**Chaetominine reduces MRP1-mediated drug resistance *via* inhibiting
PI3K/Akt/Nrf2 signaling pathway in K562/Adr human leukemia cells**

Jingyun Yao ^{1, 2}, Xing Wei ^{1, 2}, Yanhua Lu ^{1, 2,*}

¹ State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai, PR China

² Shanghai Collaborative Innovation Center for Biomanufacturing Technology, 130 Meilong Road, Shanghai, PR China

*Correspondence author

Dr. Yanhua Lu

State Key Laboratory of Bioreactor Engineering

East China University of Science and Technology

Box 283#, 130 Meilong Road, Shanghai 200237, People's Republic of China

E-mail: luyanhua@ecust.edu.cn

Tel: +86-21-64251185

Fax: +86-21-64251185

Abstract: Drug resistance limits leukemia treatment and chaetominine, a cytotoxic alkaloid that promotes apoptosis in a K562 human leukemia cell line *via* the mitochondrial pathway was studied with respect to chemoresistance in a K562/Adr human resistant leukemia cell line. Cytotoxicity assays indicated that K562/Adr resistance to adriamycin (ADR) did not occur in the presence of chaetominine and that chaetominine increased chemosensitivity of K562/Adr to ADR. Data show that chaetominine enhanced ADR-induced apoptosis and intracellular ADR accumulation in K562/Adr cells. Accordingly, chaetominine induced apoptosis by upregulating ROS, pro-apoptotic Bax and downregulating anti-apoptotic Bcl-2. RT-PCR and western-blot confirmed that chaetominine suppressed highly expressed MRP1 at mRNA and protein levels. But little obvious alternation of another drug transporter MDR1 mRNA was observed. Furthermore, inhibition of MRP1 by chaetominine relied on inhibiting Akt phosphorylation and nuclear Nrf2. In summary, chaetominine strongly reverses drug resistance by interfering with the PI3K/Akt/Nrf2 signaling, resulting in reduction of MRP1-mediated drug efflux and induction of Bax/Bcl-2-dependent apoptosis in an ADR-resistant K562/Adr leukemia cell line.

Keywords: chaetominine, reversal effect, chemoresistance, K562/Adr, MRP1

1. Introduction

Acquired chemoresistance is a significant obstacle for human leukemia therapeutics [1]. Recent reports suggest that chemotherapeutic resistance involves modified expression of drug transport pumps and alterations of the apoptosis pathway [1-3]. Drug efflux leading to less anticancer drug toxicity mainly relies on overexpression of ATP binding cassette (ABC) transporters such as multidrug resistance associated proteins (MRPs) and P-glycoprotein (P-gp), which are encoded by the MRP gene and MDR1 gene, respectively [4]. MRP1 is a well-recognized contributor to chemotherapy resistance, which has been identified in several drug-resistant leukemia cells. The pharmacological inhibition of MRP1 or/and related signaling pathway thus is a popular approach for overcoming drug resistance in leukemia cells [3, 5, 6].

The PI3K/Akt signaling pathway is implicated in cell progression during tumorigenesis [7]. To date, studies suggest that PI3K/Akt signaling is frequently activated in leukemia cell lines and an important regulator of proliferation, apoptosis and drug resistance [8]. Chemoresistance due to PI3K/Akt upregulation is generally maintained by Akt-dependent blockage of pro-apoptotic signaling molecules, because chemotherapeutics kill cancer cells *via* inducing apoptosis [5, 9]. Once activated, Akt promotes cell survival by activating Bcl-2 and inhibiting Bax, both from the apoptotic regulatory protein Bcl-2 family [10]. Moreover, in leukemia cells a strong relationship exists between the PI3K/Akt signaling pathway and MRP1 expression, and this association was not found for P-gp [8]. Also, chemoresistance could be reversed by LY294002, a widely-used PI3K/Akt inhibitor.

Nrf2 (NF-E2-related factor 2), a member of the cap-n-collar subfamily of transcription factors, is a master regulator of cellular response which facilitates cell survival after toxic stresses [11]. However, constitutively high expression of Nrf2 promotes cancer cell survival which is thought to be responsible for chemoresistance. Nrf2 activity is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1). Upon exposure to reactive oxygen species (ROS) or chemotherapeutic agents, the Keap1-Nrf2 complex is destroyed and Nrf2 translocates from the cytoplasm to the nucleus [12]. Accumulation of nuclear stabilized Nrf2 turns on downstream signaling such as the MRP1 gene [12]. Chemotherapeutic strategies targeting downregulation of the Nrf2 response by interfering with Keap1 or its upstream signaling pathway PI3K/Akt may sensitize resistant cancer cells to anticancer drugs.

Chaetominine is a cytotoxic alkaloid (Fig. 1A) from secondary metabolites of *Aspergillus fumigatus* CY018, an endophytic fungus [13]. Previous work suggests that chaetominine can kill human leukemia K562 cells by inducing apoptosis [14]. However, whether chaetominine can reverse drug resistance in leukemia and the molecular mechanism to explain this has not been elucidated. Thus, we investigated chaetominine-induced reversal of adriamycin-resistance in an K562/Adr leukemia cell line and investigated possible mechanisms for this.

2. Materials and Methods

2.1. Reagents

Chaetominine was extracted from the liquid submerged culture of *Aspergillus fumigatus* CY018 and the purity was 99.8% [14]. The control adriamycin (ADR,

purity > 98%) was obtained from Sigma-Aldrich (St. Louis, MO). The PI3K inhibitor LY294002 was purchased from Cell Signaling Technology (Beverly, MA). Primary antibodies of MRP1, PI3K, p-Akt (Ser473) were procured from Cell Signaling Technology, and Nrf2, Keap1, HO-1, NQO-1, NADPH primary antibodies were from Santa Cruz Biotechnology (CA, USA). Goat anti-mouse and goat anti-rabbit secondary IgG were from Beyotime Biotechnology (JS, China) .

2.2. Cell culture

Human chronic myeloid leukemia K562 cells and drug-resistant K562/Adr cells were purchased from Shanghai Institutes for Biological Sciences (SH, China) and KeyGen Biotechnology (NJ, China), respectively. Cells were cultured in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C in a humidified incubator (SANYO, Japan) with 5% CO₂. To maintain drug resistance, ADR (1000 ng/ml) was applied to K562/Adr cells until at least 2 weeks before experiments.

2.3. Cytotoxicity and multidrug resistance reversal assay

Cytotoxicity and chaetominine's potentiation of ADR cytotoxicity were measured in K562/Adr and K562 cells using an MTT assay. Briefly, cells were seeded in 96-well plates and incubated with drugs for 48 h. 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (5 mg/mL; Sigma) was added to each well and absorbance was measured on a microplate reader (SpectraMax® i3, Wals, Austria) at an experimental wavelength of 570 nm and a reference wavelength of 630 nm. IC₅₀ values for ADR and chaetominine for K562/Adr and K562 cells were calculated from

plotted data using untreated group as 100%.

A resistance index (RI) for K562/Adr cells was obtained by dividing the IC_{50} for the MDR cells by the IC_{50} for parental cells. Reversal fold (RF) values were calculated from fitting the data to $RF = IC_{50}$ of ADR alone/ IC_{50} of ADR in the presence of the chaetominine [15].

2.4. Cell apoptosis detection

To investigate whether chaetominine reversal of drug resistance in K562/Adr cells was because of induction of apoptosis, nuclear morphological changes in apoptotic cells were first assessed with Hoechst 33258 staining. Cells were placed into a 6-well culture plate and incubated with or without chaetominine for 24 h and then cells were collected in microtubes and washed with PBS. Subsequently, cells were fixed, washed, and stained according to the manufacturer's instructions in the Hoechst staining kit (Beyotime). Apoptotic cells were observed and photographed under a fluorescent microscope (Olympus BX51, Tokyo, Japan).

Apoptotic cells were measured using flow cytometry. Following the manufacturer's protocol for the annexin V-FITC/PI kit (KeyGen), cells were incubated in annexin V binding buffer and annexin V-FITC for 10 min in the dark. After centrifugation (1000 rpm, 4 min), cells were suspended in a mixture of annexin V and PI buffer. Then, apoptotic cells were counted with flow cytometry (FACS Aria; Becton Dickinson, city, state). Annexin V-FITC-positive and PI-negative cells were considered to be in early apoptosis, whereas annexin V-FITC- and PI-positive cells were considered to be in late apoptosis.

2.5. ROS measurement

ROS in K562/Adr cells were measured using 2',7'-dichlorofluorescein-diacetate (DCFH-DA; Jiancheng Biotechnology, NJ, China). After pretreatment with or without chaetominine for 24 h, cells were incubated with 10 μ M DCFH-CA for 1 h at 37°C and resuspended in PBS. ROS were quantified from the ratio of fluorescence to untreated control as measured with a microplate reader (SpectraMax® i3) at $\lambda_{Ex485}/\lambda_{Em525}$.

2.6. Intracellular accumulation of ADR

Accumulation of ADR was assessed with a standard procedure after incubating K562 and K562/Adr cells for 5 h at 37 °C in the presence of ADR (10 μ M) alone or in combination with chaetominine (10, 20 and 40 nM). Cells were collected and washed twice with ice-cold PBS and intracellular mean fluorescent intensity (MFI) associated with ADR was measured with a flow cytometer (FACS Aria) at $\lambda_{Ex485}/\lambda_{Em585}$.

2.7. Qualitative real time PCR

Total RNA was extracted with a Total RNA Extraction Kit (Keygen) and quantified with UV spectrophotometry at λ_{260} . Reverse transcription into cDNA was carried out using PrimeScript™ RT reagent Kit (Takara, Dalian, China) and PCR was performed. A real-time PCR reaction was conducted with SYBR® Premix Ex Taq™ II (Takara) and conditions for PCR amplification were 94 °C for 30 seconds, 60 °C for 30 sec, and then 72 °C for 1 min for 40 cycles. The primer sequences used were as follows:

MRP-1: 5'-CTGCTGGAGGAAGACGAAGATCCTT-3' (forward),
5'-GGTGGCTGTGCTTTGAATATGT-3' (reverse); MDR-1:

5'-TGGTTTGATGTGCACGATGTTGGG-3' (forward), 5'-AGATCAGCAGGAAAG CAGCACCTA-3' (reverse); Actin: 5'-CTACAATGAGCTGCGTGTGGC-3' (reverse), 5'-CAGGTCCAGA CGCAGGATGGC-3' (reverse). Gene mRNA was normalized to that of the internal control Actin.

2.8. Western blot

After cells were incubated with different concentrations of chaetominine, whole cell lysates and nuclear protein extracts were prepared, and protein was measured using a BCA protein assay kit (Pierce, Rockford, IL). Protein samples (20 µg/lane) containing 0.01% bromophenol blue were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The PVDF membrane was blocked with 3% BSA in TBS-Tween for 2 h and then incubated overnight with the appropriate primary antibodies (diluted with blocking buffer) at 4°C. After washing three times with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies. Proteins were measured using chemiluminescence and quantified with Quantity One software (v4.62; Bio-Rad, Hercules, CA).

2.9. Statistical analysis

Data are expressed as means $\pm$ SD. Group data were compared using one-way ANOVA with a Newman-Keuls test.

3. Results

3.1. Chaetominine treatment enhanced cytotoxicity of ADR in K562/Adr cells

MTT assay data confirmed that chaetominine inhibited cell growth in resistant

K562/Adr cells. Table 1 shows the IC₅₀ values. Resistance of K562/Adr cells to ADR and chaetominine was assessed using a resistance index (RI), which is depicted in Table 1. Data show that K562/Adr cells were resistant to ADR but sensitive to chaetominine. Chaetominine enhanced the cytotoxicity of ADR against K562/Adr cells as evidenced by a reversal-fold (RF) value and Table 2 depicts these data which confirm that chaetominine reduced K562/Adr cell resistance to ADR and reversed this resistance.

3.2. Chaetominine treatment enhanced ADR inducing apoptosis

Hoechst 33258 staining assays were used to validate chaetominine cytotoxicity in K562/Adr cells mediated by apoptosis. Untreated K562/Adr cells proliferated with rounded, normal-sized nuclei, and chaetominine-treated cells became bright and condensed, with shrunken nuclei and apoptotic bodies formation (Fig. 1B). This suggested that chaetominine could induce apoptosis in resistant leukemia cells. In addition, chaetominine's effect on apoptosis induced by ADR was measured using annexin V-FITC/PI staining and compared with the ADR group, co-treatment with chaetominine and ADR increased apoptosis in K562/Adr cells and these data appear in Fig. 1C. Thus, chaetominine may facilitate ADR-induced apoptosis in a dose-dependent manner in K562/Adr cells.

3.3. Chaetominine treatment upregulated ROS levels of K562/Adr cells

ROS are implicated in apoptosis and greater ROS may cause cancer cell apoptosis [16]. In K562/Adr cells, ROS were increased by addition of 20 or 40 nM chaetominine (Fig. 1D), indicating that chaetominine-induced apoptosis might be

ROS-dependent.

3.4. Chaetominine treatment increased intracellular ADR accumulation

Intracellular fluorescent intensity of ADR was measured with flow cytometry and Fig. 2A shows that ADR concentration in K562/Adr cells was less than in K562 cells. Then the MFI of ADR gradually increased with combination treatment of chaetominine and ADR in K562/Adr cells, indicating that chaetominine may reverse drug resistance by reducing ADR efflux in K562/Adr cells.

3.5. Chaetominine treatment depleted MRP1 mRNA and protein

To verify whether MRP1 and MDR1 drug transporter genes were implicated in chaetominine reversal of chemoresistance in K562/Adr cells, both genes were measured with RT-PCR. MRP1 and MDR1 mRNA in K562/Adr were significantly higher than in non-resistant K562 cells (Fig. 2B). When K562/Adr cells were incubated with chaetominine, both MRP1 mRNA and protein decreased in a dose-dependent manner (Fig. 3C). In contrast, MDR1 mRNA only changed with high concentrations of chaetominine (40 nM, Fig. 2B). Thus, chaetominine reversal of drug resistance may be maintained by MRP1-dependent transcriptional inhibition.

3.6. Chaetominine inhibited PI3K/Akt signaling and influenced the expression of MRP1

Inhibition of the PI3K/Akt pathway caused MRP1 downregulation and consequently reversed drug resistance in cancer cells in numerous studies [6, 15, 17, 18]. Thus, we studied the potential suppression of this pathway by chaetominine treatment. K562/Adr cells expressed PI3K and p-Akt at a high level (Fig. 3A), and

protein for both were attenuated after treatment with chaetominine (Fig. 3B). Accordingly, p-Akt and MRP1 expressions in K562/Adr cells decreased after treatment with chaetominine, and this was similar to data obtained with LY294002, the PI3K inhibitor (Fig. 3C). In addition, when K562/Adr cells were treated with LY294002 and chaetominine, inhibitory effect were more pronounced. These observations confirmed that chaetominine reversed MRP1-dependent drug resistance by regulating PI3K/Akt signaling in K562/Adr cells.

3.7. Effect of chaetominine on Nrf2 was correlative with Akt blocking

K562/Adr cells overexpressed MRP1 had an increased expression of Nrf2, whereas Keap1, a negative regulator of Nrf2, was less expressed compared to non-resistant cells (Fig. 4A). When p-Akt inhibition was induced with LY294002 pretreatment, K562/Adr cells had less Nrf2 expression which was similar to chaetominine treatment (Fig. 3C). And there is a dramatic decrease of Nrf2 expression by exposure to both LY294002 and chaetominine. Data indicate that chaetominine downregulated Nrf2 protein expressions at both the total and nuclear levels and altered Keap1 expressions slightly (Fig. 4B). Thus, chaetominine-induced Akt inhibition was directly related to the inhibitory effect on Nrf2, perhaps enhancing chemosensitivity in K562/Adr cells.

3.8. Chaetominine modulated Akt-mediated apoptotic related proteins

Constitutive PI3K signaling could contribute to apoptosis resistance and high Akt signals sensitize cancer cells to death by regulating expression of Bcl proteins such as Bcl-2 and Bax [19]. Chaetominine increased expression of pro-apoptotic protein Bax

but decreased expression of anti-apoptotic protein Bcl-2 in K562/Adr cells in a dose-dependent manner (Fig.4 C). Therefore, chaetominine triggered apoptosis in K562/Adr cells by modulating Akt-dependent regulators Bax and Bcl-2.

4. Discussion

Chemotherapeutic resistance arises due to multiple biological interactions [20] and natural products have been studied to circumvent drug resistance and improve treatment efficacy by inhibiting MDR proteins (one explored method) [21]. Chaetominine is a naturally occurring alkaloid that can induce apoptosis in a K562 leukemia cell line [14] but whether chemosensitivity of chaetominine in resistant leukemia cells occurs is unclear. Thus, we studied the effect of chaetominine on K562/Adr leukemia resistant cells and explored the possible mechanism behind this effect.

In this study, K562/Adr cells were used to examine the influence of chaetominine on cytotoxicity and sensitivity of ADR. K562/Adr resistance to ADR was confirmed by a high RI value which did not occur with the chaetominine group, indicating that K562/Adr cells were sensitive to chaetominine treatment. After co-treatment with chaetominine and ADR, IC_{50} values were measured and these are in Table 1. Enhanced sensitivity of K562/Adr to ADR was verified by increased ADR-induced apoptosis in the presence of chaetominine. Moreover, chaetominine treatment alone could induce apoptosis of K562/Adr cells by upregulation of ROS and modulation of Bax/Bcl-2, indicating that cytotoxicity of chaetominine towards ADR-resistant cells was due to reducing apoptosis resistance. Chaetominine-induced apoptosis in

K562/Adr cells was ROS-dependent and initiated by Bax/Bcl-2, data that are consistent with reports of chaetominine-induced intrinsic apoptosis in K562 cells [14].

K562/Adr cells are characterized by greater mRNA for major efflux pump genes that mediate drug resistance, MDR1 and MRP1. Chaetominine dose-dependently decreased MRP1 but not MDR1 at transcriptional and translational levels, increasing accumulation of ADR in K562/Adr cells. The PI3K/Akt pathway is a critical pathogenic signaling routes in human cancers, so it may be a promising therapeutic target [22]. PI3K and p-Akt were highly expressed in resistant K562/Adr cells but not in sensitive K562 cells and MRP1 protein decreased in association with the attenuation of PI3K and p-Akt expression in K562/Adr cells induced by chaetominine or/and LY294002 (a PI3K inhibitor) treatment. Thus, chaetominine sensitized cells by suppressing drug efflux functions of MRP1 *via* inhibiting PI3K/Akt signaling.

PI3K/Akt pathway activation contributes to chemoresistance *via* modulating multiple cellular processes including apoptosis (e.g. Bax/Bcl-2) and expression of drug transporters (MRP1) [23, 24]. Among them Nrf2 was thought to be a nuclear regulator of MRP1 and partially responsible for chemoresistance [25]. We observed that chaetominine significantly inhibited expressions of total and nuclear Nrf2 in K562/Adr cells, indicating that chaetominine might block nuclear translocation of Nrf2. Meanwhile, chaetominine' inhibitory effect on Nrf2 was associated with PI3K/Akt blockade due to depletion of Nrf2 which occurred after chaetominine or/and LY294002 treatment. Additionally, Akt blockade may be related to alteration of Bax/Bcl-2 in K562/Adr cells after chaetominine exposure.

In summary, we identified a mechanism behind chaetominine-induced reversal of drug resistance in a K562/Adr leukemia cell line, which was associated with the induction of ROS-dependent apoptosis and reduction of MRP1-mediated drug efflux *via* blockade of PI3K/Akt pathway. Moreover, chaetominine-induced Akt inhibition suppressed nuclear expression of Nrf2, altering MRP1. However, whether the effect of chaetominine on Bax/Bcl-2 modulates ROS or/and the PI3K pathway, or whether chaetominine regulates the Nrf2 pathway awaits further elucidation [15, 19, 26]. Our data provide a foundation for investigations into the potential anticancer effects of chaetominine.

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Conflict of interest

All authors have no conflict of interests.

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Figure Captions

Figure. 1 Chaetominine induced apoptosis in K562/Adr cells. (A) Chemical structure of chaetominine. (B) Morphological changes as evidenced by Hoechst 33258 staining. Apoptotic features (white arrows) induced by chaetominine were observed under fluorescent microscopy. (C) Apoptosis was measure with flow cytometry. Annexin V-FITC/PI staining of K562/Adr cells treated with chaetominine and ADR or ADR alone for 24 h. Data are means $\pm$ SD (n=3). Significant difference: *p<0.05 vs. ADR alone group. (D) ROS measured after chaetominine treatment in K562/Adr. Values are means $\pm$ SD of three independent experiments. Significant difference: *p<0.05 vs. control group.

Figure. 2 Chaetominine increased intracellular ADR in K562/Adr cells by inhibiting MRP1. (A) Intracellular ADR evaluated by flow cytometry. MFI values of each group are means $\pm$ SD (n=3). Significant difference: *p<0.05 vs. ctrl2 group (K562/Adr cell) and [#]p<0.05 vs. ctrl1 (K562 cell) group. (B) MDR1 and MRP1 mRNA measured with qRT-PCR. Data are means $\pm$ SD (n=3). Significant difference: *p<0.05 vs. ctrl2 group (K562/Adr cell). (C) MRP1 protein measured with Western blot. K562 and K562/Adr cells at left were untreated (*p<0.05 vs. K562 group) and K562/Adr cells (right) were treated with 0, 10, 20, and 40 nM chaetominine for 24 h (*p<0.05 vs. control group). Data are means $\pm$ SD (n=3). GAPDH was an internal control.

Figure. 3 Inhibitory effect of chaetominine on PI3K/Akt signaling correlated to expression of MRP1 and Nrf2. PI3K and p-Akt protein measured with Western blot

(A) in untreated K562 and K562/Adr cells (*p<0.05 vs. K562 group); (B) in K562/Adr cells treated with different concentrations of chaetominine for 24 h (*p<0.05 vs. control group). (C) p-Akt, MRP1 and total Nrf2 protein after pretreatment with LY294002 for 1 h or 20 nM chaetominine for 24 h (*p<0.05 vs. untreated control group). Data are means $\pm$ SD (n=3). GAPDH was an internal control.

Figure. 4 Chaetominine regulated expression of Nrf2/Keap1 and Bax/Bcl-2 mediated by Akt signaling. (A) Total Nrf2 and Keap1 measured with Western blot in untreated K562 and K562/Adr cells (*p<0.05 vs. K562 group). (B) Nuclear and total Nrf2 and total Keap1 in K562/Adr cells treated with different concentrations of chaetominine for 24 h (*p<0.05 vs. each control group). (C) Bax and Bcl-2 in K562/Adr cells treated with different concentrations of chaetominine for 24 h (*p<0.05 vs. control group of Bax and #p<0.05 vs. control group of Bcl-2). Data are means $\pm$ SD (n=3). GAPDH was an internal control for total protein extracts and Lamin B was control for nuclear protein extracts.

Table 1 IC₅₀ values for ADR and chaetominine.

Group	K562	K562/Adr	RI
ADR	1.1 ± 0.02 µM	41.4 ±1.0 µM	37.64
Chaetominine	33.7 ± 0.2 nM	47.9 ±1.1 nM	1.42

IC₅₀ and RI values of ADR and chaetominine were evaluated as depicted in Methods.

Data are means ± SD (n=3).

Table 2 Effect of chaetominine on ADR cytotoxicity in K562/Adr cells.

Group	Concentration (nM)	IC ₅₀ of ADR (µM)	RF of MDR
Control	—	41.4 ±1.0 µM	1.00
Chaetominine	10	24.1 ±1.0 µM	1.72
	20	15.8 ±1.0 µM	2.62
	40	10.2 ±1.0 µM	4.06

IC₅₀ and RF values were calculated as described in Methods and Data are expressed as means ± SD (n=3).

Figure 1

Figure 2

Figure 3

Figure 4