

Original article

## HERG-Lite<sup>®</sup>: A novel comprehensive high-throughput screen for drug-induced hERG risk

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### Abstract

**Introduction:** Direct block of  $I_{Kr}$  by non-antiarrhythmic drugs (NARDs) is a major cause of QT prolongation and torsades de pointes (TdP), and has made the hERG potassium channel a major target of drug safety programs in cardiotoxicity. Block of hERG currents is not the only way that drugs can adversely impact the repolarizing current  $I_{Kr}$ , however. We have shown recently that two drugs in clinical use do not block hERG but produce long QT syndrome (LQTS) and TdP by inhibiting trafficking of hERG to the cell surface. To address the need for an inexpensive, rapid, and comprehensive assay to predict both types of hERG risk early in the drug development process, we have developed a novel antibody-based chemiluminescent assay called HERG-Lite<sup>®</sup>. **Methods:** HERG-Lite<sup>®</sup> monitors the expression of hERG at the cell surface in two different stable mammalian cell lines. One cell line acts as a biosensor for drugs that inhibit hERG trafficking, while the other predicts hERG blockers based on their ability to act as pharmacological chaperones. In this study, we have validated the HERG-Lite<sup>®</sup> assay using a panel of 100 drugs: 50 hERG blockers and 50 nonblockers. **Results:** HERG-Lite<sup>®</sup> correctly predicted hERG risk for all 100 test compounds with no false positives or negatives. All 50 hERG blockers were detected as drugs with hERG risk in the HERG-Lite<sup>®</sup> assay, and fell into two classes: B (for blocker) and C (for complex; block and trafficking inhibition). **Discussion:** HERG-Lite<sup>®</sup> is the most comprehensive assay available for predicting drug-induced hERG risk. It accurately predicts both channel blockers and trafficking inhibitors in a rapid, cost-effective manner and is a valuable non-clinical assay for drug safety testing.

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### 1. Introduction

It was known for decades that antiarrhythmic drugs (ARDs) carried proarrhythmic liabilities but it came as a surprise in the 1990s when non-antiarrhythmic drugs (NARDs) including terfenadine (Seldane; Morganroth et al., 1993; Woosley, 1996) and cisapride (Propulsid; Mohammad, Zhou, Gong, & January, 1997; Rampe, Roy, Dennis, & Brown, 1997) were associated with sudden

cardiac death (SCD). These drugs had widespread usage and blockbuster sales yet safety concerns of regulatory agencies ultimately led to their withdrawal from the marketplace. Presently, SCD associated with NARDs has involved as many as nine top-selling drugs and has become a central issue for safety pharmacology, the pharmaceutical industry, and regulatory agencies.

NARD-induced SCD is associated with impaired cardiac repolarization, prolongation of the QT interval of the ECG, and torsades de pointes (TdP). To date, all of the NARDs that have produced TdP compromise the hERG potassium channel. HERG underlies the repolarizing current,  $I_{Kr}$ , and direct hERG block by the drug is thought to be the cause of

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the TdP (Kiehn, Lacerda, Wible, & Brown, 1996; Mohammad et al., 1997; Rampe et al., 1997; Roy, Dumaine, & Brown, 1996). The standard hERG cardiac safety assay measures patch clamp currents produced by expression of hERG in heterologous mammalian cells in the absence and presence of drug, and is now used routinely to test compounds for their acute effects on heterologously expressed hERG current.

Drug-induced hERG liability can be manifested by more than just direct block, however. More recently some NARDs have been shown to cause QT/TdP liabilities by inhibition of hERG trafficking rather than by direct block of the hERG channel. We showed that arsenic trioxide, used clinically to combat some forms of leukemia and associated with QT prolongation, does not block hERG acutely, but rather inhibits the movement of the channel from its site of synthesis and assembly in the endoplasmic reticulum (ER) to the cell surface (Ficker et al., 2004). We found a similar situation with pentamidine, a drug used to treat *Pneumocystis* pneumonia in the USA and a variety of parasitic diseases in the developing world (Kuryshv et al., 2005).

The current gold standard assay for hERG liability is the measurement of patch clamp currents in the presence and absence of drug. This assay is labor intensive as well as low throughput and is used for drugs that are being considered for investigational new drug (IND) submissions. High-throughput (HT) screening is necessary to identify potential hERG liability among the thousands of compounds that are under consideration for lead optimization. For economic reasons, 'fail early and fail cheap' has become a mantra for the pharmaceutical industry and a high premium has been placed on a satisfactory HT hERG assay. Automated patch clamp systems have been developed and among those currently being marketed include the PatchXpress (Axon Instruments/Molecular Devices) and IonWorks HT (Molecular Devices). The technology is advancing toward the combination of data accuracy and throughput that would be desirable for earlier screening during lead development. However, patch clamp methods measure acute hERG block and do not address the need to examine compounds for hERG trafficking liability.

There are a number of non-patch clamp methods with higher throughput that do not measure functional hERG current but act as surrogates for prediction of hERG block (Tang et al., 2001; Xu et al., 2001). They include (1) displacement of high affinity, radioactively-labeled ligand blocker (Finlayson, Turnbull, January, Sharkey, & Kelly, 2001), (2) atomic absorption measurement of rubidium ( $Rb^+$ ) flux (Terstappen, 1999) and (3) membrane potential using fluorescent voltage-sensitive dyes (Tang et al., 2001). The first method will only detect compounds that compete for the binding site of the labeled ligand and may miss entire classes of compounds that may not interact at the same site such as fluvoxamine (Milnes, Crociani, Arcangeli, Hancox, & Witchel, 2003; Mitcheson, 2003) as well as very weak

blockers such as the antibiotic erythromycin. In the non-radioactive  $Rb^+$  flux method, depolarization is initiated with high concentrations of extracellular potassium which reduce hERG block and decrease sensitivity. Sensitivity is further reduced by modulation of the inactivation properties of the hERG channel by  $Rb^+$  (Rezazadeh, Hesketh, & Fedida, 2004) as well as contributions from non-hERG channels to resting  $Rb^+$  flux and the ongoing reduction of the  $Rb^+$  equilibrium potential. The membrane potential assay has problems in common with the  $Rb^+$  flux method, and may be even more strongly influenced by the relative contributions of other non-potassium ion channel determinants of membrane potential.

There is a need in the industry for a comprehensive HT assay to monitor drug-induced hERG liability early in the drug development process. We have addressed this need with the development of a novel assay called HERG-Lite<sup>®</sup>, an antibody-based chemiluminescent assay that predicts hERG liability by monitoring the surface expression of two different hERG channels. Both cell lines express hERG channels with an HA epitope engineered into the extracellular loop spanning transmembrane domains S1 and S2. The first cell line overexpresses the wild-type channel, hERG-WT-HA, at high basal levels and is used for identification of drugs that induce trafficking inhibition and decrease surface expression. The second cell line expresses hERG containing a single point mutation (G601S) that was first reported in a family with hereditary long QT syndrome (Furutani et al., 1999). This mutation in the extracellular loop between the S5 domain and the pore generates a trafficking deficient channel (Ficker, Obejero-Paz, Zhao, & Brown, 2002; Furutani et al., 1999) that is largely retained (~90%) in the endoplasmic reticulum (ER). Consequently, G601S mutant channels show a reduction in current amplitude with the mutant channels that do reach the cell surface gating normally. Blockers of the channel act as pharmacological chaperones to stabilize the mildly misfolded G601S channels in their correct conformation and rescue channel expression by allowing export from the ER and movement to the cell surface (Ficker et al., 2002; Zhou, Gong, & January, 1999). For pure hERG blockers, the concentration dependence and magnitude of the rescue of G601S expression correlate with the potency of block. Stable expression of each tagged channel in HEK293 cells has generated cell lines that serve as biosensors for compounds with hERG risk.

One hundred compounds (50 blockers and 50 non-blockers) were tested in HERG-Lite<sup>®</sup>, and were grouped into four categories: A, B, C, and null. In the assay, nonblockers were either null (no effect on hERG) or Class A (trafficking inhibition of hERG). The blockers were distinguished as either Class B or C depending upon whether they also inhibited hERG trafficking (Class C). The identification of drugs with dual hERG risk (~40% of hERG blockers) is a novel finding made possible with HERG-Lite<sup>®</sup>.

## 2. Methods

### 2.1. Construct and cell line generation

A DNA fragment encoding a hemagglutinin (HA) tag with a short linker on either end was inserted into the coding region of the extracellular loop spanning hERG transmembrane domains S1 and S2 by overlap extension PCR, sequenced, and inserted into full-length hERG or hERG-G601S (SM) in the pcDNA3.1 (Invitrogen) vector. The insertion does not affect the electrophysiological properties or trafficking behavior of the channel (Ficker, Dennis, Wang, & Brown, 2003). Stable cell lines overexpressing the epitope tagged channels were made by transfecting HEK293 cells with linearized plasmids encoding the respective cDNAs using Fugene (Roche). Stable geneticin resistant subclones were isolated and hERG expression confirmed by Western blotting and HERG-Lite<sup>®</sup> surface expression assay.

### 2.2. HERG-Lite<sup>®</sup>

The assay spans a 2-day period to allow for overnight incubation of the cells with the drugs.

#### 2.2.1. Day 1

On the morning of day 1, the cells were plated in 96-well microtiter plates (poly-L-lysine coated; flat clear bottom black plates; BD Biosciences) at 40,000 cells per well in complete culture medium consisting of DMEM/F12 containing 10% fetal bovine serum and the antibiotics penicillin (100 units/ml), streptomycin (100 units/ml), and geneticin (0.5 mg/ml). We incorporated a fluorescent protocol on day 2 to measure the number of cells remaining in each well at the end of the assay. For this, we perform a standard curve of cell number versus fluorescence for each cell line by plating cells from 10,000 cells to 40,000 cells per well (three wells per concentration). The cells were then allowed to adhere to the plates for 5 h at 37 °C in a 5% CO<sub>2</sub> environment. The test compounds were obtained from either Sigma or Prestwick Chemical Company, and stock solutions prepared in DMSO. Drugs were diluted into serum- and antibiotic-free medium (DMEM/F12) just before addition to the cells. A minimum of three concentrations were tested: 1, 10, and 30 μM ( $n=3$  wells for each concentration), and each plate contained 6 wells with vehicle control (0.1% DMSO). The DMSO concentration was generally kept at 0.1% in test compound wells also, but DMSO concentrations up to at least 1% had no effect on basal surface expression. The culture medium was removed from the wells, and the wells rinsed with 200 μl DMEM/F12 before the drug-containing medium (100 μl) was applied. Once the drugs were added to the cells, the plates were returned to the 37 °C/5% CO<sub>2</sub> incubator.

#### 2.2.2. Day 2

After overnight incubation (~16 h), the plates were removed from the incubator and processed at room temper-

ature on the benchtop. All washes and antibody incubations were done with 100 μl per well. The wells were rinsed three times with phosphate-buffered saline (PBS) to remove the drug-containing medium, and fixed immediately in freshly prepared, ice cold 4% paraformaldehyde in PBS for 20 min. There is no permeabilization step following fixation so that antibody access was limited to the cell surface. Following fixation, the wells were rinsed with PBS, and nonspecific antibody binding sites were blocked by incubation of the cells with 1% goat serum (Sigma) in PBS (blocking buffer) for at least 30 min. The primary antibody (rat anti-HA; Roche; 1:500 dilution in blocking buffer) was added to the cells for a minimum of 2 h. After removal of the primary antibody, the cells were washed three times with blocking buffer (10 min per wash) and then incubated with the secondary antibody cocktail (horseradish peroxidase conjugated goat anti-rat IgG; Jackson Labs; 1:1000 in blocking buffer plus SYBR Green; Molecular Probes; 1:10,000) for at least 1 h. SYBR Green enters non-permeabilized cells and binds to nuclear DNA giving a measure of cell number (Myers, 1998). Following three washes with PBS, SYBR Green fluorescence was measured in a ThermoElectron Fluoroskan Ascent microplate reader (wavelengths: excitation, 485; emission, 538). To capture chemiluminescent signals, PBS was removed from the wells and replaced with freshly prepared SuperSignal<sup>®</sup> ELISA Femto Maximum Sensitivity Substrate (Pierce; 100 μl per well). The plates were immediately read in a GloRunner Luminometer (Turner Biosystems).

#### 2.2.3. Data analysis

Standard curves of SYBR green fluorescence versus cell number were generated for each cell line in each experiment, and were used to calculate the cell number per well for each of the test compound wells. The chemiluminescence values were corrected if the cell numbers differed from 40,000. Data from wells in which cell loss exceeded 75% (i.e., fewer than 10,000 cells per well at the end of the experiment) were considered too unreliable for analysis. For each cell line, the mean and standard deviation of the chemiluminescence signals in the control wells were calculated. For each test compound, the chemiluminescence signal in each well was normalized to the control to give a relative surface expression level. We judge a test compound to have significantly changed hERG-SM or hERG-WT surface expression if the test compound mean was above or below the vehicle control mean  $\pm$  three control standard deviations for that cell line.

### 2.3. Patch clamp

HERG currents were recorded at room temperature (20–23 °C) in HEK293 cells expressing untagged WT channels. Patch pipettes were filled with a solution consisting of (in mM): potassium aspartate, 130; MgCl<sub>2</sub>, 5; EGTA, 5; ATP, 4; HEPES, 10; pH adjusted to 7.2 with KOH. The following

pulse protocol was used: conditioning prepulse (+20 mV for 2 s), test pulse (−50 mV for 2 s), repeated at 10-s intervals from a holding potential of −80 mV. Peak tail current was measured during the step to −50 mV. Cells were incubated with drug until steady state was achieved or up to a maximum of 10 min. All drugs tested reached a steady state within the 10 min incubation period. After drug exposure, cisapride (500 nM) was added as a positive control to those cells that showed little or no drug block (<5% block at 10 μM). Data acquisition and analysis were performed using pCLAMP (Version 8.2; Axon Instruments). The steady state current before and after drug incubation was used to determine the percentage of current blocked by the drug. IC<sub>50</sub> values were determined by fitting percent block data from multiple test concentrations to the Hill equation with the exponent set to 1. In some cases, estimated IC<sub>50</sub> values were obtained from one test concentration, and are indicated as such in the data tables.

### 3. Results

To assess the performance of HERG-Lite<sup>®</sup> as a comprehensive screen to predict hERG risk, we tested a panel of

100 compounds that included an equal number of blockers and nonblockers. The test compounds consisted of 25 high affinity hERG blockers (IC<sub>50</sub> in patch clamp experiments <1 μM), 25 low affinity hERG blockers (IC<sub>50</sub> in patch clamp experiments >1 μM), and 50 nonblockers. Each compound was tested at a minimum of three concentrations in HERG-Lite<sup>®</sup>: 1, 10, and 30 μM (three wells per concentration per experiment). The data were normalized to the surface expression of hERG-SM or hERG-WT in the presence of vehicle control, and presented as relative surface expression. Drugs were classified into one of four groups for hERG risk (A, B, C or null) based on their behavior in HERG-Lite<sup>®</sup>. An example of the data analysis spreadsheet is shown in Table 1 for 8 of the 100 test set, and includes two representatives from each class.

#### 3.1. Behavior of hERG blockers in HERG-Lite<sup>®</sup>

All 50 drugs that block WT-hERG gave significant signals in HERG-Lite<sup>®</sup>, but could be separated into two groups based on their behavior in the assay. 60% (30/50) behaved as pharmacological chaperones by rescuing SM surface expression without decreasing surface expression of WT; these we call Class B drugs (see cisapride and

Table 1  
HERG-lite<sup>®</sup> sample data table

Drug	Test conc (μM)	HERG-SM, relative surface expression		HERG-WT, relative surface expression		Patch clamp IC <sub>50</sub> (μM)	HERG-Lite <sup>®</sup> class
		AVG	SD	AVG	SD		
Arsenic trioxide	1	1.04	0.21	0.86	0.11	NB	A
	10	0.87	0.29	0.35 <sup>a</sup>	0.06		
	30	1.39	0.37	0.27 <sup>a</sup>	0.04		
Geldanamycin	1	0.85	0.16	0.48 <sup>a</sup>	0.07	NB	A
	10	0.94	0.29	0.45 <sup>a</sup>	0.08		
	30	1.02	0.37	0.40 <sup>a</sup>	0.09		
Cisapride	1	3.88 <sup>b</sup>	0.59	1.16	0.10	0.027	B
	10	5.05 <sup>b</sup>	0.83	1.19	0.06		
	30	5.17 <sup>b</sup>	1.53	1.16	0.09		
Quinidine	1	1.22	0.26	0.97	0.06	1.07	B
	10	1.80 <sup>b</sup>	0.15	1.11	0.08		
	30	1.89 <sup>b</sup>	0.19	1.05	0.05		
Amiodarone	1	1.51 <sup>b</sup>	0.14	0.99	0.08	0.015	C
	10	1.13	0.21	0.55 <sup>a</sup>	0.13		
	30	TOXIC		TOXIC			
Thioridazine	1	0.98	0.17	0.92	0.10	0.096	C
	10	0.99	0.26	0.50 <sup>a</sup>	0.07		
	30	TOXIC		TOXIC			
Acetaminophen	1	1.05	0.29	0.96	0.04	NB	null
	10	1.06	0.31	0.96	0.07		
	30	1.14	0.18	1.01	0.06		
Aspirin	1	0.97	0.09	0.98	0.03	NB	null
	10	1.08	0.16	0.99	0.02		
	30	1.12	0.19	0.98	0.03		

NB—no block.

Average (AVG) surface expression in the presence of the test article ( $n \geq 6$  for each concentration) was normalized to the average for vehicle control, and indicated as relative surface expression  $\pm$  standard deviation (SD). TOXIC indicates that data could not be collected at this concentration due to cell loss of 75% or more.

<sup>a</sup> Indicates that the test article mean fell more than three standard deviations below the control mean.

<sup>b</sup> Indicates that the test article mean was greater than three standard deviations above the control mean.

quinidine in Table 1). The other 20 (40%) blockers produced a pronounced trafficking defect in WT surface expression that was, in some cases (see amiodarone in Table 1) but not all (see thioridazine in Table 1), accompanied by a small but significant rescue of SM surface expression; these we call Class C drugs.

### 3.1.2. Class B compounds

Class B compounds are hERG blockers that show significant rescue of hERG-SM surface expression without any detectable trafficking defects in hERG-WT in HERG-Lite<sup>®</sup> assays. Seventeen were high affinity and 13 were low affinity blockers. For comparative purposes, the compounds are grouped according to the lowest concentration at which significant rescue of hERG-SM was observed. The 17 compounds with patch clamp IC<sub>50</sub> values less than 1 μM (high affinity hERG blockers) all showed significant signals in HERG-Lite<sup>®</sup> when tested at 1 μM (Table 2). Within this group, the most potent hERG blockers were generally the most potent rescuers of hERG-SM surface expression in HERG-Lite<sup>®</sup>. With the exception of haloperidol with an average relative SM surface expression increase of 2.9-fold, compounds with IC<sub>50</sub> values of 50 nM or less increased the average relative SM surface expression more than 3-fold. Compounds with IC<sub>50</sub> values between 50 nM and 1 μM showed comparable rescue magnitudes (~1.7- to 2.9-fold). Even though there was a 5-fold difference between the IC<sub>50</sub> values of doxazosin and pergolide (0.6 μM versus 0.12 μM, respectively), the average increase in relative SM surface expression was similar (2.00 versus 2.06, respectively). The behavior of this test set predicts that unknown test compounds that show significant rescue of the SM at 1 μM without associated WT trafficking inhibition would be predicted to have IC<sub>50</sub> values less than 1 μM.

Thirteen of the Class B compounds were low affinity blockers (IC<sub>50</sub> > 1 μM). Those with patch clamp IC<sub>50</sub> values ranging from 1 to 10 μM did not produce significant SM rescue in HERG-Lite<sup>®</sup> at the 1 μM test concentration but did show significant changes at 10 μM (Table 2). Terazosin (IC<sub>50</sub> = 16.8 μM) also gave significant rescue at 10 μM. With the exception of prazosin which gave an average increase of 3.56-fold, these compounds showed modest increases in surface expression of about 1.5–1.9 fold at 10 μM. Even though there was a 9-fold difference in IC<sub>50</sub> values between quinidine and diltiazem, the average relative increases in SM surface expression were comparable (1.80 versus 1.62, respectively). With the exception of terazosin, the compounds with patch-clamp IC<sub>50</sub> values above 10 μM only produced significant increases in SM surface expression at test concentrations of ≥ 30 μM. Even though the compounds in this group cover about a 24-fold range in patch clamp IC<sub>50</sub> values, they do not show major differences in their HERG-Lite<sup>®</sup> phenotype.

Two drugs that are very weak hERG blockers (IC<sub>50</sub> values greater than 100 μM) did not give significant signals in HERG-Lite at 30 μM so were tested at 100 μM.

Table 2

Direct hERG blockers that rescue hERG-SM surface expression in HERG-Lite<sup>®</sup>

Class B Compounds			
	Patch clamp IC <sub>50</sub> (μM)	Lowest test conc (μM) with significant rescue	hERG-SM relative surface expression AVG ± SD
<i>High affinity hERG blockers (IC<sub>50</sub> &lt; 1 μM)</i>			
Astemizole	0.001	1	6.74 ± 1.19
Pimozide	0.001 <sup>a</sup>	1	7.87 ± 0.94
GBR 12909	0.001	1	3.03 ± 0.60
Fluspirilene	0.003	1	5.86 ± 1.39
Clofilium	0.007	1	11.08 ± 2.89
E4031	0.012 <sup>a</sup>	1	6.61 ± 1.44
Haloperidol	0.019 <sup>a</sup>	1	2.93 ± 0.66
Cisapride	0.027 <sup>a</sup>	1	3.88 ± 0.59
Vinpocetine	0.032	1	5.45 ± 1.29
Droperidol	0.064	1	2.53 ± 0.69
Domperidone	0.103	1	2.98 ± 0.78
Ketanserin	0.107 <sup>a</sup>	1	1.91 ± 0.45
Pergolide	0.120 <sup>b</sup>	1	2.06 ± 0.22
Verapamil	0.136 <sup>a</sup>	1	1.71 ± 0.15
Risperidone	0.226	1	1.81 ± 0.22
Amsacrine	0.230 <sup>c</sup>	1	2.33 ± 0.26
Doxazosin	0.600 <sup>d</sup>	1	2.00 ± 0.46
<i>Low affinity hERG blockers (IC<sub>50</sub> &gt; 1 μM)</i>			
Quinidine	1.07 <sup>a</sup>	10	1.80 ± 0.15
Prazosin	1.57 <sup>d</sup>	10	3.56 ± 0.59
Chloroquine	2.5 <sup>e</sup>	10	1.78 ± 0.30
Ajmaline	3.3 <sup>f</sup>	10	1.89 ± 0.26
Papaverine	7.3	10	1.70 ± 0.37
Diltiazem	9.1	10	1.62 ± 0.18
Fexofenadine	11.0	100	1.69 ± 0.19
Ranolazine	14.6	30	1.52 ± 0.13
Terazosin	16.8 <sup>d</sup>	10	1.59 ± 0.14
Sparfloxacin	30	30	1.54 ± 0.10
Disopyramide	42	30	1.61 ± 0.12
Erythromycin	115 <sup>a</sup>	100	1.60 ± 0.22
Sotalol	269 <sup>a</sup>	100	1.78 ± 0.09

All other values were obtained at ChanTest at room temperature.

<sup>a</sup> Kirsch et al., 2004.

<sup>b</sup> Hurst et al., 2003.

<sup>c</sup> Thomas, Hammerling et al., 2004.

<sup>d</sup> Thomas, Wimmer et al., 2004.

<sup>e</sup> Traebert et al., 2004.

<sup>f</sup> Estimated IC<sub>50</sub> from one test concentration.

Erythromycin (IC<sub>50</sub> = 115 μM) and sotalol (IC<sub>50</sub> = 269 μM) both produced significant rescue of hERG-SM at 100 μM (Table 2) without affecting WT trafficking. Although fexofenadine was a more potent blocker (IC<sub>50</sub> = 11 μM), it produced significant hERG-SM rescue only when a test concentration of 100 μM was used.

To summarize, the lowest concentration at which a Class B compound gives a significant signal in HERG-Lite<sup>®</sup> is indicative of its blocking potency. Based on this data set, compounds that test positive in HERG-Lite<sup>®</sup> at 1 μM will have IC<sub>50</sub> values less than 1 μM. With the exception of two drugs noted above, those that test positive in HERG-Lite<sup>®</sup> at 10 μM have IC<sub>50</sub> values from 1 to 10 μM, while those that test positive at 30 μM have IC<sub>50</sub> values between 10 and 100

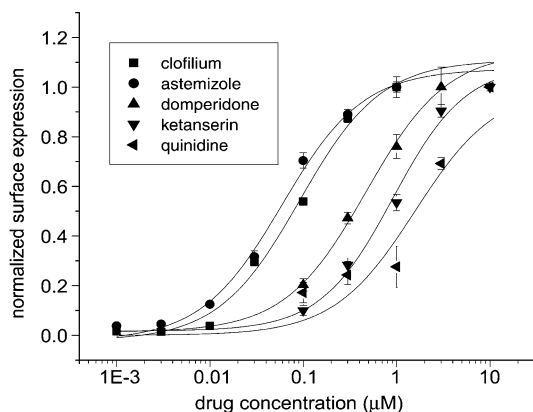
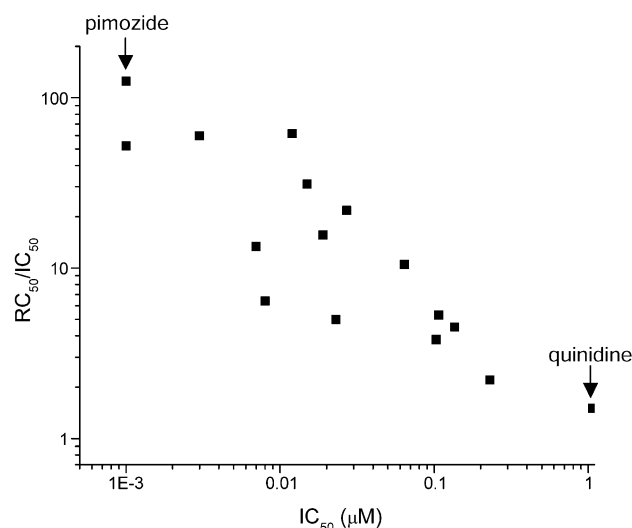


Fig. 1. HERG-SM rescue dose response curves for five Class B compounds. Rescue dose response data were normalized to control and fitted to Hill equations where the Hill coefficient was kept constant at 1.

$\mu\text{M}$ . Higher test concentrations ( $\geq 100 \mu\text{M}$ ) are required to detect weaker hERG blockers such as erythromycin and sotalol.

For Class B drugs, it is possible to calculate  $\text{RC}_{50}$  values (the concentration at which half-maximal rescue is achieved) from dose response curves for SM rescue. Fig. 1 shows hERG-SM rescue dose response curves for a subset of Class B compounds. The comparison of  $\text{RC}_{50}$  and  $\text{IC}_{50}$  values for a number of Class B compounds is shown in Fig. 2 and the relationship between  $\text{IC}_{50}$  and  $\text{RC}_{50}$  values is plotted. For the most potent blockers, there is a greater offset in the two values than seen with less potent blockers. For example, the  $\text{RC}_{50}$  for astemizole ( $\text{IC}_{50} = 1 \text{ nM}$ ) is about 50-fold greater than the  $\text{IC}_{50}$ , while the  $\text{RC}_{50}$  for quinidine ( $\text{IC}_{50} = 1.06 \mu\text{M}$ ) is only about 1.5-fold greater than the  $\text{IC}_{50}$  for block. This means that weak blockers are detected in



Drug	HERG-WT patch clamp $\text{IC}_{50}$ ( $\mu\text{M}$ )	HERG-SM $\text{RC}_{50}$ ( $\mu\text{M}$ )	$\text{RC}_{50}/\text{IC}_{50}$
astemizole	0.001	0.052	52.0
pimoziide	0.001	0.125	125.0
fluspirilene	0.003	0.179	59.7
terfenadine	0.008	0.051	6.4
clofilium	0.007	0.094	13.4
amiodarone	0.015	0.466	31.1
E4031	0.012	0.739	61.6
haloperidol	0.019	0.296	15.6
bepidil	0.023	0.114	5.0
cisapride	0.027	0.589	21.8
droperidol	0.064	0.675	10.5
domperidone	0.103	0.389	3.8
ketanserin	0.107	0.567	5.3
verapamil	0.136	0.616	4.5
amsacrine	0.230	0.500	2.2
quinidine	1.07	1.637	1.5

Fig. 2. Comparison between  $\text{RC}_{50}$  (half-maximal rescue concentration from hERG-Lite<sup>®</sup>) and  $\text{IC}_{50}$  (half-maximal inhibition determined by electrophysiology) values. Dose response relationships for rescue of hERG-G601S-HA expression were measured for 16 hERG blockers and  $\text{RC}_{50}$  values were calculated. The table compares  $\text{RC}_{50}$  values to  $\text{IC}_{50}$  values for hERG inhibition measured by patch clamp. For low affinity blockers, the  $\text{RC}_{50}$  value more closely approximates the  $\text{IC}_{50}$  as shown in the plot.

HERG-Lite<sup>®</sup> at relevant blocking concentrations. This feature of the assay ensures that both high- and low-affinity blockers are identified in HERG-Lite<sup>®</sup>.

While there is an offset between the RC<sub>50</sub> and IC<sub>50</sub> values for the most potent blockers, we have found that HERG-Lite<sup>®</sup> predicts the correct rank order for compounds within distinct chemical classes. Several examples are shown in Fig. 3. Of the three alpha 1-adrenoceptor antagonists, doxazosin, prazosin, and terazosin, the most potent hERG blocker, doxazosin (IC<sub>50</sub>=0.6 μM), rescues SM surface expression at a test concentration of 1 μM in HERG-Lite<sup>®</sup> whereas prazosin and terazosin, with respective patch clamp IC<sub>50</sub> values of 1.57 and 16.8 μM, do not show significant rescue of hERG SM until 10 μM (Fig. 3A). At this test concentration, the relative potencies of prazosin and terazosin as hERG blockers are reflected in the greater fold increase in SM surface expression shown by prazosin (3.56-fold) compared to the weaker blocker terazosin (1.59-fold). The same holds true for the fluoroquinolone antibiotics, sparfloxacin, ciprofloxacin, and ofloxacin (Fig. 3B). Of the three, sparfloxacin is the most potent hERG blocker

(IC<sub>50</sub>=30 μM) (Kang, Wang, Chen, Triggle & Rampe, 2001), and is the only one to show significant rescue of hERG SM surface expression in HERG-Lite<sup>®</sup> at test concentrations from 10 to 100 μM. The other two are very weak blockers (≥1 mM), and did not generate significant signals in HERG-Lite<sup>®</sup> at test concentrations up to 100 μM. We have also compared the behavior of the potent hERG blocker terfenadine and its weaker metabolite fexofenadine in HERG-Lite<sup>®</sup> (Fig. 3C). Although terfenadine is not a Class B compound but fits instead into the Class C group because it shows a dose-dependent trafficking inhibition of hERG-WT in addition to rescue of hERG-SM (to be described in detail below), it does show significant rescue of hERG-SM surface expression at 1 μM while its metabolite, fexofenadine, a much less potent hERG blocker, does not give a positive signal in HERG-Lite<sup>®</sup> until 100 μM.

### 3.1.2. Class C compounds

A surprising finding in the HERG-Lite<sup>®</sup> validation screen was the identification of direct hERG blockers (20 out of 50 tested) that produced trafficking defects in the

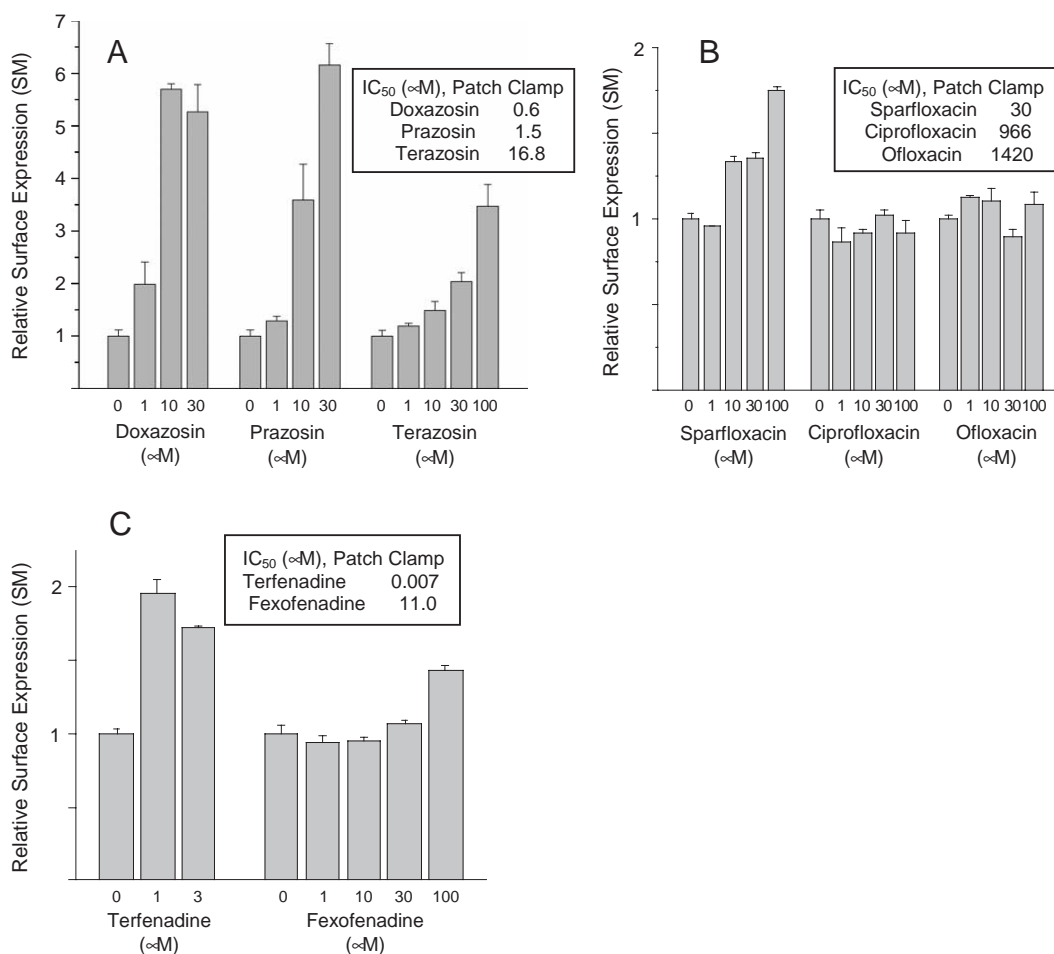


Fig. 3. Rank order of potency of hERG block is preserved in HERG-Lite<sup>®</sup> screening of compounds of distinct chemical classes. Relative surface expression of hERG-SM is plotted versus test concentration for the related compounds (A) three related alpha1-adrenoceptor antagonists, (B) three fluoroquinolone antibiotics, and (C) the antihistamine terfenadine and its metabolite fexofenadine. In all cases, the more potent blocker in the set produces significant and/or larger signals in HERG-Lite<sup>®</sup> at lower test concentrations.

hERG-WT cell line (Class C drugs). Some of these drugs show a significant rescue of hERG-SM surface expression at some concentrations with a trafficking defect in hERG-WT predominating at higher concentrations (5/20; see amiodarone in Table 1). Others produce no significant rescue of hERG-SM but show only the hERG-WT trafficking defect (15/20; see thioridazine in Table 1). More low affinity blockers (12/20) than high affinity blockers (8/20) were Class C compounds. Table 3 indicates the lowest concentration at which significant trafficking defects in hERG-WT were detected as well as the relative surface expression measured at that concentration.

### 3.2. Behavior of 50 nonblockers in HERG-Lite®

Of the 50 drugs that did not produce hERG block in patch clamp, 44 did not give a significant signal in HERG-Lite® with either cell line. Those compounds (HERG-Lite® null) are listed in Table 4 (lower). Ciprofloxacin and ofloxacin were considered nonblocking compounds for the purpose of this study as they block with IC<sub>50</sub> values ≥ 1 mM, and the highest test concentration in HERG-Lite® was

Table 3  
Direct hERG blockers that produce trafficking defects in HERG-Lite®

Class C Compounds			
	Patch clamp IC <sub>50</sub> (μM)	Lowest test conc (μM) with significant trafficking inhibition	hERG-WT relative surface expression AVG±SD
<i>High affinity hERG blockers (IC<sub>50</sub> &lt; 1 μM)</i>			
Terfenadine	0.008 <sup>a</sup>	10	0.29±0.09
Amiodarone	0.015	10	0.55±0.13
Bepidil	0.023 <sup>a</sup>	30	0.29±0.07
Thioridazine	0.096 <sup>a</sup>	10	0.50±0.07
Tamoxifen	0.111 <sup>b</sup>	10	0.33±0.12
Trifluoperazine	0.234	10	0.58±0.06
Chlorpromazine	0.370	10	0.50±0.15
Fluoxetine	0.460 <sup>a</sup>	10	0.71±0.13
<i>Low affinity hERG blockers (IC<sub>50</sub> &gt; 1 μM)</i>			
Clotrimazole	1.13 <sup>b</sup>	10	0.33±0.09
Mibefradil	1.4 <sup>c</sup>	10	0.28±0.11
Imipramine	1.9	30	0.60±0.10
Ketoconazole	1.9	30	0.54±0.07
Diphenhydramine	2.6 <sup>a</sup>	30	0.52±0.28
Mefloquine	2.6 <sup>d</sup>	10	0.36±0.16
Maprotiline	3.1	30	0.46±0.06
Desipramine	5.6 <sup>b</sup>	10	0.74±0.03
Perhexiline	7.8 <sup>c</sup>	10	0.23±0.04
Lovastatin	12.5	30	0.55±0.11
Norpropoxyphene	15.0	30	0.76±0.03
Spirolactone	23 <sup>f</sup>	10	0.57±0.04

All other values were obtained at ChanTest at room temperature.

<sup>a</sup> Kirsch et al., 2004.

<sup>b</sup> Estimated IC<sub>50</sub> from one test concentration.

<sup>c</sup> Chouabe et al., 2000.

<sup>d</sup> Traebert et al. (2004).

<sup>e</sup> Walker et al. (1999).

<sup>f</sup> Caballero et al. (2003).

Table 4  
Behavior of non-hERG blockers in HERG-Lite®

Class A compounds	Lowest test conc (μM) with significant trafficking inhibition	hERG-WT relative surface expression AVG±SD
Geldanamycin	1	0.48±0.07
Arsenic trioxide	10	0.35±0.06
Pentamidine	10	0.64±0.02
Ivermectin	10	0.34±0.09
Ethacrynic acid	10	0.70±0.08
Bufexamac	10	0.75±0.02
<b>hERG-Lite null</b>		
Enalapril		Ofloxacin**
Acetaminophen		Penicillin
Amantadine		Pinacidil
Amiloride		Pseudoephedrine
Amoxicillin		Ranitidine
Ampicillin		Resveratrol
Arterenol		Salbutamol
Aspirin		Spiramycin
Captopril		Sulfamethoxazole
Cephalexin		Sulindac
Cimetidine		Thalidomide
Ciprofloxacin**		Trimethoprim
Clindamycin		Troleandomycin
Clonidine		Warfarin
Doxycycline		Wortmannin

\*\* Compounds block hERG with IC<sub>50</sub> ≥ 1 mM (Kang et al., 2001); Maximum conc tested in HERG-Lite was 100 μM.

100 μM. The remaining 6 nonblockers all showed trafficking inhibition in hERG-WT, and are classified as Class A compounds (Table 4, upper). Geldanamycin (Ficker et al., 2003), arsenic trioxide (Ficker et al., 2004), and pentamidine (Kuryshev et al., 2005) have already been described as hERG trafficking inhibitors. This is the first report that ivermectin, ethacrynic acid, and bufexamac inhibit hERG surface expression; they do so with a potency similar to arsenic trioxide and pentamidine.

## 4. Discussion

HERG-Lite® correctly predicted hERG risk for 100 test drugs with no false positives or negatives. The drugs tested using HERG-Lite® were classified into four groups: A, B, C, and null. All 50 hERG blockers gave a significant signal in HERG-Lite®, either by rescuing hERG-SM expression (Class B) and/or inhibiting hERG-WT trafficking (Class C). Class B compounds show significant rescue of the SM at one or more of the test concentrations without showing significant trafficking inhibition of the WT at any test concentration. The lowest test concentration at which significant rescue was obtained can be related to the patch clamp IC<sub>50</sub> value. HERG-Lite® provides a relative ranking of drug blocking potency but, as with other non-electrophysiological methods for predicting hERG block, is not meant to replace patch clamp. Precise IC<sub>50</sub> values still require patch clamp analysis. The previously undescribed hERG blocker, vinpocetine, was part of this test set, and was

picked up as a Class B compound in HERG-Lite<sup>®</sup>. Vinpocetine is a potent hERG blocker (IC<sub>50</sub>=32 nM) as well as a sodium channel blocker (Zhou, Dong, Crona, Maguire, & Priestley, 2003), and is available over the counter as an herbal supplement to prevent memory loss. There are as of yet no reports in the literature indicating a risk of QT prolongation for this compound perhaps because of the associated sodium channel block.

Class C compounds are also direct channel blockers but their behavior in HERG-Lite<sup>®</sup> is very different. They may or may not show significant rescue of hERG-SM, but they all produce a significant trafficking inhibition of hERG-WT at one or more test concentrations. The 50 nonblockers were either Class A compounds or null in HERG-Lite<sup>®</sup>. Class A compounds do not block the channel directly but produce a significant trafficking defect in hERG-WT. The null class consists of compounds that are not direct blockers and do not show any significant changes in surface expression of either the SM or WT in HERG-Lite<sup>®</sup>. Class A, B, and C compounds all manifest hERG risk while null compounds do not.

A novel finding from HERG-Lite<sup>®</sup> is the substantial number of hERG blockers (~40%) that carry the additional risk of hERG trafficking inhibition. Prior to the start of this screen, we had predicted that all hERG blockers would act as pharmacological chaperones and rescue hERG-SM expression with the potency of rescue related to the potency of block. Contrary to our expectations, some blockers, even very potent ones such as terfenadine, demonstrated a pronounced trafficking inhibition of hERG-WT surface expression in HERG-Lite<sup>®</sup> rather than a predominant rescue of hERG-SM. We do HERG-Lite<sup>®</sup> screening at three test concentrations rather than one to maximize the likelihood that trafficking inhibition will be picked up for compounds in this class.

Those compounds represent a very interesting group of small molecules that possess dual hERG risk: direct channel block and trafficking inhibition. All known hERG blockers access the channel from its cytoplasmic pore, and therefore have access to the pathways for hERG synthesis, assembly, trafficking, and degradation. Blockers acting as pharmacological chaperones to rescue hERG-SM surface expression (Class B compounds) bind to the mutant channel and produce a conformation that the quality control machinery recognizes as normal. We suggest that the binding site on hERG for blockers that inhibit hERG trafficking (Class C compounds) may differ such that interaction with hERG produces channel misfolding and trafficking inhibition.

The clinical implications of the dual hERG risk of Class C compounds will have to be assessed for each compound individually, and, as a first approximation, will depend upon comparing drug plasma concentrations with the concentrations relevant for block and trafficking inhibition.

It is not only a subset of hERG blockers that inhibit WT trafficking as some nonblockers (Class A compounds) also

show decreased hERG-WT surface expression in HERG-Lite. Class A compounds are not direct blockers of hERG but produce hERG risk through trafficking inhibition of the channel. They are detected by HERG-Lite<sup>®</sup> as inhibitors of hERG-WT surface expression making them indistinguishable from Class C compounds. Consequently, any compound that produces a trafficking defect of hERG-WT in HERG-Lite<sup>®</sup> is a potential channel blocker, and standard patch clamp analysis is required to distinguish Class A and C compounds. Geldanamycin was the first compound shown to inhibit hERG trafficking, and it does so by interfering with the association of the chaperone Hsp90 with hERG in the ER (Ficker et al., 2003). Arsenic trioxide is also a proven hERG trafficking inhibitor and is associated with torsades de pointes (Ficker et al., 2004). We have recently shown that another torsadogenic drug, pentamidine, also inhibits hERG trafficking (Kuryshev et al., 2005). The remaining three compounds, ethacrynic acid, bufexamac, and ivermectin, have not previously been identified as hERG trafficking inhibitors. Ethacrynic acid is a chloride channel blocker used as a diuretic in humans, while ivermectin is widely used to treat parasitic diseases in animals. There are no published reports indicating a problem with QT prolongation with the use of either compound. Bufexamac is used as a topical drug for allergic contact dermatitis (eczema) in humans as an alternative to topical corticosteroids. Since it has minimal systemic exposure, the fact that it induces hERG trafficking defects is of greater significance as a research tool than of physiological relevance to patients. Given the cardiotoxicity of arsenic trioxide and pentamidine in patients, we propose that drugs be evaluated for their effects on hERG trafficking as part of a comprehensive in vitro assessment of hERG risk.

At present, HERG-Lite<sup>®</sup> is done manually and a trained technician can test about 50 drugs per day. The method is amenable to automation and since the chemiluminescent reaction occurs within minutes, we expect that throughput can be increased by several orders of magnitude. HERG-Lite<sup>®</sup> provides the only comprehensive assessment of drug-induced hERG risk. Since both block and trafficking inhibition are detected, HERG-Lite<sup>®</sup> is an ideal assay for detecting hERG risk during lead development.

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