Published in final edited form as:

Toxicol Appl Pharmacol. 2013 March 1; 267(2): 155–166. doi:10.1016/j.taap.2012.12.021.

Mechanism of HERG potassium channel inhibition by tetra-n-octylammonium bromide and benzethonium chloride

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Abstract

Tetra-n-octylammonium bromide and benzethonium chloride are synthetic quaternary ammonium salts that are widely used in hospitals and industries for the disinfection and surface treatment and as the preservative agent. Recently, the activities of HERG channel inhibition by these compounds have been found to have potential risks to induce the long QT syndrome and cardiac arrhythmia, although the mechanism of action is still elusive. This study was conducted to investigate the mechanism of HERG channel inhibition by these compounds using whole-cell patch clamp experiments in a CHO cell line stably expressing HERG channels. Tetra-n-octylammonium bromide and benzethonium chloride exhibited concentration-dependent inhibitions of HERG channel currents with IC₅₀ values of 4 nM and 17 nM, respectively, which were also voltage-dependent and use-dependent. Both compounds shifted the channel activation I-V curves in a hyperpolarized direction for 10–15 mV and accelerated channel activation and inactivation processes by 2-fold. In addition, tetra-n-octylammonium bromide shifted the inactivation I-V curve in a hyperpolarized direction for 24.4 mV and slowed the rate of channel deactivation by 2-fold, whereas benzethonium chloride did not. The results indicate that tetra-n-octylammonium bromide and benzethonium chloride are open-channel blockers that inhibit HERG channels in the voltage-dependent, use-dependent and state-dependent manners.

Keywords

Quaternary ammonium; Tetra-n-octylammonium bromide; Benzethonium chloride; HERG potassium channel; HERG channel blocker; Patch clamp

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Conflict of interest statement

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

Introduction

The quaternary ammonium compounds (QACs), consisting of four ethyl groups attached to a central nitrogen atom, are widely used for surface treatment and hospital/environmental disinfection (McDonnell and Russell, 1999). Tetra-n-octylammonium bromide and benzethonium chloride are both synthetic quaternary ammonium salts that are broadly used and widespread presence in the environment because of their antimicrobial and cationic surfactant properties. They have been widely used as preservative agents in vaccines and drugs, phase transfer catalyzers in industry and disinfectants in hospital (Lahtinen *et al.*, 2004) (Bearden *et al.*, 2008) (Braun *et al.*, 2010; Geier *et al.*, 2010) (Ichikawa *et al.*, 2008) (Tsubura *et al.*, 2009; Nomura *et al.*, 2010). Benzethonium chloride is also present in the baby bath, eye makeup, contact lens, personal hygiene, fragrance, hair, shaving, skin and suntan preparations as well as in fabric softening, ore flotation, corrosion inhibition and paper processing (Elder, 1984) (Anon, 2006) (Mote *et al.*, 1969). A recent report revealed that several QACs including benzethonium chloride and tetra-n-octylammonium bromide block the HERG (the human Ether-à-go-go-Related Gene) channel (Xia *et al.*, 2011). The inhibitory potency of QACs seems primarily linked to the compound lipophilicity (Choi *et al.*, 2011; Xia *et al.*, 2011), but the precise mechanism of HERG channel inhibition by QACs needs further investigation.

As a member of the large family of voltage-gated potassium channels, the HERG potassium channel plays a pivotal role in the repolarization of the cardiac action potential that regulates cardiac rhythm (Vandenberg *et al.*, 2012; Zhang *et al.*, 2012). Inhibition of HERG channel activity by the inhibitors may prolong QT interval and cause the long QT syndrome. Torsade de pointes (TdP), a rare and severe ventricular arrhythmia (Zunkler, 2006). Therefore, measurement of compound activity on HERG channels has become an important part of the drug safety evaluation process (Goineau *et al.*, 2012). Evaluation of the effect of environmental chemicals on HERG channel function can provide the information regarding the potential risks of these compounds on public health. In the present study, the potential mechanisms for HERG channel inhibition by tetra-n-octylammonium bromide and benzethonium chloride, two most potent HERG channel inhibitors found among the QACs examined (Xia *et al.*, 2011), were studied using whole-cell patch clamp experiments in a CHO cell line stably expressing HERG channels. Our results have revealed that these two compounds are potent open channel blockers which inhibit HERG channels in the use-dependent, voltage-dependent and state-dependent manners.

Materials and Methods

Reagents

Tetra-n-octylammonium bromide, benzethonium chloride and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The two quaternary ammonium compounds were first dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM, and then diluted to the desired working concentration in bath solution before experiments. Based on the reported IC₅₀ values of 0.08 μM for tetra-n-octylammonium bromide and 0.98 μM for benzethonium chloride obtained from experiments using automated Qpatch 16 (Xia *et al.*, 2011), we selected 0.0001 μM to 0.1 μM of tetra-n-octylammonium bromide and 0.001 μM to

1 μ M of benzethonium chloride for our concentration response studies. The maximal DMSO concentration in the final experimental solutions was 0.01% (v/v) that did not affect HERG currents.

Cell culture

CHO cells stably expressing HERG potassium channels were purchased from ChanTest (Cleveland, OH, USA). The cells were cultured in HAMS F-12 media (Invitrogen, Carlsbad, CA, USA) supplemented with 1 mM l-glutamine, and 10% fetal bovine serum (Hyclone, Logan, UT, USA) in a humidified incubator with 5% CO₂ at 37°C.

Whole-cell patch clamp and recordings

HERG potassium current was recorded with whole-cell patch clamp technique at room temperature (22°C). The intracellular recording solution for all experiments was (in mM): KCl 130; MgCl₂ 1; EGTA 5; Mg-ATP 5; HEPES 10; pH was adjusted to 7.2. The extracellular recording solution was (in mM): NaCl 137; KCl 4; CaCl₂ 1.8; MgCl₂ 1.0; glucose 10; HEPES 10, pH was adjusted to 7.4 (Berube *et al.*, 2001). All recordings were made using an Axopatch 200B patch clamp amplifier in conjunction with a Digidata 1400 interface (Axon Instruments, Foster City, CA, USA). Patch pipettes were fabricated from capillary glass using a Flaming/Brown micropipette puller (P-97; Sutter Instruments Co.). Patch pipette resistances were 2–4 M Ω . Cell and pipette capacitances were nulled and series resistance was compensated (85–95%) before recording. Data were acquired using pCLAMP programs (10.0; Axon Instruments).

Compound concentration response

To determine the concentration-response of two compounds on I_{HERG}, cells were depolarized from a holding potential of –80 mV to +20 mV for 4 s, then repolarized to –40 mV for 2 s, this cycle was repeated for 5 min in the absence of compound. After the HERG current became stable, the test compound was added to the bath solution from low to high concentrations cumulatively and each recorded for 5 min. The HERG tail currents were measured at –40 mV, after a step to +20 mV. The concentration–inhibition curves were fitted using the Hill equation: $B(\%) = 100/[1 + (IC_{50}/D)^n]$, where B(%) is the percent changes of HERG tail current at a compound concentration D, IC₅₀ is the concentration of quaternary ammonium compounds that produces 50% effects, and n is the Hill coefficient. Verapamil was used as a positive control compound that showed concentration-dependent suppression of HERG peak tail currents with an IC₅₀ value of 0.38 μ M and a Hill coefficient of 1.3 (Figure 1C), consistent with that previously reported (Chouabe *et al.*, 1998; Zhang *et al.*, 1999).

Use-dependent inhibition

To determine the use-dependence of HERG channel blockage by two compounds, the voltage is stepped from –80 mV to +20 mV for 4 s to inactivate the HERG channels, which was followed by channel opening at –40 mV in the absence of compounds (control run). Then, compounds were added to the bath solution. Every ten minutes later, the same protocol was repeated with the same cycle lengths (the first, second, third and fourth run).

The CHO cells were held at -80 mV without pulsing during the equilibration period. The normalized tail currents were used to investigate the use-dependency.

Voltage-dependent inhibition

The voltage-dependent inhibition of HERG channel was explored with protocols including a standard I-V protocol, a fully activated I-V protocol, and an instantaneous I-V protocol. In the standard I-V protocols, the step currents were elicited by 4s depolarizing pulses ranging from -70 to $+50$ mV and the tail currents by 2s repolarizing pulses to -40 mV. The fully activated I-V relationships were obtained as follow: a 1 s pre-pulse to $+50$ mV was applied before each of the repolarizing pulses to test potentials between -140 mV and $+20$ mV. In the instantaneous I-V protocols, the channels were first inactivated by clamping the membrane at $+50$ mV for 1s, which followed by a pre-pulse to -100 mV for 20 ms to remove inactivation without allowing sufficient time for deactivation to occur. Following the recovery pre-pulse, a family of test pulses was delivered to potentials ranging from -140 to $+20$ mV.

State-dependent inhibition

Activation—The activation currents were induced with a standard I-V protocol and tail currents were normalized to the peak values. Then, the normalized data were plotted against the pre-pulse potentials and fitted to the Boltzmann distribution $I/I_{\max} = 1 / \{1 + \exp[(V_{1/2} - V) / \kappa]\}$, where I is the HERG tail current amplitude at a prepulse potential V , $V_{1/2}$ is the voltage for half-maximal activation, and κ is a slope factor. In addition, the activation time course was fitted to a double exponential function on the currents evoked at a test potential of 0 mV from a holding potential of -80 mV.

Inactivation—The inactivation currents were elicited with a 2s depolarizing pulse to $+40$ mV to inactivate the HERG channels, followed by various repolarizing pulses to potentials from -140 mV to $+30$ mV for 5ms followed by a test pulse to $+20$ mV. In addition, the inactivation curves represent the best fits to the Boltzmann distribution: $I/I_{\max} = 1 / \{1 + \exp[(V_{1/2} - V) / \kappa]\}$, where I is the HERG tail current amplitude at a prepulse potential V , $V_{1/2}$ is the voltage for half-maximal inactivation, and κ is a slope factor. The decaying tail currents were fitted to a single exponential function for a calculation of inactivation time course.

Deactivation—The deactivation currents were induced by a test pulse to -40 mV which was preceded by a 4 s prepulse at $+20$ mV. The decay of the tail current was fitted with a biexponential function to calculate fast and slow time constants.

Recovery—The effects of compounds on the HERG channel recovery from inactivation were measured using a standard double-pulse protocol consisting of a depolarization to $+50$ mV for 1 s followed by a test pulse of -140 mV. The initial descending phase of the tail current upon hyperpolarization to -140 mV was taken as an indication of reactivation process. The currents were fitted with a single exponential function to calculate the reactivation time constant.

Data analysis

The data were presented as means $\pm$ SEM. The data were analyzed for statistical significance with Student's *t*-test or one-way analysis of variance (ANOVA) followed by the *Dunnnett's* test using SPSS software. *p* value less than 0.05 was considered significant.

Results

Concentration-dependent inhibition of HERG channel by tetra-n-octylammonium bromide and benzethonium chloride

Figure 1A and 1B showed representative traces of concentration responses of tetra-n-octylammonium bromide and benzethonium chloride on I_{HERG} . Both compounds concentration-dependently inhibited HERG channel currents. In addition, the blocked HERG channel currents by both compounds showed a minimal recovery during 10 minutes of washing, suggesting that they may be trapped in the binding sites. Benzethonium chloride exhibited a progressive inhibition rather than rapid development of HERG channel blockade compared with tetra-n-octylammonium bromide. The IC_{50} value of tetra-n-octylammonium bromide was 4 nM with a Hill coefficient of 2.45, while Benzethonium chloride was less potent with an IC_{50} of 17 nM and Hill coefficient of 0.58 (Figure 1C). The Hill coefficient was greater than 1 for tetra-n-octylammonium bromide, indicative of a positive cooperativity and/or multiple binding sites.

In addition, tetra-n-octylammonium bromide also slowed the decay of tail currents upon repolarization with a “crossover” in the tail currents (indicated by a large arrow in Figure 1A and Figure 2B). The rate of deactivation after application of tetra-n-octylammonium bromide became more sluggish compared to the control currents (Figure 8). This “crossover” phenomenon on the HERG channel tail currents was previously reported for verapamil and cocaine (Zhang *et al.*, 1999; Ferreira *et al.*, 2001) that was explained by blocking HERG channels in the open channel state. The result here suggests that tetra-n-octylammonium bromide may bind to the open state of HERG channel and slow the deactivation process.

Use-dependent inhibition of HERG channels by tetra-n-octylammonium bromide and benzethonium chloride

Use-dependent inhibition implicates that the inhibition by a compound is accumulatively enhanced when the ion channels are repetitively activated and inactivated. The use-dependent channel blockers usually bind to ion channels in the open and/or inactivated state that has a higher affinity to compounds compared with the resting state (Stork *et al.*, 2007). As shown in figure 2B and 2D, the typical traces and the normalized tail currents were recorded in 40 min after the treatment with 3 nM tetra-n-octylammonium bromide. The HERG currents did not significantly change in the presence 0.01% DMSO, a solvent used as a control (Figure 2F). During the first voltage step pulse after the application of tetra-n-octylammonium bromide, the initial peak current was modestly suppressed with a 28.8% inhibition (Figure 2D). The inhibition increased to 47.9% in the second voltage step pulse that further increased in the third cycle (66.7%) and fourth cycle (74.4%). The result

indicates that tetra-n-octylammonium bromide blocks HERG channels in a use-dependent manner.

Benzethonium chloride also exhibited use-dependency of HERG channel inhibition but the effect was weaker than that of tetra-n-octylammonium bromide (Figure 2C and 2E). The inhibition on HERG channel currents was 28.6% after the first step pulse. It progressed slightly from 36.3% inhibition in the second cycle to 46.5% inhibition in the forth cycle with a steady-state inhibition of 40.2 % reached in the third cycle.

In addition, we examined the frequency dependency of HERG channel inhibitions by these two compounds with trains of pulses at frequencies of 0.03 and 0.1 Hz. The HERG channel inhibition did not change with the alteration of frequency (data not shown), indicating that both compounds act in a frequency-independent manner.

The above data indicated that both compounds blocked HERG channels in a use-dependent manner, suggesting a preference of binding to open and/or inactivated state of HERG channels.

Voltage-dependence of HERG blockade by tetra-n-octylammonium bromide and benzethonium chloride

The inhibitory activity of a compound with the voltage-dependency usually increases with the progressive increase in depolarized potentials. We have used three I-V protocols to evaluate the voltage dependent property of the two compounds. First, a standard I-V protocol (Figure 3A and 3B) was applied to record typical currents in the absence or presence of 3 nM tetra-n-octylammonium bromide and 10 nM benzethonium chloride (Figure 3C and 3D). In the absence of the two compounds, the standard I-V curves at the end of the pulse demonstrated a strong inward rectification at potentials above 0 mV. In the presence of these two compounds, the I-V curves significantly shifted to the negative direction, suggesting that both compounds shift the channel activation towards more hyperpolarized voltages. In addition, both compounds substantially and voltage-dependently diminished the HERG currents at various test potentials (Figure 3E and 3F). When the voltages were depolarized from -70mV to $+10\text{mV}$, the inhibitions on HERG currents by tetra-n-octylammonium bromide and benzethonium chloride gradually increased from 0 to 54.1 % and 44.0 %, respectively. When the voltages further depolarized from 10 mV to 50 mV, the inhibition of HERG currents by tetra-n-octylammonium bromide reduced from 54.1 % to 43.4 %, whereas the inhibition by benzethonium chloride decreased from 44.0% to 27.5%.

Second, the fully activated HERG tail currents were recorded as the indicated protocol (Figure 4A and 4B). I-V relationships in the absence of compounds exhibited a striking inward rectification with a negative slope between -20 mV and $+20\text{ mV}$. The suppression of this fully activated HERG tail current accentuated from 35.3 to 62.6 % with increased test potentials from -140 mV to -20 mV and then reduced dramatically at more positive potentials where the negative slope occurred (Figure 4C). In contrast to it, benzethonium chloride produced a slightly increased inhibition of fully activated HERG tail current (from 39.3 to 47.1%) in test potentials between -140 and $+20\text{ mV}$ (Figure 4D).

Third, the effects of these two compounds on instantaneous I-V curve of HERG channels were examined by a protocol showed in Figure 5A and 5B that was used to remove the HERG inward rectification. The data were fitted by the single exponential function and the amplitude was plotted against test potentials. A linear I-V relationship was observed after the treatment with 3 nM tetra-n-octylammonium bromide or 10 nM benzethonium chloride, which indicated there was no inactivation during the depolarization process (Figure 5C and 5D). The inhibition by tetra-n-octylammonium bromide slightly increased from 42.1 to 49.4% in test potentials between -140 and $+20$ mV (Figure 5C). The inhibition by benzethonium chloride was in a relative constant level, from 40.0 to 43.9% in test potentials between -140 to $+20$ mV (Figure 5D).

Based on the data obtained from above three different types of I-V curves, tetra-n-octylammonium bromide exhibited a significant voltage-dependent inhibition of HERG currents in all three experiments. The inhibition of HERG currents increased with the depolarized potential but it declined as the channel rectification (indicating channel inactivation) became manifested at more positive potentials. Benzethonium chloride exhibited a voltage-dependent inhibition on the standard I-V relationship of HERG channel but not on the fully activated and instantaneous I-V relationships.

State-dependence of HERG blockade by tetra-n-octylammonium bromide and benzethonium chloride

State-dependent channel blockers often exhibit characteristic effects on channel kinetics that is a complex process when the states of channel functions are progressing sequentially from the initial closed state to open/activated state followed by inactivation and recovery from inactivation, and then back to the closed state again. The effects of these two compounds on the channel activation, deactivation, inactivation and recovery processes were determined

Activation—As shown in Figure 6 and 8, the activation curves of HERG channel currents in the presence of two compounds dramatically shifted to the negative direction. In addition, the activation process was considerably accelerated. Thus, these two compounds significantly facilitate HERG channel activation.

The control $V_{1/2}$ value (the half-maximal activation voltage) and slope factor (κ) were -8.3 ± 0.2 mV and 9.6 ± 0.2 mV, respectively. In the presence of 3 nM tetra-n-octylammonium, the $V_{1/2}$ value was -17.7 ± 0.6 mV ($p < 0.05$, $n = 13$) and κ was 7.7 ± 0.6 mV ($p > 0.05$, $n = 13$) (Figure 6A). After the treatment with 10 nM benzethonium chloride, the $V_{1/2}$ value decreased from -9.4 ± 0.4 mV (control) to -24.7 ± 0.7 mV ($p < 0.01$, $n = 7$) but the slope factor k did not change (Figure 6B). In addition, the activation time course was fitted to a double exponential function on the currents evoked at a test potential of 0 mV from a holding potential of -80 mV. The results also indicate that both compounds significantly accelerate HERG channel activation (Figure 8A). The fast and slow time constants were 418 ± 54 and 2373 ± 228 ms in the control group, respectively, while 3 nM tetra-n-octylammonium bromide treatment reduced the fast and slow time constants to 253 ± 46 and 1061 ± 126 ms ($p < 0.01$, $n = 6$), respectively. After the treatment with 10 nM benzethonium chloride, the fast and slow time constants decreased from 398 ± 54 and 2122 ± 333 ms to

168 ± 32 and 1023 ± 219 ms ($p < 0.01$, $n = 5$). Together, the results suggest that these two compounds may reach their binding sites in the open channel state because they significantly affect the channel activation kinetics.

Deactivation—Open channel blockers often cause apparent deceleration of the deactivation time course because of the time needed for drug unbinding (dissociation from the channel proteins). To test the effect of compounds on deactivation of HERG channels, tail currents were elicited by a test pulse to −40 mV which was preceded by a 4 s prepulse at +20 mV. The decay of the tail current was biexponential with fast and slow time constants of 143 ± 8 and 906 ± 44 ms in the control group, respectively. After application of 3 nM tetra-*n*-octylammonium bromide, the tail current was well fitted by two components with time constants of 268 ± 15 and 1782 ± 122 ms. These increases of both the fast and slow time constants after the treatment with tetra-*n*-octylammonium bromide were statistically significant (paired *t*-tests, $p < 0.05$, $n = 8$). In contrast, the fast and slow time constants in the presence of 10 nM benzethonium chloride were 125 ± 13 and 955 ± 42 ms that were not significantly different from 134 ± 15 and 1013 ± 45 ms in the control group ($p > 0.05$, $n = 7$). The results here suggest that the binding of tetra-*n*-octylammonium bromide stabilizes HERG channels in the open state which slows the channel closing, while benzethonium chloride has no significant effect on the channel deactivation.

Inactivation—Figure 7A and 7B showed the typical HERG inactivation currents in the absence or presence of two compounds. The control $V_{1/2}$ value and slope factor (κ) in the control group were -83.9 ± 0.9 mV and -27.0 ± 0.8 mV, respectively. After a treatment with tetra-*n*-octylammonium bromide, these constants reduced to -108.3 ± 2.8 mV ($p < 0.01$, $n = 7$) and -32.9 ± 1.5 mV ($p > 0.05$, $n = 7$), respectively (Figure 7C). In the presence of benzethonium chloride, the $V_{1/2}$ value changed from -87.8 ± 1.0 mV to -96.0 ± 1.7 mV ($p > 0.05$, $n = 7$) and the change of slope factor was also not significant (from -25.1 ± 0.8 mV to -28.7 ± 1.2 mV, $p > 0.05$, $n = 7$) (Figure 7D). The decaying tail currents from figure 7A and 7B fitted to a single exponential function for a calculation of inactivation time course. As shown in figure 8C, both compounds accelerated the decay of inactivation currents at all voltages. These results demonstrated that both compounds promoted more rapid HERG channel inactivation. Tetra-*n*-octylammonium bromide caused an apparent shift of the inactivation curve to the negative direction, whereas benzethonium chloride did not.

Recovery—The initial descending phase of the tail current upon hyperpolarization to −140 mV was taken as an indication of reactivation process and the currents were well fitted with the single exponential function. Both compounds did not change the recovery time course of HERG channels (Figure 8D), indicating that tetra-*n*-octylammonium bromide and benzethonium chloride do not stabilize channels in the inactivated state.

Discussion

Acquired long QT syndrome and resulting cardiac arrhythmias caused by drug candidates are major health concerns in the pharmaceutical industry. It has been reported that many compounds including these previously approved drugs can inhibit the HERG channel activity because of the druggability of this channel with a broad spectrum of structurally

diverse compounds. The early screen of HERG channel activity in the drug development process has effectively prevented the compounds with HERG channel inhibitory activity moving to the late stage of drug development. The screen of HERG channel activity of environment chemicals and further characterization of positive compounds may provide valuable information for evaluation of the potential toxic profile of these compounds. Tetra-n-octylammonium bromide and benzethonium chloride are both synthetic quaternary ammonium compounds that have been extensively used in industry, cosmetics and drugs/vaccines (Puziss *et al.*, 1963; Olson *et al.*, 1964; Lahtinen *et al.*, 2004; Bearden *et al.*, 2008). The toxic effects of benzethonium have been reported that triggered cell cycle arrest and apoptosis in human hematopoietic cells, Jurkat cells, neurons, glial cells, conjunctival cells and gingival fibroblasts (Yip *et al.*, 2006; Enomoto *et al.*, 2007; Braun *et al.*, 2010; Geier *et al.*, 2010; Nomura *et al.*, 2010). In addition to the HERG channel inhibition, benzethonium chloride also has found to be an inhibitor of acetylcholine esterase (AChE), choline esterase (Zaman *et al.*, 1997) and muscarinic receptors for acetylcholine (ACh) (Durieux and Nietgen, 1997) as well as $\alpha 7$ and $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptors (Coates and Flood, 2001). The study of mechanism of HERG channel inhibition by tetra-n-octylammonium bromide and benzethonium chloride can provide additional insight of structure-activity relationship of HERG channel inhibition and the molecular determinants of the interaction between the compounds and HERG channel proteins. These information and knowledge are useful for in silico modeling that can predict the potential effect of new chemical entities on HERG channels.

We found some similarities as well as differences in the electrophysiological properties of HERG channel inhibitions by tetra-n-octylammonium bromide and benzethonium chloride (Table 1). First, these two compounds are potent HERG channel blockers with IC_{50} values of 4 nM and 17 nM, respectively, which are 2 to 3 magnitudes lower than these concentrations used elsewhere (Durieux and Nietgen, 1997; Zaman *et al.*, 1997; Coates and Flood, 2001; Yip *et al.*, 2006). For example, 0.1-0.2% (2.0-4.1 μ M) of benzethonium chloride has been approved by the US Food and Drug Administration (FDA) to use in the first aid products (Edward, 2002). Ketalar, an anaesthetic for intravenous or intramuscular injection, contains 0.1 mg/ml (204.9 μ M) benzethonium chloride as the preservative (Coates and Flood, 2001). The concentration of tetra-n-octylammonium bromide used in the production of nanoparticles for drug delivery is even higher (2-200 mM) (Kanehara *et al.*, 2009; Gavia and Shon, 2012).

Second, the voltage dependent inhibition of HERG channels has been observed for both compounds although it is more significant by tetra-n-octylammonium bromide than benzethonium chloride. Both compounds have similar voltage-dependency in the standard I-V protocol (Figure 3). But the inhibitory activity of tetra-n-octylammonium bromide decreased in the channel inactivation during depolarization between -20 mV and +20 mV in the fully activated I-V protocol (Figure 4C), suggesting that it binds to the open channel and unbinds from the inactivated channel. In contrast, benzethonium chloride had a relative stable inhibition of the channel activity (Figure 4D), indicating that the inhibitory activity of this compound is not affected by the inactivated state of HERG channel. The results from experiments with the instantaneous I-V protocol which eliminated the inward rectification of HERG channel showed that both compounds constantly inhibit HERG channels (Figure 5),

suggesting a stably binding to the activated channels. These properties are compared with four known HERG channel blockers including verapamil, cisapride, ketoconazole and dofetilide in the Table 1. The binding sites of voltage-dependent HERG channel inhibitors have been reported that involve two aromatic residues, Y652 and F656 in the S6 domain of HERG channel protein. For instance, verapamil and cisapride exhibit the similar voltage-dependent property and the interactions of both compounds with the molecular determinants Y652 and F656 residues have been reported (Duan *et al.*, 2007; Kamiya *et al.*, 2008). Ketoconazole and dofetilide do not display voltage-dependent activity but Ketoconazole reportedly binds to the F656 residue (Ridley *et al.*, 2006), whereas dofetilide interacts with a serine residue in the position 620 (Ficker *et al.*, 1998).

Third, both compounds showed the use-dependency of HERG channel inhibition although tetra-n-octylammonium bromide has much notable use-dependent effect than benzethonium chloride. Generally, compounds with use-dependency such as verapamil and dofetilide belong to the group of open channel blockers of HERG channels (Zhang *et al.*, 1999; Tsujimae *et al.*, 2004). Our data indicated that tetra-n-octylammonium bromide blocks HERG channels in both open and inactivated states (Figure 2), whereas benzethonium chloride preferentially blocks HERG channels in the open states. The properties of state-dependence are further demonstrated by the effects of both compounds on the kinetics of HERG channel activation, inactivation, reactivation and deactivation (Figure 6-8). In addition, the blocked HERG currents by both compounds showed a minimal recovery during 10 minutes of washing (Figure 1), indicative of a very slow unbinding from activated channels. Similar phenomenon has been observed for the methanesulfonanilide compounds (e.g. E-4031, dofetilide, MK-499) and antimalarial drug halofantrine on HERG channels (Carmeliet, 1992; Mbai *et al.*, 2002). This phenomenon is attributed to the charged drug molecules that are “trapped” within the inner vestibule of the pore region to aromatic residues of HERG protein (Lees-Miller *et al.*, 2000; Mitcheson *et al.*, 2000b).

These two compounds exhibited significant differences in the state-dependent profile of HERG channel inhibition (Figure 6-8). Tetra-n-octylammonium bromide shifted both activation curve and inactivation curve in a hyperpolarized direction by 9.4 and 24.4 mV, respectively. It accelerated both activation and inactivation processes, and slowed the deactivation of HERG channels by ~2-fold. In contrast, benzethonium chloride did not affect inactivation curve and deactivation time constant though it shifted the activation curve in a hyperpolarized direction by 15.3 mV, and promoted more rapid activation and inactivation by ~2-fold. The state-dependency of HERG channel inhibition has been widely studied by different drugs. As shown in Table 1, verapamil, cisapride and dofetilide cause different alterations on HERG channel kinetics but all of three compounds have been demonstrated as the open channel blockers (Walker *et al.*, 1999; Zhang *et al.*, 1999; Tsujimae *et al.*, 2004), while ketoconazole preferentially interacts with the closed-channel state or as a fast open-state blocker (Dumaine *et al.*, 1998).

Our data indicate that both Tetra-n-octylammonium bromide and benzethonium chloride preferentially bind to the open channels as the HERG channel inhibitions by these two compounds exhibited a significant use-dependency (Carmeliet, 1993a; Snyders and Chaudhary, 1996; Weerapura *et al.*, 2002). Additionally, both compounds altered the kinetic

properties of HERG channels including the acceleration of channel activation (Figure 6) and decrease in the activation time constants (Figure 8). A similar shift in the gating of cardiac I_{Kr} current has been reported with the antiarrhythmic drugs including dofetilide (Carmeliet, 1992), almokalant and amiodarone (Carmeliet, 1993b), suggesting that this may be a common mechanism of action for many HERG channel blockers (Walker *et al.*, 1999; Zhang *et al.*, 1999). In addition, our data demonstrated that the inhibitions of HERG channel currents by both tetra-n-octylammonium bromide and benzethonium chloride were voltage-dependent as the inhibitions increased in the test potential of ~ 0 mV compared with these in -40 mV (Figure 3). This increase in HERG channel inhibition at the depolarized potential was in parallel to the increase in voltage-dependent activation of the channels, suggesting that activation or opening of HERG channels may facilitate the binding of tetra-n-octylammonium bromide and benzethonium chloride to their binding sites. The third feature of an open channel blocker is that it often causes apparent deceleration of the deactivation time constant because of reopening of the channels upon drug unbinding. As shown in Figure 1 and 2, the tail currents decayed at -40 mV was much slower in the presence of tetra-n-octylammonium bromide that crossed over with the control decaying tail currents, resulting in a “crossover” phenomenon. The similar “crossover” on HERG tail currents has been reported for some known HERG channel blockers including the antiarrhythmics quinidine (Sanchez-Chapula *et al.*, 2003) and verapamil (Zhang *et al.*, 1999), nervous system stimulant cocaine (Ferreira *et al.*, 2001) and some other quaternary ammonium compounds on the *shaker* potassium channels (Armstrong, 1971; Snyders and Yeola, 1995).

In consistent with the “crossover” phenomenon, tetra-n-octylammonium bromide slowed the deactivation rate of HERG channel tail current (Figure 8). This might be due to the time needed for compound to be dissociated from the binding site on the open channel state and diffused out of the channel pore before the activation gate can close. This phenomenon is similar to the “foot in the door” mechanism proposed for the other quaternary ammonium compounds acting on *shaker* potassium channels and AVE0118 on Kv1.5 channels (Armstrong, 1971; Yeh and Armstrong, 1978; Decher *et al.*, 2006). The properties of HERG channel inhibition by tetra-n-octylammonium bromide and benzethonium chloride are highly similar to verapamil and cisapride (Table 1). Verapamil is a known calcium channel blocker extensively used in the treatment of hypertension, stable angina and narrow QRS complex supraventricular arrhythmias. Although it is a HERG channel inhibitor *in vitro*, verapamil does not induce the long QT syndrome in human that is explained by a compensation of its inhibitory effect on calcium channels (Zhang *et al.*, 1999). In contrast, cisapride has been voluntarily removed from the market due to its side effects and less clinic effectiveness (Mohammad *et al.*, 1997). Therefore, the potential *in vivo* effects of these QACs on the QT prolongation and ventricular tachyarrhythmia have to be further evaluated.

In summary, our results demonstrate that tetra-n-octylammonium bromide and benzethonium chloride inhibit HERG channel currents in the voltage-dependent and use-dependent manners. Inhibition of HERG channels by both compounds is also state-dependent that is supported by a shifting of activation I-V curves in a hyperpolarized direction, and accelerations of the channel activation and inactivation processes. In addition, tetra-n-octylammonium bromide shifts the inactivation I-V curve in a hyperpolarized direction and slows the rate of channel deactivation whereas benzethonium chloride does

not. Taken together, these data suggest that tetra-n-octylammonium bromide and benzethonium chloride are open channel blockers that approach the binding sites on HERG channel proteins through the activated channels. The differences of two compounds in the above properties suggest that their binding sites on the HERG channel may not be exactly same though their structures are similar. Nevertheless, these binding sites are most probably located in the pore region and the S6 segment of the inner cavity in HERG channels that are commonly proposed for open channel blockers (Mitcheson *et al.*, 2000a; Choi *et al.*, 2011; Twiner *et al.*, 2012). The further studies with molecular biology techniques such as site-directed mutagenesis are needed to characterize the precise molecular determinants of interactions between the QACs and HERG channel proteins as well as the *in vivo* experiments to assess their potential effects on the QT prolongation and ventricular tachyarrhythmia.

Acknowledgments

This work was supported by “Hundred Talents Program” of the Chinese Academy of Sciences.

Abbreviations

CHO	Chinese hamster ovary
HERG	human ether-a-go-go related gene
IC₅₀	concentrations of half-maximal inhibitory effects
κ	slope factor
QACs	quaternary ammonium compounds
V_{1/2}	half-maximal activation (or inactivation) voltage

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Figure 1.

Concentration-dependence of HERG blockade by tetra-n-octylammonium bromide and benzethonium chloride in HERG transformed CHO cells. Typical traces of the HERG currents recorded before and after various concentrations of tetra-n-octylammonium bromide (A) and benzethonium chloride (B). Currents were elicited from a holding potential of -80 mV to 20 mV for a period of 4s, followed by 2s repolarization to -40 mV with an interval of 30s. (C) Concentration-response curves of the HERG channel blockade by tetra-n-octylammonium bromide (n=5-12) and verapamil (positive control) and benzethonium chloride. Data (n=5-12) were averaged and fitted to the Hill equation: $B(\%) = 100 / [1 + (IC_{50}/D)^n]$, where B(%) is the percent changes of HERG tail current at a compound concentration D, IC_{50} is the concentration of quaternary ammonium compounds that produces 50% effects, and n is the Hill coefficient.

Figure 2.

Use-dependence of HERG channel blockade by tetra-n-octylammonium bromide and benzethonium chloride in HERG transformed CHO cells. (A) The voltage clamp protocol for evoking HERG currents. First, a 4 s duration voltage step to +20 mV was applied from the holding potential of -80 mV, then repolarized to -40 mV in the absence of compounds (Control run). Then, 3 nM tetra-n-octylammonium bromide or 10 nM benzethonium chloride was added to the bath solution. Every ten minutes later, the same protocol was repeated with the same cycle length (the first, second, third or fourth run). The HERG-CHO cells were held at -80 mV without pulsing during the equilibration period. Typical HERG currents measured in the absence and presence of 3 nM tetra-n-octylammonium bromide (B) and 10 nM benzethonium chloride (C). Use-dependent inhibitions by tetra-n-octylammonium bromide (D) and benzethonium chloride (E) on HERG channels ($n = 5$). The peak tail currents in the second, third and fourth run were normalized to their corresponding amplitude in the first run and plotted against time after application of the compounds. (F) The maximal DMSO concentration in the extracellular solution was 0.01% (v/v) that did not affect HERG currents ($n=6$).

Figure 3.

Standard current-voltage (I-V) relationships of HERG channel inhibition by tetra-n-octylammonium bromide and benzethonium chloride in transformed CHO cells. (A and B) Representative traces of HERG currents recorded before and after compounds exposure. In the standard I-V protocols, the step currents were elicited by 4 s depolarizing pulses from -70 to +50 mV and the tail currents by 2 s repolarizing pulses to -40 mV. The voltage steps were delivered from a holding potential of -80 mV with an inter-pulse interval of 10s. (C and D) Standard I-V relationships of HERG channel before and after the treatments with two compounds determined by a standard I-V protocol (shown in the inset of A). (E and F) The % inhibitions of the HERG step currents and peak tail currents vs. test potentials in the presence of tetra-n-octylammonium bromide and benzethonium chloride for the standard I-V protocol (n=6-13).

Figure 4.

The I-V relationships of fully activated HERG tail currents in the presence of tetra-octylammonium bromide and benzethonium chloride in HERG transformed CHO cells. (A and B) Representative traces of fully activated HERG tail currents recorded before and after the treatment with compounds. The fully activated I-V relationships were obtained as follow: a 1 s pre-pulse to +50 mV was applied before each of the repolarizing pulses to test potentials between -140 mV and +20 mV. (C and D) Fully activated I-V relationships of HERG channel and % percentage of the HERG peak tail currents vs. test potentials before and after applying compounds determined by a fully activated I-V protocol (shown in the inset of A) (n=5-6).

Figure 5.

Instantaneous I-V relationship of HERG channel blockade by tetra-n-octylammonium bromide and benzethonium chloride in HERG transfected CHO cells. (A and B) Representative traces of instantaneous HERG currents recorded before and after the compound treatment. In the instantaneous I-V protocols, the channels were first inactivated by clamping the membrane at +50 mV for 1s followed by a pre-pulse to -100 mV for 20 ms to remove inactivation without allowing sufficient time for deactivation to occur. Following the recovery pre-pulse, a family of test pulses was delivered to potentials ranging from -140 to +20 mV. (C, D) Instantaneous I-V relationships and the % HERG peak currents vs. test potentials before and after the treatments with two compounds determined by the instantaneous I-V protocol (shown in the inset of A) (n=5-8).

Figure 6. Effects of (A) tetra-n-octylammonium bromide and (B) benzethonium chloride on the activation properties of HERG channel. Currents were elicited with the standard I-V protocols and the amplitude of the HERG tail currents at a repolarized test potential of -40 mV were normalized to maximum values that were plotted against the pre-pulse potentials ($n=7-13$). The curves represented the best fits to the Boltzmann distribution: $I/I_{\max}=1/\{1+\exp[(V_{1/2}-V)/\kappa]\}$, where I is the amplitude of HERG tail current at a prepulse potential V , $V_{1/2}$ is the voltage for half-maximal activation, and κ is a slope factor.

Figure 7.

Effects of tetra-n-octylammonium bromide and benzethonium chloride on the inactivation of HERG channel. (A and B) Representative traces of HERG inactivation currents recorded before and after 3 nM tetra-n-octylammonium bromide or 10 nM benzethonium chloride exposure. (C and D) Effects of tetra-n-octylammonium bromide and benzethonium chloride on the inactivation conductance curve. To construct the inactivation curves, the voltage protocols employed included a 2 s depolarizing pulse to +40 mV to inactivate the HERG channels, followed by various repolarizing pulses from -140 mV to +30 mV for 5ms and then by a test pulse to +20 mV. The amplitude of the HERG currents at the test potential was normalized and plotted against the pre-pulse potentials. The curves represented the best fits to the Boltzmann distribution. Here, $V_{1/2}$ is the voltage for half-maximal inactivation, and κ is a slope factor ($n=7$).

Figure 8.

Effects of tetra-n-octylammonium bromide and benzethonium chloride on the kinetics of the HERG channel currents. (A) 3 nM Tetra-n-octylammonium bromide (3 nM) and 10 nM benzethonium chloride accelerated HERG activation. Activation time courses were obtained by fitting the currents elicited by a step from a holding potential of -80 mV to 0 mV for the double exponential function (fast and slow time courses). (B) Tetra-n-octylammonium bromide slowed the deactivation of HERG channel. Deactivation time constants were measured using a double-pulse protocol consisting of a depolarized to $+20$ mV followed by a test pulse of -40 mV. The decaying phase of the tail current was fitted to the double exponential function and the fast and slow components were calculated. (C) Acceleration of inactivation time courses by tetra-n-octylammonium bromide and benzethonium chloride. Currents were measured using a triple pulse protocol shown in the Figure 7. The rapid decaying currents were fitted to a single exponential function and the time constants were plotted vs. the voltage. (D) Tetra-n-octylammonium bromide and benzethonium chloride had no effects on recovery from inactivation. Recovery from inactivation was measured using a double pulse protocol consisting of a depolarization to $+50$ mV followed by a test pulse of -140 mV. The time constants derived from the fits for the first phase (descending phase or increasing inward current) with the single exponential function. (C: control, TB: tetra-n-octylammonium bromide, BC: benzethonium chloride). ** $p < 0.01$ ($n = 5-8$).

Table 1

Summary of effects of tetra-n-octylammonium bromide, benzethonium chloride and some drugs on HERG channel kinetics

	TB	BC	Verapamil (Zhang <i>et al.</i> , 1999; Duan <i>et al.</i> , 2007)	Ketoconazole (Dumaine <i>et al.</i> , 1998; Ridley <i>et al.</i> , 2006)	Cisapride (Mohammad <i>et al.</i> , 1997; Walker <i>et al.</i> , 1999; Milnes <i>et al.</i> , 2010)	Dofetilide (Snyders and Chaudhary, 1996; Tsujimae <i>et al.</i> , 2004)
IC₅₀	4 nM	17 nM	143 nM (HEK), 5.1 μ M (XO)	1.7 μ M (HEK), 49 μ M (XO)	7-72 nM	4-73 nM
Concentration-dependency	Rapid	Progressive	Progressive	Progressive	Progressive	Progressive
Voltage-dependency	++++	++	++++	-	++++	-
Use-dependency	++++	+	++++	-	Not reported	++++
State-dependency						
- Activation curve	Shifted to hyperpolarized direction (9.4 mV)	Shifted to hyperpolarized direction (15.3 mV)	Shifted to hyperpolarized direction (3.3 mV)	-	Shifted to hyperpolarized direction (7.6 mV)	Shifted to hyperpolarized direction (2.5 mV)
- τ Activation	Accelerated (++++)	Accelerated (++++)	Not reported	Not reported	-	Not reported
- Inactivation curve	Shifted to hyperpolarized direction (24.4 mV)	-	Shifted to hyperpolarized direction (21.1 mV)	-	Shifted to hyperpolarized direction (7.8 mV)	-
- τ Inactivation	Accelerated (++++)	Accelerated (++++)	-	-	Accelerated (++++)	Accelerated (++)
- τ Deactivation	Slowed (++++)	-	Slowed (++++)	Slowed (++++)	-	Slowed (++)/-
- τ Reactivation	-	-	-	-	-	-

Note: τ , time constant; TB, tetra-n-octylammonium bromide; BC, benzethonium chloride; XO: xenopus oocytes. Activity increases from “+” to “++++” and “-” indicates no activity.