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Identification of quaternary ammonium compounds as potent inhibitors of hERG potassium channels

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Abstract

The human ether-a-go-go-related gene (hERG) channel, a member of a family of voltage-gated potassium (K^+) channels, plays a critical role in the repolarization of the cardiac action potential. The reduction of hERG channel activity as a result of adverse drug effects or genetic mutations may cause QT interval prolongation and potentially lead to acquired long QT syndrome. Thus, screening for hERG channel activity is important in drug development. Cardiotoxicity associated with the inhibition of hERG channels by environmental chemicals is also a public health concern. To assess the inhibitory effects of environmental chemicals on hERG channel function, we screened the National Toxicology Program (NTP) collection of 1408 compounds by measuring thallium influx into cells through hERG channels. Seventeen compounds with hERG channel inhibition were identified with IC_{50} potencies ranging from 0.26 to 22 μM. Twelve of these compounds were confirmed as hERG channel blockers in an automated whole cell patch clamp experiment. In addition, we investigated the structure-activity relationship of seven compounds belonging to the quaternary ammonium compound (QAC) series on hERG channel inhibition. Among four active QAC compounds, tetra-n-octylammonium bromide was the most potent with an IC_{50} value of 260 nM in the thallium influx assay and 80 nM in the patch clamp assay. The potency of this class of hERG channel inhibitors appears to depend on the number and length of their aliphatic side-chains surrounding the charged nitrogen. Profiling environmental compound libraries for hERG channel inhibition provides information useful in prioritizing these compounds for cardiotoxicity assessment *in vivo*.

Keywords

cardiotoxicity; hERG; long QT syndrome; NTP 1408 library; patch clamp; qHTS; tetra-n-octylammonium bromide; thallium influx

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Conflict of interest statement

The authors declare that there are no conflicts of interest, related to this article.

Introduction

The human ether-a-go-go-related gene (hERG) channel belongs to a family of voltage-gated potassium (K^+) channels (K_V) (Vandenberg *et al.*, 2004). It was first identified and cloned in 1994 (Warmke and Ganetzky, 1994). The functional K_V channels are comprised of four subunits, each containing six transmembrane domains (S1-S6), with a pore (P) between S5 and S6 (Wray, 2004). The S4 domain, which has multiple positive charges, constitutes the voltage sensor. The S5 and S6 regions connecting with the pore loop (P) contribute to the channel pore (Vandenberg *et al.*, 2004). To date, approximately 80 different K^+ channel genes have been identified in humans (Szabo *et al.*, 2010). In the K_V channel family, there are 12 distinct subfamilies based on their amino acid sequence homology (K_V1 to K_V12) (Gutman *et al.*, 2005). According to the current K_V channel nomenclature, the gene name of the hERG channel is *KCNH2* and the channel protein is $K_V11.1$ (Gutman *et al.*, 2005). hERG channels are mainly expressed in heart, but are also expressed in many other tissues including the brain, kidney, liver, and lung (Gutman *et al.*, 2005).

By conducting the rapid delayed rectifier K^+ current (Tseng, 2001), the hERG channel plays an essential role in the proper repolarization of action potential in normal heart as well as in prevention of arrhythmias induced by ectopic depolarizations (Vandenberg *et al.*, 2004). Functional block of the hERG channel due to either genetic defects or adverse drug effects can cause the abnormal action potentials and prolongation of the QT interval (i.e., the portion of an electrocardiogram between the onset of the Q wave and the end of the T wave, representing the total time for ventricular depolarization and repolarization), which may lead to long QT syndrome (LQTS) (Tseng, 2001). Drug-induced LQTS (also referred to as acquired LQTS) has become the most common cause of drug-induced cardiac arrhythmia and sudden death (Vandenberg *et al.*, 2001). During 1990–2006, 29% of the drugs withdrawn from approval was due to their potential to prolong the QT interval and/or induce a unique and potentially fatal ventricular tachyarrhythmia known as torsade de pointes (TdP) (Shah, 2006).

Although an assessment of hERG channel activity is an important step in early drug development, it has not been a focus in the evaluation of environmental chemicals for toxicity. To date, more than 100,000 chemicals have been introduced into commerce without sufficient toxicological testing (Belpomme *et al.*, 2007). These synthetic chemicals are widely used in the energy, transportation, agriculture, food, and pharmaceutical industries, and they may cause environmental pollution via contamination of air, soil, water, and food. To meet the needs of toxicity testing in the 21st century, the National Research Council (NRC) developed a long-term vision and strategic plan for the toxicological evaluation of chemicals (NRC, 2007). In response to this NRC report, the National Toxicology Program (NTP), the NIH Chemical Genomics Center (NCGC), and the U.S. Environmental Protection Agency (EPA) Office of Research and Development formed the Tox21 partnership; the U.S. Food and Drug Administration (FDA) has recently joined this partnership (http://yosemite.epa.gov/opa/admpress.nsf/d0cf6618525a9efb85257359003fb69d/571f805f9c4ff71385257765004cdb78!OpenDocument). The goals of Tox21 are to identify mechanisms of compound action at the cellular level, prioritize chemicals for further toxicological evaluation, and develop useful predictive models of *in vivo* biological response (Collins *et al.*, 2008; Kavlock *et al.*, 2009)

To assess the effect of environmental chemicals on hERG channels as part of the Tox21 program, we screened a collection of 1408 compounds (provided by the NTP) using a cell-based thallium influx assay (Titus *et al.*, 2009) in a quantitative high throughput screening (qHTS) format (Inglese *et al.*, 2006; Xia *et al.*, 2008). Using this assay, we identified 17

compounds that reproducibly inhibited the hERG channel at IC_{50} concentrations below 10 μM in the primary screen; 12 of these compounds were confirmed using an automated patch clamp electrophysiological method. The selectivity of hERG channel inhibition of these 12 compounds was demonstrated using a voltage gated $K_V1.3$ thallium assay. In addition, the cytotoxicity of these 12 compounds in the cell line used for the thallium influx assay was evaluated. This tiered screening approach appears useful for the detection of environmental chemicals that merit more extensive evaluation for cardiotoxicity and provides useful information that might be used to develop computational models for predicting the ability of new chemical entities to induce LQTS.

Materials and Methods

Cell line and culture condition

The U-2 OS cell line (HTB-96), a human osteosarcoma cell line, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). U-2 OS cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) with Glutamax containing 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), and 50 U/mL penicillin/50 μg/mL streptomycin (Invitrogen). CHO-K1 cells (from ATCC) stably transfected with hERG cDNA (GenBank sequence NM_000238) were constructed at Cerep, Inc. (Redmond, WA, USA) and used in the automated patch clamp experiments. The cells were cultured in F-12 Kaighn's Nutrient Mixture medium (Invitrogen) plus 10% FBS at 37°C for one to three days before conducting the patch clamp experiment. A CHO DUKX cell line (Perkin Elmer, Shelton, CT, USA) stably expressing the human $K_V1.3$ voltage gated K^+ channel was maintained in MEM Alpha media containing glutamine (Invitrogen), 0.4 mg/mL Geneticin (Invitrogen), and 10% FBS (Hyclone). All the cells were maintained at 37°C under a humidified atmosphere and 5% CO_2.

Compounds

A library of 1408 compounds (1353 unique compounds, 55 compounds in duplicate) was provided by the NTP. The compounds were dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA, USA) at a stock concentration of 10 mM. The completed list of compounds in this library is provided at (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcsubstance&term=NTPHTS).

For the follow-up studies, all the compounds (see Table 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA) except trixylenyl phosphate was purchased from ChemBridge (San Diego, CA, USA) and verapamil from Tocris (Ellisville, MO, USA). The purity of these compounds was provided by the vendors.

Transduction of hERG in U-2 OS cells

For viral transduction of hERG, U-2 OS cells were transduced using BacMam-hERG K^+ channel kit (Invitrogen) as described previously (Titus *et al.*, 2009). The cells used in all experiments had been passaged no more than 25 times. Briefly, after cells grew to approximately 70– 80% confluence in a T225 flask, the culture medium in the flask was removed and replaced with 2.5 mL of hERG–BacMam virus plus 12.5 mL of phosphate buffered saline (PBS, Invitrogen) (corresponding roughly to a multiplicity of infection ratio of 100 virus particles/cell). After the cell flask was incubated for 4 hr at room temperature in the dark, the hERG–BacMam viruses in the flask were removed and the flask was washed once with 25 mL of PBS. Then, 35 mL of complete culture medium was added and the cell flask was cultured at 37°C overnight. The next day, the cells were detached and re-suspended in Opti-MEM medium (Invitrogen) containing 2% fetal calf serum (FCS;

HyClone) at a density of 6.7×10^5 cells/mL. At this stage, cells were ready for thallium influx and cell viability assays.

FluxOR thallium influx assay and qHTS

Two thousand hERG transduced cells were dispensed in a volume of 3 μL per well in 1536-well, black wall/clear bottom plates (Greiner Bio-One North America, NC, USA) using a Multidrop Combi 8 channel dispenser (Thermo Fisher, Waltham, MA, USA). After plates were incubated at 37°C for 4 hr to allow the cells to adhere to the plates, 1 μL of loading buffer in Hanks Balanced Salt Solution (HBSS) [10 mM Red 40 (Spectrum Chemicals, Gardena, CA, USA)], 2.5 mM probenecid (Invitrogen), FluxOR dye at 0.7X final concentration prepared according to the manufacturer's instructions (Invitrogen), and 20 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] was added to each well using a Multidrop Combi 8 channel dispenser. The plates were then incubated at room temperature in the dark for 1 hr. Following incubation, 23 nL of each compound, dissolved in DMSO at the top stock concentration of 10 mM, was transferred to the assay plate by a Pintool (Kalypsys, San Diego, CA, USA). To achieve a concentration of 92 μM, the maximum concentration tested, 23 nL was transferred twice from the highest concentration of the compound plate into each well of the assay plate; control plates using DMSO only at this higher concentration were also included. The plates were incubated at room temperature for 10 min in the presence of compounds and the fluorescence intensity (488 nm excitation and 520 nm emission) from each well was recorded for 10 sec, in 1 sec intervals, using an Functional Drug Screening System (FDSS) 7000 kinetic plate reader (Hamamatsu, Hamamatsu City, Japan). Next, 1 μL of stimulation buffer containing 5 mM Tl_2SO_4 (Invitrogen) and 25 mM K_2SO_4 (Invitrogen) was added into all the wells using the tip head in the FDSS 7000 kinetic plate reader and fluorescence intensity was continuously measured for 2 min at 1 sec intervals.

The final compound concentrations tested ranged from 0.59 nM to 92 μM, in 14 concentrations. The total number of plates was 18, including four DMSO-only plates (two at each final DMSO concentration of 0.46% and 0.92%). Each treatment plate included concurrent DMSO and positive control wells; the positive control was astemizole, a known hERG blocker (Maxwell and Salnikow, 2004). The controls were arrayed as follows: Column 1, concentration-response titration of astemizole from 1.4 nM to 46 μM; Column 2, 10 μM astemizole; Column 3, DMSO only; and Column 4, 5 μM astemizole. The concentration-response titration for astemizole was used to evaluate plate-to-plate consistency, based on the calculated effective concentration that inhibited a half-maximal response (IC_{50}). The NTP 1408 compound library was screened twice for hERG inhibition to identify compounds exhibiting a consistent response.

To confirm the results of the initial study, compounds with an IC_{50} of less than 10 μM were retested three times in the FluxOR thallium influx assay either in hERG transduced or wild type cells. The assay protocol was the same as described above, except that the concentration titrations were all within one 1536-well plate and the compounds were tested at 24 concentrations ranging from 11 pM to 92 μM in duplicate.

Data analysis

Primary data analysis was performed as previously described (Titus *et al.*, 2009). Briefly, the slope of fluorescence intensities versus time during the first 30 sec after addition of the stimulation buffer was calculated from the kinetic results. The slopes for each titration point were first normalized relative to the astemizole positive control (10 μM, response set at -100%) and DMSO only wells (0%), and then corrected by applying a pattern correction algorithm using compound-free control plates (DMSO plates). Concentration response

titration points for each compound were fitted to the Hill equation yielding IC_{50} concentrations and maximal inhibition (efficacy) values. The concentration response curves were sorted into four major classes (1–4) using previously published criteria (Xia *et al.*, 2009). Curve classes were further subdivided to provide more detailed classification. Briefly, the highest confidence data are from compounds in curve classes 1.1, 1.2, and 2.1; compounds with class 1.3, 1.4, 2.2, 2.3, 2.4 and 3 curves have lower confidence data. Curve class 4 compounds showed no concentration-related response and were defined as inactive in this assay. Class 3 compounds display significant activity only at the highest concentration tested; class 2 compounds have incomplete curves (i.e., no low-concentration asymptote) and class 1 compounds have complete response curves (i.e., two asymptotes). Class 1 or 2 compounds were further divided into subclasses based on efficacy and quality of fit (R^2). Compounds with high (> 80%) efficacy were designated as subclass 1.1 or 2.1 ($R^2 > 0.9$) or 1.3 or 2.3 ($R^2 < 0.9$), and compounds with low efficacies (30–80%) as subclass 1.2 or 2.2 ($R^2 > 0.9$) or 1.4 or 2.4 ($R^2 < 0.9$).

Automated patch clamp

hERG CHO-K1 cells were harvested by trypsinization and kept in CHO-S-SFM II Medium (Invitrogen) for up to 4 hours at room temperature before recording. The cells were washed and resuspended in extracellular solution (for components, see below) before being applied to the patch clamp experiments. K^+ currents of these cells were recorded using the automated whole-cell patch clamp by Qpatch16 (Sophion Biosciences Inc, North Brunswick, NJ, USA). After whole cell configuration was achieved, the cell was held at −80 mV. A 50 millisecond pulse to −40 mV was delivered to measure the leak current, which was subtracted from the tail current on-line. Then, the cell was depolarized to +20 mV for 2 sec, followed by a one-second pulse to −40 mV to reveal the hERG tail current. This treatment pattern was delivered once every 5 seconds to monitor the tail current amplitude. Intracellular solutions included (in mM): 130 KCl, 10 NaCl, 1 $MgCl_2$, 10 EGTA (ethylene glycol tetraacetic acid), 5 Mg-ATP (magnesium-adenosine 5′ triphosphate), and 10 HEPES (pH adjusted to 7.2 with KOH). Extracellular solutions included (in mM): 137 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 D(+)-glucose, and 10 HEPES (pH adjusted to 7.4 with NaOH). All the chemicals in these solutions were purchased from Sigma-Aldrich. After the whole cell configuration was achieved, the extracellular solution (control) was applied first and the cell was stabilized in extracellular solution for 5 minutes. Then, the test compound was applied from low concentrations to high concentrations cumulatively. The cell was incubated with each test concentration for 5 minutes. During the incubation, the cell was repeatedly stimulated using the voltage protocol described above, and the tail current amplitude was continuously monitored. All electrophysiological recordings were performed at room temperature.

The inhibition (%) of hERG tail current is calculated by the equation of 100 × (Control-Test Compound) / Control. Control is the mean hERG tail current amplitude from the data collected over 24 seconds in the presence of control. Test Compound is the mean hERG tail current amplitudes from the data collected over 24 seconds in the presence of the test compound at each concentration.

IC_{50} values of compounds were obtained by fitting the following equation:

$$y = \frac{100}{1 + \left(\frac{C}{IC_{50}}\right)^n}$$

where y is the mean inhibition (%) of hERG tail current amplitudes, n is Hill slope, C is the test compound concentration, and IC_{50} is the test compound concentration producing 50% inhibition of hERG tail current.

K_V1.3 assay

Stably transfected K_V1.3 cells were plated at a density of 1000 cells/well in 3 μL of media in 1536-well, black wall/clear bottom plates. After the assay plates were incubated overnight at 37°C, 1 μL of the FluxOR dye loading buffer was added into each well of these assay plates using a Multidrop Combi 8 channel dispenser. After 1 hr of incubation with the loading buffer, 23 nL of each compound dissolved in DMSO was transferred into the assay plate by a Pintool. The FDSS kinetic reader was used to measure changes in fluorescence intensity using a standard Fluo-4 filter set (488 nm excitation, 520 nm emission). After 2 min of baseline measurement in the presence of compounds, 1 μL of stimulation buffer (final concentration 4 mM Tl_2SO_4, 10 mM K_2SO_4) was delivered to each well using the tip head in the FDSS 7000 kinetic plate reader. Fluorescence intensity was measured for an additional 1 min in 1 sec intervals and 2 min in 10 sec intervals. Psoralen (CASRN: 66-97-7, Sigma-Aldrich) was used as a positive control in the assay. Raw data from maximum minus minimum fluorescence reading was normalized relative to the psoralen control (1 μM, -100%) and DMSO only wells (0%). Concentration response titration points for each compound were fitted to the Hill equation yielding IC_{50} concentrations and maximal inhibition (efficacy) values.

Cell viability assay

Cell viability after treatment with the compounds identified as consistent hERG channel inhibitors was measured using a luciferase-coupled ATP quantitation assay (CellTiter-Glo® viability assay, Promega, Madison, USA) in hERG transduced U-2 OS cells. Changes in intracellular ATP content are directly proportional to the number of metabolically competent cells after compound treatment. The cells were dispensed at 2,000 cells/5 μL/well in 1,536-well white/solid bottom assay plates using a Flying Reagent Dispenser (FRD) (Aurora Discovery, San Diego, CA, USA). The cells were incubated a minimum of 5 h at 37°C prior to the addition of compounds using the pin tool. After compound addition, plates were incubated for 30 min at 37°C, followed by the addition of 5 μL per well of CellTiter-Glo reagent. After an additional 30 min of incubation at room temperature, the luminescence intensity of the plates was measured using a ViewLux plate reader (Perkin Elmer, Shelton, CT, USA).

Results

Identification of hERG channel blockers using cell-based qHTS

To profile the NTP library of 1408 compounds (1353 unique compounds) for potential activity on the hERG channel, we used a cell-based FluxOR thallium influx assay in hERG transduced cells. In this assay, thallium ions are used as surrogates for K^+ ions to monitor the activity of the hERG K^+ channel. The screen was carried out over 14 concentrations ranging from 0.59 nM to 92 μM in a qHTS format. Astemizole, a known hERG channel blocker, was used as the positive control to monitor assay performance and plate-to-plate variations during the screen. In both primary runs, the astemizole concentration response curves reproduced well in all 18 plates, including the four DMSO control plates. In the first run, the astemizole IC_{50} value was 56 ± 15 nM (mean ± standard deviation), the average signal-to-background ratio was 4.6 ± 0.2, the average coefficient of variation (CV) was 7.6 ± 3.3 (%), and the average Z′ factor (Zhang *et al.*, 1999) was 0.77 ± 0.04. Assay performance was similar in the second run (data not presented).

To evaluate assay and data reproducibility, the NTP 1408 compound library was screened in this thallium influx assay twice, on two different days. Among the 55 duplicate compounds in the library, the 10 duplicate compounds that showed hERG channel inhibition in the first run did so also in the second run, resulting in a 100% concordance rate. Of the 1353 unique NTP compounds, 88 (6.5%) hERG channel inhibitors with potent and moderate activity (curve classes 1.1, 1.2, and 2.1 in at least one run) were identified (Table 2). The IC_{50} values for these 88 unique compounds in these two runs correlated well (R = 0.85) (Fig 1). The distributions of curve class and potency for these compounds are listed in Table 2. Of these 88 compounds, 19 (1.4% of the 1353 unique NTP compounds) had an IC_{50} <10 μM, including one compound that had an IC_{50} less than 1 μM, in the first run of primary screening. These 19 compounds (Table 3) were purchased from commercial vendors for further study.

Confirmation of hERG channel blockers

Of the 19 compounds retested in the cell-based FluxOR thallium influx assay, 17 showed activity in the confirmation study that was similar to the activity observed in the primary screen, and two did not (Table 3). These two unconfirmed compounds had low efficacy (42–47%) in the primary screen (Table 3). The concentration response curves of these 19 compounds are provided in Supplementary Fig 1. For the 17 confirmed compounds, IC_{50} values in the primary screen (average of both runs) and confirmation studies were well correlated (R=0.88, Table 3). The most potent compound among the 17 confirmed compounds was tetra-n-octylammonium bromide, which had average IC_{50} values of 0.38 μM in the primary qHTS and 0.26 μM in the confirmation study. The next most potent compounds were verapamil (average IC_{50} = 3.4 μM), zinc pyrithione (average IC_{50} = 3.4 μM), and benzethonium chloride (average IC_{50} = 3.6 μM); IC_{50} values provided are from the confirmation study. These 17 compounds were also tested for thallium influx in the parental cells, which have few endogenous K^+ channels (Titus *et al.*, 2009); all were inactive in the parental cells except 2-aminoanthracene, curcumin, D&C red dye 27, and zinc pyrithione, suggesting that the inhibitory effect of these four compounds on thallium influx may not be specific to hERG channels.

To confirm the inhibitory effect of these 17 compounds on the hERG channel, we tested them using an automated whole cell patch clamp electrophysiology method, a method that is comparable to the conventional patch clamp method (gold standard) for characterization of hERG channel activity *in vitro* (Kiss *et al.*, 2003; Tao *et al.*, 2004). Among the compounds tested in the automated whole cell patch clamp experiment (Table 3 and Supplementary Fig 2), tetra-n-octylammonium bromide remained the most potent with an IC_{50} of 0.08 μM (Fig 2), followed by verapamil (IC_{50} 0.2 μM), and benzethonium chloride (IC_{50} 0.98 μM). As expected, the four compounds that showed a non-specific inhibitory effect in untransduced parental cells had much lower potency in the whole cell patch clamp experiment. For example, 2-aminoanthracene and D&C red dye 27 had IC_{50} values greater than 100 μM in the patch clamp assay compared to IC_{50} values of 17 and 7 μM, respectively, in the thallium influx assay. Curcumin and zinc pyrithione had IC_{50} values of 22 and 33 μM, respectively, in the patch clamp assay, but they had IC_{50} values of 4.4 and 3.4 μM, respectively, in the thallium influx assay. The IC_{50} values of 12 of the other 13 compounds correlated well (R = 0.77) between the thallium influx assay and the patch clamp experiment, confirming their inhibitory effect on the hERG channel. Only one compound, trixylenyl phosphate, did not inhibit hERG channel activity in the patch clamp experiment; the potency (IC_{50} of 16 μM) of this compound was relatively low in the thallium influx assay and, therefore, trixylenyl phosphate may have weak and inconsistent activity across these assays. The discordance between the potency of these compounds in the thallium influx assay and the patch clamp assay might be due to the color of these compounds. Colored compounds in solution will

absorb light, which will reduce the fluorescence signal generated in the thallium influx assay. Results of these experiments indicate that the thallium influx assay can be used as a primary screen and false positives can be eliminated by the electrophysiological experiment in the confirmation stage.

In addition, the cytotoxicity of these 12 compounds, after a 30-minute treatment period, was evaluated in a cell viability assay that measures intracellular ATP content. Four of the 12 compounds -- benzethonium chloride, domiphen bromide, malachite green oxalate, and tetra-n-octylammonium bromide -- showed low levels of cytotoxicity, with IC_{50} values of 79, 65, 31, and 34 μM, respectively, and maximum inhibition of cell viability of 34%, 33%, 72%, and 50%, respectively. However, these compounds were much more potent in blocking hERG channel, with IC_{50} values ranging from 0.26 to 4.8 μM, suggesting that the ability of these compounds to inhibit the hERG channel is not due to cytotoxicity. The other eight compounds were not cytotoxic at concentrations up to 92 μM.

Inhibition of quaternary ammonium compounds on hERG channel

In this study, we found that benzethonium chloride, domiphen bromide, and tetra-n-octylammonium bromide significantly inhibited hERG channel activity in both the thallium influx assay and the patch clamp experiment. Notably, all three compounds are quaternary ammonium compounds (QACs). Therefore, to further investigate the effect of QACs on the hERG channel activities, we purchased four more QAC analogs: benzyltrimethylammonium chloride, cetyltrimethylammonium bromide, decamethonium dibromide, and didecyl dimethyl ammonum chloride. We found the greatest potency in hERG channel blocking activity for QACs with at least two long aliphatic side chains surrounding the charged nitrogen (Fig 3), such as tetra-n-octylammonium bromide (IC_{50}, 0.24 μM in the thallium influx assay, and 0.08 μM in the patch clamp experiment) and didecyl dimethyl ammonum chloride (IC_{50}, 1.7 μM in thallium influx assay, and 0.65 μM in patch clamp experiment). In contrast, benzyltrimethylammonium chloride and decamethonium dibromide, both of which are trimethyl ammonium ions with the charged nitrogen(s) at the end of the structure, were inactive in both the thallium influx assay and the patch clamp experiment (Fig 3).

QACs are often used as powerful disinfectants in medical and food industries (Heir *et al.*, 1999; McDonnell and Russell, 1999) due to their detergent (surfactant) properties. To investigate if the inhibitory action of the QACs on the hERG channel was related to their cell membrane disruption capability, we included digitonin, a well characterized detergent, as a control in this study. We found that digitonin had no inhibitory effect on hERG channel activity at 1 μM (the highest noncytotoxic concentration) in the patch clamp experiment. In the thallium influx assay, digitonin had minimal inhibition on hERG channel activity, with an IC_{50} of 40 μM. These results suggest that the inhibition of the QACs on the hERG channel is likely unrelated to their detergent action.

Compound selectivity for hERG channel blockers

To further examine the selectivity of the 12 hERG channel blockers identified in the thallium influx assay and confirmed in the patch clamp experiment in the current study, we tested compound activity on the voltage gated $K_V1.3$ K^+ channel by measuring thallium influx in $K_V1.3$ stably transfected cells. We found that 11 of the 12 compounds tested had low potency (IC_{50} > 11 μM) or no activity for $K_V1.3$ channel inhibition (Table 4). Among these were five compounds -- 1.3-diphenylguanidine, malachite green oxalate, o,p′-DDT, quinidine, and reserpine -- that had either IC_{50} values >30 μM or were inactive in the $K_V1.3$ assay. Among the compounds that showed $K_V1.3$ inhibition, tetra-n-octylammonium bromide had the highest selectivity for the hERG channel over the $K_V1.3$ channel (42-fold potency difference), followed by reserpine (11.8-fold), verapamil (8.8-fold), malachite green

oxalate (6.5-fold), and benzethonium chloride (5-fold). In contrast, domiphen bromide and o,p′ –DDT showed no selectivity between channel types, and tamoxifen (2.7-fold), and tricresyl phosphate (2.7-fold) had low selectivity.

Discussion

In the present study, we used a cell-based thallium influx assay in qHTS format to evaluate the effect of a large set of environmental chemicals on hERG channel activity. The processes involved in conducting the screening, confirmation, and follow-up studies are summarized as a flow chart in Fig 4. Seventeen unique compounds were identified in the NTP 1408 compound library that strongly inhibited hERG channel activity, with IC_{50} values determined in the confirmation study ranging from 0.26 to 22 μM. Twelve of these compounds were confirmed as hERG blockers in an automated whole cell patch clamp assay; these 12 compounds were negative for thallium influx activity in the non-hERG transduced cells. Among these 12 confirmed hERG channel blockers, tetra-n-octylammonium bromide, a quaternary ammonium compound (QAC), was the most potent. Therefore, we investigated the structure-activity relationship of a QAC series on hERG inhibition and found that the potency of hERG inhibition activity for this class of compounds depended on the number and length of the aliphatic side-chains surrounding the charged nitrogen. Lastly, we found that 11 of these 12 hERG blockers had either low potency (IC_{50} > 11 μM) or no activity in the $K_V1.3$ channel inhibition assay, indicating the selectivity of these compounds for the hERG channel.

Drug-induced QT interval prolongation is a major health concern for both cardiac and non-cardiac drugs. Among the 12 confirmed hERG channel blockers in the NTP 1408 compound library, three compounds -- quinidine (Perrin *et al.*, 2008), verapamil (Chouabe *et al.*, 1998), and tamoxifen (Thomas *et al.*, 2003; Titus *et al.*, 2009) -- have previously been reported to inhibit the hERG channel. Quinidine is used to treat a variety of cardiac arrhythmias primarily by blocking rapid inward sodium current (Grace and Camm, 1998), but clinical use of quinidine has been linked to cardiac QT prolongation through inhibition of the hERG K^+ channel (Grace and Camm, 1998). Verapamil, an L-type Ca^{2+} channel antagonist, has been clinically used in the treatment of cardiovascular diseases including hypertension (Richard, 2005). In the previous study, we found that verapamil effectively blocked L-type Ca^{2+} channels (IC_{50} of 0.7 μM) in HEK 293 cells stably transfected with L-type Ca^{2+} channels (Xia *et al.*, 2004). In addition to blocking L-type Ca^{2+} channels, verapamil has been found to block the hERG channel by binding to the helix residue Y652 and F656 in the S6 transmembrane domain (Duan *et al.*, 2007). However, it has been shown that verapamil did not affect QT prolongation because the block of the L-type Ca^{2+} channel compensates the effect from the block of hERG channel (Ca^{2+} channels conduct inward current, whereas hERG channels conduct outward current). Therefore, the verapamil induced TdP in humans has not been reported (Redfern *et al.*, 2003). Tamoxifen is a well-known estrogen receptor antagonist that is used in the treatment of breast cancer. However, in clinical trials, tamoxifen was linked to QT interval prolongation (Trump *et al.*, 1992). Also, tamoxifen has been shown to inhibit hERG current in electrophysiology voltage clamp experiments (Thomas *et al.*, 2003) and to block hERG channels in thallium influx assays (Titus *et al.*, 2009).

In the present study, we identified two drugs, econazole nitrate (IC_{50} of 7.2 μM in the thallium influx assay and 3.5 μM in the patch clamp assay) and reserpine (IC_{50} of 4.9 μM in the thallium influx assay and 1.9 μM in the patch clamp assay), as hERG channel blockers, which to our knowledge has not been reported previously, but is consistent with their therapeutic action. Econazole nitrate, an imidazole derivative, is one of the topical antifungal medications used clinically in the treatment of superficial mycoses of the skin (Veraldi and

Milani, 2008). Previous studies have shown that econazole blocks the Ca^{2+}-dependent K^+ channel in human red blood cells (Alvarez *et al.*, 1992) and inhibits receptor-operated calcium channels in human neutrophils (Montero *et al.*, 1991). Econazole has also been reported to relax phenylephrine- and KCl-induced contraction in rat isolated aorta rings by inhibiting calcium entry via L-type Ca^{2+} channels (Tunctan *et al.*, 2000). Reserpine, an indole alkaloid, has been used as an antihypertensive drug (Shamon and Perez, 2009).

QACs, including tetraethylammonium (TEA), have been widely used for decades as classic voltage-gated K^+ channel blockers, with IC_{50} values ranging from 0.6 to 129 mM in electrophysiological studies (Stanfield, 1983; Pongs, 1992). The block of voltage-gated K^+ channels by TEA and other QACs occurs not only at the external region of the pore, but also via an internal TEA-binding site in the S5 region of the channel (Pongs, 1992). Due to their high lipophilicity, these compounds can easily cross the hydrophobic core of the plasma membrane to interact with the internal S5 region of the K^+ channel. However, to date, block of the hERG channel by these compounds has not been reported. In the present study, we found in our initial screen that the QACs, such as benzethonium chloride, domiphen bromide, and tetra-n-octylammonium bromide, significantly blocked the hERG channel. The binding of these compounds to the hERG channel may occur via the internal binding site because we found that the potency of the QACs for hERG channel inhibition is directly related to the number and length of the aliphatic side-chains. Among the QACs we screened, tetra-n-octylammonium bromide, with four long, aliphatic side-chains, was the most potent. Potency decreased in compounds that contain short, aromatic side chains or trimethylammonium heads, where the charged nitrogen is “exposed” at the end of the structure. For example, decamethonium dibromide and benzyltrimethylammonium chloride did not inhibit the hERG channel, presumably because the charged nitrogen(s) in these compounds are exposed. Compounds with more and longer aliphatic side chains, where the charged nitrogen is hidden in the middle of the structure, are more lipophilic and thus, they can more easily cross the plasma membrane and bind to the internal site of the hERG channel. To validate our QAC structure-activity hypothesis, we calculated the Log D values (Figure 3B) using Pipeline Pilot 7.0 (Accelrys, Inc., San Diego, CA, USA) for the seven QACs and found a significant correlation ($R = 0.95$, $p = 0.001$) between lipophilicity and their potency for inhibiting the hERG channel.

This study identified several compounds, including 1,3-diphenylguanidine, malachite green oxalate, o,p′-DDT, and tricresyl phosphate, as hERG channel blockers. Some of these compounds have been widely used in agriculture and industry, and have significant human exposure potential, although there have been no reports associating exposure to these compounds with QT interval prolongation. For example, 1,3-diphenylguanidine has been used as a primary and secondary accelerator in the vulcanization of rubber (NTP, 1995). Bempong and Hall (1983) reported that this compound decreased sperm count and altered sperm morphology in rodents, although these observations were not confirmed in an independent study (Koeter *et al.*, 1992). Recently, 1,3-diphenylguanidine was reported to be involved in the development of allergic contact dermatitis (Piskin *et al.*, 2006). Malachite green, a triarylmethane dye, is widely used as a parasiticide in the aquaculture industry and is also used as a food and clothing coloring agent (Srivastava *et al.*, 2004). It has been reported to be carcinogenic, mutagenic, and teratogenic, and to induce respiratory toxicity (Srivastava *et al.*, 2004). We found, using a qHTS cell viability assay, that malachite green oxalate is highly cytotoxic to a number of different human and rodent cell types (Xia *et al.*, 2008). DDT (dichlorodiphenyltrichloroethane) is one of the most well-known synthetic pesticides. DDT and related chlorinated pesticides have been banned from agricultural use in the United States since 1972, but due to their persistence in the environment, they remain a significant public health concern. Due to the lipophilic nature of these chemicals, they are stored in lipid-rich tissues such as liver, brain, and adipose tissue. DDT is highly toxic to

various organ systems including neurological, immunological, endocrinological, cardiovascular, respiratory, gastrointestinal, and other systems (Crinnion, 2009). It has been reported that DDT also causes cardiac arrhythmias, such as Q-T prolongation and repetitive ventricular tachyarrhythmias of the TdP type, after patients were poisoned with this chemical (Ludomirsky *et al.*, 1982). DDT is also known to modulate Na^+ channels of nerve cell membranes at exposure levels that cause hyperexcitatory symptoms in animals (Song *et al.*, 1996). Results with DDT in the current study suggest that hERG channel inhibition by DDT (IC_{50} values of 22.2 μM in the thallium influx assay and 2.6 μM in the patch clamp assay) may be a factor in DDT-associated cardiac arrhythmias. Tricresyl phosphate (TCP), an organophosphate compound, has been used as a plasticizer, lubricant, hydraulic fluid, paint additive, oil additive, dust suppressant, and in other commercial applications. However, most commercial uses of TCP have been halted due to its toxicity, particularly neurotoxicity (Winder and Balouet, 2002).

In summary, we have identified several known and novel hERG channel blockers in the NTP 1408 library. In the present study, we used a cell-based thallium influx assay as the primary screen, in combination with secondary assays including the whole cell patch clamp assay. In the next steps, the characterization of the hERG blockers identified in the current study would likely include QT prolongation studies in animals and investigations of the mechanisms of action (e.g., effects on ionic permeation/selectivity and/or activation, deactivation, or inactivation kinetics of hERG channels). We conclude that the approach used in this study will allow for the efficient identification and profiling of hERG channel blockers in anticipated future screenings of large collections of environmental chemicals and drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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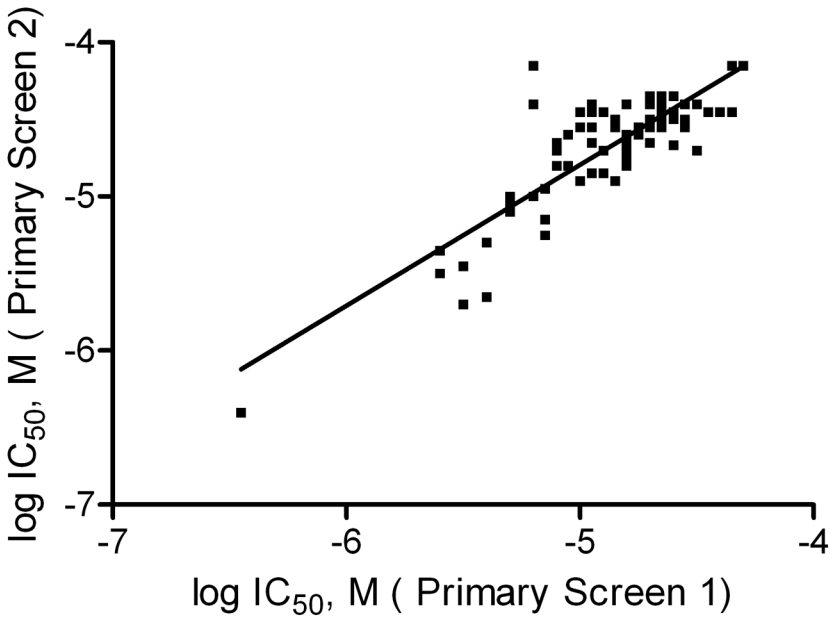


Fig 1.

qHTS reproducibility of the FluxOR thallium influx assay. The NTP 1408 compound library was screened twice in hERG transduced cells at two separate times. Linear correlation of IC_{50} values from 88 compounds with concentration response curves in two independent screenings yielded an average R of 0.85.

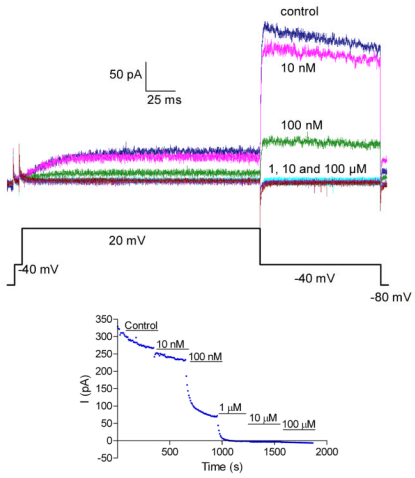


Fig 2.

Inhibitory effect of tetra-n-octylammonium bromide on hERG tail current measured in an automated whole cell patch clamp experiment. A. Representative electrophysiology recording from one automated patch clamp experiment. The voltage protocol used to induce the hERG current is shown at the bottom. B. The current vs. time plot (I–T plot) of the experiment from A.

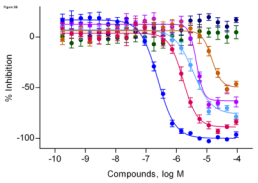
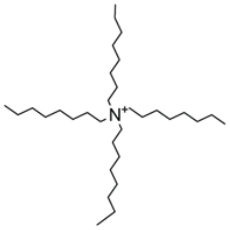
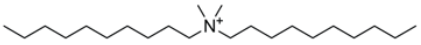


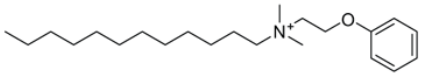
Figure 3B



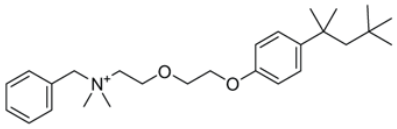
● Tetra-N-octylammonium bromide
IC_{50} = 0.24 μM (Thallium influx)
IC_{50} = 0.08 μM (Patch clamp)
LogD = 11.58



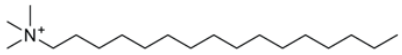
● Didecyl dimethyl ammonum chloride
IC_{50} = 1.7 μM (Thallium influx)
IC_{50} = 0.65 μM (Patch clamp)
LogD = 7.10



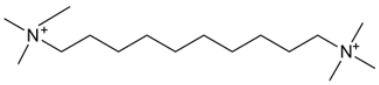
● Domiphen bromide
IC_{50} = 4.3 μM (Thallium influx)
IC_{50} = 1.5 μM (Patch clamp)
LogD = 5.58



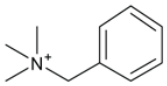
● Benzethonium chloride
IC_{50} = 3.6 μM (Thallium influx)
IC_{50} = 0.98 μM (Patch clamp)
LogD = 4.82



● Cetyltrimethylammonium bromide
IC_{50} = 14.5 μM (Thallium influx)
IC_{50} > 100 μM (Patch clamp)
LogD = 5.77



● Decamethonium dibromide
Inactive (Thallium influx)
IC_{50} > 100 μM (Patch clamp)
LogD = 1.13



● Benzyltrimethylammonium chloride
Inactive (Thallium influx)
IC_{50} > 100 μM (Patch clamp)
LogD = 0.55

Fig 3.

Quaternary ammonium compounds. A. Concentration response curves of the compounds from the ammonium series in the thallium influx assay in hERG cells. B. Structures of these compounds are shown with compound names, IC50 values, and logD values in the thallium influx assay and the whole cell patch clamp experiment.

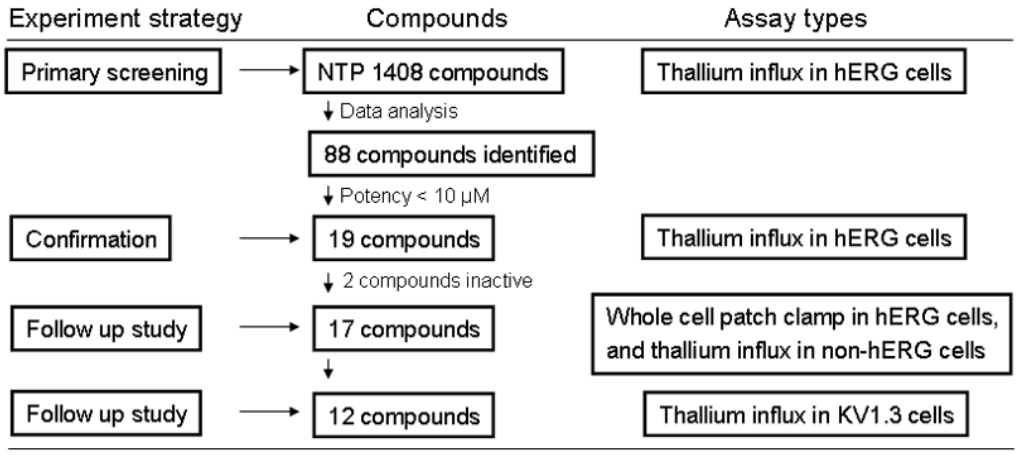


Fig 4.

A flowchart of identification of hERG channel blockers. Eighty-eight compounds with class 1.1, 1.2, and 2.1 curves were identified from primary screening. Nineteen compounds with IC_{50} values less than 10 μM were selected based on the first run of primary screening for further studies. Seventeen of nineteen compounds were confirmed in thallium influx assay, and further tested in a whole cell patch clamp experiment. Twelve of these compounds were confirmed as hERG channel blockers in a whole cell patch clamp experiment and had no off target activity in the non-hERG transduced cells. Nine of the twelve compounds had either low potency or no activity in the $K_V1.3$ channel inhibition assay.

Table 1

Compounds used in the confirmation and follow-up studies

Compound	CASRN	Purity (%)
1,3-diphenylguanidine	102-06-7	99.5
2-aminoanthracene	613-13-8	96
3,3,5-trimethylcyclohexyl salicylate	118-56-9	NP
4′-N-hexyl-4-biphenylcarbonitrile	41122-70-7	98
astemizole	68844-77-9	98
benzethonium chloride	121-54-0	99
benzyltrimethylammonium chloride	56-93-9	97
cetyltrimethylammonium bromide	57-09-0	100
curcumin	458-37-7	80
D&C red dye 27	18472-87-2	NP
decamethonium bromide	541-22-0	99
didecyl dimethyl ammonium chloride	2390-68-3	98
digitonin	11024-24-1	63
domiphen bromide	538-71-6	97
econazole nitrate	24169-02-6	99
malachite green oxalate	2437-29-8	NP
o,p′-DDT	789-02-6	NP
quinidine	56-54-2	89
reserpine	50-55-5	98
tamoxifen, citrate salt	54965-24-1	99
tetra-n-octylammonium bromide	14866-33-2	98
tricresyl phosphate	1330-78-5	90
trixylenyl phosphate	25653-16-1	90
verapamil	152-11-4	99
zinc pyrithione	13463-41-7	95

Abbreviations: CASRN = Chemical Abstracts Services Registry Number; DDT = 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane; NP = not provided by vendors

Table 2

Potency (IC_{50}) distribution of hERG inhibitors in the primary qHTS

IC_{50} (μM)	Number of inhibitors in curve class		
	1.1	1.2	2.1
<1.0	1	0	0
1.0 – <10.0	11	7	0
10.0 – 100.0	15	47	7
Total	27	54	7

IC_{50} values (concentration of half the maximal inhibition) and efficacy (inhibition of thallium influx as a % of positive control) were calculated from the concentration response titration points for individual compound. There were four major classes (1–4) based on the concentration response curves using previously published criteria (Xia *et al.*, 2009). Curve classes were further subdivided to provide more detailed classification. Briefly, the highest confidence data are from compounds in curve classes 1.1, 1.2, and 2.1; compounds with class 1.3, 1.4, 2.2, and 3 curves have lower confidence data. Curve class 4 compounds showed no concentration-related response and were defined as inactive in this assay.

Table 3

Potencies and efficacies of hERG blockers identified from primary qHTS (thallium influx), confirmation (thallium influx), and patch clamp assays

Compound	qHTS (two runs)		Confirmation		Patch clamp
	IC_{50}	Efficacy	IC_{50}	Efficacy	IC_{50}
1,3-diphenylguanidine	3.4 ± 0.3	56 ± 7	6.9 ± 3	39 ± 12	13
2-aminoanthracene*	6.5 ± 2.1	61 ± 3	17.5 ± 26	67 ± 19	>100
3,3,5-trimethylcyclohexylsalicylate	23 ± 23	52 ± 6	Inactive		ND
4-N-hexyl-4′-cyanobiphenyl	8.2 ± 2.6	39 ± 6	Inactive		ND
benzethonium chloride	2.8 ± 0.5	91 ± 1	3.6 ± 1.4	77 ± 9	0.98
curcumin*	2.6 ± 0.9	129 ± 4	4.4 ± 1.4	140 ± 4	22
D&C red dye 27*	4.5 ± 0.7	119 ± 5	7.0 ± 2.5	173 ± 41	>100
domiphen bromide	7.0 ± 2.8	78 ± 0.6	4.3 ±1.0	62 ± 11	1.5
econazole nitrate	9.1 ± 2.9	109 ± 8	7.2 ± 2.8	112 ± 11	3.5
malachite green oxalate	7.1 ± 0	97 ± 3	4.8 ± 0.8	108 ± 7	1.9
o,p′-DDT	17 ± 11	56 ± 2	22.2 ± 4.1	58 ± 8	2.6
quinidine	11 ± 5.6	107 ± 25	13.6 ± 5.4	110 ± 13	2.1
reserpine	7.5 ± 3.5	125 ± 23	4.9 ± 1.7	118 ± 7	1.9
tamoxifen, citrate salt	6.4 ± 1.0	106 ± 22	8.3 ± 0.8	110 ± 4	4.2
tetra-n-octylammonium bromide	0.38 ± 0.03	102 ± 4	0.26 ± 0.1	100 ± 3	0.082
tricresyl phosphate	14 ± 9	82 ± 11	9.7 ± 1.6	68 ± 16	8.8
trixylenyl phosphate	39 ± 45	67 ± 12	15.9 ± 11	43 ± 15	>100
verapamil	3.1 ± 1.2	105 ± 1	3.4 ± 0.8	100 ± 8	0.2
zinc pyrithione*	3.5 ± 1.4	155 ± 9	3.4 ± 0.3	178 ± 7	33

Abbreviations: DDT = 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane; ND = not determined

*Inhibition was found in wild type cells.

Each value of potency (IC_{50}, μM) and efficacy (inhibition of thallium influx as a % of positive control) is the mean ± SD of the results from two runs of primary screening and from three experiments, with each concentration tested in duplicate, in the confirmation assay.

Table 4

Effect of hERG channel blockers on thallium influx in hERG-transduced and $K_V1.3$ cells

Compound	hERG		K_V 1.3		Selectivity
	IC_{50}	Efficacy	IC_{50}	Efficacy	Fold
1,3-diphenylguanidine	6.9 ± 3	39 ± 12	Inactive		
benzethonium chloride	3.6 ± 1.4	77 ± 9	18±6	93 ± 20	5
domiphen bromide	4.3 ± 1.0	62 ± 11	5.2 ± 0.6	102 ± 22	1.2
econazole nitrate	7.2 ± 2.8	112 ± 11	30 ± 2	104 ± 10	4.2
malachite green oxalate	4.8 ± 0.8	108 ± 7	31	89	6.5
o,p′ –DDT	22.2 ± 4.1	58 ± 8	35	61	1.6
quinidine	13.6 ± 5.4	110 ± 13	Inactive		
reserpine	4.9 ± 1.7	118 ± 7	58 ± 18	78 ± 7	11.8
tamoxifen, citrate salt	8.3 ± 0.8	110 ± 4	22 ± 3	108	2.7
tetra-n-octylammonium bromide	0.26 ± 0.1	100 ± 3	11 ± 8	72 ± 16	42
tricresyl phosphate	9.7 ± 1.6	68 ± 16	26 ± 7	68 ± 17	2.7
verapamil	3.4 ± 0.8	100 ± 8	30 ± 3	88 ± 12	8.8

Abbreviations: DDT = 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane

Each value of potency (IC_{50}, μM) and efficacy (inhibition of thallium influx as a % of positive control) from thallium influx assays in hERG-transduced and $K_V1.3$ cells is the mean ± SD from two to three experiments.