Investigating The Impact Of Homoharringtonine On K562 Cell Viability And ER Stress Pathways

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Abstract. This study investigated the effects of Homoharringtonine (HHT) on K562 cell proliferation and endoplasmic reticulum (ER) stress. The inhibitory effect of HHT was assessed using the CCK-8 assay to calculate IC_{50} values. Flow cytometry evaluated cell cycle distribution post-HHT exposure, while Proteostat dye assessed protein aggregation. Expression levels of XBP1s and related markers (BIP, CHOP, IRE1α) were measured to analyze ER stress. Results indicated that HHT significantly reduced K562 cell viability, yielding an IC50 value of 28.53 nM. HHT treatment caused cell accumulation in the G0/G1 phase, indicating cell cycle arrest. It also activated ER stress pathways, leading to increased levels of XBP1s, BIP, and CHOP. The combination of HHT with the ER stress inhibitor 4-PBA alleviated HHT-induced ER stress, enhancing its anti-tumor effects. This study demonstrates that HHT inhibits K562 cell proliferation while activating ER stress pathways, suggesting that modulating ER stress may enhance its therapeutic efficacy in myeloid leukemia. Further research is required to elucidate the underlying mechanisms.

Keywords. Homoharringtonine; Myeloid leukemia; Endoplasmic Reticulum Stress; Unfolded Protein Response.

1 Introduction

Myeloid leukemia (ML) is a malignant clonal disorder characterized by the aberrant differentiation of hematopoietic cells, resulting in uncontrolled proliferation and infiltration into the bloodstream and extramedullary tissues[1, 2]. Homoharringtonine (HHT), a natural alkaloid derived from the genus Cephalotaxus[3], has been utilized for over 30 years as a cytotoxic agent in the treatment of myelogenous leukemia[4]. HHT exerts its anti-leukemia activity by inhibiting proliferation and inducing apoptosis in leukemia cells[5, 6].

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The unfolded protein response (UPR) is an endoplasmic reticulum (ER) stress-induced signaling cascade that plays a critical role in the selection, adaptation, and survival of cancer cells[7, 8]. Increasing evidence indicates that ER stress and UPR play key roles not only in stress avoidance[9], but also in cell survival, and proliferation[10]. Persistent UPR activation has been linked to enhanced pro-survival signaling and chemotherapy resistance in leukemic cells[11, 12].

However, it remains unclear whether HHT-induced disruption of protein homeostasis can induce ER stress and how this induced ER stress relates to the anti-leukemia activity of HHT. This study aims to evaluate the efficacy of HHT against K562 cells, including its effects on cell proliferation and ER stress.

2 Materials and Methods

2.1 Cell Lines and cell culture

The human leukemia cell line K562 was obtained from the Chinese Academy of Sciences Cell Bank in Shanghai, China. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% sodium pyruvate.

2.2 Chemical reagents and antibodies

HHT (Sunny Biotech Co., Ltd, CAT#16150602, Shanghai) and 4-Phenylbutyric acid (4-PBA) (MedChemExpress, HY-A0281, USA) were both dissolved in DMSO and stored at -80 °C in the dark. GRP78 and CHOP were obtained from Affinity Biosciences, while α-tubulin was purchased from Cell Signaling Technology.

2.3 Cell Viability Assay

Cell viability was assessed using the enhanced cell counting kit-8 (CCK-8, Beyotime, Shanghai, China). K562 cells (8×104) were inoculated into a 96-well plate and treated with vehicle (DMSO) or HHT at concentrations of 5, 10, 20, 40 and 80 nM. Cells were incubated for 48 hours, and each concentration was tested in triplicate. After incubation, CCK-8 solution was added to each well and the plates were incubated for an additional 4 hours. The absorbance was measured at 450nm using a multifunctional microplate reader.

2.4 Agarose Gel ElectrophoresisII Viability Assay

Accurately weigh the agarose powder and dissolve it in 1×TAE electrophoresis buffer. Add GelRed nucleic acid stain and mix thoroughly before pouring into a gel tray. Allow the gel to solidify, then remove the comb and place the gel in the electrophoresis chamber. Load 5μL of each sample, set the voltage to 130 V, and monitor bromophenol blue migration to determine when to endpoint. Finally, visualize the gel to assess bands and compare them with a DNA ladder to determine fragment sizes.

2.5 Proteostat Aggresome Detection

The PROTEOSTAT Aggresome detection kit (ENZ-51035, ENZO, USA) was utilized according to the manufacturer's instructions. Glass coverslips were pre-treated with poly-L-lysine and placed in a 6-well plate. K562 cells were seeded at a density of 1×106 cells per well and incubated for 16 hours with HHT at various concentrations. Following the treatment, cells were imaged using a laser scanning confocal microscope with a 60x oil immersion objective.

2.6 Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from cells after drug administration using FastPure Cell/Tissue Total RNA Isolation Kit V2 (7E2712C4, Vazyme, Nanjing). The relative expression levels of the gene were analyzed by the ΔΔCt method. The sequences of primers were as follows: XBP1: forward 5'-TTACGAGAGAAAACTCATGGC-3' and reverse 5'-GGGTCCAAGTTGTCCAGAATGC-3'; XBP1s: Forward 5'-TCTGCTGAGTCCGCAGCAGG-3' and reverse 5'-CTCTAAGACTAGAGGCTTGG-3'; CHOP: forward 5'-ACCAAGGGAGAACCAGGAAACG-3' and reverse 5'-TCACCATTCGGTCAATCAGAGC-3'; BIP: forward 5'-CTGTCCAGGCTGGTGTGCTCT-3' and reverse 5'-CTTGGTAGGCACCACTGTGTTC-3' ACTB: forward 5'-TGGCACCACACCTTCTACAA-3' and reverse 5'-CCAGAGGCGTACAGGGATAG-3'.

2.7 Western blotting

K562 cells were treated with vehicle or varying concentrations of HHT for 48 hours. Cells were lysed with RIPA (01731189, EpiZyme, Shanghai) buffer containing 1 mM PMSF (WBO114-2, WELLBIO, Hunan). Proteins were separated by SDS-PAGE (PG113, EpiZyme, Shanghai) and transferred to PVDF membranes (1620177, BIO-RAD, USA). Membranes were blocked with 5% nonfat milk for 1.5 hours at 37 ° C, then incubated overnight at 4 ° C with primary antibodies. After three washes with TBST, secondary antibodies were added for 1 hour at room temperature. Protein expression was detected using an ECL kit (P0018, Beyotime, China) and analyzed with the Bio-Rad ChemiDoc MP imaging system and ImageJ software.

2.8 Statistical analysis

All the results were expressed as the mean ± SEM. Differences between two groups were analyzed using Student’s t-test and multiple comparisons test was quantified by ANOVA with pair-wise comparisons. A value of $P<0.05$ was considered to be significant. All statistical analysis was determined using GraphPad Prism 10.1.2. ImageJ 2.9.0 and Flowjo 10.8.1 were used for image analysis.

3 Results

3.1 Assessment of K562 Cell Response to Homoharringtonine

We initially performed the CCK8 assay to assess the sensitivity of K562 cells to HHT exposure. As shown in Figure 1A, treatment with HHT at concentrations ranging from 5 to 160 nM led to a significant reduction in cell viability, confirming HHT's efficacy in this

malignant leukemia cell line. The calculated half-maximal inhibitory concentration (IC_{50}) for HHT in K562 cells was determined to be 28.53 nM, indicating a strong anti-proliferative effect. Further analysis of growth curves, depicted in Figure 1B, demonstrated a time- and concentration-dependent inhibition of cell proliferation following HHT treatment, suggesting that prolonged exposure enhances the drug's effectiveness.

To elucidate the mechanisms of HHT-induced growth inhibition, we conducted flow cytometric analysis to evaluate cell cycle distribution. The results revealed a concentration-dependent accumulation of K562 cells in the G0/G1 phase post-treatment, while the percentage of cells in the G2/M phase was significantly reduced (Fig 1C and D). These findings suggest that HHT primarily exerts its anti-proliferative effects by inducing cell cycle arrest at the G0/G1 phase.

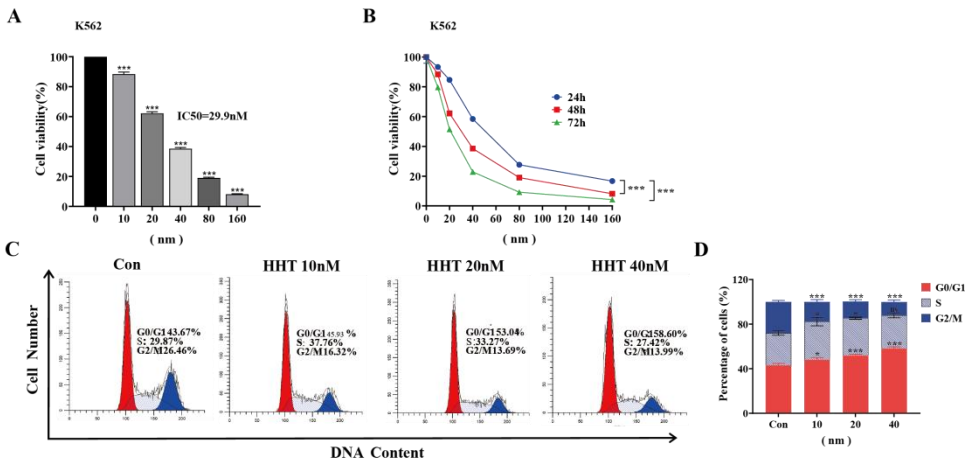

Fig. 1. HHT inhibits the proliferation of leukemia cells.

(A) The IC_{50} of HHT for K562 cells after 48 hours was determined to be 28.53 nM. (B) Cell viability in K562 cells was evaluated over time using the CCK-8 assay. (C and D) Cells treated with 0, 10, 20, and 40 nM HHT for 48 hours were stained with PI, and cell cycle distribution was analyzed by flow cytometry. Data are presented as mean ± SD (n = 3). n.s. $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Statistical significance was assessed using a two-tailed unpaired t-test or one-way ANOVA.

3.2 HHT Mediates Activation of the UPR in K562 Cells

The ER functions as a dynamic organelle with numerous essential cellular roles[13]. Under pathological conditions, insufficient nutrient availability can lead to uncontrolled protein synthesis, resulting in the aggregation of unfolded or misfolded proteins within the ER lumen and the subsequent activation of the UPR[14, 15]. In our study, we utilized Proteostat dye to detect aggregated proteins in K562 cells, revealing a significant increase in misfolded proteins compared to the control group (Figure 2A). Additionally, we found that homoharringtonine (HHT) activates XBP1, a key downstream target of the UPR. This activation results in a concentration-dependent increase in the expression of the spliced isoform XBP1s, with peak expression observed at 16 hours, followed by a decrease at 24 hours.

The UPR is initiated in response to the accumulation of unfolded or misfolded proteins in the ER lumen and involves several critical checkpoint proteins, including BIP, CHOP, IRE1α, and XBP1s[16]. Consistent with our earlier findings, qPCR results showed that the mRNA levels of these molecules increased with higher concentrations of HHT, with

expression beginning to rise at 8 hours and peaking at 16 hours (Figure 2C). Similarly, protein levels also indicated that ER stress markers increased with the dosage of HHT. Collectively, these results suggest that HHT may trigger the unfolded protein response by promoting protein aggregation, thereby activating the UPR pathway and facilitating cellular adaptation to stress conditions.

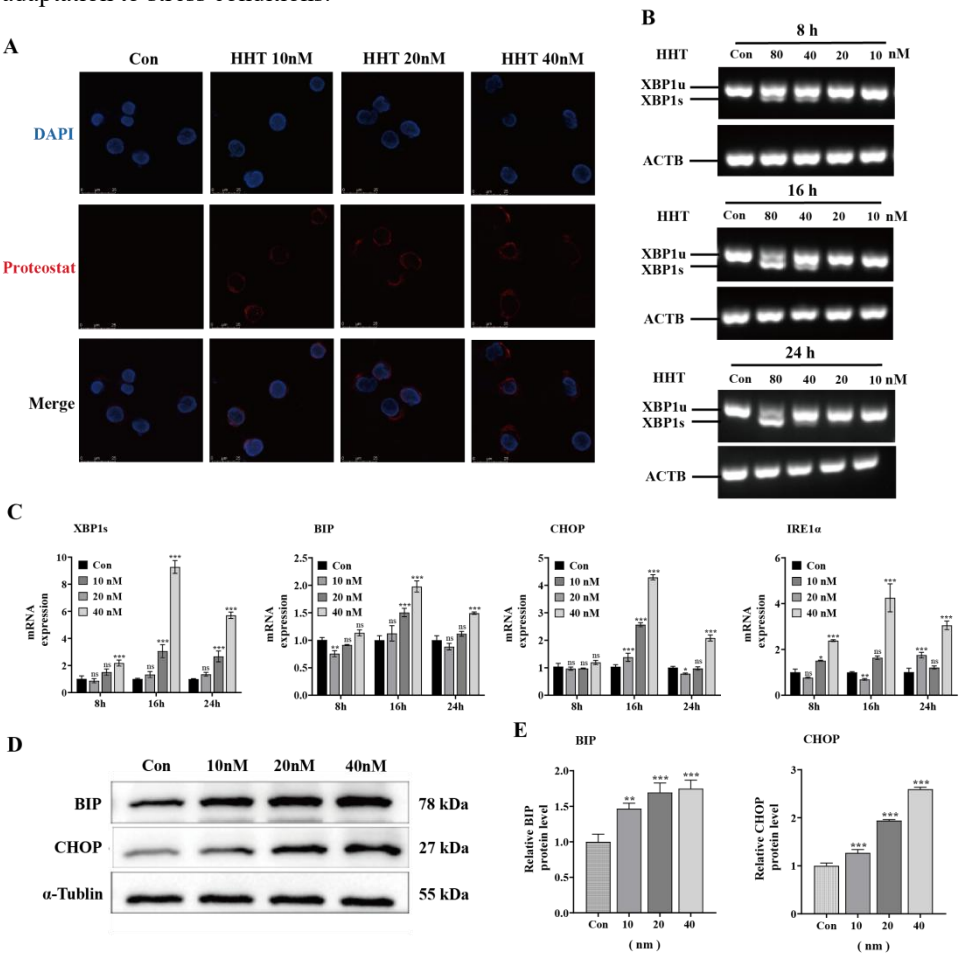

Fig 2. HHT induces ER stress in K562 cells.

(A) The expression of protein aggregates after 16 hours of HHT treatment was evaluated, with aggregates stained using Proteostat (red) and cell nuclei stained with DAPI (blue), with MG132 as a positive control. (B) XBP1s bands induced by HHT at concentrations of 0, 10, 20, 40, and 80 nM were analyzed at various time points (8 h, 12 h, and 16 h) using agarose gel electrophoresis. (C) K562 cells treated with 0, 10, 20, and 40 nM HHT for 8 h, 12 h, and 16 h had the expression levels of XBP1s, BIP, CHOP, and IRE1 quantified using real-time quantitative PCR. (D and E) Western blot analysis assessed the protein levels of BIP and CHOP after exposure to 0, 10, 20, and 40 nM HHT for 16 h. Data are presented as mean ± SD (n = 3). n.s. $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Statistical significance was assessed using a two-tailed unpaired t-test or one-way ANOVA.

3.3 Enhancement of Antitumor Efficacy of Homoharringtonine by ER Stress Inhibition in K562 Cells

ER stress and the unfolded protein response (UPR) serve as critical adaptive mechanisms in cellular stress, playing significant roles in the development of drug resistance and immune evasion[17]. In our study, we aimed to determine whether pharmacological inhibition of ER stress could enhance the antitumor efficacy of homoharringtonine (HHT) in K562 cells. To investigate this, we combined HHT with 4-PBA, a selective inhibitor of ER stress previously reported to have effects in various cancer models[18].

As illustrated in Figures 3A and 3B, the increase in protein levels of BIP and CHOP induced by HHT was significantly reversed by the addition of 4-PBA, indicating that 4-PBA effectively mitigates the ER stress response triggered by HHT. Subsequently, we treated the cells with a fixed ratio of HHT to 4-PBA (1:25000) across a range of concentrations. Notably, the co-administration of HHT and 4-PBA exhibited a significantly enhanced inhibitory effect on cell viability compared to either agent used alone (Figure 3C). Additionally, cell cycle analysis revealed that the combination of HHT and 4-PBA resulted in a more pronounced G0/G1 phase arrest compared to HHT alone, with statistically significant differences observed(Fig 3D and E). These findings suggest that the combination of HHT and 4-PBA not only alleviates ER stress but also potentiates the antitumor activity of HHT in K562 cells.

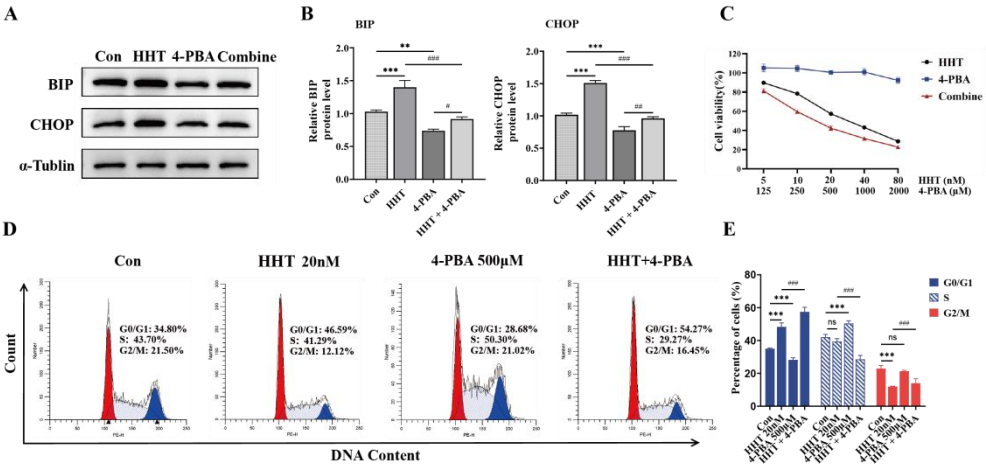

Fig 3. Effects of HHT and 4-PBA Combination on Cell Viability in K562 Cells.

(A-B) Cells were pretreated with 500 μM 4-PBA for 4 hours, then treated with 0 or 20 nM HHT for 48 hours. Western blot analysis was performed to assess changes in BIP and CHOP protein levels.(C) K562 cells were treated with various doses of HHT and 4-PBA combined at a fixed ratio, and the changes in cell viability were assessed after 48 hours of incubation. (D-E) Flow cytometry evaluated the inhibitory effects of the HHT and 4-PBA combination on K562 cell proliferation. Data are presented as mean ± SD (n = 3). n.s. $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Statistical significance was assessed using a two-tailed unpaired t-test or one-way ANOVA.

4 Discussion

HHT is a protein translation inhibitor used to treat patients with AML and CML[19] . It has received FDA approval for adult patients with chronic phase (CP) or accelerated phase (AP) CML who exhibit resistance or intolerance to two or more tyrosine kinase inhibitors (TKIs)[20]. Clinical evidence supports the efficacy of HHT in treatment-resistant CML,

particularly in cases with the BCR-ABL T315I mutation[21, 22]. Mechanistically, HHT inhibits protein synthesis by binding to a specific site on the ribosome, particularly affecting short-lived proteins such as c-myc, myeloid cell leukemia sequence 1 (Mcl-1), and cyclin D1[23]. Previous studies have reported that HHT activates ER stress-related genes, including IRE1 and PERK, in hepatocellular carcinoma cells[24]. However, the presence of a similar effect in leukemia cells remains unclear. Our findings indicate that HHT suppresses the proliferation of K562 leukemia cells primarily through cell cycle blockade. Nonetheless, further investigation is required to elucidate whether HHT impedes the translation of cyclins by inducing ER stress and to understand how this stress may influence HHT's anti-leukemic activity.

ER stress is crucial for maintaining cellular homeostasis through adaptive responses, particularly via the UPR[25]. During ER stress, multiple signaling pathways, including CHOP and XBP1, are activated, which are essential for cell survival[26, 27]. Targeting ER stress and the UPR pathway may disrupt the stress adaptation mechanisms of cancer cells[28]. ER stress dictates cell fate depending on context and signal strength. Prolonged and severe pharmacological ER stress can initiate caspase-mediated cell death through IRE1 α - and PERK-dependent pathways[29]. Specifically, IRE1α-mediated activation of JNK inhibits the anti-apoptotic protein BCL-2 while enhancing the pro-apoptotic protein BIM, thereby promoting cell death[30]. Concurrently, mammalian cells have evolved multiple adaptive mechanisms to limit pro-apoptotic UPR outputs[31]. For instance, mouse embryonic fibroblasts (MEFs) subjected to sustained low-grade pharmacological ER stress exhibit resilience to subsequent ER insults, likely attributable to increased stability of pro-survival BIP mRNA and decreased stability of pro-apoptotic CHOP mRNA[32]. If cells can effectively manage pro-apoptotic UPR outputs, ER stress may confer survival advantages during tumor progression[33]. Our study reveals that HHT induces protein aggregation and triggers ER stress, activating key markers in the UPR pathway, such as BIP, CHOP, and the spliced variant of XBP1. However, the specific role of HHT-induced ER stress in promoting leukemia cell death versus providing a protective effect requires further investigation.

4-PBA is an FDA-approved drug used for urea cycle disorders[34]. It acts as a chemical chaperone and an ER stress inhibitor[18]. Our findings show that the combination of low concentrations of HHT with 4-PBA further inhibits DNA replication and impairs the proliferation of K562 cells, indicating that HHT-induced ER stress might confer a protective role in these cells. CHOP, a critical regulator of the ER stress response, is upregulated during prolonged ER stress and significantly promotes apoptosis in stressed cells[30]. Concurrently, GRP78, a major ER chaperone that is stress-inducible, is also upregulated in various cancers and is associated with aggressive growth, invasiveness, and therapeutic resistance[35, 36]. Under normal conditions, GRP78 is primarily localized in the ER, but during ER stress, it can undergo alternative splicing to produce a cytosolic isoform that regulates UPR signaling and promotes leukemic cell survival[37]. The interplay between CHOP and GRP78 highlights the delicate balance between cell survival and death in leukemic cells, emphasizing the need for further investigation into how these pathways interact with HHT and 4-PBA in modulating cell fate.

Therefore, we will conduct in vitro experiments to rigorously validate the inhibitory effects of the HHT and 4-PBA combination on leukemia cell proliferation, while elucidating the underlying mechanisms involved. Furthermore, we will employ animal models for in vivo studies to comprehensively assess both the therapeutic efficacy and safety of this combination, thereby facilitating the translation of our findings into potential clinical applications.

5 Conclusion

This study demonstrates that HHT inhibits the proliferation of K562 cells and activates ER stress pathways. The combination of HHT with 4-PBA, an inhibitor that mitigates induced ER stress, enhances the effects of HHT. Our results suggest that targeting ER stress and the UPR pathway may offer a novel strategy to improve the therapeutic efficacy of HHT in the treatment of myeloid leukemia.

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