Pharmacological Research xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Dehydroevodiamine and hortiamine, alkaloids from the traditional Chinese herbal drug *Evodia rutaecarpa*, are I_{Kr} blockers with proarrhythmic effects *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 24 August 2017
Received in revised form 9 February 2018
Accepted 20 February 2018
Available online xxx

Keywords:
Evodia
Dehydroevodiamine
Hortiamine
hERG
Action potential duration
Early afterdepolarization

ABSTRACT

Evodiae fructus is a widely used herbal drug in traditional Chinese medicine. *Evodia* extract was found to inhibit hERG channels. The aim of the current study was to identify hERG inhibitors in *Evodia* extract and to investigate their potential proarrhythmic effects. Dehydroevodiamine (DHE) and hortiamine were identified as I_{Kr} (rapid delayed rectifier current) inhibitors in *Evodia* extract by HPLC-microfractionation and subsequent patch clamp studies on human embryonic kidney cells. DHE and hortiamine inhibited I_{Kr} with IC_{50}s of 253.2 ± 26.3 nM and 144.8 ± 35.1 nM, respectively. In dog ventricular cardiomyocytes, DHE dose-dependently prolonged the action potential duration (APD). Early afterdepolarizations (EADs) were seen in 14, 67, 100, and 67% of cells after 0.01, 0.1, 1 and 10 μM DHE, respectively. The proarrhythmic potential of DHE was evaluated in 8 anesthetized rabbits and in 8 chronic atrioventricular block (cAVB) dogs. In rabbits, DHE increased the QT interval significantly by 12 ± 10% (0.05 mg/kg/5 min) and 60 ± 26% (0.5 mg/kg/5 min), and induced Torsade de Pointes arrhythmias (TdP, 0.5 mg/kg/5 min) in 2 rabbits. In cAVB dogs, 0.33 mg/kg/5 min DHE increased QT duration by 48 ± 10% ($P < 0.05^*$) and induced TdP in 2/4 dogs. A higher dose did not induce TdP. In human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), methanolic extracts of Evodia, DHE and hortiamine dose-dependently prolonged APD. At 3 μM DHE and hortiamine induced EADs.

hERG inhibition at submicromolar concentrations, APD prolongation and EADs in hiPSC-CMs and dose-dependent proarrhythmic effects of DHE at micromolar plasma concentrations in cAVB dogs should increase awareness regarding proarrhythmic effects of widely used *Evodia* extracts.

1. Introduction

Herbal drugs are one of the cornerstones of traditional Chinese medicine (TCM). Wu Zhu Yu, the dried and nearly ripe fruit of *Evodia rutaecarpa*, is among the most popular and widely used herbal drugs in TCM. It is used as an analgesic, anti-emetic, for treatment of headache, gastrointestinal disorders, and menstrual complaints, or by means of external application against mouth ulcers [1]. However, in contrast to the elaborate preclinical safety evaluations [2,3]

Abbreviations: DHE, dehydroevodiamine; I_{Kr}, rapid delayed rectifier current; APD, action potential duration; EAD, early afterdepolarization; cAVB, chronic atrioventricular block; TdP, Torsade de Pointes arrhythmia; hiPSC-CM, human induced pluripotent stem cell-derived cardiomyocytes; TCM, traditional Chinese medicine; hERG, human Ether-a-go-go Related Gene; HEK, human embryonic kidney; MD, molecular dynamics; BVR, beat-to-beat variability of repolarization; STV, short-term variability; LV, left ventricle; RV, right ventricle; MAP, monophasic action potentials; EB, ectopic beats; VSD, voltage-sensitive dye; LED, light-emitting diode; PMT, photomultiplier; DMSO, dimethylsulfoxide; WT, wild type; SEB's, single ectopic beats; MEB's, multiple ectopic beats; FDA, Food and Drug Administration; CiPA, Comprehensive in vitro Proarrhythmia Assay.

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https://doi.org/10.1016/j.phrs.2018.02.024

to which new drug molecules are subjected, herbal drugs are considered as safe on the basis of empirical knowledge from use over centuries. This may be an issue, in particular, with herbal drugs containing pharmacologically potent molecules, such as is the case for some TCM herbs. Use of some TCM herbal preparations have been associated with severe side effects, and even deaths due to organ failure, as was the case with a slimming product containing *Aristolochia fanchi*, an herbal drug with meanwhile well-understood nephrotoxic liabilities [4].

DHE is the major alkaloid of *Evodia rutaecarpa,* and is known to have cardiovascular effects. DHE causes bradycardia, hypotension, and vasorelaxation [5–7]. More detailed electrophysiological studies revealed interference with pacemaker activity and with several ion currents in the heart. In pacemaker cells from rabbit sinoatrial tissue, diastolic depolarization was inhibited, and the spontaneous cycle length increased [8]. Furthermore, DHE increased the APD in these cells, an effect that was also shown in rabbit papillary muscle, and in isolated guinea pig atrial and ventricular cardiomyocytes [8,9]. In isolated guinea pig cardiomyocytes, DHE (0.1 μM) inhibited outward potassium currents (delayed rectifier) by 50%, and higher concentrations resulted in additional blockage of inward calcium and sodium currents [9]. It is currently not known whether inhibition of hERG (human Ether-a-go-go Related Gene) contributes to DHE-induced prolongation of the cardiac APD.

hERG encodes the α-subunit of the rapid delayed rectifier K^+ channel which can be mainly found in human myocardium, where it plays a central role in the repolarization phase of the cardiac action potential [10–12]. Inhibition of repolarizing outward potassium currents (especially hERG channel inhibition) and APD prolongation are considered as risk factors for TdP, even though the relation between the factors is not very strong [13]. Therefore, investigation of possible effects on the surface ECG, and direct determination of proarrhythmic potential in suitable large animal models is recommended. We here identified DHE and hortiamine as potent hERG channel inhibitors in *Evodia* extracts. The effects of DHE (the major alkaloid in *Evodia*) on APD were evaluated in isolated cardiomyocytes from cAVB dogs. Anesthetized rabbits and cAVB dogs were used to evaluate whether the ion blocking actions of DHE indeed translate to QT prolongation and proarrhythmia *in vivo*. Additionally, proarrhythmic effects of Evodia extracts, DHE and hortiamine on APD were studied in hiPSC-CMs.

2. Methods

2.1. Electrophysiology and molecular modeling

2.1.1. Patch clamp studies on HEK 293 cells expressing hERG, $Na_V1.5$ and $Ca_V1.2$ channels

HEK (human embryonic kidney) 293 cells stably expressing hERG (a kind gift of Dr. January, University of Wisconsin-Madison, WI, USA) and $Na_V1.5$ channels (ChanPharm GmbH, Vienna, Austria) were cultured and harvested as previously described [14,15]. Currents through hERG and $Na_V1.5$ channels stably expressed in HEK 293 cells were studied within 8 h of harvest in the whole-cell configuration of the planar patch clamp technique (NPC-16 Patchliner®, Nanion Technologies GmbH, Munich, Germany) [16,17] making use of an EPC 10 patch clamp amplifier (HEKA, Lambrecht/Pfalz, Germany). Currents were low-pass filtered at 10 kHz using the internal Bessel filter of the EPC-10 and sampled at 25 kHz. The extracellular bath solution for hERG current recordings contained (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, D-Glucose*H_2O 5 and HEPES 10 (pH 7.4 with NaOH). The intracellular solution for hERG current recordings contained (in mM): KCl 50, NaCl 10, KF 60, EGTA 20 and HEPES 10 (pH 7.2 with KOH). The extracellular bath solution for sodium current recordings contained (in mM): NaCl 20, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 0.75, choline chloride 120 and HEPES 5 (pH 7.4 with NaOH). The intracellular solution for sodium current recordings contained (in mM): CsF 120, CsCl 20, EGTA 5 and HEPES 5 (pH 7.4 with CsOH). NPC-16 Patchliner® was used for drug applications. The PatchMaster software v.2.65 (HEKA, Lambrecht/Pfalz, Germany) was used for data acquisition. The voltage protocol for hERG current recordings (see inset in Fig. 1C) was designed to simulate voltage changes during a cardiac action potential with a 300 ms depolarization to +20 mV (analogous to the plateau phase of the cardiac action potential), a repolarization for 300 ms to −50 mV (inducing a tail current) and a final step to the holding potential of −100 mV. The decreases in the resulting tail current amplitudes were taken as a measure of block development during a pulse train. Sodium currents were recorded in response to 10 ms pulses (0.2 Hz) from a holding potential of −120 mV to 0 mV, and the $Na_V1.5$ channel block was estimated as the decrease in the peak current amplitude during a pulse train.

For barium current measurements through $Ca_V1.2$ channels, HEK 293 cells were transfected and cultured as previously described [18]. Barium currents through $Ca_V1.2$ channels transiently expressed in HEK 293 cells were studied 36–48 h after transfection by manual patch clamp technique making use of an Axopatch 200A patch clamp amplifier (Molecular Devices, Inc., Sunnyvale, CA, USA). Currents were filtered at 5 kHz and sampled at 10 kHz. The extracellular bath solution for I_{Ba} recordings contained (in mM): $BaCl_2$ 20, $MgCl_2$ 1, choline chloride 90 and HEPES 10 (pH 7.4 with methanesulfonic acid). Patch pipettes with resistances of 1–4 MΩ were made from borosilicate glass (Harvard Apparatus, Cambridge, UK) and filled with intracellular solution that contained (in mM): CsCl 145, $MgCl_2$ 3, EGTA 10 and HEPES 10 (pH 7.25 with CsOH). Drugs were applied to cells under voltage clamp using a microminifold perfusion system. The pClamp software package v.10.0 (Molecular Devices, Inc., Sunnyvale, CA, USA) was used for data acquisition. Barium currents were recorded in response to 50 ms pulses (0.2 Hz) from a holding potential of −80 mV to +10 mV, and the $Ca_V1.2$ channel block was estimated as the decrease in the peak current amplitude during a pulse train.

2.1.2. Voltage clamp studies on Xenopus oocytes expressing hERG channels

For details see Supplemental Material.

2.1.3. Patch clamp and voltage clamp data analysis

Origin software v.7.0 (OriginLab Corp., Northampton, MA, USA) was employed for analysis and curve fitting. The cumulative concentration-inhibition curves were fitted using the Hill equation: $I_{hERG,drug}/I_{hERG,control} = (100 - A)/(1 + (C/IC_{50})^{nH}) + A$ in which IC_{50} is the concentration at which hERG inhibition is half-maximal, C is the applied drug concentration, A is the fraction of hERG current that is not blocked, and n_H is the Hill coefficient [19].

2.1.4. Docking and MD (molecular dynamics) simulations

Coordinates of the identified hERG blockers were generated with Gaussview 5 (Gaussian, Inc., Wallingford, CT, USA). The thoroughly validated homology model of the open hERG pore (model 6 of Stary et al. [20]) was used as starting point for docking analyses. Docking was performed with the program GOLD v5.2 (Cambridge Crystallographic Data Centre, Cambridge, U.K.) using the GOLD scoring function. The coordinates of the geometric center calculated among the Y652 and F656 residues were taken as binding site origin and both side chains were treated as flexible. The modeling software Pymol (Molecular Graphics System, Version 1.4.1; Schrodinger, LLC) was used to visualize and examine the interactions of DHE with the hERG cavity. The most frequent binding mode was used as a starting conformation for MD simulations. Simulations were performed with Gromacs v. 4.5.4 [21] as described

Please cite this article in press as: I. Baburin, et al., Dehydroevodiamine and hortiamine, alkaloids from the traditional Chinese herbal drug *Evodia rutaecarpa*, are I_{Kr} blockers with proarrhythmic effects *in vitro* and *in vivo*, Pharmacol Res (2018), https://doi.org/10.1016/j.phrs.2018.02.024