Homoharringtonine enhances the effect of imatinib on chronic myelogenous leukemia cells by downregulating ZFX

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Abstract. Homoharringtonine (HHT) and imatinib have a synergistic effect in the clinical treatment of chronic myeloid leukemia (CML). The purpose of the present study was to explore the underlying mechanisms by which HHT enhanced imatinib sensitivity. K562 CML cells were treated with HHT and imatinib separately or in combination. Cell viability was detected by Cell Counting Kit-8 assay; apoptotic rates and protein expression levels of phosphorylated-tyrosine (p-Tyr) and p-CRK like proto-oncogene, adaptor protein (p-Crkl) were analyzed by flow cytometry; zinc-finger protein, X-linked (ZFX) overexpression plasmid was transfected to cells using electroporation; western blotting was used to detect the protein expression levels of PI3K, aKT, p-aKT and ZFX; and reverse transcription-quantitative PCR was used to measure ZFX mRNA expression levels. The results demonstrated that HHT and imatinib co-treatment had significant effects of proliferation inhibition and apoptosis induction on K562 CML cells compared with imatinib alone. Co-treatment also significantly downregulated the expression levels of p-Tyr, p-Crkl, PI3K and p-Akt compared with imatinib or HHT treatment. In addition, HHT downregulated ZFX mRNA and protein expression. ZFX overexpression reversed cell sensitivity to imatinib and HHT and also reduced the HHT-induced imatinib sensitization by increasing p-Akt expression. In conclusion, HHT may enhance the effect of imatinib on CML cells by downregulating ZFX.

Introduction

Chronic myeloid leukemia (CML) is characterized by the formation of the Philadelphia (Ph) chromosome, which occurs in pluripotent hematopoietic stem cells (1). This translocation generates the Bcr activator of rhoGeF and GTPase (BCR)-ABL fusion gene that encodes p210Bcr-aBl protein (2). The oncoprotein exhibits constitutive tyrosine kinase activity and serves a fundamental role in the formation of CML (3). Imatinib, a tyrosine kinase inhibitor (TKI), is the upfront treatment for Ph+ CML (4,5). However, drug resistance is a major reason for relapsed and refractory CML following the termination of imatinib treatment (6), and a number of mechanisms of resistance are independent of p210 Bcr-aBl upregulation and mutation status (7,8). Therefore, it is important to develop a technique to improve the therapeutic effects of imatinib.

Homoharringtonine (HHT) is a plant alkaloid with antitumor properties that is derived from trees of the genus Cephalotaxus; it has been widely used in China for the treatment of hematological malignancies since the 1970s (9,10). HHT has served an important role in the treatment of CML, both prior to the widespread use of TKIs, and at present following the development of TKIs resistance (11-13). The FDA has approved HHT for CML refractory to TKIs (1). The anti-leukemic mechanism of HHT is based on the inhibition of protein synthesis (10). HHT reduces p210Bcr-aBl protein expression level in Bcr-aBl+ cells independently of Bcr-aBl mutational status (13-15). HHT also has a synergistic relationship with imatinib in clinical therapy (16), but the working mechanism is poorly understood.

Zinc-finger protein, X-linked (ZFX) gene is on the mammalian X chromosome and is a transcriptional regulator involved in the maintenance of embryonic and hematopoietic stem cells (17,18). Homoharringtonine (HHT) is a plant alkaloid with antitumor properties that is derived from trees of the genus Cephalotaxus; it has been widely used in China for the treatment of hematological malignancies since the 1970s (9,10). HHT has served an important role in the treatment of CML, both prior to the widespread use of TKIs, and at present following the development of TKIs resistance (11-13). The FDA has approved HHT for CML refractory to TKIs (1). The anti-leukemic mechanism of HHT is based on the inhibition of protein synthesis (10). HHT reduces p210Bcr-aBl protein expression level in BCR-ABL+ cells independently of BCR-ABL mutational status (13-15). HHT also has a synergistic relationship with imatinib in clinical therapy (16), but the working mechanism is poorly understood.

The zinc-finger protein, X-linked (ZFX) gene is on the mammalian X chromosome and is a transcriptional regulator involved in the maintenance of embryonic and hematopoietic stem cells (17,18). Previous studies suggest that ZFX serves a pivotal role in tumorigenesis of multiple types of cancer, including lung cancer, gastric cancer, breast cancer, malignant glioma and leukemia (19-23). Additionally, ZFX participates in drug resistance in hepatocellular carcinoma (24,25). The results of our previous study also demonstrated that ZFX may involve in the regulation of cell proliferation and imatinib resistance in CML (26). Thus, the present study investigated the effects and mechanisms of HHT facilitating imatinib
sensitivity in K562 human CML cells. The results indicated that HHT may enhance the effects of imatinib on CML cells by downregulating ZFX expression.

Materials and methods

Cell culture. K562 human CML cells were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultivated in RPMI-1640 medium supplemented with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37°C. A range of concentrations of imatinib (Selleck Chemicals) or HHT (Bio-Techne) were added to the cells during experiments.

Transfection. To overexpress ZFX, the human ZFX sequence was amplified and cloned into the pEGFP-C1 expression plasmid by Shanghai GeneChem Co., Ltd. Cell transfection was performed by electroporation. Typically, 7x10⁵ cells and pEGFP-C1-ZFX or empty vector were electroporated using a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Inc.) with 250 V voltage and 950 µFd electric capacity. Cells were subsequently resuspended in RPMI-1640 medium and cultured for 24-72 h.

Cell viability assay. Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay was used to examine cell viability following different treatments. K562 cells were seeded in a 96-well plate (8x10³ cells/well), cultured for 24 h and treated with different concentrations of imatinib or HHT for 24 or 48 h at 37°C. Subsequently, 10 µl CCK-8 solution was added to the plate and the cells were incubated for 2 h at 37°C. Absorbance at 450 nm was measured using a microplate reader (BioTek instruments, Inc.). HHT treatment or ZFX overexpression may affect cell viability; therefore, the relative viability of drug-treated cells was normalized to DMSO-treated cells to eliminate a false-positive effect.

Colony formation assay. Drug-treated K562 cells were cultured in a two-layer soft agar system as previously described (27). Single-cell suspensions were washed with RPMI-1640 medium, enumerated and plated into a 12-well plate (1 ml/well; Single -cell suspensions were washed  with r PMi-1640 in a two-layer soft agar system as previously described (27). Drug-treated K562 cells were cultured for 10 days at 37°C, the colonies (≥40 cells for each) were counted under an inverted microscope (magnification, x100; Olympus Corporation).

Apoptotic assay. Apoptosis was detected using the Annexin V-FITC/propidium iodide (PI) Apoptosis Detection kit (BD Biosciences) according to the manufacturer's protocol. Stained cells were analyzed using a flow cytometer (BD Biosciences), and cells were separated into normal, early apoptotic, late apoptotic and dead cells. The relative ratios of early apoptotic cells were analyzed by FlowJo software (version 10; FlowJo LLC).

Phosphorylated-tyrosine (p-Tyr) protein assay. Drug-treated K562 cells were fixed with 1% paraformaldehyde (BD Biosciences) for 30 min and permeabilized with 3% saponin (BD Biosciences) for 1.5 h (both at room temperature). Subsequently, the cells were stained using phycoerythrin-conjugated p-Tyr (1:1,000; cat. no. 558008) or p-CRK like proto-oncogene, adaptor protein (p-CrkL) (1:1,000; cat. no. 560788) antibodies (both BD Biosciences). Following washing with PBS, cells were recovered in 3% saponin and submitted to flow cytometric analysis (BD Biosciences), and mean fluorescence intensity (MFI) was recorded by FlowJo software (version 10) to observe the levels of p-Tyr and p-CrkL proteins.

Western blot analysis. Cells were lysed using RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The protein concentration was determined using a bicinchoninic acid assay, and equal amounts (20-30 µg) of total protein were separated by 6-10% SDS-PAGE and transferred onto PVDF membranes, which were then blocked with 5% skim milk for 2 h at room temperature. The primary antibodies against ZFX (1:1,000; cat. no. ab115998; Abcam), β-Actin (1:1,000; cat. no. ab8224; Abcam), α-3XK (1:1,000; cat. no. 05-212; Merck KGaA), AKT (1:1,000; cat. no. 07-383; Merck KGaA) and p-AKT (1:1,000; cat. no. 04-736; Merck KGaA) were used to incubate the membranes overnight at 4°C. Following incubation with secondary goat anti-mouse (1:2,000; cat. no. ab6789; Abcam) or goat anti-rabbit (1:2,000; cat. no. ab6721; Abcam) antibody for 2 h at room temperature, blots were visualized using ECL Detection Reagent (cat. no. P0018; Beyotime Institute of Biotechnology) and analyzed using Image Lab software (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from cells using TRizol® (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed with FastQuant RT kit (Tiangen Biotech Co., Ltd.). PCR was performed in triplicate with SuperReal PreMix Plus (Tiangen Biotech Co., Ltd.) using the Real-Time PCR Detection System (Roche Molecular Systems, Inc.). qPCR was conducted at 95°C for 5 min, followed by 40 cycles at 95°C for 10 sec, 65°C for 20 sec and 72°C for 30 sec. The primer sequences for ZFX and β-Actin were as follows: ZFX, forward 5'-GGGACTCTACAGTGCCGATT-3', reverse 5'-TGGATATCAGGACAGGAGGA GTGCAGAG-3'; β-Actin, forward 5'-CTCATCTGGCCTTC GTCTG-3' and reverse 5'-GCTGTCACCTTACCGTTC-3'. Relative mRNA levels were normalized to β-Actin expression using the 2^(-ΔΔCq) method (28).

Statistical analysis. SPSS 19.0 software (IBM Corp.) was used for statistical analysis. Data are expressed as the mean ± SD. Differences between two groups were assessed by Student's t-test. Statistical differences among multiple groups were analyze by one-way analysis of variance followed by a Bonferroni post hoc test. Each experiment was repeated for at least three times. P<0.05 was considered to indicate a statistically significant difference.

Results

**HHT facilitates imatinib sensitivity in CML cells.** The effects of HHT on K562 cell viability were tested by CCK-8 assay. Following treatment for 24 or 48 h, HHT reduced cell viability in a dose- and a time-dependent manner (Fig. 1A). A HTT concentration of 10 nM was selected for subsequent
experiments, as this concentration produced a small inhibitory effect at 24 h that would still enable the detection of further sensitization effects. 24-h co-treatment with 10 nM HHT and a range of concentrations of imatinib resulted in significantly greater inhibition of cell viability compared with imatinib alone (Fig. 1B). The results of a cloning experiment demonstrated the additive effect of imatinib and HHT on the reduced ability of K562 cells to form colonies compared with either drug alone (Fig. 1C). In addition, compared with HHT-alone or imatinib-alone groups, 24-h co-treatment with HHT and imatinib significantly increased the early apoptotic rate of K562 cells (Fig. 1D). An imatinib concentration of 0.2 µM imatinib was selected, as this concentration produced a small inhibitory effect upon which further sensitization could be observed, like for HTT.

**Effects of HHT combined with imatinib on tyrosine kinase activity in CML cells.** Tyrosine kinase activity may be reflected by p-Tyr protein expression levels in Bcr-Abl+ cells (29). Co-treatment of cells with HHT and imatinib for 24 h significantly decreased the MFI of p-Tyr staining compared with either treatment alone (Fig. 2A). p-CrkI, which has been...
inhibit cMl cell growth through the Pi3K/aKT pathway (26).

The difference in Pi3K expression was observed between the HHT treated and control groups. Western blotting was performed to detect expression levels of Pi3K, Akt, and p-Akt in K562 cells treated with HHT or imatinib for 48 h. CTRL, control; HHT, homoharringtonine; p-, phosphorylated; ZFX, zinc-finger protein, X-linked.

To test whether the effect of HHT on cMl cells was associated with ZFX overexpression, K562 cells were co-treated with HHT and imatinib. The results of our previous study demonstrated that ZFX silencing may enhance the effects of HHT in enhancing the effect of imatinib on CML cells. Therefore, the expression levels of Pi3K and p-Akt in K562 cells (Fig. 3).

However, no difference in total Akt was detected. No notable difference in Pi3K expression was observed between the HHT and co-treatment groups.

HHT downregulates ZFX expression levels in CML cells. The results of our previous study demonstrated that ZFX silencing may inhibit CML cell growth through the Pi3K/AKT pathway (26). To test whether the effect of HHT on CML cells was associated with ZFX expression, RT-qPCR and western blot analyses were performed; the results revealed that the mRNA and protein expression levels of ZFX were dose-dependently decreased by HHT treatment for 24 h in K562 cells (Fig. 4A and B). In addition, HHT and imatinib co-treatment downregulated ZFX protein expression levels (Fig. 3).

Overexpression of ZFX induces imatinib resistance in CML cells. To validate the effects of ZFX on CML cell response to imatinib, K562 cells transfected with either ZFX-GFP or empty GFP plasmids were incubated with imatinib for 24 h. ZFX mRNA and protein expression levels were successfully increased following transfection with the ZFX-GFP plasmid (Fig. 4C and D). The results of the CCK-8 assay indicated that overexpression of ZFX decreased the sensitivity of K562 cells to imatinib (Fig. 4E). In addition, overexpression of ZFX reversed the inhibitory effects of imatinib on colony formation in K562 cells (Fig. 4F).

Overexpression of ZFX attenuates the effects of HHT on CML cells. Overexpression of ZFX reversed the HHT-induced inhibition of proliferation and clone formation in K562 cells (Fig. 5A and B). Additionally, the effects of HHT on imatinib sensitivity were attenuated by overexpression of ZFX; the cell inhibition rate in the HHT + ZFX group was significantly increased compared with that in the ZFX group (Fig. 5C). ZFX overexpression also reversed HHT-induced decrease of p-AKT expression (Fig. 5D). Thus, the data suggested that ZFX may participate in HHT-induced imatinib sensitivity in CML cells.

Discussion

HHT treatment is effective for patients with CML and may provide an effective treatment for patients with TKI-resistant CML with BRC-ABL mutations (32,33). Thus, co-treatment with HHT and imatinib may provide a novel approach to improve the efficiency of CML treatment. The results of the present study have demonstrated a potential mechanism of HHT in enhancing the effect of imatinib on CML cells.

In the present study, HHT treatment increased CML cell sensitivity to imatinib. Co-treatment with HHT and imatinib resulted in decreases in K562 cell viability and in the number of colonies formed compared with either treatment alone. In addition, co-treatment induced apoptosis in K562 cells more effectively compared with individual treatments. These results were consistent with our previous study in imatinib-resistant CML cells (34). Therefore, HHT may facilitate imatinib sensitivity by inducing apoptosis in CML.

HHT is a broad-spectrum protein TKI that inhibits signaling protein phosphorylation by oncogenic proteins, such as Janus kinase 2 V617F and p210Bcr-aBl, thus blocking the survival signaling pathways of leukemia cells (10,35). Constitutive Try phosphorylation is the major characteristic of BCR-ABL+ cells (1). In the present study, flow cytometry was used to measure the p-Tyr and p-CrkI expression levels, which were previously demonstrated to be abnormally high in CML cells (30,36). The results of the present study demonstrated that co-treatment with HHT and imatinib decreased p-Tyr and p-CrkI expression levels compared with individual drug treatment. HHT reduces p210BCR-ABL expression in BCR-ABL+ cells (37), which may partially explain the additive interaction between HHT and imatinib.

The Pi3K/AKT signaling pathway is essential for CML cell viability, and may be an effective target for therapeutic intervention in imatinib-resistant CML (3,31). Previous studies have reported that HHT mediates myeloid cell apoptosis by inhibiting the Pi3K/AKT signaling pathway (13,38). In the present study, Pi3K and p-AKT expression levels were decreased by co-treatment with HHT and imatinib compared with either treatment alone. These data indicated that HHT may enhance the effects of imatinib through synergistic inhibition of the Pi3K/AKT pathway.

To further examine the crucial molecules except for p210BCR-ABL underlying the mechanisms via HHT enhances imatinib sensitivity through the Pi3K/AKT pathway, the role of ZFX, which is a known mediator of biological function in cancer cells (39-41), was investigated. ZFX mRNA and protein expression levels were downregulated by HHT. Overexpression
of ZFX reversed imatinib- or HHT-induced inhibition of cell viability. In addition, ZFX overexpression significantly weakened HHT-induced imatinib sensitization and reversed the inhibitory effect of HHT on p-AKT expression. Thus, ZFX may be responsible for abolishing the sensitization of imatinib mediated by HHT.
In conclusion, the results of the present study demonstrated that HHT may increase imatinib sensitivity of CML cells through downregulation of ZFX expression, which leads to the inhibition of PI3K/AKT pathway, thereby providing new insight into the therapeutic strategy of CML treatment.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
JW and BW drafted the manuscript. JW, BW, YS, Xl and YD collected, analyzed and interpreted the data. Yl and cW conceived and designed the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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