Peer Review File

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Reviewer A

This is an experimental research on the role of 4-PBA in the pathway between ERS and ERAD-related gene E3 ubiquitin ligase HRD1.

1) First, the title needs to indicate the experimental research design.

Response: Thank you for pointing out this issue. Title has been modified in this revision.

Changes in the text: Title: 4-phenylbutyric acid re-trafficking hERG/G572R channel protein by modulating the endoplasmic reticulum stress-associated chaperones and endoplasmic reticulum-associated degradation gene

2) Second, the abstract needs further revisions. The background did not accurately indicate the research question and why the authors hypothesized the role of 4-PBA. The methods need to specify the research questions to be answered by these procedures. The results need to quantify the findings by reporting statistics such as expression levels and accurate *P* values. The conclusion needs more detailed comments for the possible clinical implications of the findings.

Response: Abstract, background, methods，results and conclusion have been revised according to the opinions of reviewers.

Changes in the text:

Abstract: Meanwhile，many in vivo and in vitro studies have reported the efficacy of 4-PBA in diseases with inherited genetic mutations.

Background：Please visit the fourth and fifth paragraphs of the Introduction section of the manuscript.

Methods：

1) Antibodies and drugs: The aim was to prepare the reagents and drugs needed for the experiment；

2) Plasmid construction, cDNA cloning, and cell culture: The aim was to construct WT wild, hERG/G572R mutant, and WT/G572R heterozygous HEK293 cell models；

3) Whole-cell patch-clamp electrophysiological recordings: Whole-cell patch-clamp electrophysiological recordings detected the channel current（IKr/IhERG） of hERG (WT/G572R) before and after 4-PBA administration；

4) Western blot (WB): WB assay detected the protein expression of hERG (hERG/G572R、WT/G572R) before and after 4-PBA administration；

5) Quantitative real-time polymerase chain reaction (qPCR): qPCR assay detected the mRNA expression of hERG (WT/G572R) before and after 4-PBA administration；

6) Co-immunoprecipitation (co-IP): CO-IP assay was used to discover the interaction of hERG (WT/G572R) with ER molecular chaperone GRP78, GRP94, and ERAD genes HRD1.

Results：

1）In addition, the results suggested that the hERG (WT/G572R) tail current was substantially increased after drug treatment (17.201 vs. 23.133 in 0 mV, P=0.0070; 19.428 vs. 26.647 in 10 mV, P=0.0003; 20.061 vs. 27.375, in 20 mV, P<0.0001; Figure 2B,2C);

2).....（0.2873 vs. 0.7807，P＝0.0269）.....（0.5597 vs. 1.345， P＝0.0011）.....（0.01662

vs. 2.376， $P<0.0001$）.....

3) As shown in Figure 4A-4D, in the heterozygous WT/G572R cell line, ATF6 （0.8160 vs. 0.5268， ns）, GRP78 （1.081 vs. 0.8079， $P=0.0373$）, and GRP94 （1.232 vs. 0.9250， $P=0.0144$） also decreased at the protein level after applying 5 mM 4-PBA. As shown in Figure 4E-4I, compared with the wild WT, the heterozygous WT/G572R resulted in an mRNA up-regulation of ATF6 （0.01248 vs. 0.05161， $P<0.0001$； 0.05161 vs. 0.02098， $P<0.0001$）, GRP78 （0.06956 vs. 0.3444， $P<0.0001$； 0.3444 vs. 0.1684， $P=0.0034$）, GRP94 （0.1227 vs. 0.2589， $P=0.0397$； 0.2589 vs. 0.1808，ns）, CRT (0.1149 vs. 0.2125，ns； 0.2125 vs. 0.1635，ns) and CNX (0.05440 vs. 0.1569， $P<0.0001$； 0.1569 vs. 0.1338，ns), all of which were down-regulated after treatment with 5 mM 4-PBA for 24 hours.

4) As shown in Figure 5A-5C, heterozygous WT/G572R of Mendelian genetic disease alleles caused a significant increase in E3 ubiquitin ligase HRD1 compared with wild WT, and HRD1 decreased at both mRNA （0.001685 vs. 0.0008124， $P=0.0001$） and protein （1.264 vs. 0.9836， $P=0.0062$） levels after 4-PBA treatment.

Conclusion: 4-PBA is expected to be developed as a novel therapeutic method for LQT2 （hERG/G572R）， but more large-scale multicenter trials are needed to verify it. With the deepening of research, further understanding of 4-PBA will provide new ideas for treating LQT2.

3) Third, the introduction of the main text needs to specify the research hypotheses of this study and how the findings have the potential to improve the clinical management of LQTS.

Response: Thank you for pointing out this issue. We have corrected all in this revision.

Changes in the text: In light of the mechanism of ERS caused by hERG mutant proteins and the promising therapeutic effects of the small molecule 4-PBA, in this study, We hypotheses hERG/G572R is capable of being regulated by 4-PBA to re-transport the mutant protein and used experimental analysis to investigate the influence of transport-deficient hERG/G572R mutant protein on ERS-related pathways and ERAD gene.

4) Fourth, at the beginning of the methodology in the main text, the authors need to have an overview of the experimental procedures and the underlying questions to be answered. In statistics, please ensure $P<0.05$ is two-sided.

Response: Thank you for pointing out this issue. We have corrected all in this revision. Meanwhile, we ensure $P<0.05$ is two-sided in statistics.

Changes in the text: In the section of overview of the experimental procedures and statistics

5) Finally, please consider to review cite this relevant paper: Zhang G, Wang B, Cheng S, Fan H, Liu S, Zhou B, Liu W, Liang R, Tang Y, Zhang Y. KDELR2 knockdown synergizes with temozolomide to induce glioma cell apoptosis through the CHOP and JNK/p38 pathways. Transl Cancer Res 2021;10(7):3491-3506. doi: 10.21037/tcr-21-869.

Response: Thank you for pointing out this issue. We have corrected all in this revision.

Changes in the text: 20. Zhang, G., et al., KDELR2 knockdown synergizes with temozolomide to induce glioma cell apoptosis through the CHOP and JNK/p38 pathways. Transl Cancer Res, 2021. 10(7): p. 3491-3506.

Reviewer B

The paper titled “4-PBA re-trafficking hERG/G572R channel protein by modulating the endoplasmic reticulum stress-associated chaperones” is interesting. 4-PBA corrects hERG channel transport defects by inhibiting excessive ERS and the endoplasmic reticulum-

associated degradation (ERAD)-related gene E3 ubiquitin ligase HRD1. Additionally, 4-PBA improved hERG/G572R channel current. 4-PBA is expected to be developed as a new treatment method for LQT2. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) How to evaluate the role of the ubiquitin proteasome pathway in the degradation of mutant hERG proteins? How to apply it to this study? Suggest adding relevant content.

Response: Thank you for pointing out this issue. As shown in Figure 1 in the introduction to this article, many studies have reported that mutations cause endoplasmic reticulum stress（ERS）and that endoplasmic reticulum stress（ERS）can induce the endoplasmic reticulum-associated degradation (ERAD) pathway, and HRD1 is one of the main pathways for the degradation of misfolded endoplasmic reticulum (ER) mutation proteins in eukaryotic cells. And ER is a quality control (ERQC) system, and misfolded mutant proteins are retained in the endoplasmic reticulum, degraded and eliminated through the ERAD pathway. In the meantime，in Figure 1 and Table 3 of this article, it can be seen that ERAD includes the ubiquitin-proteasome pathway (UPP) and autophagy-lysosomal pathway (ALP）.

Changes in the text:

1）hERG protein misfolding and hERG protein unfolding after gene mutation (G572R) causes an ERS response.

2）ERS maintains homeostasis in the cell, ERAD degrades excess unfolded proteins, and E3 ubiquitin ligase HRD1 is one of the critical genes in ERAD.

3）ER can sense certain stimuli inside and outside the cell, and misfolded, unfolded, and unassembled proteins can be degraded through the ERAD pathway, which is one of the critical mechanisms for maintaining homeostasis. ERAD includes the ubiquitin-proteasome pathway (UPP) and autophagy-lysosomal pathway (ALP). UPP includes ubiquitin, E1 ubiquitin-activating enzyme (UBA), E2 ubiquitin-conjugating enzyme (UBC), E3 ubiquitin-ligating enzymes, 26S proteasomes, and deubiquitinating enzymes (DUBs).

2) It is recommended to perform double immunofluorescence protein tagging to examine the cellular trafficking of mutant subunits.

Response: Thank you for your advice. Unfortunately, due to time and personnel limitations, double immunofluorescence protein tagging was not conducted in this experiment.

Changes in the text: no changes in the text

3) What is the author's next research plan? It is recommended to add relevant content to the discussion.

Response: In the discussion section of this paper, three potential major therapeutic pathways for hERG/G572R are written, namely molecular chaperone, ERAD, and apoptosis pathway. For details on these three pathways, please refer to Table 2, Table 3, and Table 4.

Changes in the text: Please see the entire discussion section of the manuscript.

4) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Development and validation of endoplasmic reticulum stress-related eight-gene signature for predicting the overall survival of lung adenocarcinoma, Transl Cancer Res, PMID: 35966313”. It is recommended to quote the article.

Response: Thank you for pointing out this issue. We have cited it in this revision.

Changes in the text: 21. Lin, L. and W. Zhang, Development and validation of endoplasmic reticulum stress-related eight-gene signature for predicting the overall survival of lung adenocarcinoma. Transl Cancer Res, 2022. 11(7): p. 1909-1924.

5) What is the potential contribution of post-ER quality control pathways to the disease phenotype? Suggest adding relevant content.

Response: Thank you for pointing out this issue. The content of endoplasmic reticulum quality control (ERQC) pathway has been added to the second and third paragraphs of the background section of this revised manuscript.

Changes in the text:

1）Moreover, misfolded, incompletely folded, and improperly assembled mutant proteins are recognized by ER quality control pathway and cause ERS response.

2）When hERG mutations are co-expressed with WT subunits to form heterotetramers, the dramatic improvement [PD > Per-Arnt-Sim domain (PASD) > C-linker/cyclic nucleotide-binding homology domain (CNBHD)] pharmacology (E4031) correction to escape ER quality control systems [such as ER-associated degradation (ERAD)] is now understood to be more common than previously thought. Most of the transport-deficient but correctable mutations are located in the PD between S5 and S6 (3).

6) How can the results of this study help to develop therapeutic strategies against LQT2? It is recommended to add relevant content.

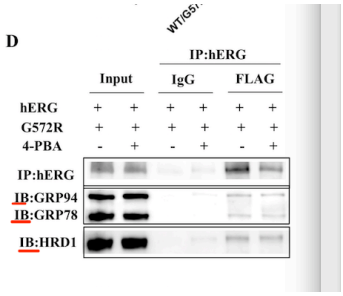
Response: The hERG/G572R mutant protein was divided into 135 KDa immature protein and 155 KDa mature protein. The 155 KDa mature protein was a functional and normally transported membrane channel protein. After 5mM 4-PBA treatment, the mature 155 KDa protein of hERG/G572R was increased, accompanied by the recovery of potassium channel current (IKr/I_{hERG}). This section has been added to the conclusion of the revised manuscript.

Changes in the text: Our work provides preliminary evidence that 4-PBA corrects hERG channel transport defects by inhibiting excessive ERS and ERAD-related gene E3 ubiquitin ligase HRD1. Additionally, 4-PBA increasedhERG/G572R mature functional channel protein and improved channel current. 4-PBA is expected to be developed as a new treatment method for LQT2（hERG/G572R）. but more large-scale multicenter trials are needed to verify it. With the deepening of research, further understanding of 4-PBA will provide new ideas for treating LQT2.

Reviewer C

1. Figure 5D

Please check whether “IB” and “IP” are correct.



732 expression in WT, WT/G572R, and WT/G572R + 5 mM 4-PBA cell lines. (B)
733 Statistical graph of HRD1 protein expression in WT, WT/G572R, and WT/G572R + 5
734 mM 4-PBA cell lines. (C) Statistical analysis of HRD1 mRNA expression in WT,
735 WT/G572R and WT/G572R + 5 mM 4-PBA cell lines. (D) Co-IP of GRP78, GRP94,
736 HRD1, and hERG (WT/G572R)-FLAG channel. WT vs. WT/G572R; WT/G572R vs.
737 WT/G572R + 4-PBA. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. hERG,
738 human ether-à-go-go-related gene; 4-PBA, 4-phenylbutyric acid; HRD1,
739 3-hydroxy-3-methylglutaryl coenzyme A reductase degradation protein 1; WT, wild
740 type; WB, western blot; IB, immunoblotting; co-IP, co-immunoprecipitation; mRNA,
741 messenger RNA.

Response: Thank you for pointing out this issue. It has been revised in the revised manuscript.

2. The citation of Ref. 15 in the main text was missing. Please indicate where you would like to cite Ref. 15 in the main text.

Response: Ref. 15 is quoted on the far right of the line in Table 2 with content of $Hsp100_S$ (100KDa).

3. The authors mentioned "studies...", while only one reference was cited. Change "Studies" to "A study" or add more citations. Please revise. Please number references consecutively in the order in which they are first mentioned in the text.

Studies have shown that 90% of hERG mutations inhibit kv11.1 transport from the endoplasmic reticulum (ER) to Golgi and cell surface membrane by destroying subunit folding and assembly (1).

Previous studies have reported that chemical and pharmacological chaperones, such as TUDCA, reduced degradation and/or promoted plasma-membrane localization of defective subunits in cyclic nucleotide-gated (CNG) channel mutations (13).

Response: It has been revised in the revised manuscript.