Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;34:691-702
DOI: 10.1159/000363034
Published online: August 18, 2014
Accepted: June 23, 2014

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1421-9778/14/0343-0691$39.50/0

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Original Paper

Berberine Induces hERG Channel Deficiency through Trafficking Inhibition

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Key Words
Berberine • hERG • Arrhythmia • Long QT Syndrome • Unfolded protein response • Endoplasmic reticulum stress response

Abstract
Aims: The human ether-a-go-go-related gene (hERG) encodes the α subunit of the IKr, which plays an essential role in repolarization of action potentials. hERG channels are targeted by various pro-arrhythmic drugs. Berberine (BBR) was previously found to acutely inhibit hERG currents and prolong action potential duration. The present study aimed to determine long-term effects of BBR on the expression of 135kDa/155kDa hERG and the mechanism. ***Methods and Results:*** hERG expression was assessed by western blot. Mature hERG (155 kDa) was reduced, whereas ER-located hERG (135 kDa) was increased by BBR. This indicated that hERG was restricted to the ER and that BBR disrupted channel trafficking. To determine the mechanism of trafficking inhibition, we performed western blot and immunoprecipitation to test folding of hERG by assessing interaction between hERG and Hsp90/Hsp70. Both the expression of Hsp90 and its interaction with hERG were strongly decreased by BBR. These data suggest that BBR reduces channel folding to induce trafficking inhibition. Western blot and confocal imaging were used to further detect whether the unfolded protein response (UPR) was activated. Active ATF6, a marker of the UPR, was activated by BBR. Calnexin and calreticulin, chaperones that are activated by ATF6 to assist channel folding, were also elevated and increasingly colocalized with hERG. These data also demonstrate that the UPR was activated. Immunoprecipitation and western blot assays were performed after BBR treatment to examine ubiquitination and degradation, common endpoints of the UPR. We found that the ER-restricted hERG was ubiquitinized and degraded in the lysosomes and proteasomes. ***Conclusion:*** Our study demonstrates that BBR induces hERG channel deficiency by inhibiting channel trafficking after incubation for 24h. Trafficking inhibition activated the UPR, and the ER-restricted hERG was ubiquitinized and degraded in lysosomes and proteasomes.

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Introduction

The human ether-a-go-go-related gene (hERG) encodes the α subunit of the IKr channel, which plays an essential role in phase III repolarization of the action potential. hERG channel deficiency can slow repolarization, prolong the QT interval, cause long QT syndrome (LQTS) and induce serious manifestations such as torsade de pointes and sudden cardiac death [1]. Unfortunately, various drugs have been reported to cause hERG channel deficiency. Thus, the FDA has ruled that the effect of various drugs on hERG channels should be tested. Drugs primarily affect hERG channels in two ways: by acutely blocking hERG current and/or chronically dysregulating hERG expression. For example, dofetilide and cisapride are proarrhythmic due to their acute blockade of hERG channels [2, 3]. Arsenic trioxide, pentamidine and desipramine cause LQTS by altering hERG expression [4-6]. Importantly, most of these drugs alter hERG expression by interfering with its trafficking. However, although up to 40% of all hERG blockers exert combined hERG blockade and trafficking inhibition, few compounds have been fully characterized at the cellular level [4].

Berberine (BBR) is an isoquinoline alkaloid that is used clinically to treat bacillary diarrhea and to lower lipids along with simvastatin [7]. Previous studies reported that BBR acutely blocks the hERG channel and prolongs action potential duration (APD) in Xenopus oocytes [8]. We recently found that long-term incubation of BBR reduces expression of the hERG protein in rat ventricular tissue [9]; however, the mechanism is still unknown.

In this study, our results demonstrated that BBR caused hERG channel deficiency by inhibiting trafficking and subsequently increasing degradation after incubation for 24h. Due to these effects of BBR on hERG channels, we further showed that BBR prolongs the APD and the QT interval in guinea pigs.

Material and Methods

Animal Care

All animal care and experimental protocols were in accordance with the ethical standards of the Declaration of Helsinki and its later amendments. All animal experiments were approved by the institutional animal care and ethics committee of Harbin Medical University. Guinea pigs (200-300 g) were killed humanely by cervical dislocation. A total of 60 guinea pigs (bought from experimental animal center of the second affiliated hospital of Harbin Medical University) were used in the experiments.

Cell Acquisition and Cell Culture

Human embryonic kidney 293 (HEK293) cells (Chinese Peking Union Medical College, Peking, China) and HEK293 cells stably expressing the wild-type hERG gene (HEK293/hERG, kindly provided by Professor Zhiguo Wang) were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone, Logan, Utah, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, TBD, Tianjin, China). For HEK293/hERG cells, the medium was also supplemented with 400 $\mu g.ml^{-1}$ gentamycin (G418, Invitrogen, USA).

Guinea pigs of either gender (250–300 g) were sacrificed by cervical dislocation after anesthetization with pentobarbital (40 $mg.kg^{-1}$, i.p.). Their hearts were quickly retrogradely perfused on a constant pressure Langendorff perfusion apparatus with Ca^{2+}-containing Tyrode's solution at 37°C until the effluent was clear of blood. Then, the heart was perfused with Ca^{2+}-free Tyrode's solution until the heart stopped beating and then perfused with type II collagenase (Worthington Biochemical Corporation) and bovine serum albumin (Huashun Biotechnology). After the heart had softened, it was removed, minced and preserved in KB solution for analysis.

Cellular Electrophysiology

Current and action potential recordings were performed as described previously [10]. Briefly, after incubation of BBR for 24h, HEK293/hERG cells were trypsinized, centrifuged and stored at 4°C in bath solution for 1h to stabalize seperated-single cells. The cells were seeded for 20 minutes in a recording chamber on a microscope (Olympus IX-70, Japan) and superfused with bath solution at a rate of 1.0 $ml.min^{-1}$. The whole-

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cell configuration was formed by applying a pipette with a tip resistance of 1-3 MΩ. The cell membrane was sucked to form a whole-cell configuration after offsetting the junction resistance. The whole-cell capacitance and 30% resistance were compensated, and the leak currents were subtracted. Then, the current was recorded with an Axonpatch-200B amplifier (Axon Instruments, USA). The action potentials in guinea pig cells were recorded after overnight culturing in M199 medium (Sai Mo Fei biochemical corporation). The recordings were performed with a myocyte pipette solution. pClamp 9.2 (Axon Instruments, USA) was used to control the protocols. Experiments were performed at room temperature.

Western Blot Analysis

Cells were placed on ice and washed 3 times with 3 ml ice-cold PBS. Then, 60 μl RIPA (Bi Yun Tian, China) and 0.6 μl PMSF (Shenneng Bocai, China) were added to the plates, and the cells were scraped from the plates and transferred into tubes. After ultrasonic oscillation 3 times, the cell lysates were centrifuged at 13,500 rpm·min^{-1} for 15 min. The supernatants were collected and total protein concentration was determined by Bradford's method. After adding loading buffer (Beyotime), the samples were boiled and separated by SDS-PAGE. The proteins were then transferred to a PVDF membrane, incubated with 5% nonfat milk (Becton Dickinson Company) for 2 h at room temperature. Subsequently, the membrane was probed with antibodies against hERG (Santa Cruz, USA), calnexin (Abcam, ab22683), calreticulin (Abcam, ab22595), Hsp70 (Enzo Life Science, ADI-SPA-810), Hsp90 (Enzo life science, ADI-SPA-835), ATF-6 (Active Motif 40962), cleaved ATF6 (Abcam, ab122897) and actin (Zhongbin Jinqiao, China) overnight at 4°C on a shaker. The membranes were washed 3 times with 0.05% TBST and incubated with secondary antibodies (Li-CoR) for 1 h in the dark at room temperature. After the membranes were washed 3 times with 0.05% TBST, the bands were detected with the Odyssey instrument (American Gene Corporation). The blots were analyzed and quantified with Scion Image.

Immunoprecipitation

A total of 1×10^{7}.l^{-1} HEK293/hERG cells were washed 3 times with PBS on ice and transferred to a 1.5 ml tube. Cells were centrifuged at 3000 rpm.min^{-1} for 5 min, and 300 μl RIPA plus 3 μl PMSF was added to the pellet. The uniformly mixed suspension was placed on ice for 10 min and then centrifuged at 12000 rpm.min^{-1} for 10 min. The supernatant protein concentration was measured using Bradford's method. Two micrograms of anti-hERG, Hsp70 or Hsp90 antibody was added and mixed with protein respectively, and the mixture was placed on a 360° shaker overnight at 4°C. Subsequently, 40 μl of beads (Santa Cruz A-G SC-2003) was added to the mixture, and the tubes were placed on a shaker overnight at 4°C. The mixture was centrifuged at 1500 rpm.min^{-1} for 5 min, and the pellet was washed 6 times with TBST on ice. Finally, 100 μl loading buffer was added to the pellet, and the tubes were boiled for 10 min and centrifuged. The supernatants were collected and separated by SDS-PAGE, transferred to a PVDF membrane, blocked with 5% nonfat milk, incubated with anti-hERG, anti-Hsp90, anti-Hsp70 or anti-ubiquitin (Santa Cruz, sc-8-17) antibody and then secondary antibody. The blots were analyzed and quantified using Scion Image.

Immunofluorescence

HEK293/hERG cells were diluted to a concentration of 0.25×10^{5}.l^{-1} and grown on poly-lysine-coated (BOSTER-AR0003) sterile coverslips overnight. Cells were then exposed to control conditions or 10 μM BBR for 24 h. Coverslips were washed 3 times with ice-cold PBS, fixed in ice-cold 4% paraformaldehyde/PBS (Kemi Ou Chemical Developmental Center, China) for 15 min. After fixation, the cells were washed, permeabilized with 0.4% Triton-100 (Hua Shun Biotechnology) and blocked with 10% goat serum (BOSTER-08C05A09) for 1 h at room temperature. Permeabilized cells were incubated with dilutions of different primary antibodies (Grp78, Santa Cruz) at 4°C overnight without shaking. Then, the cells were washed and incubated with diluted Alexa Fluor 488 or 594 (1:500, Molecular Probes) for 2 h in the dark at room temperature. The nucleus was stained with DAPI (1:100, Bi Yun Tian-C1005) for 5 min. After washing and mounting, the coverslips were imaged with a Nikon Eclipse or an Olympus confocal microscope.

Electrocardiogram Record

Guinea pigs (200-300 g) were used for electrocardiogram (ECG) recordings. Guinea pigs were fed for 3 days for adaption. After intragastric administration of 27.1 mg.kg^{-1} BBR (this dose was based on the dose 0.9 g.d^{-1} of BBR used in clinical on patients), saline or vehicle, guinea pigs were anesthetized with

1.5 g.kg^{-1} urethane (Sigma, 101188735), and ECGs were recorded using the BL-NewCentury (Peking Zhong Shi Di, China) for 3 h. ECGs were recorded again 24 h after intragastric drug administrations. Corrected QT intervals (QTc interval) at different time points were measured with the BL-NewCentury Microscope software and analyzed.

Data Analysis

The data are expressed as the means ± standard error of the mean (S.E.M). Unpaired two-tailed Student's t test and two-way ANOVA were used to compare significances between the means. P values<0.05 were considered statistically significant.

Materials

The solutions used were of the following compositions (mM):

(1) Ca^{2+}-containing Tyrode's solution: NaCl 136, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 10, glucose 10 and HEPES 10 (pH 7.4);

(2) Ca^{2+}-free Tyrode's solution: NaCl 136, KCl 5.4, $MgCl_2$ 10, glucose 10 and HEPES 10 (pH 7.4);

(3) KB solution: glutamic acid 70, taurine 15, KCl 30, HEPES 10, $MgCl_2 \cdot 6H_20$ 0.5, Glu 10, EGTA 0.5, KH_2PO_4 10;

(4) Bath solution: NaCl 136, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 10, glucose 10 and HEPES 10 (pH 7.4);

(5) Pipette solution: KCl 130, $MgCl_2$ 1, EGTA 5, Mg-ATP 5, GTP 0.1 and HEPES 10 (pH 7.3);

(6) Myocyte pipette solution: K-gluconate 119, KCl 15, $MgCl_2$ 3.75, EGTA 5, HEPES 5, K-ATP 4, phosphocreatine 14, Tris-GTP 0.3 and creatine phosphokinase 50 U.ml^{-1} (pH 7.2).

Stock solutions of BBR (Sigma), lactacystin (Abcam) and bafilomycin (Abcam) were dissolved in DMSO. The stock solution of BBR used for gavage was dissolved in 6.4 g.L^{-1} CMC-Na/PBS. An equivalent amount of vehicle was used for the vehicle control.

Results

BBR reduces the expression of mature hERG protein by trafficking inhibition

To determine the long-term effect of BBR on the expression of hERG channels, hERG expression and location were examined by western blot and immunofluorescence. Fig. 1A and B show that mature-155 kDa hERG is decreased after incubation with 1 μM and 10 μM BBR for 24 h. The band densities were 78.95% ± 4.58% (1 μM) and 49.18% ± 5.48% (10 μM) of the control level. Interestingly, immature-135 kDa hERG was increased by 1.31- (1 μM) and 1.78-fold (10 μM). The total hERG protein was not changed.

The 135 kDa hERG protein is formed after it is translated and glycosylated at the ER. The 155 kDa hERG protein is formed after the 135 kDa hERG is trafficked to the Golgi for a second glycosylation. A reduction in the 155 kDa hERG protein and an increase in the 135 kDa hERG protein suggests that trafficking from the ER to the Golgi is inhibited. Consistent with these findings, Fig. 1C shows that co-localization between hERG protein (red) and Grp78 (green, ER marker protein) is enhanced after incubation with 10 μM BBR for 24 h (shown as stronger yellow staining). Together, these results suggest that BBR decreases the expression of mature hERG by inhibiting hERG channel trafficking.

BBR decreases hERG current in a concentration- and voltage-dependent manner

To test whether reduction of mature hERG protein causes a dysfunction in hERG current, hERG currents were recorded by patch-clamp. Fig. 2 shows that hERG current is decreased in a concentration- and voltage-dependent manner by BBR. In the treated condition, at 40 mV, only 55.00% ± 2.90% (1 μM) and 15.47% ± 1.23% (10 μM) of the hERG current remained compared with the control condition.

Trafficking deficiency is caused by impaired channel folding

Because channel folding plays an essential role in channel trafficking, we used western blot and immunoprecipitation to investigate folding of the hERG channel to clarify the

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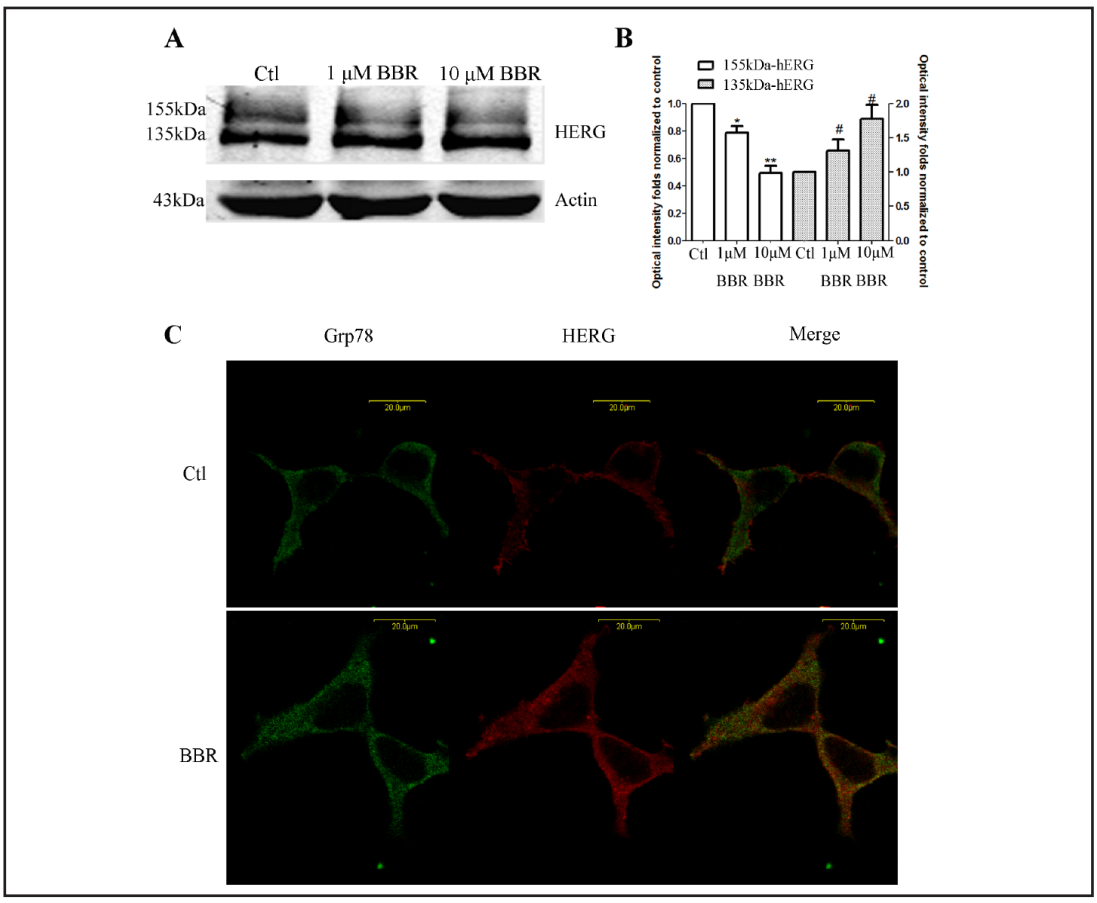


Fig. 1. BBR inhibits hERG channel trafficking after incubation for 24h. (A and B) Western blot results and statistics for hERG expression after control or 1 μM/10 μM BBR incubation for 24 h. 155 kDa hERG was concentration-dependently inhibited, while 135 kDa hERG was increased, which indicates an impaired trafficking process. *P<0.05, **P<0.01, #P<0.05, significantly different from their respective controls, n=5. (C) Example of an immunofluorescence image showing the ER localization of hERG protein. Red and green represent hERG and Grp78 (an ER marker protein), respectively. There's an overload of hERG protein at ER after 10 μM BBR incubation (as the stronger yellow staining showed).

mechanism for the BBR-induced trafficking defect of hERG channels. Fig. 3A shows the effect of BBR on the expression of heat shock protein 90 (Hsp90) and heat shock protein 70 (Hsp70), two essential chaperones responsible for channel folding. Hsp90 levels were decreased; only 75.00% ± 5.65% and 48.25% ± 2.33% of the control levels of Hsp90 remained after 1 μM and 10 μM BBR incubation, respectively. Hsp70 was not changed. Immunoprecipitation showed that the amount of hERG protein interacting with Hsp90 was decreased, while the amount of hERG protein interacting with Hsp70 was increased (Fig. 3B).

Together, these results suggest that the BBR-induced trafficking deficiency of hERG channels is caused by impaired folding. Impaired channel folding was due to a disordered interaction between hERG protein and chaperones.

The unfolded protein response is activated by BBR

To determine the response after impaired folding, the unfolded protein response (UPR) was investigated. The UPR is a process that prevents the accumulation of unfolded proteins in the ER. Fig. 4A shows that cleaved active transcription factor 6 (ATF6) (~50 kDa), a marker protein of the UPR, is activated by BBR. Increased cleaved ATF6 (~50 kDa) was generated from ATF6 (~90 kDa), and ATF6 (~90 kDa) was decreased proportionally.

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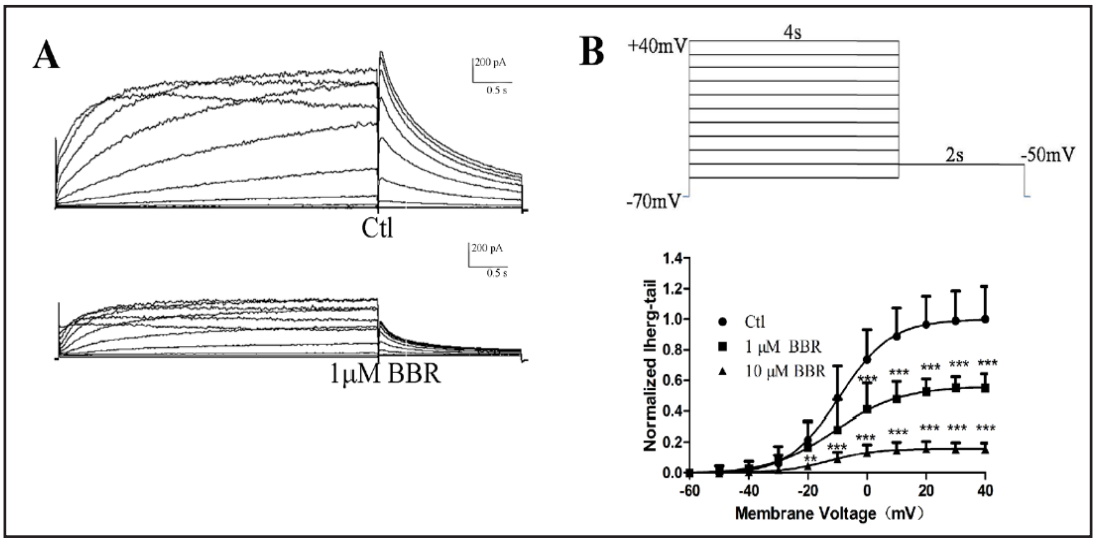


Fig. 2. BBR decreases hERG current after incubation for 24h. (A) Examples of hERG current under control or 1 μM BBR-treated conditions. (B) Protocol and I-V curve of hERG current. BBR concentration- and voltage-dependently reduced hERG current. **P<0.01, ***P<0.001, significantly different from control, n=10.

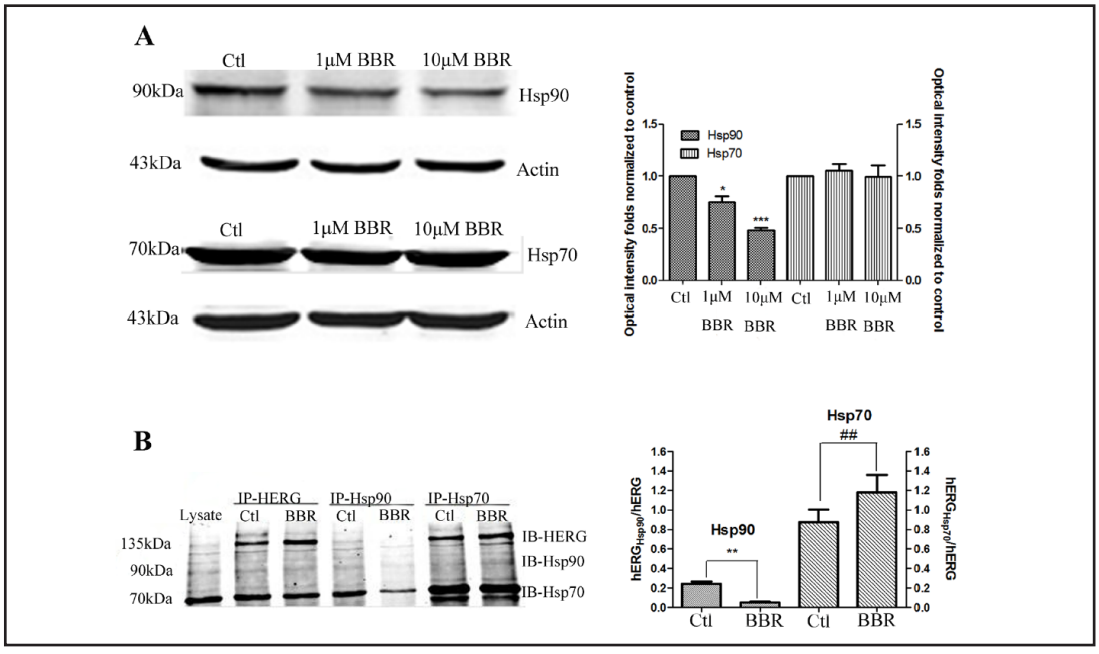


Fig. 3. BBR disrupt the interactions between hERG and chaperones. (A) Western blot results of Hsp90 and Hsp70 expression after incubation of BBR (1 μM and 10 μM) for 24 h. Hsp90 was concentration-dependently decreased while Hsp70 was unchanged. *P<0.05, ***P<0.001, significantly different from control, n=5. (B) Immunoprecipitation results and statistics of the interaction between hERG and Hsp90/Hsp70. The experiment was carried out by using anti-hERG, anti-Hsp70 or anti-Hsp90 antibody and immunomagnetic beads to purify interacting compounds from total protein and afterwards by using respective antibody to detect their interactions. The hERG protein interacting with Hsp90 was decreased, while the hERG protein interacting with Hsp70 was increased. **P<0.01, ##P<0.01, significantly different from their respective controls, n=3.

Fig. 4B shows that calnexin and calreticulin, two chaperones that help channel folding and that are downstream targets of cleaved-ATF6, were significantly increased. Both calnexin and calreticulin were upregulated nearly two-fold by 10 μM BBR. Consistent with

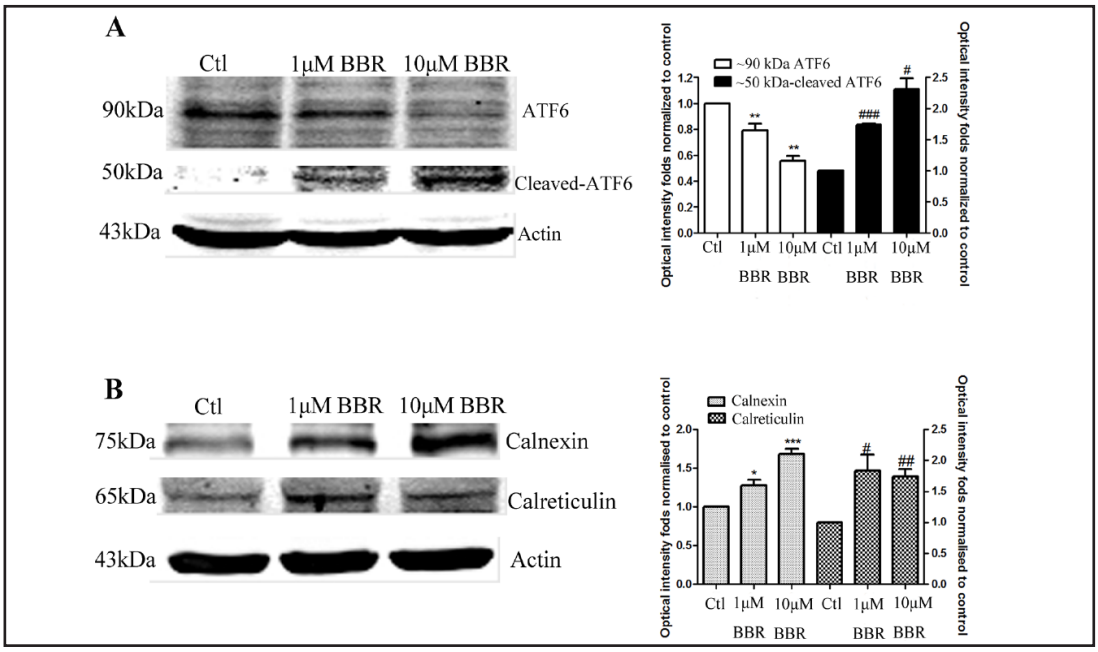


Fig. 4. Unfolded protein response is activated by BBR. (A) Expression of ATF6 (~90 kDa) and cleaved ATF6 (>50 kDa) after 1 μM or 10 μM BBR incubation for 24h. Cleaved ATF6 was activated and increased two-fold by 10 μM BBR. **P<0.01, #P<0.05, ###P<0.001, significantly different from their respective controls, n=3. (B) Western blot results and statistics of calnexin and calreticulin expression. BBR significantly increased the expression of both chaperones. */#P<0.05, ##P<0.01, ***P<0.001, significantly different from their respective controls, n=5.

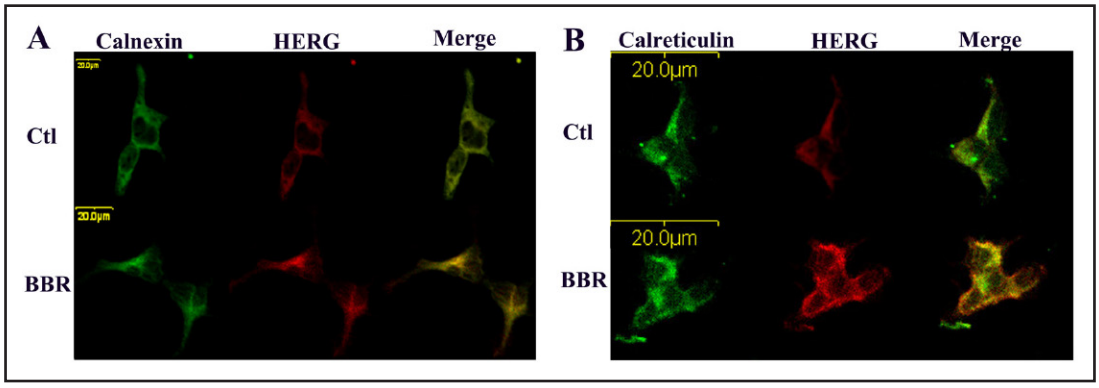


Fig. 5. BBR enhances the colocalization between hERG and calnexin/calreticulin after incubation for 24h. (A and B) Confocal microscopy demonstrates the colocalization of hERG (red) with calnexin (green) and calreticulin (green). Merged images on the right show that BBR increased the colocalization (yellow) between hERG and each chaperone.

these results, Fig. 5 shows that colocalization (yellow) between hERG (red) and calnexin (green) or calreticulin (green) were both enhanced. Together, these results suggest that the UPR is activated by BBR.

Disordered hERG channels are degraded in proteasomes and lysosomes

To determine the fate of disordered hERG protein, lactacystin (proteasome inhibitor) and bafilomycin (lysosome inhibitor) were added to the cells. Fig. 6A shows that 10 nM bafilomycin restored the decreased 155 kDa hERG from 64.19% ± 1.39% to 85.75% ± 2.91%, which suggests that lysosomes mediate 155kDa hERG degradation. Increased 135 kDa hERG

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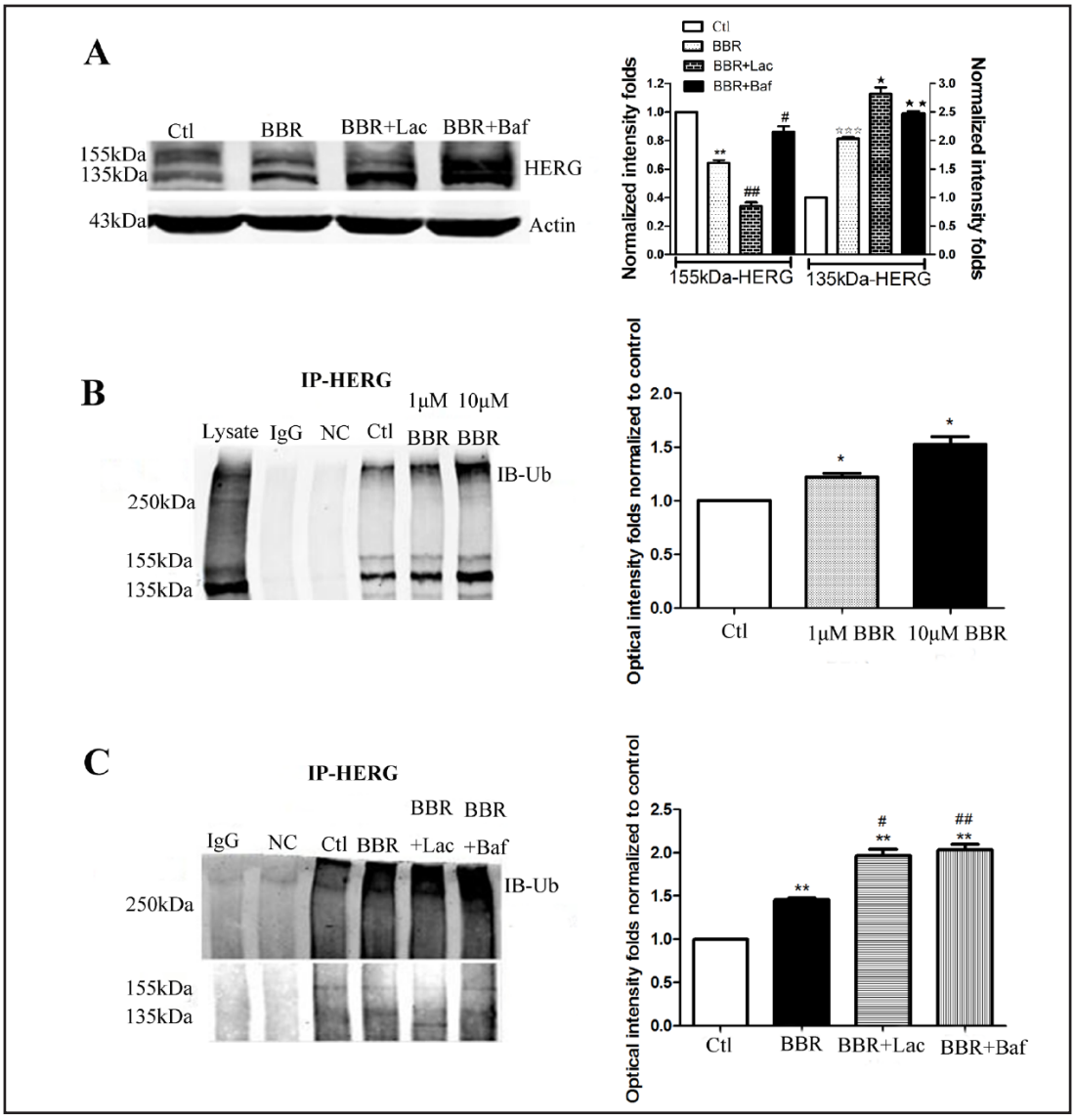


Fig. 6. Defective hERG protein is degraded in the lysosome and the proteasome. (A) hERG expression after incubation of control, BBR and BBR plus lactacystin or bafilomycin for 24h. The proteasome inhibitor lactacystin (20 μM) and the lysosome inhibitor bafilomycin (10 nM) further increased the elevated 135 kDa hERG, indicating degradation of defective hERG by proteasomes and lysosomes. Bafilomycin restored the reduced 155 kDa hERG, indicating lysosome-mediated degradation of 155 kDa hERG. **P<0.01, ☆☆☆P<0.001, significant different from their respective controls. #P<0.05, ##P<0.01, ★P<0.05, ★★P<0.01, significantly different from BBR, n=3. (B) Immunoprecipitation results of hERG ubiquitination. Both 1 μM and 10 μM BBR significantly increased the ubiquitination of hERG protein after incubation for 24h (see black band, approximately 250 kDa). *P<0.05, significantly different from control, n=3. (C) Immunoprecipitation results of the ubiquitination of hERG protein after treatment with lysosome inhibitor or proteasome inhibitor for 24h. The increased ubiquitination induced by BBR was further enhanced by lactacystin and bafilomycin, indicating that degradation was dependent on a ubiquitination-proteasome and ubiquitination-lysosome pathway. **P<0.01, significantly different from control. #P<0.05, ##P<0.01, significantly different from BBR, n=3.

levels were further elevated by 20 μM lactacystin and 10 nM bafilomycin, which suggests that both proteasomes and lysosomes mediate 135 kDa hERG degradation.

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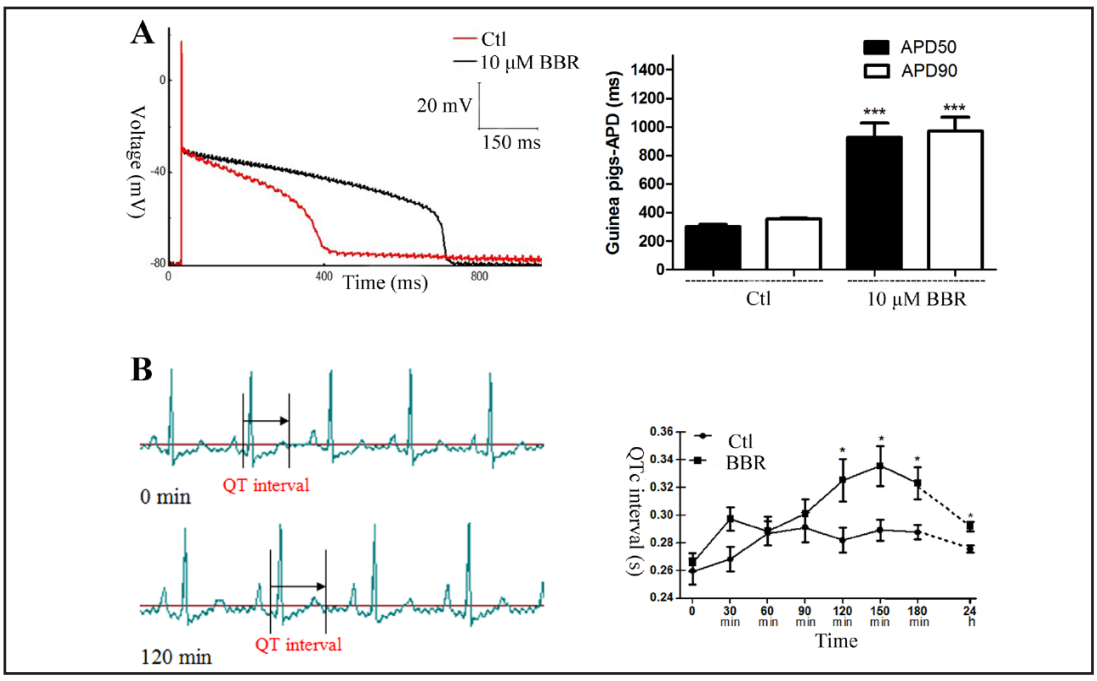


Fig. 7. BBR prolongs both the APD and the QTc interval of guinea pig myocytes. (A) Representative action potential and statistics of guinea pig myocytes after incubation of vehicle or BBR for 24h. Treatment with 10 μM BBR significantly prolonged APD_{50} and APD_{90} in guinea pig myocytes. ***P<0.001, significantly different from their respective controls, n=4. (B) Examples and statistics of the ECGs of guinea pigs after 27.1 $mg.kg^{-1}$ BBR gavage. The QTc was prolonged by BBR at 120 min. *P<0.05, significantly different from the vehicle control, n=6.

Fig. 6B shows that BBR concentration-dependently increased polyubiquitination of the hERG protein. The increased polyubiquitination was further elevated by lactacystin and bafilomycin (Fig. 6C). Together, these results suggest that disordered hERG protein is degraded in the lysosome and proteasome. Additionally, polyubiquitin is added to the hERG protein to signal its degradation.

BBR prolongs APD and QTC interval in guinea pigs

To test whether BBR could further change APD or the QT interval due to hERG channel deficiency, we performed patch-clamp and ECG recording experiments in guinea pig cells. Fig. 7A shows that the APD in guinea pig cardiomyocytes is significantly prolonged by 10 μM BBR. The APD_{50} was prolonged from 371.4±35.4 ms to 797.4±103.6 ms, and the APD_{90} was prolonged from 429.3±18.0 ms to 840.6±88.1 ms.

After gavage of 27.1 $mg.kg^{-1}$ BBR, the corrected QT interval of guinea pigs was prolonged after 120 min (Fig. 7B). The QTc intervals at 120 min, 150 min, 180 min and 24 h in the vehicle control group were 0.2821±0.0090 s, 0.2893±0.0076 s, 0.2877±0.0053 s and 0.2776±0.0029 s, respectively. The intervals in the BBR gavage group were 0.3255±0.0154 s, 0.3357±0.0147 s, 0.3233±0.0116 s and 0.2948±0.0036 s, respectively. Together, these results suggest that BBR prolonged both the APD and the QT_C interval in guinea pigs.

Discussion

In these studies, we determined that BBR induces a reduction in mature hERG expression through a mechanism that involves inhibition of trafficking. Specifically we found the following: (1) Trafficking inhibition is caused by defect of interaction with chaperone

proteins; (2) The UPR is subsequently activated; (3) Disordered hERG channels are degraded in the proteasomes and lysosomes; (4) Polyubiquitin is added to hERG channels to signal their degradation.

hERG channels are translated and initially glycosylated at the ER (molecular weight of hERG protein is 135 kDa). After folding properly with the assistance of chaperones, hERG channels are trafficked to the Golgi for secondary glycosylation (molecular weight of hERG protein is 155 kDa) and then further trafficked to the membrane [11]. In our study, a reduction in 155 kDa hERG and an increase in 135 kDa hERG were observed after treatment with BBR (Fig. 1A and B), which illustrates that trafficking from the ER to the Golgi was impaired. In fact, increasing evidence shows that drugs affect hERG channels by interfering with their trafficking [5, 12]. Numerous studies have shown that misfolding of hERG channels is most often the cause of defects in trafficking. Hsp70 and Hsp90 are two key chaperones that assist in channel folding. Hsp70 at the ER binds to the initial hERG, while Hsp90 in the cytoplasm binds to the terminal hERG to assist in folding [13]. In our study, 1 μM and 10 μM BBR reduced Hsp90 but had no effect on Hsp70 (Fig. 3A). The level of hERG protein interacting with Hsp90 was decreased, while the level of hERG protein interacting with Hsp70 was increased by BBR (Fig. 3B). There may be two explanations for these results: (1) Decreased Hsp90 directly reduces the interaction between terminal hERG and Hsp90, and redundant hERG is retained in the ER thus increases the interaction between initial hERG and Hsp70. This mechanism is similar to that of arsenic trioxide [14]; (2) BBR may bind to hERG channels and change their conformation. Thus, the interaction between initial hERG and Hsp70 is prolonged, and less hERG enters the terminal conformation phase to interact with Hsp90. This mechanism is similar to that of pentamidine [15]. Although BBR may alter hERG channel folding by both mechanisms, we speculate that explanation (1) may be the primary mechanism.

The UPR, which indicates an ER stress response, is a process that prevents the accumulation of unfolded protein in the ER and provides a mechanism by which cells can rapidly adapt to alterations such as disruptions in protein folding [16]. In fact, the ER stress response is often activated when hERG channel trafficking is defective [14, 17, 18]. Among the three pathways (ATF6, IRE-1 and PERK) of UPR, ATF6 is suggested to be the initial respondent [19]. There are two forms of ATF6: the inactive 90 kDa form and the active-cleaved 50 kDa form. The cleaved ATF6 shall enter the nuclus to initiate its target genes and thus increase expresssion of chaperones which assist hERG channel folding [16, 19]. For example, calnexin and calreticulin are two ER marker proteins and chaperones which are regulated by ATF6 to assist hERG channel folding [14]. In our study, hERG channel trafficking was impaired by BBR. Consistently, we found that a decrease of inactive ATF6 and an increase of active-cleaved ATF6 were induced by 1 μM and 10 μM BBR (Fig. 4A). In addition, calnexin and calreticulin were both up-regulated (Fig. 4B). Fig.5 consistently shows that co-localization between calnexin or careticulin and hERG were enhanced especially co-localization around the nucleus (main location area of ER). These results indicate that UPR is activated by BBR-induced trafficking deficiency of hERG channel.

Misfolded and trafficking-defective proteins retained in the ER are eventually degraded by a process termed ER-associated degradation [17]. Earlier studies suggested that misfolded and trafficking-defective proteins are degraded by the ubiquitination-proteasome way [20]. In fact, increasing research suggests that both the proteasome and lysosome participate in the degradation of trafficking-defective hERG protein [4, 21, 22]. The results of our study support these findings. As inhibitors like bafilomycin has no effect on hERG expression [4], we performed experiments to compare hERG expression between BBR group and BBR plus inhibitor group. Both the lysosome inhibitor and proteasome inhibitor further increased the restricted 135 kDa hERG that was induced by BBR (Fig. 6A). This would aggravate burden of UPR thus decreased efficacy of folding assistance and finally induced further decrease of 155 kDa (as is shown in proteasome inhibitor group). Unexpectedly, the lysosome inhibitor restored the decreased 155 kDa hERG that was induced by BBR. We inferred that BBR could not just inhibit hERG trafficking but also might increase lysosome-mediated 155 kDa hERG degradation. Furthermore, BBR indeed induced ubiquitination of hERG protein

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in our study (Fig. 6B). Fig. 6C suggested that BBR stimulates hERG protein degradation by the ubiquitination-proteasome pathway similar to desipramine [4, 21], and BBR stimulates degradation by the ubiquitination-lysosome pathway similar to ceramide [23].

Importantly, BBR prolonged APD in guinea pig myocytes after incubation for 24h (Fig. 7A). This result is consistent with the idea that the prolonged APD is caused by hERG channel deficiency. In fact, previous studies showed that BBR acutely blocks both L- and T-type calcium channels [24]. In addition, BBR has been shown to inhibit Iks and Ik1 [8, 25]. Because Ica, Ikr, Iks and Ik1 are the main currents responsible for the repolarization of action potentials, we conclude that the prolonged APD caused by BBR incubation is due to combined effects on various currents. Furthermore, BBR gavage prolonged the QT interval of guinea pigs in vivo (Fig. 7B). Based on the finding that the QT interval was prolonged after 120 min, we speculate that the prolongation of the QT interval by BBR is a combination of acute and long-term effect (trafficking deficiency of hERG). BBR induced trafficking deficiency of hERG and prolongation of APD/QTc interval provide supports to an increasing number of studies demonstrating the pro-arrhythmic potential and genetic toxicity of BBR on heart [26, 27].

There are some shortcomings of our paper. On one hand, it's not clear whether trafficking inhibition caused by BBR is specifically to hERG channel or not. On the other hand, rescuing strategies of BBR-induced hERG channel deficiency are needed to be developed in the future.

In summary, the results of our study are the first to demonstrate the effect of BBR on hERG channels after incubation for 24h, and they show that BBR induces hERG channel deficiency by inhibition of trafficking. On account of the close relevance between trafficking inhibition-induced hERG deficiency and cardiotoxicity, our study suggests a high pro-arrhythmic potential of BBR and provides useful information for the use and development of BBR as a medication.

Disclosure Statement

There are no conflicts of interest.

Acknowledgements

This work was funded by the National Natural Science Foundation of China [no. 31173050 and 30973530], the Key Program of the National Natural Science Foundation of China [no. 81230081] and the Funds for Creative Research Groups of The National Natural Science Foundation of China [81121003].

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