Comparative Effects of Liensinine and Neferine on the Human Ether-a-go-go-related Gene Potassium Channel and Pharmacological Activity Analysis

Zeng-Xiang Dong^{*,1}, Xin Zhao^{*,1}, Dong-Fang Gu¹, Yuan-Qi Shi¹, Jia Zhang¹, Xing-Xia Hu¹, Mei-Qin Hu¹, Bao-Feng Yang^{1,2} and Bao-Xin Li^{1,2}

¹Department of Pharmacology, Harbin Medical University, Harbin, ²State-Province Key Laboratory of Biopharmaceutical Engineering, Harbin, *Zeng-Xiang Dong and Xin Zhao made equal contributions to this study

Key Words

hERG Channel • Liensinine • Neferine • LQTS • Structure-Activity Relationship

Abstract

Liensinine and neferine, a kind of isoquinoline alkaloid, can antagonize the ventricular arrhythmias. The human ether-a-go-go-related gene (hERG) is involved in repolarization of cardiac action potential. We investigated the effects of liensinine and neferine on the biophysical properties of hERG channel and the underlying structure–activity relationships. The effects of liensinine and neferine were examined on the hERG channels in the stable transfected HEK293 cells using a whole-cell patch clamp technique, western blot analysis and immunofluorescence experiment. The pharmacokinetics and tissue distribution determination of liensinine and neferine in rats were determined by a validated RP-HPLC method. Liensinine and neferine induced decrease of current amplitude in dose-dependent. Liensinine reduced hERG tail current from 70.3 ± 6.3 pA/pF in control group to 56.7 ± 2.8 pA/pF in the 1 μ M group, 53.0 ± 2.3 pA/pF (3 μ M) and 17.8 ± 0.7 pA/pF (30 μ M); the corresponding current densities of neferine-

treated cells were 41.9 ± 3.1 pA/pF, 32.3 ± 3.1 pA/pF and 16.2 ± 0.6 pA/pF, respectively. Neferine had binding affinity for the open and inactivated state of hERG channel, liensinine only bound to the open state. The inhibitory effects of liensinine and neferine on hERG current were attenuated in the F656V or Y652A mutant channels. Neferine distributed more quickly than liensinine in rats, which was found to be in higher concentration than liensinine. Both liensinine and neferine had no effect on the generation and expression of hERG channels. In conclusion, neferine is a more potent blocker of hERG channels than liensinine at low concentration (<10 μ M), which may be due to higher hydrophobic nature of neferine compared with liensinine. Neferine may be safety even for long-term treatment as an antiarrhythmic drug.

Copyright © 2012 S. Karger AG, Basel

Introduction

Human ether-a-go-go-related gene (hERG) encodes the α -subunit of the rapid delayed rectifier potassium channel (IKr), which plays a central role in terminal repolarization of human ventricular myocytes [1]. Block of hERG channels or its native current (IKr) can lead to

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2012 S. Karger AG, Basel
1015-8987/12/0294-0431\$38.00/0

Accessible online at:
www.karger.com/cpb

Dr. Bao-Xin Li
Department of Pharmacology
(State-Province Key Laboratory of Biopharmaceutical Engineering)
Harbin Medical University, #157 Baojian Road, Harbin 150081 (China)
Tel. +86 451 8667 1354, Fax +86 451 86667511, E-Mail libx64@hotmail.com

Human long-QT (LQT) syndrome which can be associated with syncope and sudden death [2, 3]. LQTS has become an important liability for clinically available drugs and developmental compounds. This has led to several drug withdrawals and restrictions of use [4].

However, hERG has been regarded as an important target for the pharmacological treatment of cardiac arrhythmias by class III antiarrhythmics [5]. Recent investigations indicate that berberine and resveratrol have anti-arrhythmic effects for their inhibitory actions on hERG channels [6, 7]. Thus, interference with hERG channels seems to be the main mechanism explaining both the therapeutic actions and the potential cardiotoxicity of the structurally diverse compounds. Tremendous progress has been made toward drug-induced LQTS mechanisms, which may be attributed to direct hERG block by a diverse set of small organic molecules [8-10]. To better understand the structure-activity relationships of hERG channel-blocking drugs, it was necessary to elucidate the structural conditions, which keep the delicate balance between antiarrhythmic and proarrhythmic potential.

Liensinine (lien) and neferine (nef), a kind of isoquinoline alkaloid, extracted from the seed embryo of *Nelumbo nucifera* Gaertn, have a wide range of biological activities, including anti-arrhythmias, anti-hypertension, relaxation on vascular smooth muscle, etc [11, 12]. Accumulating line of evidence supported that liensinine and neferine were effective in improving arrhythmias *in vivo* and *in vitro* models [13-15]. They blocked L-type calcium channel current and prolonged action potential duration. Up to date, the exact antiarrhythmic mechanism of liensinine and neferine remains unclear and little information is available in the literature about hERG channels blockade by liensinine or neferine and their underlying mechanisms. Therefore, the aim of this study was to investigate the effects of liensinine or neferine on hERG channels. As liensinine and neferine share a similar pharmacophore (Fig.1), much less is known about structure-activity relationships between the compounds, we also compared the pharmacokinetics and tissue distribution of liensinine or neferine in an attempt to reveal the underlying structure-activity relationships.

In this study, we aimed to compare: (a) the effects of liensinine or neferine on the amplitude and kinetics of currents in hERG channels stably expressed in human embryonic kidney (HEK293) cells, which were evaluated using a whole-cell patch clamp technique; (b) molecular determinants of liensinine and neferine binding to hERG channel in mutant hERG channels expressed in HEK293 cells, which were measured using whole-cell patch clamp

Fig. 1. Structures of liensinine (A), neferine (B).

technique; (c) pharmacokinetics and tissue distribution of liensinine or neferine in rat, which were determined using a validated reversed phase high-performance liquid chromatographic (RP-HPLC) method; and (d) the effects of liensinine or neferine on hERG protein levels in HEK293 cells, which were achieved by western blot analysis and immunofluorescence experiment.

Materials and Methods

hERG -Expressing Cell Lines

HEK293 cells that stably expressed the wild-type hERG gene (provided by Professor Zhiguo Wang, Montreal Heart Institute, Montreal, Canada). Y652A (tyrosine to alanine substitution at position 652) and F656V (phenylalanine to valine substitution at position 656) mutations were generated by site directed mutagenesis of wild-type hERG cDNA. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, UT) supplemented with 10 % (v/v) fetal calf serum (FCS, Gibco, NY) at 37°C in a humidified atmosphere of 5 % CO₂. Stably transfected cells were then selected with 200 μM geneticin (G-418, Gibco, NY).

Reagents

Liensinine and neferine (purity of ≥ 98%, National Institute for the Control of pharmaceutical and Biological Products, Beijing, China) were dissolved in a little HCl and diluted with deionized water (pH=6.8 with NaOH) to obtain a 20 mM stock solution, which were stored at -20°C.

Electrophysiological Recordings

hERG current was recorded from cells using a standard whole-cell patch-clamp technique. Cells were superfused with Tyrode's solution containing (in mM): 136 NaCl, 5.4 KCl, 5 HEPES, 1MgCl₂·6H₂O, 1CaCl₂ and 10glucose (pH 7.4 with NaOH). Patch pipettes contained (in mM): 130 KCl, 1MgCl₂·6H₂O, 10 HEPES, 5 Mg-ATP, 5 EGTA and 0.1 GTP (pH 7.3 with KOH) and had resistances of 2–4 MΩ. Recordings were taken at room temperature using an Axopatch-200B amplifier (Axon Instruments, Foster City, CA) controlled by pCLAMP 9.2. Data were low-pass filtered at 1 kHz. Junction potentials were offset before formation of the giga-seal in extracellular solution. After giga-seal, the membrane was ruptured by gentle suction to establish the whole-cell configuration. The whole-cell capacitance and resistance were compensated (typically 75% ~ 85%) and the leak currents were subtracted.

In order to allow for difference in cell size, currents amplitudes were normalized to cell capacitance. Cell capacitance was read from the whole cell parameter in front panel of amplifier by adjusting to eliminate the capacitance currents in the seal test model. The mean capacitance of cells included in this experiment was 24.3±7.6 pF, and the access resistance (R_a) was 7.3±1.8 MΩ (n=10). The measured currents were normalized by whole-cell capacitance and presented as the current density (pA/pF).

The current-voltage relationships for the dose-dependent current were elicited from a holding potential of -80 mV by 4-s test pulses between -60 mV and +40 mV in 10 mV steps every 8 s. Each test pulse was followed by a repolarization step to -50 mV, which evoked large, slowly decaying outward tail-currents. To measure steady-state inactivation relationships, channels were inactivated at a holding potential of +40 mV for 2.5 s, before being recovered from inactivation at various potentials from -120 to +20 mV in 10 mV increments for 12 ms, then the resulting peak outward current at constant +20 mV as a measure of steady-state inactivation were recorded.

The effect on the onset of inactivation of the hERG current was investigated using a three-pulse protocol. The channel was initially held at -80 mV. The channel was depolarized to +40 mV for 2.5 s to inactivate the channels. A 15-ms repolarizing step to -100 mV was applied to recover inactivated channels to the open state. Following the recovery prepulse, a series of test pulses were delivered to potentials ranging from -120 to +30 mV for 90 ms. To determine recovery from inactivation, the fully activated I-V protocol was used: a 2.5 s depolarizing pulse to +40 mV to inactivate the hERG channels, followed by varying repolarizing pulses to test potentials from -120 to +30 mV for 90 ms. Graphical fits of the data were made using previously described standard equations [16, 17].

Plasma Pharmacokinetics and Tissue Distribution Determination

The animal protocols used were reviewed and approved by the Animal Care and Ethics Committee of the Harbin Medical University (Harbin, China). Wistar rats (225–250 g) which were provided by the Harbin Medical University Animal Center (Harbin, China) were given food and water ad libitum. They

were housed two to five per cage and maintained in a temperature-controlled colony room with a 12-h light/dark schedule. The rats received a 25 mg/kg dose of liensinine or neferine by tail vein injection.

For plasma pharmacokinetic studies, blood samples (250 µl) were collected at 0.083, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 h after injection. The blood samples were transferred to a 1.5 ml polypropylene tube containing calparine, and the plasma was separated from whole cells by centrifugation at 15000 rpm for 10 min and then stored at -20°C until analysis. For the tissue distribution studies, rats were decapitated at 30 min, whole brain, heart, lung, liver, spleen, fat, and kidney were quickly dissected free and stored in polypropylene tubes until analysis.

Samples were analyzed for liensinine or neferine by high performance liquid chromatography (HPLC) method. A 200 µL of sample was added to an 10-ml glass screw top tube containing 2 µg of dauricine as internal standard, and the tube was agitated at high speed on a mechanical shaker for 30 s. 3.0 ml of acetonitrile was added, and the tube was agitated for 30 s and centrifuged at 15000 rpm for 10 min. The top organic layer was transferred to a clear glass screw top tube and evaporated by N₂ at 40°C. The resulting residue was reconstituted with 100 µL of mobile phase and the tube was agitated for 30 s and centrifuged at 15000 rpm for 10 min. The top organic layer was transferred to crimp-top vials, and injected into a HPLC by auto-injection.

The HPLC system consisted of two pumps (LC-10AD), a UV-vis spectrophotometer detector (SPD-10AD) operated at wavelength of 282 nm, and a data processor (C-R4A) (Shimadzu, Kyoto, Japan). The analytical column was a Cosmosil ODS C18 column (250 mm x 4.6 mm, 5 µm particle size). A 1:2:2 v/v/v solutions of methanol/ acetonitrile/ triethylamine (0.2%) were used as the mobile phase. The mobile phase was filtered, degassed and pumped at a flow rate of 1.0 ml/min. Standard curves were constructed from spiked blank blood or tissue prepared under the conditions described above. Range of concentrations for the standard curve was between 0.5 and 50 µg/ml of the appropriate drug.

The concentration of drug in each unknown sample was determined by solving the linear calibration curve equation for each corresponding drug/internal standard ratio. For plasma and tissue distribution assays, the regression coefficient (r)> 0.99, Inter- and intra-assay variability were acceptable with no more than 10% coefficient of variation. Recovery of all compounds from tissue was >80%.

Western Blot Analysis

The expression of high levels of hERG protein was monitored using western blot experiments. Drug was diluted and added to hERG-HEK cells for 24 h at 37°C before analysis by Western blot. The cells were washed using ice-cold phosphate-buffered saline, and total protein was extracted. Protein (150 µg) per sample was separated using SDS-PAGE, transferred onto nitrocellulose membranes (Stratagene, La Jolla, CA) and incubated with primary antibodies against GAPDH (Affinity Reagents) and specific polyclonal rabbit anti-hERG antibody (Santa Cruz Biotechnology, CA) at 1:100 dilution. Goat

Fig. 2. Dose-dependent blockade of hERG channel. (A) Representative hERG current traces in control and in the presence of liensinine or neferine were recorded using the pulse protocol shown. I–V relationships for the tail current in the absence and presence of liensinine (B) or neferine (C). At drug concentrations of 1, 3, 10 and 30 μM , the tail-current amplitude were measured for each voltage were normalized to whole-cell capacitance to obtain the current densities (pA/pF). Average values were described as mean $\pm$ SEM, $n=10$ HEK293 cells that stably expressed HERG channels. * $P<0.05$, ** $P<0.01$ vs. control.

anti-rabbit Alexa Fluor 700 (dilution 1:2000, Molecular Probes, Eugene, OR) was used as a secondary antibody. The Odyssey infrared fluorescent scanning system (LI-COR, Lincoln, NE) was used to detect membrane proteins. The band densities were quantified by densitometry, using Scion Image software (Scion, Frederick, MD). Data were normalized to GAPDH.

Immunofluorescence

After 24 h of incubation in DMEM with or without drugs, culture media was removed and hERG-HEK cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After washes with PBS, cells were permeabilized for 1 h with 0.4% Triton X-100 in PBS, blocked in 5% bovine serum albumin for 5 h at room temperature, and incubated overnight at 4°C with primary antibody (rabbit anti-hERG, 1:100, Santa Cruz Biotechnology, CA). After rewarming, cells were incubated in the dark with the secondary antibody (goat anti-rabbit Alexa Fluor 488, 1:100, Molecular Probes, Eugene, OR) for 2 h at room temperature. Samples were washed with PBS before mounting onto the microscopic slides for examination using laser confocal microscopy (Olympus, Japan). Control and treated cells were cultured and prepared for microscope in parallel.

Statistical Analysis

Data were presented as mean $\pm$ standard error of the mean (SEM), unless otherwise specified. Statistical comparisons were

made using unpaired Student's t-test. P-values <0.05 were interpreted as being statistically significant. Plasma concentration versus time data from each individual experiment was evaluated by noncompartmental analysis as well as a two-compartment model using the DAS (Drug and Statistics for Windows) version 2.1 program (Kontron Elektronik GMBH, Germany).

Results

Concentration Dependence of hERG Channel Blockade

The effect of liensinine or neferine on the hERG current-voltage (I–V) relationship was shown in Fig. 2. Currents were recorded first under control conditions, and then liensinine or neferine was added into the bath for 10 min, with the cell kept at the holding potential, before current recording commenced in the presence of drug (Fig. 2A).

Liensinine and neferine blocked hERG tail current in a dose-dependent manner (Fig. 2B and C). At drug concentrations of 1, 3, 10 and 30 μM , the tail-current amplitude at 10 mV were measured. Liensinine reduced

Fig. 3. Voltage-dependent blockade of hERG channel. (A) Normalized I–V relationships for step currents before and after 30 μM drug, as determined according to the same protocol shown in Fig. 2. (B) Normalized I–V relationships for tail currents before and after 30 μM drug, as determined according to the same protocol shown in Fig. 2. (C) Voltage-dependent activation curves for the control and following exposure to drug, as calculated from the normalized peak tail current amplitudes before and after application of drug. Smooth curves were best fits of the data to a Boltzmann function. $n=10$ batches of cells for each group. * $P<0.05$, ** $P<0.01$ vs. control.

Fig. 4. Effect on steady-state and onset of inactivation of the hERG current. (A) Representative current tracing in control and in the presence of liensinine or neferine for steady-state inactivation using a double-pulse protocol with varying interpulse repolarization levels (from -120 mV to $+20$ mV). (B) With the instantaneous I–V protocol, current for onset of inactivation were recorded. (C) Normalized steady-state inactivation curves before and after exposure to 30 μM drugs. (D) The time course of the development of inactivation obtained by fitting the exponential function to the decaying current traces during the third pulse of the protocol, was plotted against membrane potentials. (E) Onset-of-inactivation curves before and after drugs. (F) Time constant for onset of inactivation before and after exposure to drugs. $n=10$ batches of cells for each group. * $P<0.05$, ** $P<0.01$ vs. control.

hERG tail current from the control value of 70.3 ± 6.3 pA/pF to 56.7 ± 2.8 pA/pF in the 1 μM group ($P>0.05$), 53.0 ± 2.3 pA/pF in the 3 μM group ($P<0.05$), 27.5 ± 4.6 pA/pF in the 10 μM group ($P<0.01$) and 17.8 ± 0.7 pA/pF in the 30 μM group ($P<0.01$); the corresponding current densities of neferine-treated cells were 41.9 ± 3.1 pA/pF ($P>0.05$), 32.3 ± 3.1 pA/pF ($P<0.05$), 25.7 ± 3.9 pA/pF ($P<0.01$) and 16.2 ± 0.6 pA/pF ($P<0.01$), respectively. Thus, compared with the control, 3, 10, 30 μM liensinine and 3, 10, 30 μM neferine had statistically significant inhibitory effects on $I_{\text{hERG-tail}}$. When the low concentration (<10 μM), neferine was a more potent blocker of hERG channels than liensinine.

Voltage Dependence of hERG Channel Blockade

Fig. 3A showed the IV relationships of hERG step current obtained in the absence and in the presence of 30

μM liensinine or neferine, as determined according to the same protocol shown in Fig. 2. In the absence of drug, the IV relationship exhibited the characteristic bell-shape

Fig. 5. Effects on recovery from inactivation of the hERG current. (A) In the fully activated I–V protocol, representative current in control and in the presence of liensinine or neferine was obtained by depolarization to +40 mV for 2.5 s to reach a steady-state level before repolarization to potentials from -120 to +30 mV. (B) The peak current density before and after 30 μ M drug during repolarization steps were plotted against voltage. (C) Time constant for recovery from inactivation obtained by fitting a single exponential to the initial increase in tail-current amplitude, was plotted against membrane potential. n=10 batches of cells for each group. * P <0.05, ** P <0.01 vs. control.

that increased from -40 mV to 0 mV and, due to the fast C-type inactivation of hERG channels; it decreased with further depolarization. Liensinine or neferine at a concentration of 30 μ M inhibited the hERG tail current at all test potentials. The blockade was enhanced with stronger depolarization between -10 and +40 mV (Fig. 3B). This change of liensinine was similar with neferine.

Modulation of hERG Channel Kinetics

Previous study showed that drugs that blocked ion channels often altered the voltage dependence or kinetics of channel gating. To compare the quality of liensinine and neferine, therefore, we examined the effects of liensinine and neferine on the voltage dependence of activation and rectification, and on the kinetics of inactivation and de-inactivation. The activation curves were constructed by normalizing the tail currents recorded with the protocol used in Fig. 2. The normalized data were plotted against the test pulse potentials and fitted to the Boltzmann function (Fig. 3C). The activation curves showed that the threshold voltage for hERG current activation was close to -50 mV and that it was fully activated with voltage steps to +10 mV. The rate of activation was similar before and after exposure to

30 μ M liensinine. $V_{1/2}$ values were -16.2 ± 0.2 mV in the control and -16.7 ± 0.7 mV in liensinine ($P > 0.05$); the corresponding k values were 7.9 ± 0.2 and 6.5 ± 0.6 , respectively ($P > 0.05$). However, 30 μ M neferine altered the activation properties: the $V_{1/2}$ and k values were -28.4 ± 2.1 mV ($P < 0.01$) and 6.4 ± 1.0 ($P < 0.05$).

Fig. 4A showed representative current tracing for steady-state inactivation using a double-pulse protocol. In Fig. 4C, the inactivating outward current amplitude was normalized and plotted against the test pulse potential, giving the steady-state inactivation curve. This curve could be fitted with a Boltzmann distribution, yielding inactivation $V_{1/2}$ and k values. $V_{1/2}$ shifted lightly after exposure to 30 μ M liensinine, from -69.0 ± 0.8 mV in the control to -72.1 ± 1.3 mV ($P > 0.05$). After treatment with 30 μ M neferine, $V_{1/2}$ shifted to -93.2 ± 2.7 mV ($P > 0.05$). The corresponding k values were -21.8 ± 0.7 in the control, -20.1 ± 1.2 in 30 μ M liensinine, and -29.7 ± 1.6 in 30 μ M neferine (all $P > 0.05$ vs. control). The time course of the development of inactivation was also assessed. Exponential curve fitting of the inactivation time-course yielded time-constant values plotted against voltage in Fig. 4D. The time constants of steady-state inactivation were smaller in the presence of 30 μ M liensinine ($P < 0.05$) than in the absence of the drug, as neferine.

Fig. 6. Liensinine and neferine inhibit hERG current by assessing the Y652 and F656 amino-acid residues. (A–C) Representative current traces of wild type (WT)-, Y652A- and F656V-hERG channels in the absence (Con) and presence (Lien) of 100 μ M liensinine. (D) Summary of mean current remaining with 100 μ M neferine, 300 μ M neferine, 100 μ M liensinine, 300 μ M liensinine, inhibition of Y652A- and F656V-hERG by the indicated compounds was significantly weaker ($*P<0.05$, $**P<0.01$) than inhibition of WT-hERG. Normalized tail current = current densities (pA/pF) (drug) current densities (pA/pF) (control). $n=6$ batches of cells for each group.

Fig. 7. Pharmacokinetics and tissue distribution determination in rats. (A) Mean plasma concentration-time curves after intravenous administration of liensinine or neferine in rats. After treatment with drug, blood samples were collected and examined using HPLC method. The concentration of drug in each unknown sample was determined by solving the linear calibration curve equation for each corresponding drug/internal standard ratio. $n=6$ batches of rats for each group.

(B) Distribution of tissue after intravenous administration of liensinine or neferine in rats. After tail vein injection of 25 mg/kg dose of liensinine or neferine for 30 min, tissue samples were collected and examined using HPLC method. The concentration of drug in each unknown sample was determined by respective standard curves. $*P<0.05$, $**P<0.01$.

The effect on the onset of inactivation of the hERG current was investigated using a three-pulse protocol. With the instantaneous I–V protocol, current for onset of inactivation were recorded (Fig. 4B). The onset-of-inactivation curves in the absence and presence of 30 μ M liensinine or neferine were shown in Fig. 4E. The time constant for the onset of inactivation was obtained by fitting a single exponential function to the decaying current traces during the third pulse of the protocol. Fig. 4F showed that inactivation was accelerated in the presence of 30 μ M liensinine or neferine at test potentials ranging -10 and +30 mV.

To determine recovery from inactivation, the fully activated I–V protocol shown in Fig. 5A was used. The prepulse potential at +40 mV was positive enough to induce

full conductance of the hERG channels but also rendered a large number of the channels in the inactivated state. The peak currents before and after 30 μ M drug during repolarization steps were plotted as a function of voltage (Fig. 5B). The time constant for recovery from inactivation was determined by fitting a single exponential function to the initial increase in tail-current amplitude at potentials between -60 and -20 mV (Fig. 5C). 30 μ M liensinine or neferine decreased the time constant of recovery from inactivation.

Molecular Determinants of Liensinine and Neferine Block of I_{hERG}

To study if the liensinine and neferine interact with the putative binding domain, we analyzed their action on

Fig. 8. Western blot analysis of the expression of hERG channel protein in stably transfected HEK293 cells. (A) Two hERG protein bands were detected in the western blot analysis of hERG-transfected HEK293 cells: a 135 kDa band representing the core-glycosylated, immature form of the channel protein located in the endoplasmic reticulum, and a 155 kDa band representing the fully glycosylated, mature form of the channel protein located in the plasma membrane. Representative image: lane 1, control group; lane 2, 1 μ M Lien group; lane 3, 3 μ M Lien group; lane 4, 10 μ M Lien group; lane 5, 30 μ M Lien group. (B) Data were normalized to GAPDH. (C) After treatment with drug for 24 h, cells were examined using immunofluorescence method. Control and treated cells were cultured and prepared for microscopy in parallel. (1) control group; (2) 1- μ M Lien group; (3) 3- μ M Lien group; (4) 10- μ M Lien group; (5) 30- μ M Lien group. n=6 batches of cells for each group.

selected hERG channel mutants (Y652A and F656V). We compared the current inhibition of wild type (WT) and mutants Y652A and F656V by all compounds. Fig. 6 showed representative currents recorded from HEK293 cells expressing WT (Fig. 6A), Y652A (Fig. 6B) and F656V (Fig. 6C) in the absence and presence of 100 μ M liensinine. Fig. 6D illustrated the effects of the liensinine and neferine on mutants Y652A and F656V. The drug concentrations were chosen to induce 90% inhibition of WT hERG channels (range: 100–300 μ M). The mutant F656V channel almost completely lost sensitivity to all compounds, while the Y652A channel showed a reduced sensitivity to liensinine and neferine.

Pharmacokinetics of Liensinine and Neferine

Concentrations of the liensinine and neferine were measured in plasma at various time points after tail vein injection 25 mg/kg of drug. As shown in Fig. 7A, there were mean plasma concentration-time curves of liensinine and neferine. Rapid absorption of both two compounds was apparent by the absence of points in the absorption phase. Average peak levels of two drugs occurred within 5 min of injection. The distribution half-life ($t_{1/2}$) of

liensinine (0.22 ± 0.04 h) was greater than the half-life of the neferine (0.21 ± 0.03 h), the corresponding k values were 1.52 ± 0.84 and 1.94 ± 0.59 , respectively.

Tissue Distribution of Liensinine and Neferine

For the tissue distribution studies, samples were analyzed for liensinine or neferine by HPLC method. Table 1 showed levels of each compound measured in rat tissue at 30 min after by tail vein injection of 25 mg/kg dose of liensinine or neferine. Distribution patterns of each drug were quite different from each other. For liensinine, the order of tissue concentration at 30 min was kidney > lung > heart > liver > spleen > fat. The neferine was distributed with the order lung > kidney > spleen > heart > liver > fat (Fig. 7B).

Effects on hERG Protein Levels

Western blot analysis was used to identify the effect of drugs on hERG protein expression in HEK cells stably transfected with hERG channel (Fig. 8). Antibody directed against the N terminus (anti-N) was used to probe for hERG protein. The anti-N antibodies recognized two bands of hERG protein in transfected cells: an upper band

with an apparent molecular mass of 155 kDa and a lower band with an apparent molecular mass of 135 kDa (Fig. 8A). To test the effects of two drugs on the expression of hERG channels, hERG-transfected HEK293 cells were treated with 0 (control), 1, 3, 10 and 30 μ M of either drug for 24 h. Western blot analysis was then performed. There was no significant difference in the expression of hERG protein between the liensinine or neferine-treated groups and the control group after 24h (Fig. 8B).

The localization of hERG channel proteins was then studied by immunofluorescence experiments. Cells were plated for 24 h, fixed, and labeled with anti-hERG antibody. Photographs were then taken with a confocal microscope and merged. As shown in Fig. 8C, there was no significant difference in fluorescence intensity between 1, 3, 10, and 30 μ M liensinine or neferine-treated groups (The data was not shown.) and control group. This was coincident with the results from western blot analysis.

Discussion

In previous studies, it had been demonstrated that liensinine could antagonize the ventricular arrhythmias induced by aconitine in rats, ouabain in guinea pigs and coronary occlusion–reperfusion in rats [13]. Moreover it markedly prevented the guinea pigs from developing ventricular fibrillation induced by adrenaline. In other studies, neferine could markedly inhibit the ventricular arrhythmias induced by aconitine in rats [18], myocardial ischemic damage in dogs [19]. They found liensinine and neferine prolonged the action potential duration and inhibited the Ca^{2+} current. The rate of repolarization is determined by a team effort of several ion currents such as L-type calcium current, the rapid delayed rectifier K^+ current. Although these studies suggested an antiarrhythmic action of liensinine and neferine in animals, little is known about their electrophysiologic effects on I_{Kr} channel. Based on these results of animal studies, firstly, we had compared the effects of liensinine and neferine on hERG channels using a whole-cell patch clamp technique in this paper.

In present study, liensinine and neferine inhibited hERG tail current in a dose-dependent manner. Both liensinine and neferine were potential hERG channel blockers. Single hERG channels are either closed, open or inactivated, depending on transmembrane voltage [20]. Different drugs exhibit various affinities for the three states, such as most drugs [21–23], bind preferentially to the open and/or inactivated channel states. In our

Tissue	Concentration / $\mu\text{g/mL}$	
	Liensinine	Neferine
Heart	2.90 ± 0.33	6.95 ± 1.91
Liver	2.52 ± 0.28	4.66 ± 1.35
Spleen	2.31 ± 0.14	$10.95 \pm 2.04^*$
Lung	5.09 ± 0.58	$55.91 \pm 8.12^{**}$
Kidney	6.45 ± 1.87	$23.62 \pm 4.65^*$
Fat	0.18 ± 0.03	—
Brain	—	—

Table 1. Drug concentration in rat tissues after intravenous administration of liensinine or neferine (mean $\pm$ SEM, n=6). * $P < 0.05$, ** $P < 0.01$.

experiment neferine shifted the activation and inactivation curve in a negative direction, but not liensinine (Fig. 3C and Fig. 4C). These results suggest that neferine has a higher binding affinity for the inactivate state. In the fully activated I–V protocol (Fig. 5), where the full conductance of the hERG channels were induced, liensinine or neferine inhibited hERG current even at a hyperpolarizing potential of -120 mV. These findings suggest that both drugs bind to hERG channels that are in the open state and voltage-dependence of hERG channels inhibition by neferine is more notable. We also observed drugs caused obvious decrease of the time constant of onset of inactivation and recovery from inactivation (Fig. 4F and Fig. 5C). These findings suggest that both drugs tend to become ‘trapped’ inside the channel on open state or bind to activation gate of hERG channels that are in the closed state. In addition, the time constants after exposure to neferine were smaller than that after liensinine. These results suggest that neferine has a higher binding affinity than liensinine.

The structural requirement for drug binding in hERG channels was characterized in detail recently [24, 25]. Alanine scanning mutagenesis revealed that the putative binding sites of most hERG inhibitors comprise three residues at the base of the selectivity filter (Thr623, Ser624 and Val625) and four on the S6 transmembrane helix (G648, Y652, F656 and V659) [20, 26]. Aromatic amino-acid residues (Y652 and F656) in the S6 domain were the most important molecular determinants of drug binding [27], which were thought to mediate direct block of I_{hERG} . To study if the liensinine and neferine interact with the putative binding domain, we have analyzed their action on selected hERG channel mutants (Y652A and F656V). In present study, the inhibitory effect of liensinine and neferine on hERG current was almost completely abolished in the F656V mutant channels and partially

attenuated in the Y652A mutant channels (Fig. 6D). The result clearly shows that the binding sites (Y652 and F656) in the pore-S6 region of hERG mediate the direct liensinine and neferine-induced current block. So salient features that can be speculated: (i) liensinine and neferine access the channel from the cell interior after crossing the cell membrane; (ii) a large inner cavity and the aromatic amino-acid residues Y652 and F656 may interact with blocking drugs by cation- π and π -stacking interactions [27]; (iii) liensinine and neferine tend to inhibit open of the activation gate; (iv) channel inactivation may act, directly or indirectly, to stabilize binding of neferine and (v) on channel closure liensinine and neferine can be retained within the channel.

Published pharmacophore models of hERG channel blockers typically have three important chemical features, (a) hydrophobic group, (b) ring aromatic group, and (c) hydrogen bond acceptor lipid group [28-30], where hydrophobic nature is a key factor effecting on drugs crossing the cell membrane and accessing the channel from the cell interior. To study the antiarrhythmic QSAR of liensinine derivatives, six derivatives were synthesized via acylation and etherification on its two phenol hydroxyl groups. Through the arrhythmic model induced by aconitine in rats, the antiarrhythmic effects of these derivatives were evaluated. They found that the higher antiarrhythmic activities were showed when the phenol hydroxyl groups of liensinine were substituted by the lipophilic groups [31]. The structures of liensinine and neferine are similar. The only difference is that neferine has more a methyl than liensinine (Fig.1). Overall, the methyl as hydrophobic group, improves the hydrophobic nature of neferine, which may be the structure basis that neferine has stronger hERG inhibition compared with liensinine. To further investigate the hydrophobic and distribution nature *in vivo* of liensinine and neferine, we used HPLC method to analyze plasma pharmacokinetics and tissue distribution from rat (Fig. 7). Much like the case with the electrophysiological assessments, the tissue distribution data was difficult to place within the context of currently available literature because of the differences in dose and analysis method of drug. Nonetheless, qualitative comparisons could be made between liensinine and neferine in our results.

Liensinine and neferine were quickly absorbed and reached peak levels within the first 5 min of tail vein injection. In addition, the distribution half-life of neferine obtained in the study was 0.21 ± 0.03 h, which was smaller than distribution half-life of liensinine (0.22 ± 0.04 h), the corresponding k value of neferine (1.94 ± 0.59) was

greater than liensinine (1.52 ± 0.84). It indicated that the distribution rate of neferine was faster than liensinine. Organ distribution patterns are complicated by several factors. The most important determinants of organ distribution appear to be lipid solubility, perfusion and extraction efficiency of each tissue, and inherent tissue affinity for the drug [32]. As shown in Fig. 7B, the neferine was found to be higher in concentrations than the liensinine in rat organs. The results of the study prove that the hydrophobic nature of neferine is stronger than liensinine, which is coincident with the results from electrophysiological analysis. Moreover, the result of tissue distribution showed that the levels measured in rat heart was 4.7 ± 1.2 μ M for liensinine versus 11.1 ± 6.8 μ M for neferine after 30 min by tail vein injection of 25 mg/kg dose of drug. From the electrophysiological and tissue distribution results, we can suggest that low concentration liensinine and neferine $C < 10$ μ M is more significant *in vitro* and *in vivo* research.

WT hERG channel protein consists of two bands (135 and 155 kDa) on western blot analysis, corresponding to the immature and mature forms of hERG channel protein, respectively [33]. The 135-kDa band is the core-glycosylated form located in the endoplasmic reticulum, and the 155-kDa band is the complex glycosylated which is synthesized in the endoplasmic reticulum and transported to the cell surface via the Golgi apparatus. Previous studies have shown that some drugs, such as arsenic trioxide and pentamidine inhibit hERG protein trafficking rather than directly inhibiting the channel action [34, 35]. On the other hand, some drugs not only directly block hERG channel, but also indirectly disrupt channel protein trafficking. Ketoconazole, fluoxetine and norfluoxetine inhibit both ion conductivity through the hERG channels and protein trafficking [33, 36]. The findings add complexity to understanding the mechanisms that cause pro-arrhythmic and antiarrhythmic effects. In the present study, beside the acute effects of drugs for the direct hERG channel block as stated above, the chronic effects were observed at concentrations 1, 3, 10, and 30 μ M of liensinine and neferine for the hERG channel protein expression. When the cells were cultured for 24 h in the absence or presence of neferine, there was no significant effect on the expression of hERG protein (The data was not shown.). This was coincident with our previous results [37]. Compare to neferine, the effect of liensinine on hERG protein expression was studied with both western blot analysis and laser-scanning confocal microscopy method. The result showed that both the 135 kDa band and the 155 kDa band were present and there

was no significant difference between the absence and presence of liensinine (Fig. 8B and C). These findings indicate that both of liensinine and neferine do not affect the processing of the core-glycosylated, surface membrane expression of the hERG polypeptide. In other words, for long time application of liensinine and neferine, there is no effect on the synthesis and expression of hERG protein.

hERG has been regarded as an important target for the treatment of arrhythmias. Such as amiodarone is a potent class III antiarrhythmic agent, which inhibits IKr [38]. It has been used both in the treatment of acute life-threatening arrhythmias as well as the chronic suppression of arrhythmias. In our study, liensinine and neferine are potent in inhibiting hERG current. So liensinine and neferine may be development for the treatment of arrhythmias.

In summary, liensinine and neferine are potent in inhibiting hERG channels by changing the channel kinetics.

Because of higher hydrophobic nature of neferine compared with liensinine, neferine is a more potent blocker of hERG channels at low concentration (<10 μ M). Furthermore, our findings provide insight to compare liensinine and neferine for further clinical exploration of these drugs. Because of the comprehensive effects of gating altering, pharmacokinetics, tissue distribution and protein levels, neferine may be a more potent drug using for anti-arrhythmia even for long-term treatment.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (no. 30973530, 81173050 and 31000512) and Creative Research Groups of the National Natural Science Foundation of China (no.81121003).

References

- Curran ME, Splawski I, Timothy KW, Vincent GM, Green ED, Keating MT: A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. *Cell* 1990;80:795-803.
- Raschi E, Vassina V, Poluzzi E, Ponti, FD: The hERG K⁺ channel: target and antitarget strategies in drug development. *Pharmacol Res* 2008;57:181-195.
- Stringer J, Welsh C, Tommasello A: Methadone-associated Q-T interval prolongation and torsades de pointes. *Am J Health-Syst Ph* 2009;66:825-833.
- Deisemann H, Ahrens N, Schlobohm I, Kirchhoff C, Netzer R, Moller C: Effects of common antitussive drugs on the hERG potassium channel current. *J Cardiovasc Pharm* 2008;52:494-499.
- Tsujiama K, Suzuki S, Yamada M, Kurachi Y: Comparison of kinetic properties of quinidine and dofetilide block of HERG channels. *Eur J Pharmacol* 2004;493:29-40.
- Li BX, Yang BF, Zhou J, Xu CQ, Li YR: Inhibitory effects of berberine on Ik1, Ik, and HERG channels of cardiac myocytes. *Acta Pharmacol Sin* 2001;22:125-131.
- Zhao WX, Qi ZP, Bai YL, Zhang Y, Zhao XL, Lu YJ, Li BX, Yang BF: Effect of resveratrol on function of HERG K⁺ channels. *Chin Pharmacol Bull* 2007;23:865-869.
- Farkas AS, Nattel S: Minimizing repolarization-related proarrhythmic risk in drug development and clinical practice. *Drugs* 2010;70:573-603.
- Hong HK, Park MH, Lee BH, Jo SH: Block of the human ether-a-go-go-related gene (hERG) K⁺ channel by the antidepressant desipramine. *Biochem Biophys Res Commun* 2010;394:536-541.
- Schramm A, Baburin I, Hering S, Hamburger M: hERG channel inhibitors in extracts of coptidis rhizoma. *Planta Med* 2011;77:692-697.
- Yang Y, Shui QL, Zeng XR, Liu ZF, Zhou W, Li ML: Effects of neferine on potassium channels in guinea pig ventricular cells and porcine coronary artery smooth muscle cells. *Chin J Pharmacol Toxicol* 2000;14:405-410.
- Tang XQ, Cao JG: Review on the pharmacological research of neferine. *Chin Pharmacol Bull* 2004;20:8-10.
- Wang JL, Nong Y, Jiang MX: Antiarrhythmic action of liensinine. *Acta Univ Med Tongji* 1992;2:317-319.
- Wang JL, Zong XG, Yao WX, Jiang MX: Effects of neferine on I_{Na}⁺, I_{Ca-L} and steady-state outward K⁺ current of ventricular myocytes. *Chin Pharmacol Bull* 1999;15:357-360.
- Wang JL, Zong XG, Jiang MX: Effects of Liensinine on I_{Na}⁺, I_{Ca-L} and steady-state outward K⁺ current of rat ventricular myocytes. *Acta Univ Med Tongji* 1999;28:496-499.
- Du X, Lu D, Daharsh ED, Yao A, Dewoody R, Yao JA: Dimethyl sulfoxide effects on hERG channels expressed in HEK293 cells. *J Pharmacol Toxicol Methods* 2006;54:164-172.
- Milnes JT, Crociani O, Arcangeli A, Hancox JC, Witchel HJ: Blockade of HERG potassium currents by fluvoxamine: incomplete attenuation by S6 mutations at F656 or Y652. *Br J Pharmacol* 2003;139:887-898.
- Xia GJ, Liu YF, Lu FH: Effects of methyl-liensinine on experimental arrhythmias. *Acta Univ Med Tongji* 1986;3:200-202.
- Guo ZB, Li Q, Cao HY, Xu Z: Antiarrhythmic efficacy of neferine assessed by programmed electrical stimulation in a canine model of electropharmacology. *J Chin Pharm Sci* 2002;11:35-42.
- Sanguinetti MC, Tristani-Firouzi M: hERG potassium channels and cardiac arrhythmia. *Nature* 2006;440:463-469.

- 21 Zitron E, Kiesecker C, Scholz E, Luck S, Bloehs R, Kathofer S, Thomas D, Kiehn J, Kreye VA, Katus HA, Schoels W, Karle CA: Inhibition of cardiac HERG potassium channels by the atypical antidepressant trazodone. *N-S Arch Pharmacol* 2004;370:146-156.
- 22 Zitron E, Karle CA, Wendt-Nordahl G, Kathofer S, Zhang W, Thomas D, Weretka S, Kiehn J: Bertosamil blocks HERG potassium channels in their open and inactivated states. *Br J Pharmacol* 2002;137:221-228.
- 23 Karle CA, Kreye VA, Thomas D, Rockl K, Kathofer S, Zhang W, Kiehn J: Antiarrhythmic drug carvedilol inhibits HERG potassium channels. *Cardiovasc Res* 2001;49:361-370.
- 24 Kamiya K, Mitcheson JS, Yasui K, Kodama I, Sanguinetti MC: Open channel block of HERG K channels by vesnarinone. *Mol Pharmacol* 2001;60:244-253.
- 25 Sanchez-Chapula JA, Navarro-Polanco RA, Culbertson C, Chen J, Sanguinetti MC: Molecular determinants of voltage-dependent human ether-a-go-go related gene (HERG) K channel block. *J Biol Chem* 2002;277:23587-23595.
- 26 Mitcheson JS: hERG potassium channels and the structural basis of drug-induced arrhythmias. *Chem Res Toxicol* 2008;21:1005-1010.
- 27 Mitcheson JS, Chen J, Lin M, Culbertson C, Sanguinetti MC: A structural basis for drug-induced long QT syndrome. *Proc Natl Acad Sci USA* 2000;97:12329-12333.
- 28 Choe H, Nah KH, Lee SN, Lee HS, Jo SH, Leem CH, Jang YJ: A novel hypothesis for the binding mode of HERG channel blockers. *Biochem Biophys Res Commun* 2006;344:72-78.
- 29 Aronov AM: Common pharmacophores for uncharged human ether-a-go-go-related gene (hERG) blockers. *J Med Chem* 2006;49:6917-6921.
- 30 Garg D, Gandhi T, Gopi MC: Exploring QSTR and toxicophore of hERG K⁺ channel blockers using GFA and HypoGen techniques. *J Mol Graph Model* 2008;26:966-976.
- 31 Luo SD, Yang J, Wang H, Cai HS: Studies on synthesis and antiarrhythmic quantitative structure-activity relationships of liensinine derivatives. *Chin Pharm J* 2002;37:702-705.
- 32 Thai DL, Yurasits L, Rudolph GR, Perel JM: Comparative pharmacokinetics and tissue distribution of the d-Enantiomers of para-Substituted methylphenidate. *Analogs Drug Metab Dispos* 1999;27:645-50.
- 33 Rajamani S, Eckhardt LL, Valdivia CR, Klemens CK, Gillman BM, Anderson CL, Holzem KM, Delisle BP, Anson BD, Makielski JC, January CT: Drug-induced long QT syndrome: hERG K channel block and disruption of protein trafficking by fluoxetine and norfluoxetine. *Br J Pharmacol* 2006;149:481-489.
- 34 Ficker E, Kuryshev YA, Dennis AT, Obejero-Paz C, Wang L, Hawryluk P, Wible BA, Brown AM: Mechanisms of arsenic-induced prolongation of cardiac repolarization. *Mol Pharmacol* 2004;66:33-44.
- 35 Cordes JS, Sun Z, Lloyd DB, Bradley JA, Opsahl AC, Tengowski MW, Chen X, Zhou J: Pentamidine reduces hERG expression to prolong the QT interval. *Br J Pharmacol* 2005;145:15-23.
- 36 Takemasa H, Nagatomo T, Abe H, Kawakami K, Igarashi T, Tsurugi T, Kabashima N, Tamura M, Okazaki M, Delisle BP, January CT, Otsuji Y: Coexistence of hERG current block and disruption of protein trafficking in ketoconazole-induced long QT syndrome. *Br J Pharmacol* 2008;153:439-447.
- 37 Gu DF, Li XL, Qi ZP, Shi SS, Hu MQ, Liu DM, She CB, Lv YJ, Li BX, Yang BF: Blockade of HERG K⁺ channel by isoquinoline alkaloid neferine in the stable transfected HEK293 cells. *Naunyn Schmiedebergs Arch Pharmacol* 2009;380:143-151.
- 38 Kiehn J, Thomas D, Karle CA, Schols W, Kubler W: Inhibitory effects of the class III antiarrhythmic drug amiodarone on cloned HERG potassium channels. *Naunyn Schmiedebergs Arch Pharmacol* 1999;359:212-219.