Restoration of mutant hERG stability by inhibition of HDAC6

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Short title: **Inhibition of HDAC6 stabilizes mutant hERG**

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ABSTRACT

The human *ether-a-go-go-related gene* (hERG) encodes the α subunit of a rapidly activating delayed-rectifier potassium (I_{Kr}) channel. Mutations of hERG cause long QT syndrome type 2 (LQT2). Acetylation of lysine residues occurs in a subset of non-histone proteins and this modification is controlled both by histone acetyltransferases and deacetylases (HDACs). The aim of this study was to clarify effects of HDAC(s) on wild-type (WT) and mutant hERG proteins. WThERG and two trafficking-defective mutants (G601S and R752W) were transiently expressed in HEK293 cells, which were treated with the pan-HDAC inhibitor Trichostatin A (TSA) or the HDAC6-selective inhibitor Tubastatin A (TBA). Both TSA and TBA increased protein levels of WThERG and induced expression of mature forms of the two mutants. Immunoprecipitation showed an interaction between HDAC6 and immature forms of hERG. Coexpression of HDAC6 decreased acetylation and, reciprocally, increased ubiquitination of hERG, resulting in its decreased expression. siRNA against HDAC6, as well as TBA, exerted opposite effects. Immunochemistry revealed that HDAC6 knockdown increased expression of the WThERG and two mutants both in the endoplasmic reticulum and on the cell surface. Electrophysiology showed that HDAC6 knockdown or TBA treatment increased hERG channel current corresponding to the rapidly activating delayed-rectifier potassium current (I_{Kr}) in HEK293 cells stably expressing the WT or mutants. Three lysine residues (K116, K495 and K757) of hERG were predicted to be acetylated. Substitution of these lysine residues to arginine eliminated HDAC6 effects. In HL-1 mouse cardiomyocytes, TBA enhanced endogenous ERG expression, increased I_{Kr}, and shortened action potential duration. These results indicate that hERG is a substrate for HDAC6. HDAC6 inhibition induced acetylation of the 3 lysine residues in hERG which counteracts ubiquitination

leading to stabilization of hERG. HDAC6 inhibition may be a novel therapeutic option for LQT2.

Key Words: hERG, HDAC6, acetylation, ubiquitination, LQT2.

1. Introduction

The human ether-a-go-go-related gene (*hERG*) encodes the α subunit of a rapidly activating delayed-rectifier K^+ channel which plays a critical role in repolarization of the cardiac action potential (AP)[1, 2]. Mutations in the gene result in long QT syndrome type 2 (LQT2) which causes life-threatening arrhythmia. Most mutations cause impaired maturation and/or trafficking of the channel protein, leading to its ubiquitination and subsequent degradation by the proteasome[3]. HERG is synthesized as a 135-kDa immature form in the endoplasmic reticulum (ER). After full glycosylation in the Golgi apparatus, it is transported to the cell surface as a 155-kDa mature form[4]. The sarcolemmal ionic channel current density depends on the channel protein level on the plasma membrane which is governed by both transcriptional and post-transcriptional mechanisms[5, 6].

Several potential biochemical and molecular strategies from both transcriptional and post-transcriptional mechanistic aspects have been reported to rescue mutant hERG[7]. Histone deacetylases (HDACs) modulate acetylation of histone as well as non-histone proteins[8]. Eighteen mammalian HDACs are grouped into four classes based on their structures[9, 10]. Recent studies showed that HDAC inhibitors enhanced acetylation of the epithelial Na^+ channel (ENaC) and thereby increased the channel density on the cell surface[11]. Silencing HDAC7 or treatment with HDAC inhibitors stabilized △F508 cystic fibrosis transmembrane regulator (CFTR), suggesting a therapeutic value of HDAC

inhibitors for channelopathy. HDAC7 appeared to alter expression of multiple proteins that affect △F508 CFTR folding and trafficking[12]. Class Ⅱb HDACs include HDAC6 and 10, both of which predominantly localize in the cytosol and regulate acetylation of non-histone proteins in the cytoplasm[13, 14]. HDAC6 expressed in cardiomyocytes of human, mice and canine has been found to be involved in atrial fibrillation and hypertension[15-17]. These studies indicate that acetylation of channel proteins is one of the post-translational modifications to regulate the ion channel density.

Here, we investigated effects of HDAC inhibition on expression of wild-type (WT) and LQT2-related mutant hERG in HEK293 cells, as well as on endogenous ERG in HL-1 mouse cardiomyocytes.

2. Materials and Methods

Detail experimental procedures are available in the Online Supplemental Material.

2.1. Cell culture and transfection

HEK293 cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (JRH) and penicillin/streptomycin/geneticin at 37℃ with 5% CO_2. HL-1 mouse cardiomyocytes were maintained as previously described[5]. Human embryonic stem cells (KhES-1) obtained from Institution for frontier medical sciences, Kyoto University, were cultured and differentiated into cardiomyocytes as previously reported[18].

The expression construct pcDNA3/hERG-FLAG was engineered by ligating an oligonucleotide encoding a FLAG epitope to the carboxyl terminus of hERG cDNA. Missense mutations, G601S and R752W were introduced into pcDNA3/hERG-FLAG by site-directed mutagenesis. One and all of three lysine residues at amino acids 116, 495 and 757 of hERG were replaced by arginine using site-directed mutagenesis for the

development of the single lysine (1K) and triple lysine (3K) mutants, respectively; each substitution was confirmed by direct sequencing. The plasmids were transfected into HEK293 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total amount of cDNA was adjusted using vector cDNA.

2.2. Drugs

Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), Scriptaid, Tubastatin A (TBA) and SB939 (pracinostat) were purchased from Sigma, Cayman Chemical, Biomol, Bio vison and Abcam Biochemicals, respectively. The stock solutions were prepared in DMSO and drugs were added to the culture medium 24 h after transfection. For chase experiments, 48 h after transfection, cycloheximide (60 μg/ml) was added to the culture medium and cells were harvested at indicated time points.

2.3. Small interference RNA (siRNA)

siRNA targeting HDAC6 and scrambled control siRNA were used. Sequences of siRNA are HDAC6 (sense: 5'-UUAAUCGUCGCAGUUCUCU-3'; antisense: 5' -AGAGAACUGCGACGAUUAA- 3') and scramble (sense: 5'-GAAGCGAGAUAUCCCUGACTT-3'; antisense: 5'-GUCAGGGAUAUCUCGCUUCTT-3'). Cells were transfected with siRNA using lipofectamine 2000, according to the manufacturer's instructions.

2.4. Immunoblotting and immunoprecipitation

Cells were harvested 48 h after transfection and lysed by sonication in a buffer (PBS supplemented with 1% polyoxyethylene octyiphenyl ether (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 1.5 mM aprotinin, 21 mM leupeptine, 15 mM pepstain and

1 mM phenylmethylsulfonylfluoride)[5]. Proteins were separated on SDS-PAGE and electrotransferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in PBS plus 0.1% Tween and immunoblotted with a primary antibody. The blots were developed by using an ECL system (Amersham, Biosciences, Piscataway, NJ, USA). For immunoprecipitation (IP), proteins were incubated with protein G agarose (Pharmacia, Uppsala, Sweden) bound with individual antibody for 16 h at 4 °C, washed and followed by elution of bound proteins by heating at 37°C for 20min in SDS-PAGE sample buffer. The bound proteins were analyzed by SDS-PAGE followed by immunoblotting. Band densities were quantified using a NIH image J software. The image densities of Western blotting were quantified and normalized to their image densities of ß-actin level and control.

2.5. Immunofluorescence

HEK293 cells were transfected with pcDNA3/hERG-FLAG together with pDsRed2-ER (Clontech), pDsRed-Monomer-Golgi (Clontech) or pPM-mKeima-Red (BML) constructs. 24 h after transfection, cells were fixed with 4% paraformaldehyde/PBS and then permeabilized with 0.5% Triton X-100. They were incubated for 1 h at room temperature with a primary antibody (FLAG, 1:1000). After blocking in 3% albumin, bound antibody was visualized with Alexa Fluor 488 conjugated mouse secondary antibody (1:2000), and images were collected with a TCS SP2 confocal microscope (Leica, Tokyo, Japan).

2.6. Electrophysiological recordings

HEK293 cells stably expressing hERG-FLAG were transfected with a scrambled siRNA or an siRNA against HDAC6, or the cells were treated with DMSO (Vehicle) or TBA. 24 h after the transfection or addition of TBA, hERG currents were measured at 37℃ using whole-cell patch-clamp techniques with an Axopatch-200 amplifier (Axon instrument, USA). HL-1 cells were also used for the measurement of the rapidly activating delayed-rectifier potassium (I_{Kr}) and APs. Procedures for the current measurements in HEK293 and HL-1 cells were essentially the same as described previously[6].

2.7. Real-time reverse transcription-PCR (RT-PCR)

RNAs were extracted from HEK293 cells using an RNeasy Plus mini kit (QIAGEN). cDNA was synthesized using SuperScript™ II reverse transcriptase (Gibco BRL). Quantitative RT-PCR (qRT-PCR) was performed with 7900HT Fast Real-Time PCR System, according to the manufacturer's instructions (Applied Biosystems, CA). Primers used are as follows:

HERG forward : GGGCTCCATCGAGATCCT;

reverse: AGGCCTTGCATACAGGTTCA.

β-actin forward : GCACCCAGCACAATGAAGA;

reverse: CGATCCACACGGAGTACTTG.

Data analysis was performed with the SDS software version 3.2 (Applied Biosystems).

2.8. Statistical analysis

All data are presented as mean ± SEM. For statistical analysis, Student's *t*-test and repeated measures analysis of variance (two-way ANOVA) were used, with $p<0.05$ being considered statistically significant.

3. Results

3.1. Pan-HDAC inhibitor increased expression of WThERG and trafficking-deficient mutants

We first examined effects of pan-HDAC inhibitors TSA, SAHA and Scriptaid on WThERG-FLAG and two trafficking-deficient mutants (G601S-FLAG, R752W-FLAG) expressed in HEK293 cells. G601S and R752W mutations are located in a pore-region and an intracellular loop, respectively (Fig.1A). On anti-FLAG immunoblots (IB), WThERG-FLAG gave an immature core-glycosylated form at 135-kDa and a fully glycosylated mature form at 155-kDa, whereas the two mutants gave only the immature form. All of the inhibitors increased both the mature and immature forms of the WT. Similarly, these drugs increased the immature form of the mutants and induced expression of the mature form (Fig.1B). HERG was recovered in the detergent-soluble fraction, regardless of the drug treatment, suggesting that the HDAC inhibitors did not change protein solubility (data not shown).

3.2. Selective inhibition of HDAC6 increased expression of WThERG and trafficking-deficient mutants via post-translational modification

Previous studies demonstrated that hERG maturation is facilitated by Hsp90 which is a substrate of HDAC6[4, 19]. We examined effects of an HDAC6 selective inhibitor TBA, to determine the subtype of HDAC that regulates hERG expression. TBA exerted the same enhancing effects as TSA on the WT and mutant hERG expression (Fig. 1C); it enhanced the WT expression in a concentration-dependent manner (Fig. 1S). These HDAC inhibitors did not alter Hsp90 or Hsp/Hsc70 protein levels (Fig. 2S). To confirm the role of HDAC6 in hERG maturation, the half-life of hERG was determined by cycloheximide

chase assay in the absence or presence of TSA or SB939. Both TSA and SB939 are pan-HDAC inhibitors, but SB939 does not inhibit HDAC6[20]. The half-life of the 135-kDa hERG was prolonged by TSA (6.7 ± 0.9 h and 10.8 ± 1.2 h in the absence and presence of TSA, respectively; n=6-8) but was not affected by SB939 (6.5 ± 1.2 h) (Fig. 2A).

Next, we examined whether there was any physical interaction between HDAC6 and hERG. The anti-HDAC6 immunoprecipitates (IPs) contained the immature form of the WT (Fig. 3S). Conversely, endogenous HDAC6 was detected in the anti-FLAG IPs. The interaction between hERG-FLAG and HDAC6 was further supported by double immunostaining of hERG-FLAG and HDAC6 (Fig. 4S). A small interfering (si) RNA targeting HDAC6 recapitulated the effects of TBA on the WT and two mutants (Fig. 2B). Coexpression of HDAC6-FLAG decreased the two forms of the WT and the immature form of the two mutants (Fig. 2C).

Intracellular localization of hERG-FLAG was analyzed by immunofluorescence. The immunoreactivity of the WT was localized in the endoplasmic reticulum (ER) and the Golgi apparatus as well as on the cell membrane; siRNA against HDAC6 increased the signals of the WT in all of these cellular components (Fig. 5S A and B). The immunoreactivities of the two mutants were mainly localized in the ER but not on the cell membrane; siRNA against HDAC6 also increased the signals of the two mutants in the ER, Golgi as well as on the cell membrane (Fig. 2D).

To see whether HDAC6 affects the levels of functional hERG channels on the cell membrane, we measured hERG currents in HEK293 cells stably expressing the WT or the

mutants. Depolarization pulses activated time-dependent outward currents corresponding to I_{Kr} in HEK293 cells expressing the WThERG. These currents were completely inhibited by 10 μM E4031. E4031-sensitive currents were obtained by digital subtraction of the currents in the presence of E4031 from the currents in its absence. Substantial decreases in peak currents and loss of I_{Kr} tail currents were observed in cells expressing the mutants (Fig. 3A). TBA treatment (24 h) as well as siRNA against HDAC6 increased the peak and tail currents mediated by the WT. They also enhanced the peak currents mediated by G601S and R752W. The tail currents mediated by G601S and R752W became detectable by TBA treatment and knockdown of HDAC6 (Figs. 3B-E).

To examine whether HDAC inhibitors affect transcription of *hERG*, we performed real-time PCR (Fig. 6S A-C). The pan-HDAC inhibitor TSA increased the WT and the two mutant *hERG* mRNA, whereas the selective HDAC6 inhibitor TBA and siRNA targeting HDAC6 failed to alter their levels. These results suggest that HDAC6 causes post-translational modifications of hERG.

3.3. HDAC6 regulated acetylation and ubiquitination of hERG

Lysine residue is a target of both acetylation and ubiquitination and degradation of the immature form of hERG depends on its poly-ubiquitination[4, 9]. To obtain direct evidence for the role of HDAC6 in hERG degradation, we examined the levels of hERG ubiquitination and acetylation. Ubiquitination levels of the two mutant hERGs were higher than that of the WT (Fig. 4A). An siRNA against HDAC6 and TBA treatment significantly decreased ubiquitination of the WT and the two mutants (Fig. 4A, B). The anti-acetylated lysine IPs contained the immature form of the WT and the two mutants,

indicating acetylation of the immature forms. Knockdown of HDAC6 (Fig. 4A) and TBA treatment (Fig. 4B) increased the level of acetylation of the WT and the two mutants. Coexpression of HDAC6-FLAG decreased the acetylation level of the WT and the two mutants with increases of ubiquitination (Fig. 4C). These results indicated reciprocal regulation of the two types of modifications, acetylation and ubiquitination.

To obtain evidence that HDAC6 affects proteasomal degradation of hERG immature forms, we examined effects of a proteasome inhibitor MG132 on hERG expression. MG132 (6 μM) increased the immature form of the WT. It also prevented HDAC6-induced decreases of the immature form of hERG proteins (Fig. 4D). To further confirm HDAC6 effects on hERG stability, we examined HDAC6 effects on the half-life of the WT by chase experiments (Fig. 5A). The half-life of the 135-kDa immature form (6.7 ± 0.9 h in the control) was shortened to 4.4 ± 0.6 h when cotransfected with HDAC6-FLAG, but was prolonged by knockdown of HDAC6 and TBA treatment to 10.5 ± 0.9 h and 10.7 ± 1.5 h, respectively (Fig. 5B).

Since HDAC6 harbors the ubiquitin zinc finger domain (BUZ) [21], we tested whether the BUZ domain and its activity are involved in the HDAC6 effects on hERG. A mutant HDAC6 lacking the BUZ domain (△BUZ) or lacking deacetylase activity (inactive M) failed to decrease the level of WThERG-FLAG significantly (Fig. 5C), indicating that the BUZ domain and the deacetylating activity of HDAC6 are indispensable for the HDAC6 effects on hERG-FLAG stability.

3.4. Identification of acetylated lysines in hERG

The posttranslational modification database PHOSIDA (http:// www.Phosida.org), predicted 5 potential acetylation sites (lysine 116, 495, 525, 638 and 757) in hERG. Three lysine residues at the amino acids 116, 495 and 757 are located on the cytosolic face of the protein (Fig. 1A). Since the lysine residues 525 and 638 are in the transmembrane domain and in the pore-region, respectively, HDAC6 located in the cytoplasm is unlikely to act on these residues. Thus, we focused on lysine 116, 495 and 757.

To verify that the residues 116, 495 and 757 are involved in acetylation and ubiquitination of hERG, we generated a series of mutants with single (1K), or triple (3K) lysine to arginine substitutions. When the 1K or the 3K mutant was expressed in HEK293 cells, they gave both 135-kDa and 155-kDa forms. The protein level of the 3K mutant was higher than that of the WT (Fig. 6A). Coexpression of HDAC6-FLAG with these mutants decreased the expression in an order of the WT> 1K > 3K (Fig. 6A). In contrast, TBA treatment increased the levels of these proteins in the same order (Fig. 6B). Expression of the 3K mutant was not significantly affected by coexpression of HDAC6 or TBA. To examine whether all the three lysine residues are involved in the acetylation, we expressed the 3K mutant or each of the three 1K mutants in HEK293 cells. The acetylated immature forms of the three 1K (116, 495 and 757) were detected, whereas no clear bands appeared in the 3K, suggesting that the three lysine residues are acetylation sites (Fig. 6C). Moreover, siRNA knockdown of HDAC6 had no effect on ubiquitination or acetylation levels of the 3K mutant (Fig. 7A). Similarly, both TBA treatment and HDAC6 coexpression were ineffective (Fig. 7B, C). Chase experiments revealed that the half-life of the immature form of the 3K mutant (9.2 ± 0.4 h) was longer than that of the WT. Coexpression of HDAC6, knockdown of HDAC6 or TBA treatment did not significantly alter the half-life of the immature form of the 3K mutant (Fig. 5A and B).

3.5. Inhibition of HDAC6 enhanced ERG expression in HL-1 mouse cardiomyocytes

To see whether HDAC inhibition affects expression of endogenous ERG, we examine effects of TSA and TBA on ERG expression in HL-1 mouse cardiomyocytes. Both TSA and TBA enhanced ERG expression (Fig. 8A). TSA, but not TBA, increased the acetylated histone H3 level, indicating that HDAC6 is not involved in epigenetic modification of ERG expression. We next recorded I_{Kr} in HL-1 cells. As shown in Fig. 8B, depolarizing pulses activated time-dependent outward currents which were completely abolished by 10 μM E4031. TBA treatment caused significant increases in E4031-sensitive currents. Since the ERG current I_{Kr} is responsible for repolarization of the cardiac AP and is the dominant outward current in HL-1 cells[6], we examined whether TBA treatment shortened AP duration (APD) (Fig. 8C). Application of TBA significantly shortened the APD at 90% repolarization (APD_{90}) without affecting resting membrane potentials (-75.1±1.2 in control and -76.5±1.1 in with TBA, p=0.48). APD_{90} values in control (DMSO) and TBA treatment were 160.4±6.2 and 98.8±10.6 ms, respectively (Fig. 8C). 1 μ M E-4031 which is the concentration to completely block I_{Kr} in HL-1 cells[22] prolonged APD_{90} in HL-1 cells treated by DMSO and TBA (APD_{90}=210±6.7, 217±8.1, respectively. p=0.57). The percentage of APD_{90} shortening in absence of E-4031 compared with that in presence of E-4031 is more prominent in TBA treated HL-1 cells, indicating the contribution of increased I_{Kr} to APD_{90} (Fig. 11S).

4. Discussion

4.1. HDAC6 regulates hERG channel expression

HDAC class ⅡB including HDAC6 and HDAC10 are predominantly located in the cytoplasm[16,23]. Although both HDAC6 and HDAC10 interact with the immature form of hERG (Fig. 3S, 7S A), selective inhibition of HDAC6, but not HDAC10, increased the WT and mutant hERG expression (Fig. 7S B). The pan-HDAC inhibitor TSA, but not another pan-HDAC inhibitor SB939 that does not inhibit HDAC6, stabilized hERG (Fig. 2A). HDAC6 unaltered hERG transcripts (Fig. 6S). These findings demonstrate that HDAC6, but not HDAC10, regulates hERG channel expression via post-translational modification. TBA treatment and knockdown of HDAC6 increased hERG peak and tail currents mediated by WT. However, the tail currents mediated by the mutants remained small. The faster activation kinetics of the mutant currents than that of WT; thus, very small tail currents of the mutants may be accounted for by their very rapid deactivation.

HDAC6 deacetylates non-histone proteins located in the cytosol, such as microtubules and cortactin to regulate cell motility and cell adhesion[8,24] . Inhibition of HDAC6 induced acetylation of Hsp90 decreasing its interaction with client proteins, resulting in their ubiquitination and degradation[19]. Since heat shock proteins regulate WT and mutant hERG maturation[4, 6], one may assume that HDAC6 regulates hERG stability via modifications of these proteins. However, Hsp90, Hsp70/hsc70 protein levels were unaltered by HDAC6 inhibitor treatment (Fig. 2S). An additional knockdown of Hsp70 by siRNA against Hsp70 together with TBA treatment did not abolish the effects of TBA on hERG proteins (Fig. 8S), suggesting that HDAC6 effects on hERG is not mediated by heat shock proteins.

4.2. Acetylation of hERG enhances its stability via inhibition of ubiquitination

Inhibition of HDAC6 resulted in enhanced acetylation and reduced ubiquitination of

the immature form of hERG leading to prolongation of its half-life (Fig. 4A-C, Fig. 5A and B). HDAC6 has two deacetylase domains and a c-terminus zinc finger motif (ZnF-UBP) [21]. The ZnF-UBP finger binds to ubiquitin or ubiquitinated proteins, leading to their degradation. Both the deacetylase and ubiquitin-binding activities of HDAC6 are required for acetylation and ubiquitination processes[25]. The catalytically inactive HDAC6 possesses a normal BUZ domain and may still facilitate ubiquitination-induced degradation. This may be a reason why the catalytically inactive HDAC6 still weakly reduced hERG-FLAG level (Fig. 5C). △BUZ HDAC6 may deacetylate hERG but was unable to facilitate hERG ubiquitination and reduce its expression level.

Lysine residues serve as targets of multiple covalent modifications including ubiquitination and acetylation[26]. Their acetylation in the epithelial Na^+ channel reduced channel ubiquitination and degradation[11]. Similarly, tumor suppressor p53 was stabilized by acetylation[27]. Two lysine residues are reciprocally modified by acetylation or ubiquitination in controlling stability of Smad7[28]. The lysine residues in hERG may also be reciprocally targeted by ubiquitination and acetylation.

It has been reported that ubiquitin itself is a substrate for acetylation at its lysine residues[29]. Acetylation of ubiquitin suppresses polyubiquitin chain elongation at ubiquitination sites of proteins including hERG. Thus acetylation of ubiquitin by HDAC6 inhibition may also be involved in the HDAC6 effect on the stability of hERG.

4.3. The acetyl-conjugation sites in hERG

There are 27 lysine residues in the cytoplasmic face of hERG and residues at 116, 495 and 757 are targeted for acetylation. The 3K mutant hERG showed a higher expression level than WT and 1K mutants. The ubiquitination level was lower in the 3K mutant than

in WT, suggesting that the substitution for the lysine residues stabilizes hERG. The substitution of the three lysine residues abolished the effects of HDAC6 on hERG (Fig. 7A-C) and attenuated the concentration-dependent effect of MG132 on ubiquitnated hERG level (Fig. 9S), indicating that HDAC6 regulates hERG stability mainly via the three lysine residues. Since weak acetylation has been found in the 3K hERG (Fig.7) and coexpression of HDAC6 partially reduced 3K hERG expression (Fig. 6A), we cannot rule out that other lysine residues in hERG might also be targeted for deacetylation.

HDAC 6 inhibition resulted in increased endogenous ERG expression in HL-1 mouse cardiomyocytes and hERG expression in cardiomyocytes derived from human ES cells (Fig. 10S). TBA treatment increased I_{Kr} and shortened APD in HL-1 cells. To study HDAC inhibition effects on I_{Kr} and APD in native cardiomyocytes is more precisely. It is the limitation of current study and needed to be studied in the future. It has been reported that HDAC inhibitors possess cardiotoxicity including QT interval prolongation[30]. Some pan-HDAC inhibitors mediated a delayed onset of effects on the beating properties of human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs). The pan-inhibitors altered the genes related to ion channels protein trafficking and insertion into the cell membrane[31]. TBA decreased the beat rate of hiPS-CMs only at the highest concentration (>10 μM) due to loss of selectivity resulting inhibition of additional HDAC isofroms. However, we found that TBA at 2 μM or more effectively enhanced hERG protein expression (Fig. 1S). HDAC6-deficient mice lack any pathological phenotype and are indistinguishable from normal mice[32]. Therefore inhibition of HDAC6 is a feasible pharmacological target for the treatment of LQT2 resulting from trafficking-defective mutant hERG.

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Disclosures

None

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Figure legends

Figure 1. hERG channel structure and effects of HDAC inhibitors on WT, G601S and R752W hERG-FLAG.

A, Diagram of a single α subunit of hERG protein. Locations of LQT2-associated mutations G601S and R752W (closed ellipses), and predicted acetylated lysine residues K116, K495 and K757 (open ellipses) are shown.

B, C, Effects of HDAC inhibitors. HEK293 cells were transiently transfected with either pcDNA3/WThERG-FLAG, G601S-FLAG or R752W-FLAG, together with pEGFP and treated with TSA (0.1 μM), SAHA (5 μM), Scriptaid (1 μM) or DMSO (V) for 24 h (**B**) (n=5-7). TBSA, a HDAC6-selective inhibitor, is 8 μM(**C**) (n=5). Shown are representative blots. Image densities of the immature and mature forms of hERG bands normalized to those in cells

treated with DMSO (V) (*P< 0.05; † P<0.01 vs V).

Figure 2. Effects of an HDAC6 inhibitor on the levels of hERG-FLAG expression.

A, Effects of HDAC inhibitors on hERG stability. Cells transiently expressing WThERG-FLAG were chased for the indicated time after administration of cycloheximide together with DMSO, TSA (0.1 μM) or SB939 (0.15 μM) for 24 h. Shown are the representative blots presenting time-dependent changes in the density of hERG-FLAG. The density of 135-KDa hERG-FLAG was normalized to the density at time 0 and β-actin. Bar graph shows half-life of hERG proteins. (n=4-6, *P<0.05 vs hERG-FLAG with DMSO (V).

B and C, HEK293 cells were transfected with WThERG-FLAG, G601S-FLAG or R752W-FLAG plasmids together with a scrambled siRNA or an siRNA against HDAC6 (**B**), or with HDAC6-FLAG (**C**). Shown are representative blots. Densities of the bands were quantified and normalized to those in the absence of siRNA against HDAC6 (**B**) or exogenous HDAC6 (**C**). *P<0.05; †P< 0.01 vs scrambled siRNA (**B**) or absence of HDAC6-FLAG transfection (**C**) (n=5-6).

D, Effects of knockdown of HDAC6 on intracellular localization of hERG-FLAG. Cells were transfected with G601S or R752W-FLAG plasmids together with a scrambled siRNA or an siRNA against HDAC6, and were co-transfected with pDsRed-ER(ER), pDsRed-Monomer Golgi (Golgi) or pPM-mKeima-Red (Mem). All the cells were stained with anti-FLAG and Alexa Fluor 488-conjugated mouse secondary antibody (green). Shown are representative images obtained by a confocal microscope. A histogram shows the ratios of Alexa 488/DsRed-ER, DsRed-Monomer-Golgi and PM-mKeima-Red fluorescence. The signals of the mutant hERG proteins merged with those of the marker proteins were normalized to those with the scrambled siRNA. (n=6,*P<0.05; †P<0.01 vs scrambled siRNA).

Figure 3. Effects of coexpression or knockdown of HDAC6 on hERG currents.

A, Effects of TBA and an siRNA against HDAC6 on hERG currents. Shown are representative E4031-sensitive currents traces recorded from cells stably expressing WThERG-FLAG, G601S-FLAG or R752W-FLAG with a scrambled siRNA (None) or an siRNA against HDAC6 or in the presence of 8 μM TBA. Currents were recorded in the absence and presence of 10 μM E4031; E4031-sensitive currents were obtained by digital subtraction. Inserts above the currents mediated by the mutants are expanded scale views of the tail currents.

B-E, Average current-voltage relationships of peak (**B**) and tail currents (**C**) for WThERG (n=10-17), (**D-E**) are peak currents for G601S and R752W, respectveily (n=10-15). **P*<0.05, †*P*<0.01 vs with TBA or siRNAHDAC6.

Figure 4. Levels of acetylation and ubiquitination of WT and mutant hERG-FLAG with coexpression or knockdown of HDAC6 or TBA treatment.

A-**C,** HEK293 cells expressing WThERG-FLAG, G601S-FLAG or R752W-FLAG and pEGFP were transefected with a scrambled siRNA or an siRNA against HDAC6 (n=5) (**A**), treated with 8 μM TBA (n=5-7) (**B**), or co-transfected with HDAC6-FLAG (n=6-7) (**C**). Anti-hERG and anti-acetylated lysine IPs were subjected to IB with anti-ubiquitin (ub) and anti-FLAG antibody, respectively (**A**, **B**). Anti-hERG IPs were subjected to IB with anti-ubiquitin and anti-acetylated lysine antibody, respectively(**C**).

D, Cells were transfected with pcDNA3/WThERG-FLAG, pEGFP, and either HDAC6-FLAG plasmids or pcDNA3. Cells were treated with 6 μM MG132 36 h after transfection for overnight. Shown are representative blots and the densities of 135-KDa hERG normalized to those in the absence of HDAC6 and MG132 (n=6). †P<0.01 vs HDAC6 (-)

and MG132 (-).

Figure 5. Effects of HDAC6 inhibition and coexpression on degradation of WT and triple lysine mutant (3K) hERG-FLAG.

A, HEK293 cells transiently expressing WT or 3K mutant hERG-FLAG were transfected with pcDNA3 (V), HDAC6-FLAG or an siRNA against HDAC6, or were treated with 8 μM TBA. The cells were chased for the indicated time after addition of cycloheximide. Shown are the representative blots and time-dependent changes in the densities of 135-KDa bands (n=5-7). *P<0.05, †P<0.01vs hERG-FLAG with DMSO/ scrambled siRNA/pcDNA3.

B, Bar graph shows the half-lives of hERG proteins. *P<0.05, †P<0.01 vs WT with V (n=5-7).

C, Effects of the mutant HDAC6 on hERG expression. Cells were transfected with WThERG-FLAG, pEGFP, and either WT (HDAC6-FLAG), deacetylase inactive (inactive M-FLAG) or BUZ domain-eliminated (△BUZ-FLAG) HDAC6. Shown are representative blots. **I**mage densities of hERG-FLAG normalized to hERG levels in the cells transfected with pcDNA3 (n= 5, †P<0.01 vs with pcDNA3).

Figure 6. Effects of HDAC6 and TBA on 3K and 1K mutant hERG-FLAG expression.

A, B, HEK293 cells were transfected with WT, 3K or 1K (K116R, K495R or K757R) hERG-FLAG plasmids together with pEGFP and pcDNA3 or HDAC6-FLAG (**A**) (n=3), treated with 8 μM TBA for 24 h (**B**) (n=4). Shown are representative blots. Image densities of hERG proteins normalized to those of β-actin and hERG in the cells transfected with pcDNA3 (**A**) or treated with DMSO (**B**).*P<0.05 vs HDAC6-FLAG (-)/ TBA (-).

C, HEK293 cells were transiently expressed 3K or 1K (K116R, K495R or K757R) hERG-FLAG. Anti-acetylated lysine IPs were subjected to IB with anti-FLAG antibody (n=3).

Figure 7. Effects of TBA and coexpression or knockdown of HDCA6 on 3K hERG-FLAG expression, acetylation and ubiquitination.

A-**C,** HEK293 cells were transfected with WT or 3K hERG-FLAG together with an siRNA against HDAC6 (**A**) (n=7) or HDAC6-FLAG (**C**) (n=5), or were treated with 8 μM TBA (**B**) (n=5). Cell extracts were subjected to IB against indicated antibodies. Anti-hERG IPs were subjected to IB with anti-ubiquitin and anti-acetylated lysine antibody (n=5-7).

Figure 8. Effects of HDAC6 inhibition on ERG expression, I_{Kr} and APD.

A, HL-1 cells were treated with TBA (8 μM), TSA (0.1 μM) or DMSO (V) for 24 h. The cell lysates were subjected to IB with indicated antibodies. Densities of bands were quantified and normalized to those in cells treated with DMSO (V) (n=5). *P<0.05, †P<0.01 vs V.

B, Effects of TBA on I_{kr} . Whole-cell membrane currents were recorded from HL-1 cells treated with 8 μM TBA or vehicle in absence and presence of E4031 (10 μM). E4031-sensitive currents were obtained by digital subtraction. Current-voltage relationships of peak currents during depolarizing pulses and tail currents during repolarizing pulses are shown at the bottom (n=15-19).*$P<0.05$, †P<0.01 vs none.

C, Effects of TBA on APD in HL-1 cells.

APs were recorded 24 h after TBA or vehicle (V) treatment in absence and presence of 1 μM E-4031. APD_{90} values are summarized as a bar graph. †P<0.01 vs V.