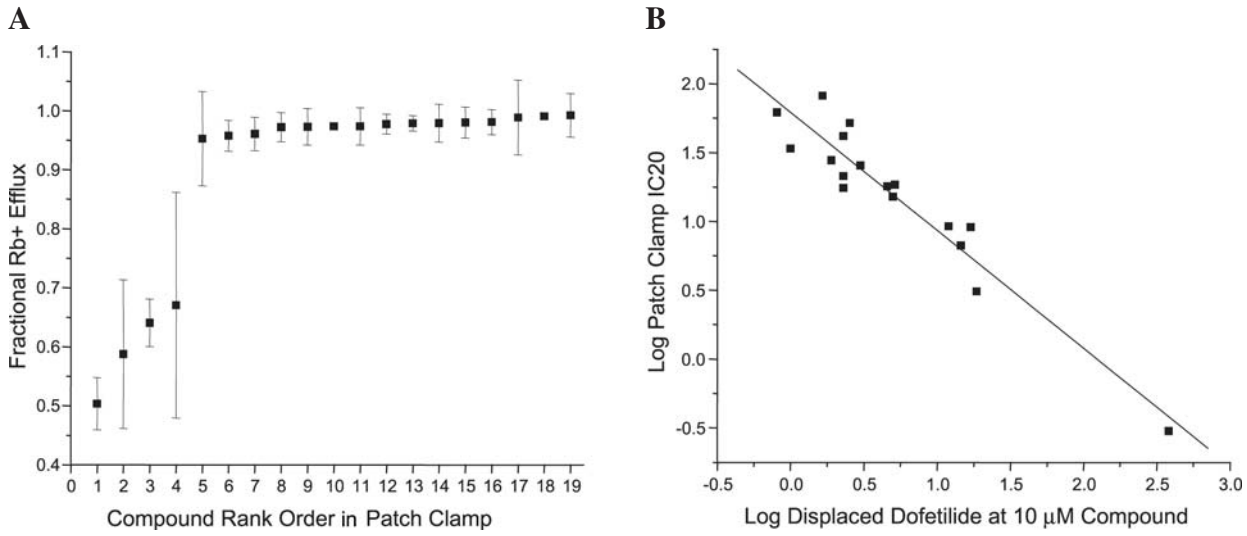

FIG. 4. Predictability of rank order using higher-throughput assays. (**A**) Rank order comparison of percent rubidium efflux after incubation with 10 μM test compound versus historic rank order determined by patch clamp electrophysiology. Patch clamp rank order is indicative of concentration necessary to cause 20% inhibition of current measured in human *ether-a-go-go* (hERG)-expressing human embryonic kidney-293 cells. Inability to differentiate compounds that cause mild hERG inhibition in the rubidium efflux assay (15 out of 19 compounds) suggests the lack of sensitivity of this assay compared to electrophysiology (n = 4 wells per compound using rubidium assay). (**B**) There is a linear relationship between the radioactive dofetilide assay compared to electrophysiology for the 19 compounds tested at 10 μM, in contrast to the rubidium efflux assay. Data are transformed to the logarithm of the concentration necessary for 20% current inhibition in the patch clamp assay versus the logarithm of displaced radioactive dofetilde concentration during incubation of hERG-expressing membranes with 10 μM test compound.

Discussion

hERG block induces long QT syndrome

Studies done by Sanguinetti and Jurkiewicz[25,26] first described two components to the mammalian delayed rectifier potassium current: a rapid (I_{Kr}) and slow (I_{Ks}) component. The rapid component of the delayed rectifier potassium current was later linked to a specific gene, similar to that which encodes a potassium channel in *Drosophila*.[27,28] Mutations in the genes encoding for these and various other ionic currents can lead to congenital long QT syndromes.[5,29,30] Genetic disorders are not the only culprits behind alterations in the electrical activity of the heart. Another form of long QT syndrome is acquired by the use of various pharmaceutical compounds.[8,25,31] The sensitivity of I_{Kr} to specific compounds allowed for its dissociation from other overlapping potassium currents.[25]

Several regulatory bodies have instituted guidelines requiring the screening of compounds for QT liability. Ideally, a new chemical entity should be evaluated *in vivo* and *in vitro* for possible QT prolongation, as extended episodes of arrhythmia can lead to *torsades de pointes* and sudden cardiac death. The inherent cost of bringing a drug to market, from initial structure–activity relationship determination to clinical trials and marketing, emphasizes the need to develop efficient methods for preclinical screening.[32] As illustrated by the cases of cisapride, risperidone, and astemizole, preclinical screening might have prevented the development of these compounds and allowed for the allocation of resources to more promising analogs. Our results support the princi-

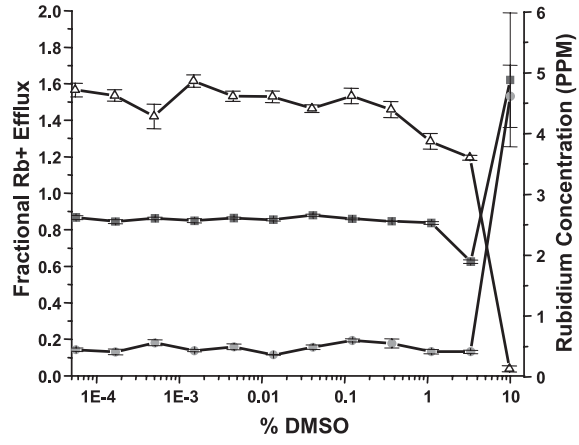

FIG. 5. Effects of dimethyl sulfoxide (DMSO), used as a solvent for compound formulation. At concentrations greater than 1.25%, DMSO causes erratic fractional efflux in unstimulated, and depolarized human *ether-a-go-go*-expressing cells (–●– = basal/unstimulated, –■– = active/depolarized). Evaluation of the total rubidium, in parts per million (PPM), per well (right axis; open triangles, –△– = rubidium content), suggests the abrupt loss of rubidium at concentrations higher than 1.25%. Data are expressed as mean ± SEM (n = 4 wells for active, n = 3 wells for unstimulated per concentration).

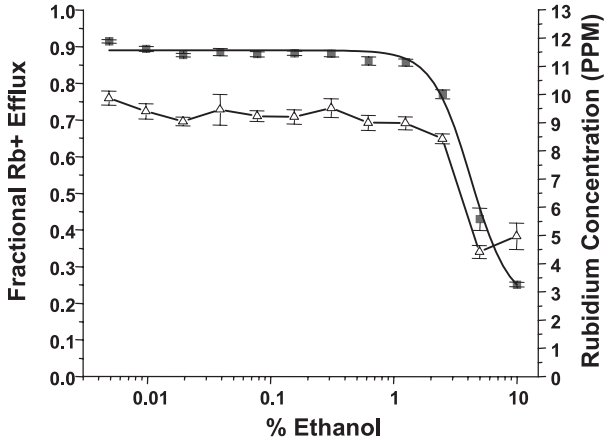

FIG. 6. Effects of ethanol, used as a solvent for compound formulation. At concentrations greater than 1.25%, ethanol causes a decrease in fractional rubidium efflux (–■– = active efflux). Evaluation of the total rubidium content, in parts per million (PPM), per well (right axis; –△– = rubidium content), suggests an abrupt loss of rubidium at concentrations higher than 1.25%. Data are expressed as mean ± SEM (n = 7 wells per concentration).

ple of nonradioactive rubidium efflux to measure potential inhibition of hERG channel current; however, we find the sensitivity of the assay in comparison to other HTS tools to be a limitation with respect to the determination of potency.

Rubidium efflux assay controls

Conventional methods for hERG screening involve labor-intensive patch clamp electrophysiology. While this assay is the most sensitive in determining possible effects of compounds on hERG blockade, it requires specialized training and skills and is very low throughput. Therefore, a higher-throughput assay would be an attractive option for preclinical screening of compounds for QT liability. The use of radioactive rubidium as an analog for potassium in flux-based assays has been well characterized for decades.[21,33–36] Rubidium is preferentially loaded into the cell by the Na^+/K^+ pump when extracellular potassium is replaced by rubidium. The recent advent of a nonradioactive form of this assay by Terstappen[18] has increased its popularity because of its increased throughput, lack of radioactivity, and functional analysis of channel activity.

The experiments highlighted in our study support the use of the rubidium efflux assay to measure hERG channel activity. In comparison to wild-type HEK-293 cells, hERG-transfected cells allow approximately 65–70% greater efflux upon depolarization of 5 min or longer in duration. These results are comparable to those found by others using the radioactive, and nonradioactive rubidium efflux assay.[17,18,21] Interestingly enough, our data suggest the passive diffusion of rubidium from both wild-type and hERG-expressing HEK-293 cells. The source of the efflux (~22%) from these cells was not determined, although possibilities include active hERG channels and inward rectifier potassium channels as well as other nonspecific cation transporters.

Because of its potent inhibition of hERG current, cisapride has become a common reference agent for assay validation. Literature values for inhibition of I_{Kr} using patch clamp methods and in homologous cell lines have ranged from 5 nM[37] to 45 nM.[17] IC_{50} values obtained for the inhibition of hERG channel function by cisapride using the rubidium efflux assay averaged 397.8 ± 37.3 nM. Although the multiple experiments we conducted testing cisapride produced a high level of precision, the accuracy of the assay comes into question when comparing results to conventional patch clamp electrophysiology. Our results suggest a 10–20-fold rightward shift in the concentration–response curve IC_{50} in comparison to patch clamp measurements, which is similar to previously published results.[17] This observation becomes problematic when studying compounds that are (1) relatively insoluble and (2) weak blockers of the hERG channel. To reach concentrations upwards of 10-fold higher than what is necessary for equivalent block using electrophysiology is not always possible because of solvent saturation and decreased solubility. This problem is confounded by the fact that the relative shift in the IC_{50} is unknown for different chemical structures and pharmaceutical classes of compounds. Therefore, generalization of a decrease in sensitivity (a shift in IC_{50}) might cause an increase in false-negative results.

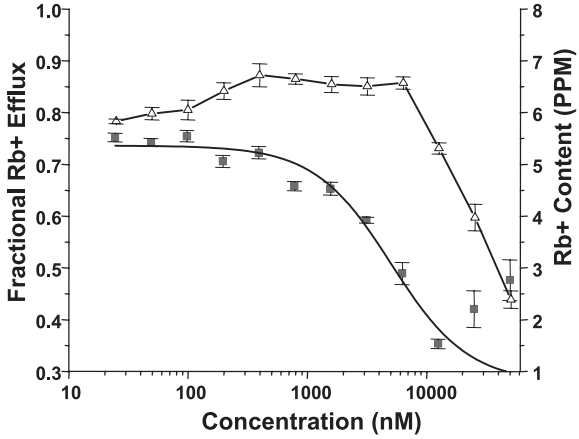

FIG. 7. Concentration–response curve for the neuroleptic agent thioridazine. Concentrations greater than 6.25 μM cause an increase in error for each data point, while concentrations higher than 12.5 μM lead to a gradual increase in human *ether-a-go-go* rubidium efflux (–■– = active efflux). Analysis of the total rubidium, in parts per million (PPM), found in each well of the assay plate (right axis; –△– = rubidium content) suggests a loss of rubidium at concentrations higher than 6.25 μM due to the potential cytotoxic effects of thioridazine. Data are expressed as mean ± SEM (n = 7 wells per concentration).

Thorough validation of a novel high-throughput assay requires the testing of reference agents with known inhibitory properties. To this end, we tested several drugs, some of which have been on the market and have been retracted by the Food and Drug Adminstration because of hERG and QT liability. The relative potency of these compounds to block the hERG channel is summarized in Table 1. Unlike fluorescent indicators, a tool commonly used for HTS, the rubidium efflux assay is insensitive to compound/reagent interactions. Therefore, one can avoid the artifacts associated with dye quenching and with photobleaching. Other than that for astemizole, the rank order of compound potency we observed using the rubidium efflux assay matches the published IC_{50} values using patch clamp electrophysiology. In the case of astemizole, differences may be due to the reported use and voltage dependence of blockade of this compound.[20,38,39]

The dofetilide binding assay provides a high-throughput screen for evaluation of hERG interaction by compounds. The advantages of this assay are its throughput, relative ease of use, low cost, ability to automate, and the fairly high sensitivity in comparison to electrophysiology.[40,41] Since the displacement of dofetilide binding is a nonfunctional assay, one might theoretically find false-negatives with compounds that bind at sites other than that occupied by the radiolabeled dofetilide molecule. Therefore, the measurement of channel function provided by the rubidium efflux assay represents a more physiological characterization of channel inhibition.

Others have reported the rightward shift found in compound potencies using the rubidium efflux assay in comparison to electrophysiology, indicating that the shift is intrinsic to the assay.[17,21,40] Possible reasons for the decrease in compound potency may include the supraphysiological concentrations of extracellular potassium used to chemically depolarize the cells. Indeed, Sanguinetti *et al.*[8] have shown that increased extracellular potassium causes an increase in the conductance of the hERG channel. An increase in the extracellular potassium also reduces the time course of channel inactivation.[19] Although increasing extracellular potassium induces a depolarizing shift in the membrane potential, increasing the open probability of the hERG channel, studies have shown that decreasing the $[K^+]_o$ from 65 m*M* to 40 m*M* causes a leftward shift in the potency of E-4031 to inhibit the channel.[21] Modulation of channel properties by extracellular potassium may reduce the affinity of compounds for possible binding sites within the channel vestibule. These shifts in relative potency between the rubidium efflux and electrophysiological assays are further confounded by the observation that different families of compounds show different relative shifts in potency. Although we chose to induce depolarization with 50 m*M* $[K^+]_o$, it is clear that altering the extracellular potassium concentration has dramatic effects on compound-dependent inhibition of the channel.

In addition, the use of rubidium as the charge carrier in place of potassium also modulates the inhibitory effects of compounds on the hERG channel. In a previous study evaluating the rubidium efflux assay, Rezazadeh *et al.*[42] found that increasing extracellular Rb^+ while measuring hERG current caused upwards of threefold rightward shifts in compound potency as measured by patch clamp electrophysiology.

Finally, "state-dependent" inhibitory properties of compounds also lead to deviations in potency measurement in the different assays. Conventional patch clamp electrophysiology allows for the measurement of the biophysical properties of the hERG channel, because the precise control of membrane potential forces the channel to cycle through various configurations. Normally, depolarization leads to the opening of available channels. The hERG channel then transitions to an inactivated state. The predominant mechanism of channel block is binding of the compound to the inner vestibule of the channel. In some instances, such as for the case of dofetilide, blockade of the channel occurs when the channel is in the open and, subsequently, the inactive state.[43,44] The rubidium efflux assay limits the cycling, or "use," of these transitions states and therefore might underestimate the potency of compounds. The dofetilide displacement binding assay is even more limited in this regard, as the channel is exposed to no potential gradient across the membrane. Rather, the displacement assay specifically measures the affinity, and competition, of compounds for the dofetilide binding domain of the channel vestibule. One would suspect a greater rightward shift in potency determination compared to electrophysiology for this assay; however, previous studies have highlighted the correlation between IC_{50} values generated by patch clamp and displacement binding assays.[45,46]

Rubidium efflux assay unknowns

In order to test the capabilities of the rubidium efflux assay, several unknown compounds were selected from the internal compound library, which have already been screened using electrophysiology and the dofetilide displacement binding assay. A high dose concentration (10 μM) was chosen to determine potential hits. Out of the 19 potential hERG blockers, only four were considered hits using the rubidium efflux assay. The rest of the compounds fell within 1 SD from the maximum rubidium efflux used as a control measurement. However, the dofetilide displacement assay produced a linear correlation between IC_{50} values on a log scale. These results mimic a situation that is more realistic in terms of preclinical screening. Often, no electrophysiological data are available in the initial phases of drug screening, so predicting a relative shift in the IC_{50} during the formulation of test reagents is not possible. The standard high dose approach is used to determine whether a compound

has hERG blocking properties. To use concentrations much higher would confound any predictions of electrophysiological potency, which, in the end, is the ultimate goal.

Rubidium efflux assay and potential effects of cytotoxicity on results

During our evaluation of the rubidium efflux assay, we characterized whether conventional solvents used to formulate test reagents could alter results when used at high concentrations by inducing leakage of rubidium from the cells. In this case, we studied DMSO and ethanol. Increasing concentrations of DMSO greater than 1% final concentration caused erratic increases in efflux from depolarized HEK-293/hERG cells as well as unstimulated cells. Values for stimulated cells exceeded positive controls, which delimit the maximum efflux. Upon further evaluation, our results indicate a loss of total rubidium in wells treated with greater than 1% DMSO. This finding was calculated by adding the rubidium concentration found in the supernatant and the lysate. Fortunately, collection of both of these values during every experiment allows one to gain insight as to possible effects of solvents as well as test compounds, which may be independent of channel blocking properties. Ethanol seemed to decrease hERG channel activity in a dose-dependent manner when cells were treated with concentrations exceeding 1%. Similarly, when the total rubidium in each well was calculated, we observed drastic losses of rubidium at higher concentrations, most likely due to the cytotoxic effects of ethanol.

An interesting observation was also made after routine treatment of cells with thioridazine. This neuroleptic agent caused a dose-dependent decrease in hERG function, followed by an abrupt increase in efflux at high concentrations. Upon further investigation, it was evident that at higher concentrations of thioridazine, there seems to be a loss of total rubidium from each well. The total DMSO concentration (the solvent used for formulation) was calculated to be 0.5%—well below cytotoxic levels. These results are consistent with thioridazine becoming cytotoxic to the HEK cells at concentrations greater than 12.5 μM. Further, these results are supported by toxicity studies conducted in a hepatoma cell line, in which the 50% effective concentration value for cytotoxicity was in the micromolar range.[47]

Conclusions

In conclusion, there is an unmet need for higher-throughput assays targeted towards assessment of QT liability. The rubidium efflux assay allows for the rapid screening of compounds in a noninvasive manner. However, our results suggest that the lack of sensitivity of the rubidium efflux assay, in comparison to classical electrophysiology, hinders its ability to accurately screen for hERG blockade. Moreover, the dofetilide displacement assay, albeit less physiological than the rubidium efflux assay, is better suited as a preclinical indicator of hERG inhibition.

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