Olmutinib Reverses Doxorubicin Resistance in ETS1-Overexpressing Leukemia Cells

ABE 1  Jiansheng Zhong
AB 2  Jinnli Zhang
BF 3  Xiaoyang Yu
CD 1  Xing Zhang
CD 1  Linping Dian

Corresponding Author: Jiansheng Zhong, e-mail: jianshengz@163.com
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Background: Drug resistance is a major problem in the treatment of leukemia with doxorubicin (Dox), and the erythroblastosis virus E26 oncogene homolog 1 (ETS1) gene is associated with drug resistance. Olmutinib is a third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) reported to play a role in reversing multidrug resistance (MDR) in cancer cells. The objective of this study was to investigate whether olmutinib could reverse Dox resistance in leukemia cells overexpressing ETS1.

Material/Methods: Human chronic myelogenous leukemia cell line K562 and its Dox-resistant cell line K562/ADR were used. Western blot and qPCR detected the expression of ETS1 and ABCB1. Cell proliferation was measured by cell counting kit-8 and methyl thiazolyl tetrazolium. Cell apoptosis was observed by western blot and flow cytometry. A nude mice K562/ADR xenograft model was used to investigate the inhibitory effects of olmutinib on tumor growth in vivo.

Results: The mRNA and protein expressions of ETS1 and ABCB1 were up-regulated in Dox-resistant leukemia cell line K562/ADR. We overexpressed ETS1 in both cell lines, finding that olmutinib inhibited the cell viability of K562 and K562/ADR in a concentration-dependent manner. The cytotoxicity of Dox to ETS1-overexpressing K562/ADR cells was enhanced by olmutinib. Olmutinib also promoted apoptosis of K562 and K562/ADR cells compared with Dox treatment alone. In vivo, olmutinib enhanced the inhibitory effects of Dox on ETS1-overexpressing K562/ADR cell xenograft growth.

Conclusions: Our results suggest that the novel EGFR TKI olmutinib enhances the sensitivity of ETS1-overexpressing leukemia cells to Dox.

MeSH Keywords: Doxorubicin • Drug Resistance • Leukemia • Proto-Oncogene Protein c-ets-1

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Background

Leukemia is a malignant clonal disease of the hematopoietic stem cells. It can be divided into acute and chronic leukemia according to the degree of differentiation and the length of the natural course. According to the types of cells involved, it can be divided into myeloid leukemia and lymphoblastic leukemia. Chemotherapy is the crucial method to treat leukemia, and anthracyclines such as doxorubicin (Dox) are the most commonly used chemotherapeutic drugs. Dox kills leukemia cells by inhibiting DNA transcription and replication and RNA synthesis, and by destroying the DNA and protein structure of leukemia cells, but the occurrence of drug resistance and side effects seriously impacts the treatment efficacy and duration of Dox [1].

Multidrug resistance (MDR), known as the resistance of cancer cells to multiple classes of anticancer drugs, is one of the crucial clinical obstacles in cancer chemotherapy leading to the failure of chemotherapy in cancer patients [2]. Although a combination of drugs may effectively induce the death of cancer cells, this method frequently results in undesired cytotoxicity. Thus, a great deal of attention has been focused on identifying the genetic and molecular mechanisms of MDR. Until now, the mechanisms of MDR have been intensively studied, and the increased efflux of anti-cancer drugs by membrane-embedded transporters is considered the predominant cause of MDR [3]. P-glycoprotein (P-gp/MDR1), belonging to the ATP-binding cassette (ABC) superfamily and encoded by ABCB1, is the most established drug transporter in humans. P-gp exists in almost all malignant tumor cells and reduces drug concentrations by pumping drugs out of cells [4]. A number of important anticancer agents, such as anthracyclines (daunorubicin, Dox), vinca alkaloids, and paclitaxel, are substrates of MDR1.

In recent years, it was reported that overexpression of the erythroid blast crisis virus E26 oncogene homolog 1 (ETS1) gene was associated with drug resistance in breast cancer [5]. Further studies indicated that the silencing of ETS1 can effectively reverse Dox or cisplatin resistance in breast carcinoma cells and sorafenib resistance in hepatocellular carcinoma cells [6–9]. Wei et al. found that inhibiting ETS1 expression significantly reduced the mRNA and protein expression levels of MDR1 (P-gp), and simultaneously increased the sensitivity of Dox-resistant breast cancer cells [6]. Another study confirmed that high ETS1 expression levels may up-regulate MDR1 gene transcription and contribute to the development of resistance [5,8]. In addition, a search of the GEO database found that, compared with the leukemia cell K562, the expression of ETS1 in Dox-resistant cells (K562/ADR cells) was significantly up-regulated (GSE93942). Furthermore, several publications showed that ETS1 was up-regulated in the blood cells from chronic myeloid leukemia patients at diagnosis, in contrast to that of healthy controls, and played a critical role in leukemic transformation [10,11]. All of these findings implicate the potential role of ETS1 in drug resistance in leukemia.

Olmutinib (HM61713/BI1482694) is an oral, novel third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) that selectively inhibits EGFR mutations. It was developed by Hanmi Pharmaceutical Co. Ltd and approved in South Korea in May 2016 for the treatment of patients with locally advanced or metastatic EGFR T790M mutation-positive non-small cell lung cancer who were pretreated with an EGFR TKI [12]. It was recently suggested that olmutinib significantly increases the sensitivity of BCRP-overexpressing cells to chemotherapy drugs, including Dox. Olmutinib not only interacted directly with BCRP but also acted as a competitive inhibitor, thus inhibiting chemotherapeutic drug efflux and increasing intracellular drug accumulation [13,14]. However, whether olmutinib could increase the sensitivity of leukemia cells to Dox remains to be elucidated.

We aimed to investigate the effects of olmutinib on ETS1-overexpressing leukemia cell proliferation and Dox resistance to these cells.

Material and Methods

Cell lines and cell culture

Human chronic myelogenous leukemia cell line K562 and its Dox-resistant cell line K562/ADR were obtained from Shanghai Meixuan Biotechnology Co., Ltd (Shanghai, China). Cells were incubated in Roswell Park Memorial Institute-1640 medium (Gibco, USA) and supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C in a humidified atmosphere of 5% CO₂.

Plasmids construction and cell transfection

The full-length DNA of ETS1 was synthesized by Thermo Scientific (USA) and cloned into pcDNA3.1 vector (forward, 5’-TAAGTGAGGTGCTGAGAGCAG-3’ and reverse, 5’-CCCCAAAGGGTAGCAA-GGT-3’). pcDNA3.1 empty vector was designed as a negative control. The pcDNA3.1-ETS1 and pcDNA3.1 were amplified by transforming into DH5α competent cells and then transfected into K562 and K562/ADR cell lines using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions.

Cell proliferation assay

The cytotoxicity of olmutinib (MedChemExpress, USA) was detected by a cell counting kit-8 (CCK-8) (Beyotime, China) following the manufacturer’s instructions. Briefly, cells were seeded...
in 96-well plates at a density of 10^4 to 10^5 cells per well. After incubation under normal conditions for 24 h, various concentrations of 0.1, 1, 10, and 100 μM were added to medium and incubated for 24 h. Then, 10 μL of CCK-8 solution was added to each well and incubated for 1 to 4 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, USA).

Methyl thiazolyl tetrazolium (MTT) assay (Beyotime, China) was utilized to detect the concentration-dependent effect of Dox on the K562 and K562/ADR cells with or without ETS1 overexpression. In short, cells were seeded in 96-well plates for 24 h, and then different concentrations of olmutinib (0.25, 0.5, and 1 μM) were added 1 h before adding a series of different concentrations of Dox. After incubation for 48 h, 20 μL MTT solution was added to each plate. Afterward, the MTT medium was discarded, and the remaining formazan crystals were dissolved with DMSO. The cell viabilities were tested at 570 nm with a microplate reader (Thermo Fisher Scientific, USA). The half inhibitory concentration (IC50) of Dox was calculated from the survivorship curve using GraphPad Prism 6. The fold-reversal of IC50 was obtained by dividing the IC50 of cells treated with Dox alone by the IC50 of cells treated with Dox in the presence of olmutinib.

RT-qPCR

The mRNA levels of ETS1 and ABCB1 were detected by RT-qPCR. In brief, the total RNA of K562 and K562/ADR cells was extracted using a TRIzol regent kit (Thermo Fisher Scientific, USA) and reverse-transcribed to cDNA using a PrimeScript RT reagent Kit (Takara Biotechnology, China). The qPCR was performed using TB Green Fast qPCR Mix (Takara Biotechnology, China) in a thermal cycler system (Applied Biosystems, USA). The qPCR was performed at least 3 times. Differences between the 2 groups were determined using student’s t-test, and the P value < 0.05 was considered as statistical significance.

Statistical analysis

All data were expressed as mean±SD, and each experiment was repeated at least 3 times. Differences between the 2 groups were determined using student’s t-test, 3 groups were analyzed using ANOVA and LSD-t-tests, and the P value < 0.05 was considered as statistical significance.

Results

ETS1 and ABCB1 were up-regulated in Dox-resistant leukemia cell line

To determine whether ETS1 and ABCB1 were involved in Dox resistance in leukemia, we assessed their protein and mRNA expression in the K562/ADR cell line. Consistent with the database

Flow cytometry

The ratio of apoptotic cells was assessed by flow cytometry using an Annexin V-FITC kit. Briefly, cells were gently washed twice with PBS, digested with 0.25% trypsin, and gently suspended in Annexin V binding buffer. After incubation with Annexin V-FITC/PI, flow cytometry was performed using a flow cytometer (Becton Dickinson, USA). Data were analyzed by flow cytometry software and the apoptosis rate was calculated from the ratio of Q2 plus Q3.

Nude mice K562/ADR xenograft model

Athymic nude mice (4–6 weeks, 16-18 g in weight) were purchased from Vital River (Beijing, China). Briefly, about 1×10^7 K562/ADR cells that overexpressed ETS1 were collected and implanted subcutaneously under the shoulders of nude mice (5-8 weeks) to establish a K562/ADR cell xenograft model. When the average tumor diameter reached 0.5 cm, the mice were randomly divided into 4 groups and treated as follows: i) saline (p.o., q2d×12); ii) olmutinib (30 mg/kg, p.o., q2d×12); iii) Dox (2 mg/kg, i.p., q2d×12); iv) olmutinib (30 mg/kg, p.o., q2d×12) given 1 h before Dox injection)+Dox (2 mg/kg, i.p., q2d×12). The body weights of animals and the 2 perpendicular diameters (A and B) of each tumor were measured every 2 days. The tumor volume (V) was calculated with this formula: \( V = \frac{1}{2} \times A \times B \times \frac{3}{2} \times \frac{4}{3} \times \frac{2}{3} \times \frac{2}{3} \). After 30 days, the animals were euthanized and the tumors were excised and weighed.
and previous studies [15,16], results showed that EST1 and ABCB1 were significantly up-regulated in Dox-resistant leukemia cells (Figure 1), indicating the stimulative effect of ETS1 and ABCB1 on Dox resistance in leukemia.

Olmutinib inhibited Dox resistance of ETS1-overexpressing leukemia cells in vitro

Next, to observe the effect of olmutinib on overcoming Dox resistance in leukemia, we overexpressed ETS1 in chronic myelogenous leukemia cell line K562 and its Dox-resistant cell line K562/ADR, and cells that transfected with empty plasmids were used as negative control. Results from western blot analysis and RT-qPCR verified the success of ETS1 overexpression (Figure 2A–2C).

The cytotoxicity of olmutinib on K562 and K562/ADR cells was detected. Cells with or without ETS1-overexpression were exposed to 0.1, 1, 10, and 100 μM olmutinib, and the cell viability was tested by CCK-8. As shown in Figure 2D, olmutinib inhibited the cell viability of both cell lines in a concentration-dependent manner. Also, olmutinib at the concentration of 10 μM began to exert obviously inhibitory effects on cell viability, so we chose ≤1 μM olmutinib for the next reversal experiments in vitro. The IC\textsubscript{50} values of Dox in K562 and K562/ADR cells with or without ETS1 overexpression in the absence or presence of 0.25, 0.5 and 1μM olmutinib are displayed in Table 1.

Olmutinib decreased the IC\textsubscript{50} values of both K562 and K562/ADR cells in a concentration-dependent manner. The sensitivity of ETS1-overexpressing Dox-resistant cells (K562/ADR Oe-ETS1) to Dox was significantly enhanced compared with that of their parental cells (K562 Oe-ETS1).

Olmutinib promoted Dox-induced apoptosis in ETS1-overexpressing leukemia cells in vitro

To observe whether olmutinib could restore the ability of Dox to induce apoptosis in Dox-resistant leukemia cells, we treated normal leukemia cells and Dox-resistant leukemia cells with 10 μM Dox, and the cell apoptosis with or without synergistic treatment of 1 μM olmutinib was compared. On the one hand, ETS1 overexpression in the K562 cell line weakened the apoptosis rate under Dox treatment, which confirmed our finding that ETS1 exerted a stimulative effect on Dox-resistance (Figure 1). However, the synergistic treatment of olmutinib partially recovered the apoptosis rate induced by Dox. On the other hand, the cytotoxicity of Dox in the Dox-resistant leukemia cell line K562/ADR was lower than that in its parental cell line K562. In addition, the presence of olmutinib also enhanced the apoptosis rate of ETS1-overexpressing cells treated with Dox (Figure 3).

At the same time, the overexpression of ETS1 increased the expression of anti-apoptotic protein Bcl-2, while it decreased

Figure 1. The expression of ETS1 and ABCB1 in K562 and K562/ADR cell lines. (A) Representative immunoblot analysis of ETS1 and ABCB1. (B) Relative protein expression of ETS1 in K562/ADR cell line (n=3). (C) Relative protein expression of ABCB1 in K562/ADR cell line (n=3). (D) Relative mRNA expression of ETS1 in K562/ADR cell line (n=3). (E) Relative mRNA expression of ABCB1 in K562/ADR cell line (n=3). ** P<0.001 vs. K562 cell line.
the expression of pro-apoptotic protein Bax both in the K562 and K562/ADR cells that were exposed to Dox. The treatment of olmutinib partially abolished the effects of ETS1 on Bcl-2 and Bax protein expression (Figure 4), indicating its recuperative effect on Dox-induced cell apoptosis in both leukemia cells and Dox-resistant leukemia cells.

**Olmutinib potentiated the efficacy of Dox in ETS1-overexpressing leukemia cell xenograft model in vivo**

An ETS1-overexpressing K562/ADR cell xenograft model was established in athymic nude mice to estimate whether olmutinib could reverse the resistance of leukemia to Dox in vivo. As shown in Figure 5, there was an obvious inhibition of tumor growth in animals under the olmutinib and Dox synergistic treatment group, compared with that in the other monotherapy groups. The mean tumor weights were 2.43±0.09 g, 2.38±0.16 g, 2.38±0.22 g, and 1.82±0.98 g in the saline, olmutinib, Dox, and
olmutinib reversed ETS1-mediated Dox resistance in vitro. This study is the first to investigate the reversal effect of olmutinib on Dox resistance in leukemia. Our results revealed that olmutinib not only enhanced the cytotoxicity of Dox but also promoted Dox-induced apoptosis in both ETS1-overexpressing K562 cells and K562/ADR cells. In addition, olmutinib potentiates the antitumor activity of Dox without causing additional side effects in vivo.

MDR is one of the major causes of poor outcomes and even failure of chemotherapy in various cancers including leukemia. Cancer cells that are originally sensitive to a monotherapy later become resistant to multiple anticancer drugs. It is believed that several mechanisms can give rise to MDR, such as increased efflux and reduced uptake of anticancer drugs, alteration in drug target, metabolic detoxification, and imbalance in DNA damage repair functions. As a cytotoxic agent, Dox is the first-line of chemotherapy for leukemia, but the resistance of leukemia cells to Dox limits its efficacy.

Table 1. Effect of olmutinib on enhancement of doxorubicin.

| Compound          | IC50 (μM) (fold-reversal) |
|-------------------|---------------------------|
|                   | K562 Oe-NC | K562 Oe-ETS1 | K562/ADR Oe-NC | K562/ADR Oe-ETS1 |
| Dox               | 0.143±0.008 (1.00) | 0.197±0.009 (1.00) | 0.202±0.009 (1.00) | 2.432±0.131 (1.00)*** |
| +Olm 0.25 μM      | 0.136±0.018 (1.04) | 0.183±0.009 (1.07) | 0.184±0.01 (1.11) | 1.331±0.123 (1.71)*** |
| +Olm 0.5 μM       | 0.113±0.016 (1.22) | 0.168±0.097 (1.18) | 0.153±0.031 (1.21) | 0.501±0.107 (4.66)*** |
| +Olm 1 μM         | 0.061±0.009 (1.94) | 0.114±0.024 (1.73) | 0.109±0.063 (1.42) | 0.233±0.054 (10.08)*** |

The fold-reversal of MDR (values given in parentheses) was calculated as described in Material and Methods. Data are shown as the mean±standard deviation (SD) of 3 independent experiments. *** P<0.001 significantly different from K562 Oe-ETS1 group.

Figure 3. The effect of olmutinib on apoptosis rate in K562 and K562/ADR cell lines overexpressing ETS1. Cell apoptosis of K562 (A) and K562/ADR (B) cells in different groups was assessed by flow cytometry (n=3). *** P<0.001 vs. Oe-NC+Dox; ** P<0.01 and ### P<0.001 vs. Oe-ETS1+Dox. Oe-NC – overexpression-negative control; Oe-ETS1 – overexpression-ETS1; Dox – doxorubicin; Olm – olmutinib.

Discussion

This study is the first to investigate the reversal effect of olmutinib on Dox resistance in leukemia. Our results revealed that olmutinib not only enhanced the cytotoxicity of Dox but also promoted Dox-induced apoptosis in both ETS1-overexpressing K562 cells and K562/ADR cells. In addition,
Interestingly, previous studies have clarified that ETS1 plays an important role in MDR of other cancers, and silencing it can reverse drug resistance [5–8]. Based on our findings, we speculated that ETS1 exerted an accelerating effect on Dox resistance in leukemia. So, we overexpressed ETS1 in both K562 and K562/ADR cell lines for the next experiment.

Besides antitumor activity, olmutinib was reported to act as a competitor of BCRP to enhance the chemotherapy sensitivity and the accumulation of Dox and rhodamine. Here, we showed that olmutinib strengthened the cytotoxicity of Dox on K562 and K562/ADR cell lines, which was implied by the result that the IC_{50} values of Dox in ETS1-overexpressing K562/ADR cells were decreased by olmutinib in a concentration-dependent manner. Apoptosis rate of tumor cells is an indicator for evaluating the antitumor effect of Dox, and, as expected, olmutinib enhanced the ability of Dox to induce apoptosis in leukemia cells. On the one hand, the apoptosis rate was increased by olmutinib compared with Dox treatment alone. On the other hand, olmutinib decreased the expression of anti-apoptotic protein Bcl-2 while it improved the expression of pro-apoptotic protein Bax.

To confirm the enhancing effect of olmutinib on the anti-tumor efficacy of Dox in vivo, we generated an ETS1-overexpressing A

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\caption{The effect of olmutinib on protein expression of Bcl-2 and Bax in K562 and K562/ADR cell lines overexpressing ETS1. (A) Representative immunoblot analysis together with relative protein expression of Bcl-2 and Bax in K562 cells (n=3). (B) Representative immunoblot analysis together with relative protein expression of Bcl-2 and Bax in K562/ADR cells (n=3). *** P<0.001 vs. Oe-NC+Dox; * P<0.05, ** P<0.01 and *** P<0.001 vs. Oe-ETS1+Dox. Oe-NC – overexpression-negative control; Oe-ETS1 – overexpression-ETS1; Dox – doxorubicin; Olm – olmutinib.}
\end{figure}

ABC transporter-mediated drug efflux is the most important mechanism of MDR, and a great deal of research has been devoted to exploiting ABC transporter inhibitors to overcome ABC transporter-mediated MDR. Until now, 3 generations of MDR have been developed. The first-generation MDR transporter inhibitors, including verapamil [17], cyclosporine A, trifluoperazine, quinidine, and progesterone, were quickly replaced by the second-generation inhibitors due to low therapeutic response and high cell toxicity [18]. Based on the structure of first-generation inhibitors, the second-generation inhibitors were designed to acquire higher potency, specificity, and lower cell toxicity. However, drug-drug interactions with anticancer drugs limited their application [4,19]. The third-generation inhibitors were developed to enhance potency of reversing MDR and overcome the defects of the previous 2 generations of inhibitors [20]. Some of them had already been tested in clinical trials, but few agents displayed remarkable effects of improving patient overall survival or response rate [21].

To handle these disadvantages, we shed light on other targets of MDR, and found that similar to with ABCB1, the expression of ETS1 was up-regulated in Dox-resistant leukemia cells, although the level was lower than that of ABCB1 (Figure 1). Interestingly, previous studies have clarified that ETS1 plays an important role in MDR of other cancers, and silencing it can reverse drug resistance [5–8]. Based on our findings, we speculated that ETS1 exerted an accelerating effect on Dox resistance in leukemia. So, we overexpressed ETS1 in both K562 and K562/ADR cell lines for the next experiment.

Besides antitumor activity, olmutinib was reported to act as a competitor of BCRP to enhance the chemotherapy sensitivity and the accumulation of Dox and rhodamine. Here, we showed that olmutinib strengthened the cytotoxicity of Dox on K562 and K562/ADR cell lines, which was implied by the result that the IC_{50} values of Dox in ETS1-overexpressing K562/ADR cells were decreased by olmutinib in a concentration-dependent manner. Apoptosis rate of tumor cells is an indicator for evaluating the antitumor effect of Dox, and, as expected, olmutinib enhanced the ability of Dox to induce apoptosis in leukemia cells. On the one hand, the apoptosis rate was increased by olmutinib compared with Dox treatment alone. On the other hand, olmutinib decreased the expression of anti-apoptotic protein Bcl-2 while it improved the expression of pro-apoptotic protein Bax.

To confirm the enhancing effect of olmutinib on the anti-tumor efficacy of Dox in vivo, we generated an ETS1-overexpressing...
K562/ADR cell xenograft model and concluded that olmutinib enhanced the efficacy of Dox on an ETS1-overexpressing Dox-resistant leukemia cell transplantation model in vivo. However, because of olmutinib’s potential to result in skin and gastrointestinal toxicities despite its excellent anticancer activity in various lung cancer cell lines with EGFR mutations, including the T790 M [22,23], and the cardiotoxicity of Dox as a long term schedule [24], we recommend further in vivo or clinical experiments of the clinically combined application of olmutinib and Dox. In addition, we know that the action of ETS1 is to promote production of blood vessels around the cancer tissues [25]. Whether the effects of olmutinib were dependent on blocking MDR1 expression remains to be elucidated. Therefore, the potential mechanisms and the effect of olmutinib on the circulation system or blood flow and MDR1 expression will be the focus of our subsequent research.

**Conclusions**

In conclusion, the present study demonstrated that the novel EGFR TKI-olmutinib was able to enhance the sensitivity of ETS1-overexpressing leukemia cells to Dox and reverse leukemia resistance to Dox in vivo. Our findings provide a potential therapeutic approach to overcome Dox resistance.

**Statement of Ethics**

The authors declare that this study was approved by the ethics committee of the Jinan University.

**Conflict of interest**

None.
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