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Original Article

Homoharringtonine enhances idarubicin-induced apoptosis via regulation of the mitochondrial pathway

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Abstract: Objective: The detailed mechanism of the efficacy of Homoharringtonine (HHT) in anti-leukemia remains unclear. This study is to investigate the synergistic effects and mechanism of HHT when combined with Idarubicin (IDA), on K562 cells derived from the blast crisis of CML. Method: K562 and HUVEC cell lines were maintained and treated with IDA and HHT alone or in combination, cell viability assay and flow cytometry analysis were performed to detect the synergistic effects. The expression of active-caspase-3, PARP-1 and cleaved-PARP-1 in K562 cells were detected by Western blotting. The production of reactive oxygen species (ROS) and depolarization of mitochondrial membrane potential (MMP) were measured by flow cytometry. Result: HHT had synergistic effects when combined with IDA to suppress leukemia growth via apoptosis. Mechanistically, HHT and IDA combination treatment promoted the production of ROS and depolarization of MMP, the expression levels of active-Caspase-3 and cleaved-PARP-1 were significantly higher than that of the HHT or IDA group. Based on both proliferation and MMP, the apoptotic effect was attenuated when pre-treated with the ROS scavenger N-acetyl-cysteine (NAC). Conclusion: Taken together, we find HHT potentiates IDA-induced apoptosis and this synergistic combination has potential as a treatment strategy for AML patients.

Keywords: Homoharringtonine, idarubicin, mitochondria pathway, K562 cells, apoptosis

Introduction

Chronic myeloid leukemia (CML) is a clonal malignant hematologic diseases, characterized by the molecular genetic alterations in hematopoietic stem cells. Disease progression is characterized in three distinct phases: chronic phase, accelerated phases and blast crisis [1]. According to studies, clonal evolution and blast crisis result from persistent activation of BCR-ABL1 and genomic instability. The later is due to oxidative stress and reactive oxygen species [2, 3] induced DNA damage in combination with impaired DNA repair [4]. Although tyrosine kinase inhibitor (TKI) imatinib has significantly improved the prognosis and survival in CML patients [5], targeting the blast crisis of CML remains a challenge as patients have low remission and high fatality rates. There are currently few strategies for patients that lack response to TKI treatment that results from clonal progression and drug resistance [6], thus identifying additional treatment options would greatly benefit this patient population.

Homoharringtonine (HHT) is a plant cephalotaxine alkaloid that drives anti-leukemia activity by reducing protein synthesis and promoting apoptosis [7]. While HHT exhibits anti-leukemic activity in the treatment of CML, AML and MDS [8, 9], in combination with cytarabine and granulocyte colony-stimulating factor (G-CSF) (HAG regimen), or with aclarubicin and cytarabine (HAA regimen), it also enhances drug sensitivity in multidrug resistance tumor cells [10, 11]. HHT has relatively mild extra-medullary and no anthracycline-like cardiac toxicities, which makes this an ideal treatment for elderly patients [12]. Additionally, some studies demonstrate HHT-sensitivity is mechanistically distinct to other anti-leukemic agents, while synergy is observed with other chemotherapeutic drugs

as well [12-15]. However, due to its myelosuppression effects, the use of HTT has been limited [16, 17]. Idarubicin (IDA) is a highly effective anthracycline antineoplastic derivative of daunorubicin (DNR). It is widely used to treat haematological malignancies and has shown less toxicity and higher intracellular uptake relative to DNR [18, 19]. This makes IDA more suitable in patient care relative to DNR, as it may promote complete remission (CR) in subsets of patients [20]. However, cumulative dose-related cardiac damage [21, 22] can occur post IDA-administration, and this is thought to result from generation of reactive oxygen species (ROS).

In this study, we investigated the synergistic potential of HHT and IDA to limit the side effects while maintaining efficacy in treating CML blast crisis derived K562 cells. Our findings suggest clinical investigation of these as a combined chemotherapeutic strategy.

Materials and methods

Cell cultures

K562 and HUVEC cell lines were obtained from Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco, NY, USA). Cells were incubated at 37°C, 5% CO_2 incubator (SANYO, Japan). IDA (zadevos) (Pfizer, NY, USA) was dissolved in physiological saline as a 100 mM stock solution and was stored in aliquots at -20°C. Homoharringtonine was provided by Minsheng Pharmaceutical (Hangzhou, China).

Cell viability assay

K562 and HUVEC cells were seeded on 96-wells plate and incubated with different concentrations of IDA and HHT alone or in combination for 24 h. These respective cells were next incubated with 0.5 mg/ml 3 (4, 5 dimethylthiazol 2 yl) 2, 5 diphenyltetrazolium bromide (MTT; Sigma Aldrich) solution for 4 h. The supernatant was centrifuged at 1000 rpm and discarded. The formazan blue was dissolved in 150 µl DMSO. Optical density was measured at 490 nm with a microplate reader (Rayto. ShenZhen, China) and relative proliferation was determined at percent viability, with vehicle treated cells as 100% viable. IC50 values of the drug for each group were identified utilizing GraphPad Prism 6 software after generating a dose response curve.

Flow cytometry analysis

Apoptosis was analyzed by flow cytometry (Beckman Coulter, USA) using PE Annexin-V Apoptosis Detection Kit (BD Biosciences, CA, USA) according to manufacturer's instructions. Briefly, K562 cells were seeded on 6-wells plate (10^6 cells/well) and treated with only HHT (0.14, 1.4 and 14 ng/ml), IDA (0.2, 0.1, and 0.04 ng/ml) for 6 h or HHT (0.14 ng/ml) combined with IDA (0.02 ng/ml) for 24 h, respectively. Untreated cells served as controls. The cells were collected and washed once with 1× PBS and suspended in 100 µl 1× binding buffer. The suspended cells were then stained with 5 µl PE conjugated annexin V antibody and 5 µl 7-AAD in 1× annexin-binding buffer followed by incubation in dark for 15 min. Cells were then washed in 1× annexin binding buffer and resuspended in 500 µL binding buffer prior to flow cytometry analysis.

Western blot analysis

K562 cells were seeded on 6-wells plate (10^6 cells/well) and treated with only HHT (0.14 ng/ml), IDA (0.2 ng/ml) for 6 h or HHT (0.14 ng/ml) combined with IDA (0.2 ng/ml) for 12 h, Caspase inhibitors (Z-VAD-FMK, Ac-DEVD-FMK and Z-LETD-FMK) were added in advance for 2 h in the groups that received combination treatments. Cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-Cl (pH 7.5), and 150 mM NaCl) in the presence of 1 mM PMSF and phosphatase inhibitor (Cocktail). Cell lysates were subjected to SDS/polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane using a semi-dry transfer apparatus (Bio-Rad, CA, USA). Membranes were incubated with specific antibodies (Abcam, USA) diluted in 5% skimmed milk and 10 mM PBS, 0.05% Tween 20 (PBST) buffer at 4°C overnight with continuous shaking. Signals were visualized using X explorer. β-actin was used as a loading control.

Measurement of intracellular ROS production

The production of ROS in K562 cells was measured using ROS Analysis Kit (KeyGEN, Nanjing,

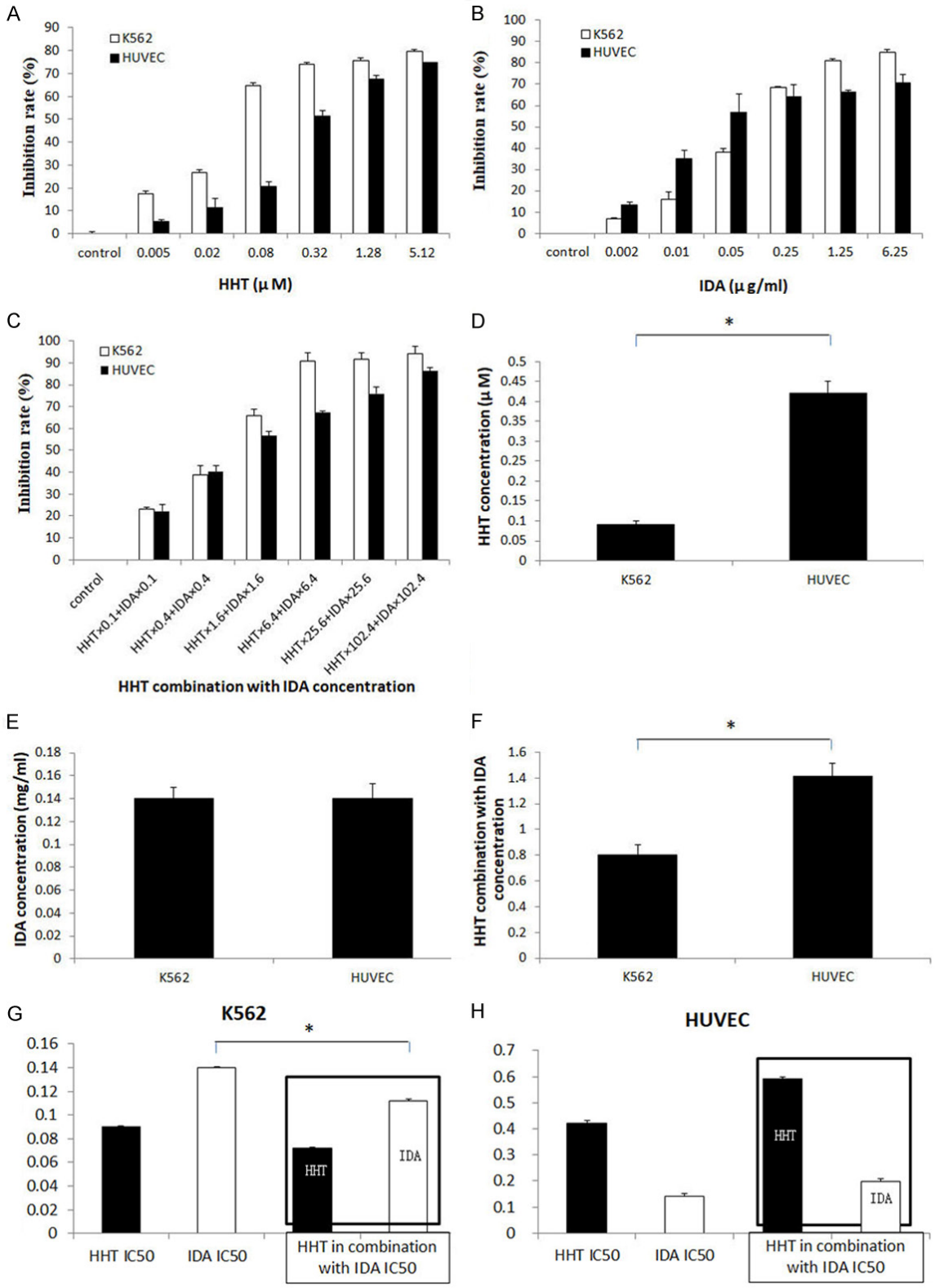

Figure 1. HHT and IDA combinations differentially inhibit proliferation of K562 and HUVEC cells. The inhibitory effects (A-C) and IC50 values (D-H) of HHT and IDA combinations on K562 and HUVEC cells were evaluated by MTT assay. Individual or combination HHT and IDA treatments consisted of treatments at their respective IC50 concentrations. Cell inhibition rate was expressed as the percentage of untreated control. Values are expressed as means ± SD, n = 3. *P<0.05.

China) according to manufacturer's instructions. K562 cells were cultured in the 6-wells plate (10^6 cells/ml), and pre-treated with N-acetyl-cysteine (NAC) for 1 h followed by treatment with HHT or IDA at IC50 concentrations or co-treated with both for 12 h. The fluorescent probe H2DCFDA was diluted with PBS at the final concentration of 10 mM. The assays were conducted in a 1 mL mixture with an equal volume of H2DCFDA staining solution and cell suspension, and then incubated at 37°C, 5% CO_2 incubator for 60 min. The cell mixtures were washed twice with preheated RPMI-1640, and then incubated at 37°C, 5% CO_2 for 30 min. The assays were measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm by flow cytometry.

Mitochondrial membrane potential analysis

The Mitochondrial membrane potential in K562 cells was measured using Fluorochrome Rhodamine-123 (KeyGEN, Nanjing, China) according to manufacturer's instructions. K562 cells were first cultured in six-well plates (10^6 cells/mL), after 1 h of pre-treatment with NAC, they were treated with HHT, IDA at IC50 concentrations alone or both for 12 h. Cells were subsequently harvested and then incubated with 1 mL Rhodamine-123 (10 μg/mL) staining solution at 37°C, 5% CO_2 for 30 min, then washed twice in RPMI-1640. Cells were resuspended in RPMI-1640 and incubated at 37°C, 5% CO_2 for 60 min. The assays were measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm by flow cytometry.

Statistical analysis

For statistical analysis, all data were expressed as means ± SD and were representative of triplicate samples. Statistics were compared using Graphpad Prism 6 software. Normally distributed groups were compared by Paired t-test and one-way ANOVA. $P<0.05$ * was considered statistically significant.

Results

Combined IDA and HHT treatment significantly reduced viability of CML cells

To determine the potential synergy of HHT and IDA treatments against CML, we examined response of the K562 cells to individual and combined treatments relative to a normal human cell lines (HUVEC). We utilized MTT assays to determine relative viability in each case. Results demonstrate that both HHT and IDA suppressed proliferation of K562 and HUVEC cells in a dose-dependent manner (**Figure 1A** and **1B**). K562 cells were more sensitive relative to HUVEC cells in low concentrations of HHT (under 0.08 μmol/L) (**Figure 1A**). However, HUVEC cells were more sensitive to low concentrations of IDA (0.05 μg/mL) (**Figure 1B**). In K562 cells, the IC50 concentrations of HHT and IDA was 0.09 μmol/L, 0.14 μg/mL respectively as single agents (**Figure 1D** and **1E**). In comparison, cell viability assays on HUVEC cells gave IC50 concentrations of 0.42 μmol/L, 0.14 μg/ml for HHT and IDA as single agents (**Figure 1D** and **1E**). However, upon co-treatment with HHT and IDA, we observed significant reduction of the IC50 concentrations in K562 cells (**Figure 1C**, **1F** and **1G**). In contrast, this was increased in HUVEC cells (**Figure 1C**, **1F** and **1H**). Overall the combined therapy improved our ability to target CML cells but not normal cells.

IDA and HHT promote apoptosis

Anthracycline can induce severe DNA damage that directly causes apoptosis without activation of DNA repair pathways. PE-AnnexinV/7-AAD double staining confirmed that even in higher concentrations (10 and 100-fold of IC50), HHT modestly induced apoptosis of K562 cells (**Figure 2A**). We also observe that treatment with IDA at IC50 or 2-fold diluted concentrations significantly increased late apoptosis (**Figure 2B**). However, when treated with 10-fold dilution IDA, we observed that cells largely resided in early phase apoptosis and had low rates of late phase apoptosis. However, when this concentration of IDA was supplemented with IC50 concentrations of HHT, we observed increased rates of late phase apoptosis ($P<0.05$) (**Figure 2C**). Our results indicate that HHT significantly enhances apoptosis in low doses of IDA.

HHT and IDA treatment promotes Caspase-3 activation and cleavage of PARP-1

To evaluate the signaling pathways involved in apoptosis, we next examined key apoptotic markers upon treatment with HHT and IDA. As a DNA damage sensor, Poly-ADP-ribose polymerase-1 (PARP-1) plays important roles in DNA repair, and can be cleaved by caspases in response to DNA damage induced apoptosis.

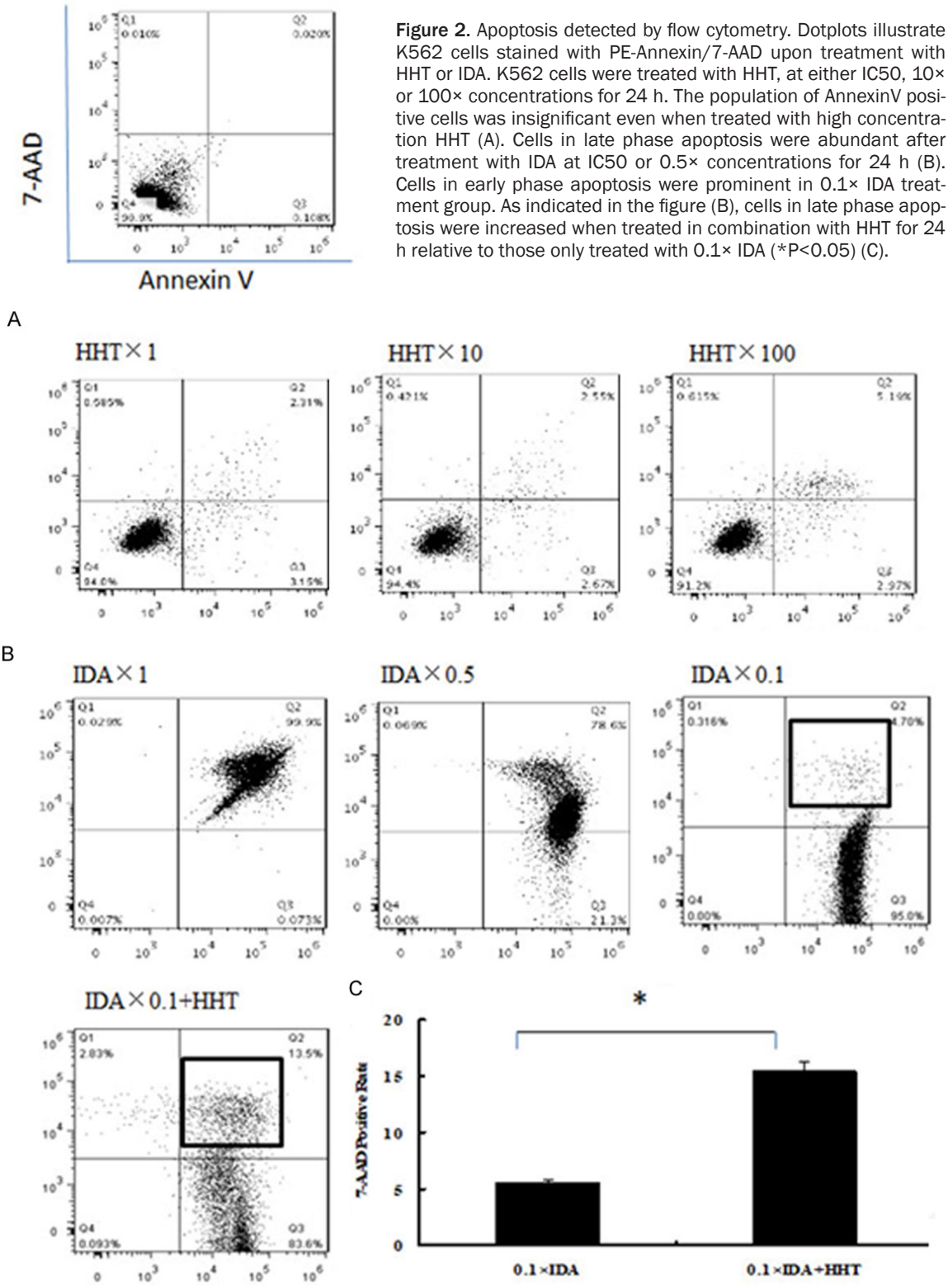

Figure 2. Apoptosis detected by flow cytometry. Dotplots illustrate K562 cells stained with PE-Annexin/7-AAD upon treatment with HHT or IDA. K562 cells were treated with HHT, at either IC50, 10× or 100× concentrations for 24 h. The population of AnnexinV positive cells was insignificant even when treated with high concentration HHT (A). Cells in late phase apoptosis were abundant after treatment with IDA at IC50 or 0.5× concentrations for 24 h (B). Cells in early phase apoptosis were prominent in 0.1× IDA treatment group. As indicated in the figure (B), cells in late phase apoptosis were increased when treated in combination with HHT for 24 h relative to those only treated with 0.1× IDA (*P<0.05) (C).

As showed in **Figure 3A**, we noticed that upon using immuno-blots to analyze expression of active-caspase-3, PARP-1 and cleaved-PARP-1 that 12 h treatment of HHT or IDA led to significantly higher levels of active-caspase-3 relative to control group. In each case, the expression level of un-cleaved PARP-1 was diminished as we observed more cleaved-PARP-1. When cells were treated with combinations, the levels of actived-caspase-3 and cleaved-PARP-1 were

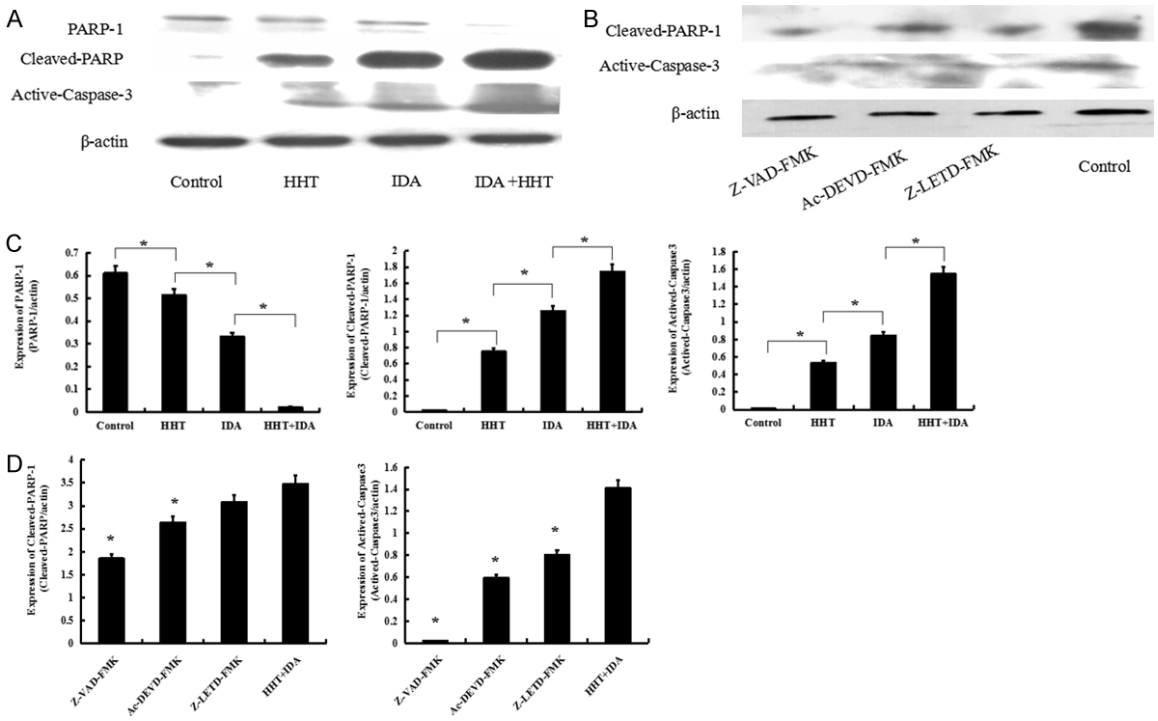

Figure 3. Treatment with HHT and IDA activated Caspase-3 activity and led to cleavage of PARP-1. K562 cells were treated with HHT and IDA in IC50 concentration for 12 hours, and total protein extracted was used for immuno-blot analysis. Actived-Caspase-3 and Cleaved-PARP-1 (A and C) were up-regulated in response to HHT and IDA treatment, and the expression levels were further increased in the combination group. Prior to HHT combined with IDA treatment, pan-Caspase inhibitor (Z-VAD-FMK), Caspase-3 inhibitor (Ac-DEVD-FMK) or Caspase-8 inhibitor (Z-LETD-FMK) were added, the expression of actived-Caspase-3 and cleaved-PARP-1 (B and D) were significantly attenuated by the pre-treatment of inhibitors, (*P<0.05, compared with combination group.)

further increased relative to single drug treated groups.

To demonstrate the necessity for the Caspase-3 pathway in HHT and IDA induced apoptosis, we utilized the peptide-based pan-caspase inhibitor, Z-VAD-FMK, the Caspase-3 specific inhibitor, Ac-DEVD-FMK, as well as the Caspase-8 specific inhibitor, Z-LETD-FMK. Cells were pretreated with inhibitors, prior to the treatment of HHT and IDA. As indicated in **Figure 3B**, this blocked Caspase-3 activity and cleavage of PARP-1 relative to the combination groups. Our data indicate that IDA activates the Caspase signal pathway and HHT can enhances this apoptotic activity by cleaving Caspase-3 and PARP-1 when combined with IDA.

IDA generates intracellular ROS

Studies suggest anthracycline leads to cytotoxicity by generating reactive oxygen species (ROS), which causes damage to membranes, cellular DNA and protein. H2DCFDA fluorescent probe was used to detect the product of intracellular ROS induced by IDA or HHT in K562 cells. As showed in **Figure 4A** and **4B**, HHT had small non-significant effects on ROS relative to control (P>0.05). However, ROS production was substantially increased when cells were treated with IDA or combined with HHT (P<0.05). Interestingly, the ROS level was decreased in the combination group relative to the single treatment of IDA (P<0.05). Additionally, we notice that pre-treatment of K562 cells with NAC, a ROS scavenger, significantly reduced the mean fluorescence intensity (MFI) in the IDA or combined treatment groups (P<0.05), accompanied by peak left shift in the histograms, as indicated in **Figure 4A**. The data suggested that IDA induces apoptosis by triggering the production of ROS and oxidative stress response, and administration of antioxidants or co-treatment with HHT can attenuate the accumulation of ROS.

HHT induce further depolarization of the mitochondrial membrane

To further examine the apoptosis effects associated with the HHT, we utilized Rhodamine-123

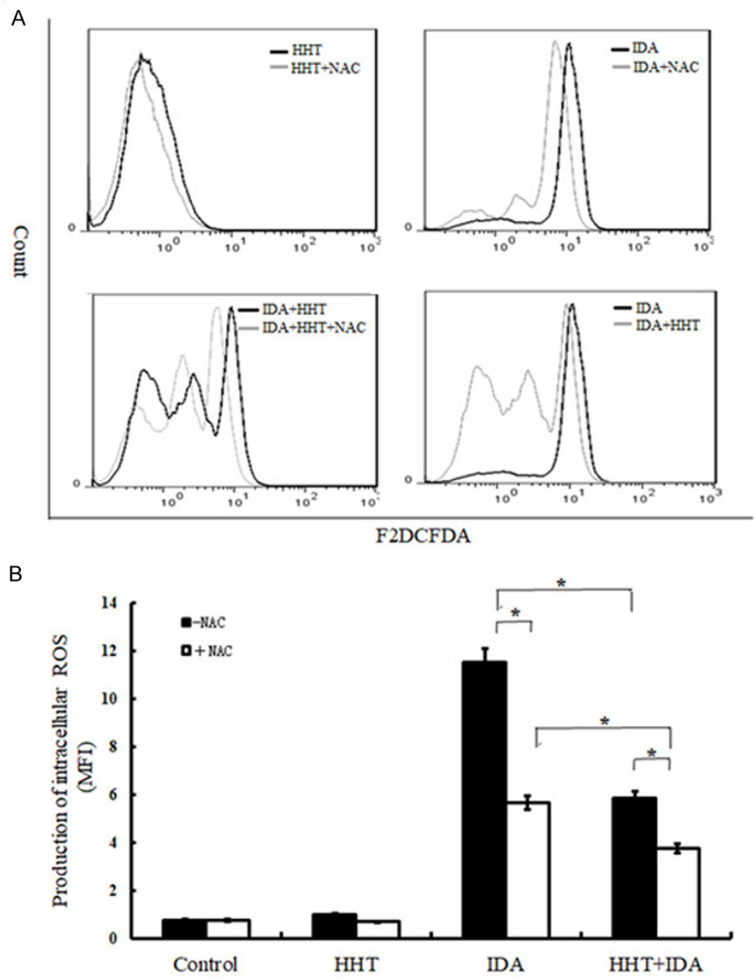

Figure 4. Drug treatment enhanced ROS production. After drug treatment, ROS levels are represented by histograms reflecting the fluorescence intensity variation. A. Histogram overlay illustrates the fluorescence intensity of K562 cells that have been exposed to HHT, IDA, HHT+IDA with or without NAC, and then stained with H2DCFDA probe. B. Mean fluorescence intensity (MFI) was significantly increased upon IDA treatment ($P<0.05$), but decreased when combined with HHT. C. MFI were significantly decreased by NAC pre-treatment relative to drug treated groups. (#$P<0.05$ or *$P<0.05$ relative to control in NAC-pretreated or untreated group, respectively).

dye to analyze the potential of mitochondrial transmembrane in K562 cells. As shown in **Figure 5A** and **5B**, significant right shifts of the MFI were observed in the distribution histogram of the NAC pretreatment groups, compared with the IDA or HHT single agent groups ($P<0.05$), but no differences were seen between the combination treatment and NAC pretreatment groups. While HHT induced further depolarization of the mitochondrial membrane relative to IDA ($P<0.05$) (**Figure 5B**), the combination treatment group promoted a slight decrease of MFI in comparison to HHT alone ($P>0.05$). Our results suggest that HHT significantly decreased the MMP, and while NAC reverses this effect induced by HHT or IDA alone, it couldn't reverse this after combined treatment (**Figure 5B**).

Discussion

HHT was first extracted in China from a Cephalotaxus alkaloid. It is now widely used in the treatment of AML and CML. HHT-based strategies have higher CR rate and patient survival relative to DA regimens in AML [23]. However, while the general toxicity is mild, there is an increased risk of death. HHT does not share cross-resistance with conventional anti-leukemic agents [13], thus it has potential as a non-toxic sensitizer for these agents.

Anthracyclines remain an important class of chemotherapeutic agents. Idarubicin (IDA) is the most successful alternative to anthracyclines [24]. The absence of the methoxy group at position 4 of IDA results in significantly enhanced lipophilicity, rapid cellular uptake, improved DNA-binding capacity and consequently greater cytotoxicity relative to other anthracyclines [25]. Unfortunately, their application in cancer treatment has been limited by cumulative dose-dependent cardiotoxicity as well as other complications.

The human CML cell line (K562) contains a reciprocal translocation between chromosomes 9 and 22 (9;22) (q34;q11) that results in a bcr/abl fusion gene and fusion protein with constitutively active tyrosine kinase activity [26-28]. In this study, we examined its susceptibility to IDA with or without HHT. We first demonstrate that HHT exhibited growth suppressive effects on K562 and normal HUVEC cells in a dose-dependent manner (**Figure 1A**). Interestingly,

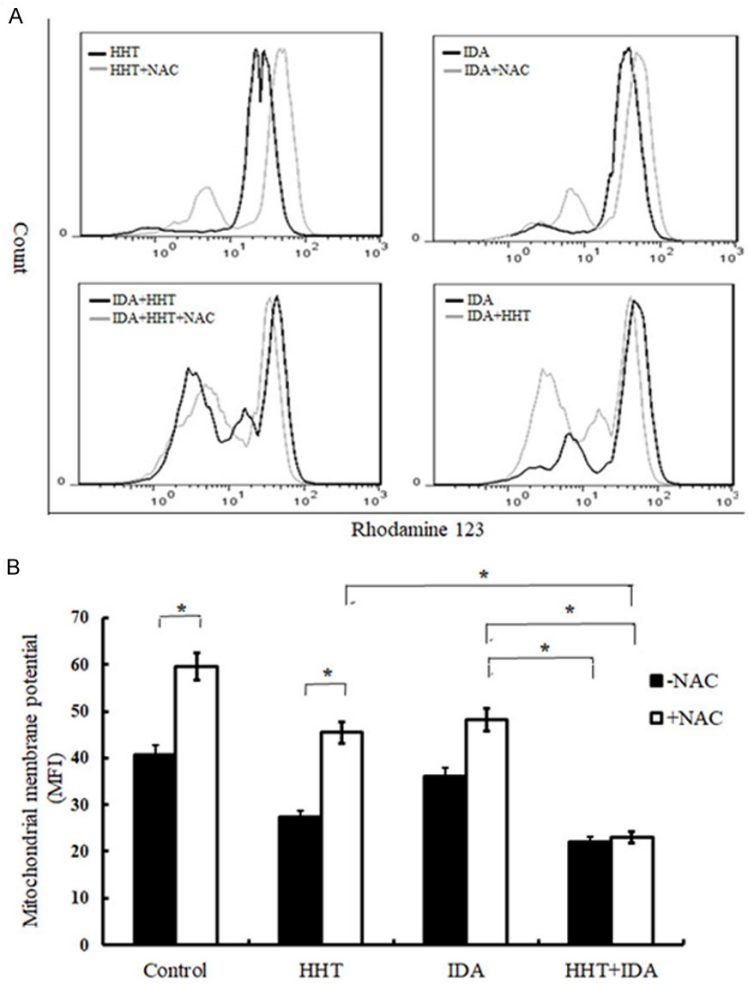

Figure 5. Treatments regulate mitochondrial transmembrane potential. Histograms reflecting the fluorescence intensity variation are shown for Mitochondrial transmembrane potential (MMP) of K562 cells. A. Histogram overlays illustrate the fluorescence intensity of K562 cells, exposed to HHT, IDA, HHT+IDA with or without NAC, and then stained with Rhodamine 123, as indicated in the figure. B. Mitochondrial transmembrane potential was significantly reduced by HHT treatment, or combinations with IDA ($P<0.05$). NAC was unable to regulate the depolarization of mitochondrial transmembrane potential in the combination group. (#$P<0.05$ or *$P<0.05$ relative to control in NAC-pretreated or untreated group, respectively).

the IC50 value was decreased in K562 cells after combined treatment of IDA with HHT (**Figure 1G**). By contrast, this was increased in normal HUVEC cells (**Figure 1H**). This cancer cell-specific cytotoxicity indicates that the combination treatment potentiates the anti-leukemic effects of IDA, and even improves the IDA tolerance in normal cells (**Figure 1F**). To further support this finding, we also found that HHT in combination with IDA effectively reduced IDA doses as shown in **Figure 1G**, which would reduce treatment related side effects in patients.

While we demonstrate a synergistic effect on apoptosis, the detailed molecular mechanisms still needs to be characterized. It is possible that HHT reduces expression of anti-apoptotic signals including BCR-ABL, p-AKT, Mcl-1 and Hsp-90 in K562 [29]. HHT also targets a broad-spectrum of protein tyrosine kinases to inhibit the phosphorylation of proliferative signaling proteins such as JAK2, STAT5 and AKT in AML cell lines [30]. Future research is necessary to examine precise mechanisms in the future.

We hypothesized that the DNA damaging effects of IDA would be enhanced when combined with HHT. Our results show that HHT induced mild apoptosis (**Figure 2A**), but enhanced the efficacy of IDA at low concentrations (**Figure 2B**). IDA generally promoted caspase-3 activation and cleavage of PARP-1, and these apoptotic signals were enhanced when co-treated with HHT (**Figure 3A**). This agreed with reports that semi-synthetic homoharringtonine induces apoptosis through decreased myeloid cell leukemia-1 (Mcl-1) expression and increased Bcl-2 cleavage, accompanied by the decrease of mitochondrial membrane potential and the release of cytochrome c [31].

Dose-related cellular injury within the cardiac myocytes can be mediated by increased oxidative stress. In this study, we also find that IDA but not HHT induced apoptosis by triggering the production of ROS mediated DNA damage and PARP-1 activation; and ROS production was attenuated by antioxidants. Studies suggest that active mutants of BCR-ABL1 and CML progression promotes increased production of ROS [32]. Thus, to circumvent apoptosis that accompanies continuous ROS production and accumulated DNA damage, BCR-ABL increases

DNA repair and to reduce oxidative DNA damage [33]. Despite its ability to enhance apoptosis, HHT reduced ROS levels in combination-treated cells (**Figure 4B**). We suspect this potentially occurs through inactivation of BCR-ABL1 and its associated DNA damage repair processes [29]. It has also been reported that the antioxidant NAC prevents DNA damage and inactivates PARP-1 to inhibit ROS production [34]. However, our results indicate that HHT related apoptosis is closely associated with decreased MMP (**Figure 5B**); this confirms previous observations from Z Cai et al. that MMP was reduced even in HHT-treated cells with low ROS levels [35]. Recently, increasing numbers of reports have indicated that HHT exhibit anti-tumor activity and sensitization effect to other agents. HHT is an efficient enhancer of TRAIL-mediated apoptosis/growth suppression of resistant human colorectal carcinoma cells as it suppresses expression of anti-apoptotic proteins Mcl-1 and cFLIP [10, 36]. HHT or velcade induced apoptosis was shown to be mediated by the up-regulation of TIEG1, down-regulation of Bcl-2 and Bcl-XL, disruption of the mitochondrial membrane potential and activation of caspase-3 [37]. Altogether, these research along with our own, suggest that HHT may activate the mitochondrial apoptotic pathway by modulating expression and localization of anti-apoptotic proteins on the mitochondrial membrane, and this overall effect may be related to inactivation of BCR-ABL1 signaling as well [7].

In conclusion, we demonstrated that HHT enhances the anti-leukemic effects of IDA, and improves the drug tolerance for normal HUVEC cells. The combination of IDA and HHT induced significant activation of caspase-3 and cleavage of PARP-1, and this effect may be mediated by HHT induced BCR-ABL1 inactivation and mitochondrial apoptotic pathway activation, enhancing IDA-induced mitochondrial membrane damage by ROS to promote subsequent apoptosis. These findings suggest that clinical investigation of HHT in combination with IDA could potentially serve as a treatment strategy for patients with blast crisis of CML. Additionally, our study supports further investigation of the potential cardio-protective effects of HHT upon IDA treatment.

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Disclosure of conflict of interest

None.

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