

Osthole shows the potential to overcome P-glycoprotein-mediated multidrug resistance in human myelogenous leukemia K562/ADM cells by inhibiting the PI3K/Akt signaling pathway

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Abstract. P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) has been reported to play a pivotal role in tumor chemotherapy failure. Study after study has illustrated that the phosphoinositide 3-kinase (PI3K)/Akt signaling cascade is involved in the MDR phenotype and is correlated with P-gp expression in many human malignancies. In the present study, osthole, an O-methylated coumarin, exhibited potent reversal capability of MDR in myelogenous leukemia K562/ADM cells. Simultaneously, the uptake and efflux of Rhodamine-123 (Rh-123) and the accumulation of doxorubicin assays combined with flow cytometric analysis suggested that osthole could increase intracellular drug accumulation. Furthermore, osthole decreased the expression of multidrug resistance gene 1 (MDR1) at both the mRNA and protein levels. Further experiments elucidated that osthole could suppress P-gp expression by inhibiting the PI3K/Akt signaling pathway which might be the main mechanism accounting for the reversal potential of osthole in the MDR in K562/ADM cells. In conclusion, osthole combats MDR and could be a promising candidate for the development of novel MDR reversal modulators.

Introduction

Chronic myeloid leukemia (CML) is a type of hematological malignancy caused by the neoplastic transformation of hematopoietic stem cells (1). CML is characterized by a reciprocal chromosomal translocation t(9;22)(q34;q11) named the Philadelphia chromosome which fuses the Abelson kinase (ABL) gene from chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22 (2). The BCR-ABL fusion oncogene is responsible for the pathogenesis of CML and is the primary molecular target for therapy with imatinib mesylate (formerly ST1571), a tyrosine kinase inhibitor (3,4). Although imatinib treatment has achieved outstanding curative efficacy in the chronic phase of CML, a significant portion of patients fail drug treatment due to intrinsic or acquired drug resistance (5). The drug resistance mechanisms involve many factors, which are mainly divided into BCR-ABL-dependent and BCR-ABL-independent mechanisms (6). Among the latter is the MDR phenotype which is dependent on the expression of drug resistance proteins represented by P-gp (7). P-gp is encoded by the MDR1 gene and belongs to the superfamily of ATP-binding cassette transporters, which powerfully pump a wide variety of structurally diverse chemotherapeutics across the membranes, resulting in a decreased intracellular drug concentration and chemotherapy bioavailability (8). It has been demonstrated that imatinib is a substrate of the P-gp pump (9). Consequently, much interest is focused on searching for drug transport antagonists for these proteins or identifying proliferation inhibitors of MDR cancer cells.

There are various PI3K families found in higher eukaryotes. To date, the class IA PI3Ks consisting of a p110 catalytic subunit and a p85 regulatory subunit have been implicated in cancer (10). Multiple growth factors such as IGF, PDGF, EGF and HGF can activate PI3K (11-14). Following PI3K activation, phosphoinositol-4,5-bisphosphate (PIP₂) is converted into the second messenger phosphoinositol-3,4,5-trisphosphate (PIP₃) (15). The serine-threonine kinase Akt containing the pleckstrin homology domain binds to PIP₃ at the plasma membrane where Akt undergoes phosphorylation at both Thr308 and Ser473 (16). The aberrant activation of the PI3K/Akt cell transduction pathway has been found in different

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Abbreviations: P-gp, P-glycoprotein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; MDR, multidrug resistance; MDR1, multidrug resistance gene 1; CML, chronic myeloid leukemia

Key words: osthole, P-glycoprotein, multidrug resistance, chronic myeloid leukemia, phosphoinositide 3-kinase

types of neoplasms, which contributes collectively to inhibit cell apoptosis and promote tumor proliferation, angiogenesis, invasion and metastasis (17). Recently, involvement of the PI3K/Akt signaling pathway in MDR has attracted increased attention. Research has highlighted that the PI3K/Akt pathway is closely associated with modulation of MDR mediated by P-gp in human leukemia, breast carcinoma, and gastric cancer (18-20).

Osthole, 7-methoxy-8-(3-methyl-2-butenyl) coumarin, is extracted from the fruit of *Cnidium monnieri* (L.) Cusson and is widely used in traditional Chinese medicine. Osthole exhibits a broad spectrum of pharmacological and biological properties such as anti-hepatitis, anti-osteoporosis, anti-inflammatory, anti-allergic and anti-mutation (21). Moreover, several publications have indicated that osthole possesses neuroprotective effects against traumatic brain injury and accumulation of β -amyloid peptide injuries (22). Osthole has recently been confirmed to exhibit effective anticancer activity either *in vivo* or *in vitro*. Nevertheless, comparative experiments suggest that osthole causes neither apoptosis nor growth inhibiting effects on normal human peripheral blood mononuclear cells and cervical cells (23). Accumulating evidence has revealed that osthole can suppress proliferation as well as enhance apoptosis, invasion and migration in human lung cancer, breast carcinoma, hepatocellular carcinoma, cervical cancer, colorectal adenocarcinoma, and glioblastoma multiforme (24-29). But to the best of our knowledge, little research has been conducted to explore the function of osthole in hematopoietic malignancies, which was only reported in the human acute promyelocytic leukemia HL-60 cell line (24). On the other hand, the efficacy and the associated molecular mechanisms of osthole in the reversal of MDR, to date, have not been examined in MDR leukemia cells.

In the present study, we utilized the human myelogenous leukemia K562/ADM cell line which is resistant to doxorubicin chemotherapy and has been extensively applied for academic studies. We investigated the intrinsic cytotoxic effect of osthole on K562/ADM cells. The reversal ability of osthole and the potential linkage of the reversal of P-gp-mediated MDR and the PI3K/Akt signaling cascade were described, which may be the major mechanisms underlying the MDR reversal potential of osthole in K562/ADM cells.

Materials and methods

Chemicals and reagents. Osthole was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Osthole was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) as a stock solution (10 mM), stored at -20°C , and diluted in RPMI-1640 medium (Hyclone Laboratories, Inc., Logan, UT, USA) to the desired concentration before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study. Doxorubicin (Melone Pharmaceutical Co., Ltd., Dalian, China) was dissolved in a stock concentration of 10 mM with ddH₂O and divided into five aliquots. Monoclonal rabbit anti-human MDR1 (cat no. 12683), polyclonal rabbit anti-human AKT (cat no. 9272) and polyclonal rabbit anti-human phospho-AKT (Ser473, cat no. 9271) were obtained from Cell Signaling Technology, Inc. (Danvers,

MA, USA). Polyclonal rabbit anti-human GAPDH (cat no. bs-2188R) was a product of Biosynthesis Biotechnology Co., Ltd. (Beijing, China).

Cell lines and cell culture. The parental drug-sensitive human CML K562 cell line was supplied by the Key Laboratory of Tumour Molecular Biology of Binzhou Medical University (Binzhou, China) and the MDR K562/ADM subline was obtained from the Department of Pharmacology, The Institute of Hematology of the Chinese Academy of Medical Sciences (Tianjin, China). The two cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone), in a humidified atmosphere of 5% CO₂ at 37°C. The K562/ADM cells were grown in the presence of 1 μM doxorubicin. Before the experiment, doxorubicin was withdrawn from the cells for two weeks.

Determination of multidrug resistance. To determine the multidrug resistance of the K562/ADM cells to chemotherapeutic agents, K562/ADM cells and the parental K562 cells (1×10^4 /well) were seeded into 96-well plates and incubated with multiple concentrations of doxorubicin, diluted in RPMI-1640, for 24 h. Each group consisted of five parallel wells. After that, 10 μl of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) (Cell Counting Kit-8, CCK-8; Dojindo Molecular Technologies, Inc., Shanghai, China) solution was added to every well and incubated for a further 4 h. Then, the absorbance at 570 nm was measured using a fluorescence spectrofluorometer (F-7000; Hitachi High-Technologies Corporation, Tokyo, Japan). After plotting the dose-effect curve, IC₅₀ values and the resistant fold obtained from the data [Resistant fold = IC₅₀ of K562/ADM cells/IC₅₀ of K562 cells] were calculated. Each experiment was carried out in triplicate.

Intrinsic cytotoxicity assay. The cytotoxicity of osthole *in vitro* was assessed using the CCK-8 assay. In short, K562/ADM cells at the logarithmic growth phase were seeded into a 96-well plate at a density of 1×10^4 cells/well. Subsequently, osthole diluted in RPMI-1640 was added. The cells were cultured in a humidified incubator in 5% CO₂ at 37°C for 24 h, and the quantity of viable cells was determined. Because the concentrations of the reversal multidrug agents we needed were neither inhibitory nor toxic (30), osthole concentrations of 5 and 15 μM were finally used to study the reversal of MDR. All analyses were carried out at least thrice independently.

Assay of the reversal efficacy. The potential of osthole to overcome MDR was evaluated by the CCK-8 method in the K562/ADM cell line. K562/ADM cells were seeded into 96-well plates ($\sim 1 \times 10^4$ cells each well). Shortly afterward, doxorubicin only or combinations with osthole (5 and 15 μM) diluted in RPMI-1640 were added. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ for 24 h, and the percentages of viable cells were appraised by the CCK-8 assay. Each group included five parallel wells. The reversal fold (RF) values were calculated using the following formula: RF = IC₅₀ of doxorubicin alone/the IC₅₀ of doxorubicin in the presence of osthole.

Table I. Determination of multidrug resistance according to the sensitivity of K562/ADM and K562 cells toward doxorubicin.

Treatment	K562/ADM IC ₅₀ (μM)	K562 IC ₅₀ (μM)	Resistant fold
Doxorubicin	90.60±1.28 ^a	2.35±0.49	38.55

^aP<0.05 vs. the K562 cells. Values are the means ± SD of triplicate experiments.

Enhancement of Rhodamine-123 (Rh-123) accumulation.

ABC transporters especially P-gp play essential roles in the efflux of chemotherapeutic drugs from tumor cells into the surrounding tissue (8). To detect the capacity of osthole on the P-gp pump, K562/ADM or K562 cells were pretreated in the absence or presence of osthole at concentrations of 5 and 15 μM in an incubator at 37°C and 5% CO₂ in air for 24 h. Then, Rh-123 at a final concentration of 5 μM was applied to the wells. After incubation for 30 min, the cells were harvested by centrifugation and washed thrice with ice-cold PBS. The intracellular mean fluorescence intensity (MFI) of Rh123 was measured using a flow cytometer (FACS FC500; Beckman Coulter, Brea, CA, USA) with an excitation wavelength of 488 nm and emission wavelength of 530 nm.

Enhancement of intracellular doxorubicin accumulation. A standard procedure was employed to monitor the intracellular accumulation of doxorubicin. Briefly, K562/ADM or K562 cells (5×10⁵/well) were seeded into 6-well plates. The cells were pretreated in the absence or presence of osthole at concentrations of 5 and 15 μM in the dark at 37°C and 5% CO₂ in air for 24 h. Then, doxorubicin (5 μM) was applied to the wells. After incubation for 1 h, the cells were collected and washed three times with ice-cold PBS. The intracellular MFI associated with doxorubicin was detected by FACS. Excitation wavelength and emission wavelength were 485 and 580 nm, separately.

RNA isolation and reverse transcription-PCR (RT-PCR) analysis. Previous to PCR, K562/ADM cells were pretreated with osthole (5, 15 μM) or RPMI-1640 for 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The purity of the total RNA was assessed from the ratio of A260/A280 by a spectrophotometer (NanoDrop 2000). RNA (1–2 μg) was applied for the synthesis of the first strand cDNA. The primers used in this experiments were designed using Primer 5 version 5.6.0 software and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The primers and probe sets were as follows: MDR1 forward, 5'-GTTGCCATTGACTGAAAGAAAGAAC-3' and reverse, 5'-ACAGGAGATAGGCTGGTTTGA-3'; 126 bp); and GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CACCTGTTGCTGTAGCCAA-3'; 121 bp). The reverse transcription reaction was implemented with PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio, Otsu, Shiga, Japan). The polymerase chain reaction amplification was performed on Eppendorf Mastercycler Personal

Table II. Effect of osthole on the sensitivity of the K562/ADM cells toward doxorubicin by CCK-8 assay.

Treatment	IC ₅₀ (μM)	RF
Doxorubicin alone	97.79±2.97	
Doxorubicin + 5 μM osthole	54.63±1.31 ^a	1.79
Doxorubicin +15 μM osthole	25.45±0.47 ^a	3.84

^aP<0.05 vs. doxorubicin alone. Values are the means ± SD of triplicate experiments. RF, reversal fold.

(Eppendorf China Co., Ltd.) by using Premix Taq™ (Takara). The reaction system contained diethyl pyrocarbonate, forward primer, reverse primer, Premix Taq and template cDNA. After 5 min of initial incubation at 95°C, cDNA was amplified in 35 cycles consisting of a 40-sec denaturation at 95°C, a 30-sec annealing at 56°C and a 40-sec elongation at 72°C. The PCR products were separated by 1.5% agarose gels (Takara Biotechnology Dalian Co., Ltd.), and stained with ethidium bromide for 15 min. The images were captured by Tanon GIS system. GAPDH served as an internal standard for quality control and quantification of the target genes.

Western blot assay. K562 and K562/ADM cells following different treatments were harvested, and the proper amount of lysis buffer (Beyotime Biotechnology, Shanghai, China) was added. Protein concentrations were determined by a bicinchoninic acid protein assay kit (Beyotime Biotechnology). Protein samples were separated on 10 or 6% SDS-PAGE gel (Beyotime Biotechnology) and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked with 5% skimmed dry milk in TBS containing 0.2% Tween-20 at room temperature for 2 h and incubated overnight at 4°C with primary antibodies against MDR1 (1:1,000), AKT (1:1,000), p-Akt (Ser473, 1:1,000) and GAPDH (1:500). After three washes in TBS/Tween buffer, the membranes were incubated in horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (1:5,000; cat no. ZB-5301; Beijing ZhongShan-Golden Bridge Technology Co., Ltd., Beijing, China) for 2 h at 37°C. Detection was performed using the FluorChem FC2 gel imaging system (Alpha Innotech, San Leandro, CA, USA). Each band density was quantified using ImageJ image processing program and normalized by GAPDH for their respective lanes.

Data analysis. Statistical analyses were carried out using SPSS13.0 software (IBM SPSS, Armonk, NY, USA). Data are expressed as the mean ± SD. Statistical comparisons were evaluated by one-way ANOVA. Statistical significance was accepted at P<0.05.

Results

Determination of multidrug resistance. Based on the CCK-8 method, it was observed that the IC₅₀ values of doxorubicin in the K562/ADM cells were markedly increased in comparison with the values in the drug-sensitive K562 cells. As indicated in

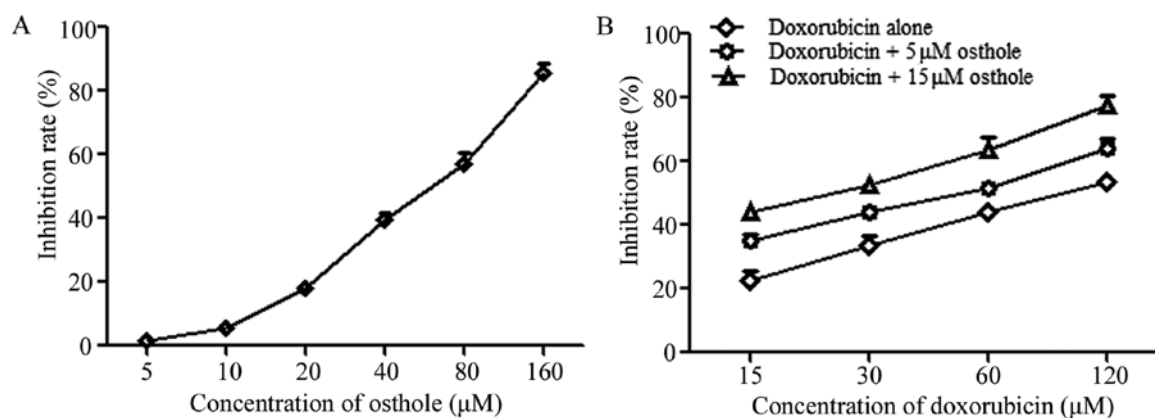


Figure 1. Inhibitory effect of osthole on K562/ADM cell proliferation. (A) K562/ADM cells were treated with osthole or RPMI-1640 for 24 h. Proliferation of cancer cells was assessed using the CCK-8 assay, and the inhibition rate (%) was calculated. (B) Effect of osthole on doxorubicin-induced cytotoxicity. Growth curves were based on data from CCK-8 assay in the K562/ADM cells treated with doxorubicin alone or in combination with osthole (5 and 15 μ M). Data are expressed as means \pm SD values of triplicate experiments.

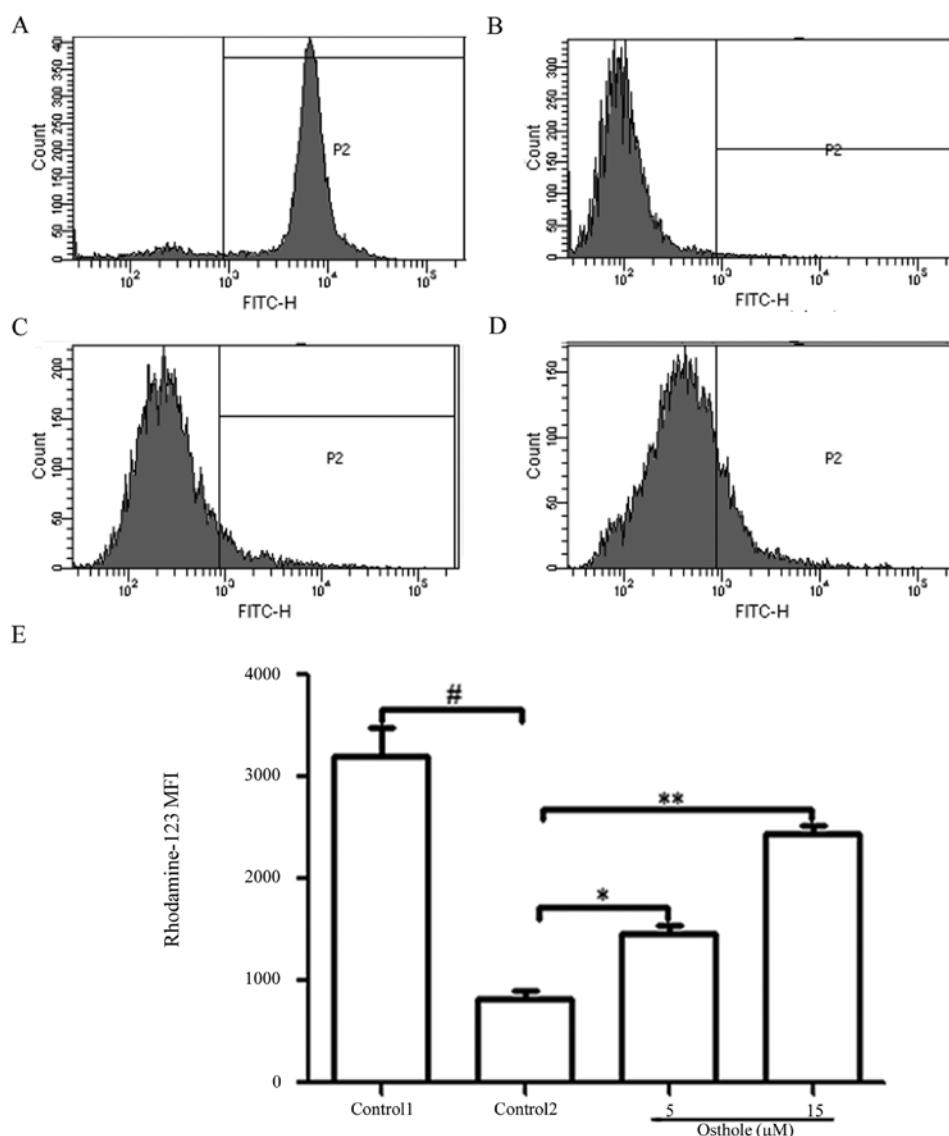


Figure 2. Effect of osthole on the intracellular accumulation of rhodamine-123 in K562 and K562/ADM cells. K562 and K562/ADM cells treated with RPMI-1640 are indicated as control1 and control2. K562 and K562/ADM cells were treated with RPMI-1640 or osthole for 24 h. Then cells were incubated with rhodamine-123 for 30 min and intracellular fluorescence was measured to assess the efflux pump function of P-gp. (A) K562 cells treated with RPMI-1640. (B) K562/ADM cells treated with RPMI-1640. (C) K562/ADM cells treated with 5 μ M osthole. (D) K562/ADM cells treated with 15 μ M osthole. (E) Graph representing the analysis of intracellular rhodamine-123 mean fluorescence intensity (MFI). Data presented are means \pm SD values from at least three independent experiments. # P <0.01 vs. control1 in K562 cells. * P <0.05 vs. control2 in K562/ADM cells. ** P <0.01 vs. control2.

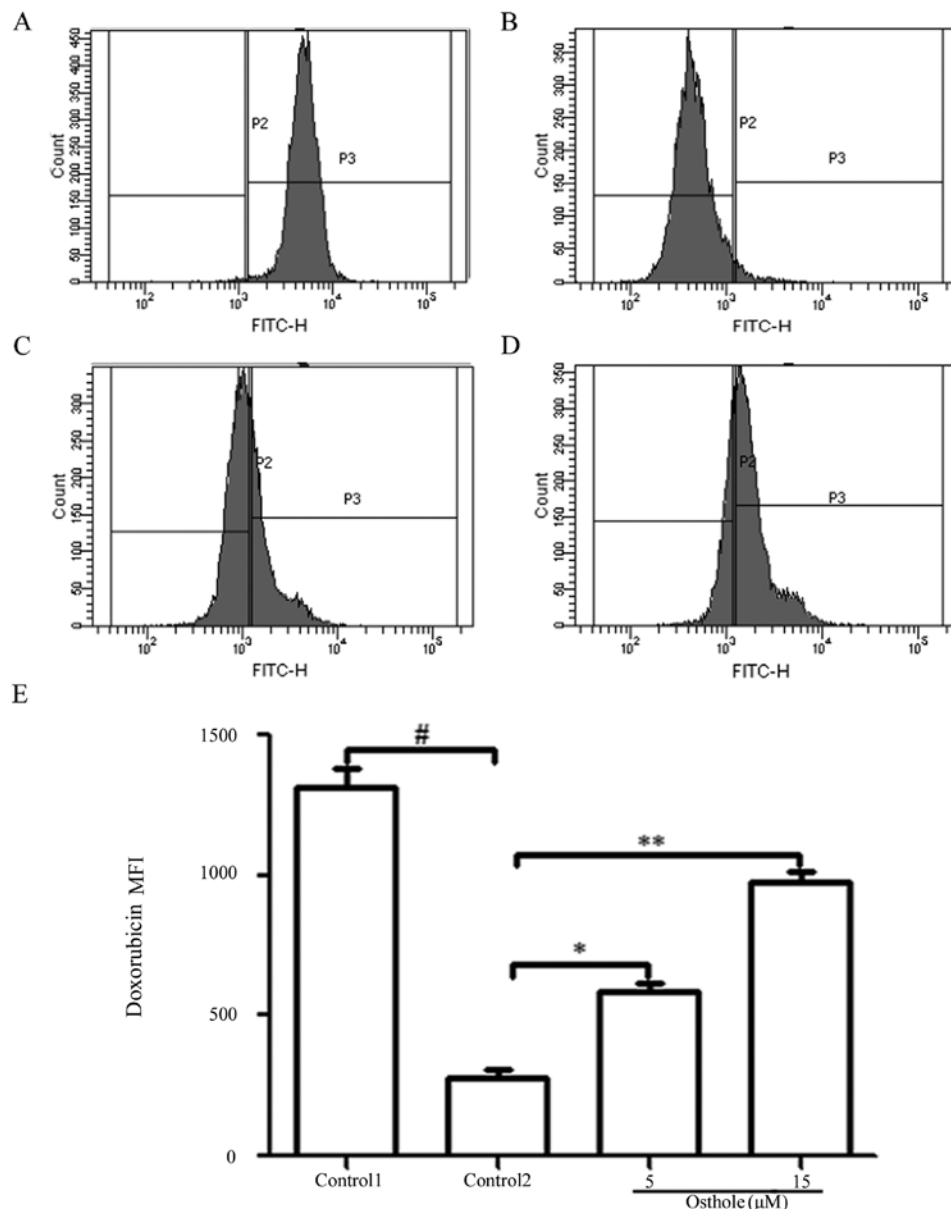


Figure 3. Osthole increases the intracellular accumulation of doxorubicin. Control1 and control2 represent K562 and K562/ADM cells treated with RPMI-1640, respectively. After K562 and K562/ADM cells were treated with RPMI-1640 or osthole for 24 h, the cells were incubated with doxorubicin for 1 h and intracellular fluorescence was measured. (A) K562 cells treated with RPMI-1640. (B) K562/ADM cells treated with RPMI-1640. (C) K562/ADM cells treated with 5 μ M osthole. (D) K562/ADM cells treated with 15 μ M osthole. (E) Graph representing the analysis of intracellular doxorubicin mean fluorescence intensity (MFI). Data are expressed as mean \pm SD of triplicate experiments. #P<0.01 vs. control1 in K562 cells. *P<0.05 vs. control2 in K562/ADM cells. **P<0.01 vs. control2.

Table I, the K562/ADM cells exhibited an ~38.55-fold increase in resistance to doxorubicin compared with the parental K562 cells.

Intrinsic cytotoxic effect of osthole in the K562/ADM cells. To acquire an indication of the potential therapeutic concentration as modulator, the CCK-8 assay was carried out to evaluate the effect of osthole on K562/ADM cells. Osthole inhibited the viability of the K562/ADM cells in a dose-dependent manner *in vitro* (Fig. 1A). As determined by the dose response curve, 5 μ M osthole exhibited no significant cytotoxicity (inhibition rate <5%) while 15 μ M osthole was weakly cytotoxic (inhibition rate 10-15%). Therefore, treatment concentrations of 5 and 15 μ M osthole were chosen in the following reversal experiments.

Reversal effect of osthole on the sensitivity to doxorubicin in the K562/ADM cells. The influence of osthole on the sensitivity of doxorubicin in the K562/ADM cells is shown in Table II and Fig. 1B. The reversal ability of the modulator, valuated by RF, was scored as follows: RF>1 implies enhanced drug sensitivity in the presence of osthole, RF=1 suggests no effect and RF<1 indicates decreased drug sensitivity; the greater the RF magnitude, the more powerful is the reversal effect (30). Osthole enhanced the sensitivity of the K562/ADM cells to doxorubicin by 1.79- and 3.84-fold at concentrations of 5 and 15 μ M, respectively. All these findings confirmed that osthole could enhance the potency of doxorubicin against K562/ADM cells, supporting the notion that osthole has a reversal effect on the drug sensitivity of K562/ADM cells.

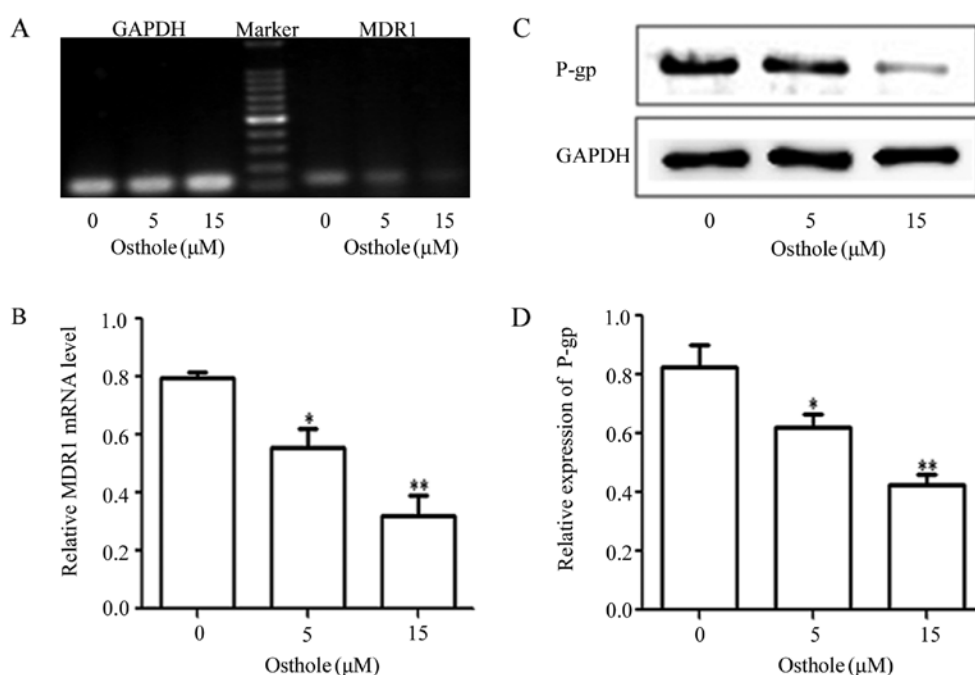


Figure 4. Osthole decreases MDR1 expression at both the mRNA and protein levels in K562/ADM cells. The MDR1 mRNA and P-gp expression following treatment with different concentrations of osthole were determined by RT-PCR and western blot analysis, separately. (A) Image of agarose ethidium bromide gel. (B) Analysis of MDR1 mRNA expression. (C) Image of SDS-PAGE. (D) Analysis of P-gp expression. Results are expressed as the mean \pm SD of triplicate experiments. * $P < 0.05$ compared with the untreated K562/ADM cells. ** $P < 0.01$ compared with the untreated K562/ADM cells.

Enhancement of Rh-123 accumulation. Flow cytometry results revealed that the intracellular accumulation of Rh-123 was evidently decreased in the K562/ADM cells in contrast with the K562 cells and the MFI was 823 ± 47.98 and 3209 ± 101.95 (Fig. 2A and B). Osthole (5 and 15 μ M) enhanced the accumulation of Rh-123 by 1.78- and 2.96-fold compared to the control group cells. The MFI of Rh-123 was 1462 ± 42.82 (osthole 5 μ M) and 2437 ± 46.47 (osthole 15 μ M) (Fig. 2C-E).

Osthole increases the intracellular accumulation of doxorubicin. The fluorescence characteristics of doxorubicin were utilized to determine the drug concentration in the cells by flow cytometry; intracellular MFI is representative of doxorubicin concentration. The detection results signified that the MFI of K562 and K562/ADM cells was 1313 ± 40.77 and 279 ± 15.53 (Fig. 3A and B). The intracellular accumulation of doxorubicin increased by 2.11- and 3.49-fold in comparison with the negative control group in the K562/ADM cells (Fig. 3C and D). These results implied that osthole elevated the sensitivity of the K562/ADM cells toward doxorubicin by increasing intracellular doxorubicin accumulation.

Osthole efficiently decreases the P-gp expression in the K562/ADM cells. K562/ADM cells express a higher level of P-gp than K562 cells (31). As shown in Fig. 4, after K562/ADM cells were treated with different concentrations of osthole, the expression of MDR1 at both the mRNA and protein levels was markedly decreased in a dose-dependent manner.

Osthole inhibits PI3K/Akt signaling pathway activity in the K562/ADM cells. According to our research, K562/

ADM cells with overexpressed P-gp demonstrated higher expression of the p-Akt protein level than the parental K562 cells (Fig. 5A and B), which revealed that osthole would be more effective in a resistant cell line when inhibiting the PI3K/Akt signaling pathway. After treatment with osthole for 24 h, there was no apparent change in the total amount of Akt, whereas p-Akt was reduced obviously (Fig. 5C and D), which was parallel to the decrease in P-gp.

Osthole downregulates P-gp expression via inhibition of the PI3K/Akt signaling pathway. To confirm the possibility that suppression of the PI3K/Akt pathway results in decreased P-gp level, we investigated the effect of the well-characterized pharmacological PI3K inhibitor LY294002 on P-gp expression in the K562/ADM cells (32). Stimulation of K562/ADM cells with LY294002 alone for 1 h led to inhibition of P-gp expression and Akt phosphorylation. In addition, combined treatment with LY294002 and osthole (pretreatment with 15 μ M osthole for 24 h, then treatment with LY294002 for 1 h) showed additive inhibitory effects on P-gp expression and Akt phosphorylation (Fig. 6A and B). That is, the inhibition of the PI3K/Akt pathway induced by osthole was responsible for the reduced P-gp expression.

Further verification of the suppression of P-gp expression via inhibition of the PI3K/Akt signaling pathway by osthole. The PI3K-specific agonist insulin-like factor-1 (IGF-1) was used to further verify the relationship between activation of the PI3K/Akt signaling pathway and P-gp expression (33). As shown in Fig. 6C, the expression of p-Akt was significantly increased by treatment with IGF-1 for 1 h. However, the increasing trend was reversed by 15 μ M osthole (pretreatment

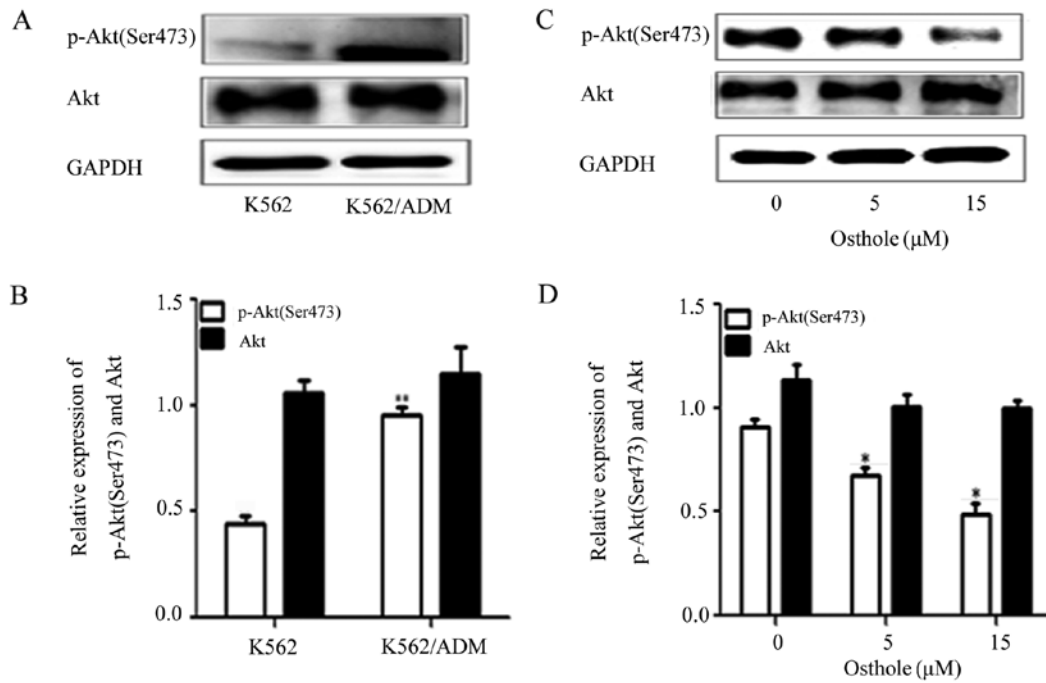


Figure 5. Osthole inhibits the PI3K/Akt signaling pathway in K562/ADM cells. (A) Expression of p-Akt(Ser473) and total Akt levels were evaluated by western blot analysis in the K562 and K562/ADM cells. (B) Analysis of protein expression. (C) K562/ADM cells were treated with different concentrations of osthole and western blot analysis was performed to detect p-Akt(Ser473) and Akt levels. (D) Analysis of protein expression. All results are expressed as the mean \pm SD of triplicate experiments. ** $P < 0.01$ compared with the K562 cells. * $P < 0.05$ compared with untreated the K562/ADM cells.

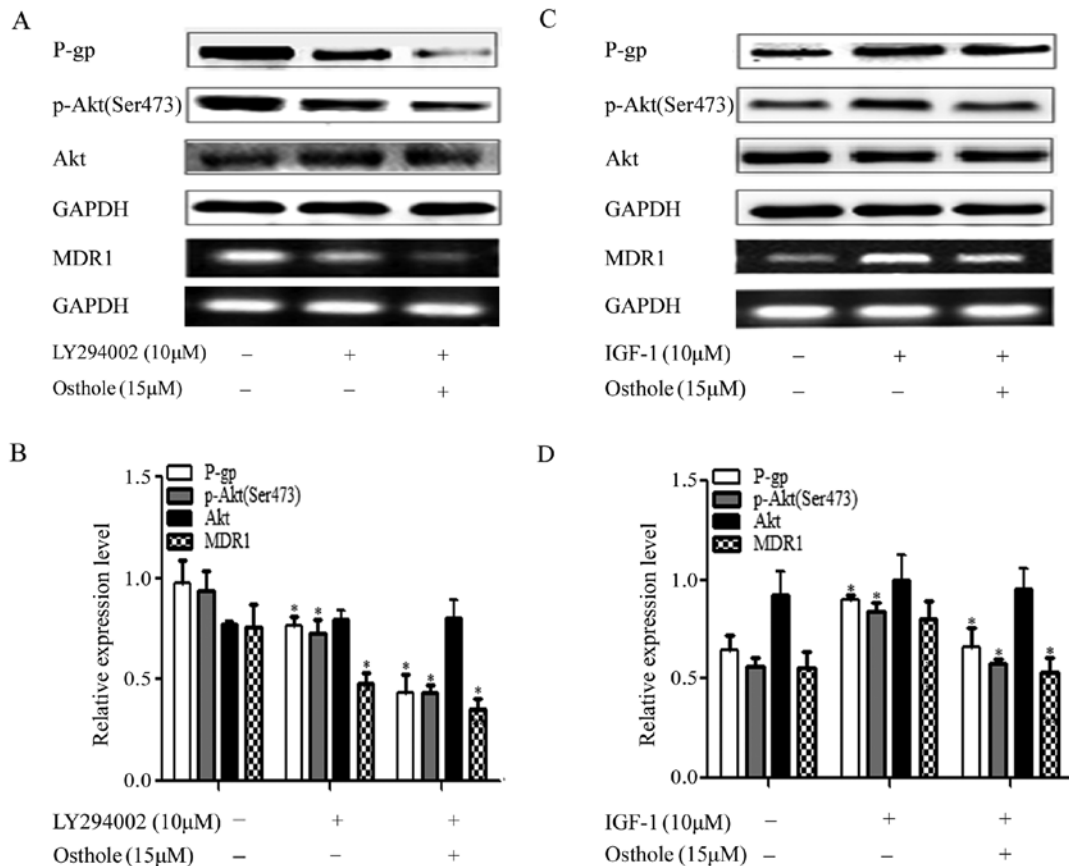


Figure 6. Osthole suppresses P-gp expression via inhibition of PI3K/Akt signaling in K562/ADM cells. (A) K562/ADM cells were either left untreated or treated with LY294002 for 1 h or combination of 15 μ M osthole for 24 h and LY294002 for 1 h. Western blot analysis was performed to detect p-Akt(Ser473) and P-gp levels. RT-PCR was used to determine MDR1 mRNA level. (B) Analysis of protein and mRNA levels. (C) Further demonstration of the suppression of P-gp expression via inhibition of PI3K/Akt signaling by osthole. K562/ADM cells were either left untreated or treated with IGF-1 for 1 h or pretreated with 15 μ M osthole for 24 h and then stimulated with IGF-1 for 1 h. Western blotting and RT-PCR were performed to detect protein and mRNA levels. (D) Analysis of protein and mRNA expression. Results are expressed as the mean \pm SD of triplicate experiments. * $P < 0.05$ compared with the control cells.

with 15 μ M osthole for 24 h, and then treatment with IGF-1 for 1 h). The same trend was found in MDR1 expression at both the transcription and translation level. These results further explain that osthole downregulated p-Akt activity, resulting in the decreased expression of MDR1.

Discussion

Multidrug resistance (MDR) mediated by drug transporters particularly P-gp has been a significant impediment to the successful treatment of cancer (34). Hence, direct blockage of the P-gp efflux pump or inhibition of its expression, thereby restoring the sensitivity of tumor cells to chemotherapeutic agents, is an ideal strategy for MDR reversal. To date, many P-gp inhibitors have been developed. Verapamil and cyclosporine A, as classical representatives of first-generation P-gp inhibitors, are combined with various types of chemotherapy regimens for many neoplasms, but the therapeutic effects are not satisfactory. Due to pharmacokinetic interaction with chemotherapeutic drugs, second-generation P-gp inhibitors such as dexverapamil, PSC 833 and VX-710 are confined in clinical use (35). Time and resources have been spent on the exploration of the third-generation P-gp inhibitors represented by Zosuquidar (LY335979), Tariquidar (XR9576), Laniquidar (R102933), Elacridar, PGP-4008, CBT-1 and HM30181. Despite the initial enthusiasm following the preliminary report of XR9576, clinical trials brought the tragic reality to light (36,37). All in all, none of these hopeful modulators has been licensed for application clinically owing to their toxicities at the high doses needed for effective use or highly unacceptable side effects (38). New reversal agents which are efficient and minimally toxic require active exploration.

In order to find novel, non-toxic, and high affinity P-gp inhibitors, more and more researchers have put emphasis on screening active components from natural sources, which are classified by some authors as fourth-generation P-gp inhibitors (37). Osthole, a natural coumarin compound, was verified to potently induce apoptosis and to attenuate the migration of tumor cells but caused no significant side effects to normal cells (23,24). Furthermore, osthole is capable of enhancing the immunity of the human body, which is a major advantage that makes it superior to existing chemotherapy drugs (21). Taken together, great expectations have been put on its potential role in drug discovery and clinical utilization.

In the present study, we evaluated the efficacy of osthole as a powerful reversal agent for overcoming drug resistance of K562/ADM cells for the first time. Cytotoxicity analysis implied that the cytotoxicity of osthole in K562/ADM cells was dose-dependent. In accordance with the experimental results, weakly cytotoxic concentrations of 5 and 15 μ M osthole were ultimately administered to the cells. Data indicated that IC_{50} values of doxorubicin were significantly decreased in the K562/ADM cells after osthole treatment, which indicated that osthole could distinctly enhance the doxorubicin-induced cytotoxicity toward K562/ADM cells. Herein, the reversal efficiency of osthole was reflected by RF which reached ~4-fold. Moreover, doxorubicin and rhodamine-123 efflux assay was exploited to assess the inhibition of P-gp transport by osthole and to estimate the interaction between osthole and this protein. The flow cytometric assay indicated that

osthole notably reversed the accumulation deficit and blocked the cellular efflux of P-gp substrates doxorubicin and rhodamine-123 in a concentration-dependent manner. To reveal the mechanisms, the MDR1 expression at both the transcription and translation levels were examined. The mRNA as well as the protein expression levels of MDR1 were significantly suppressed in the K562/ADM cells when treated with 5 and 15 μ M osthole (Fig. 4). In summary, osthole partially reversed MDR of K562/ADM cells by inhibiting the cellular efflux function of P-gp and downregulating the expression level of MDR1, thus elevating intracellular chemotherapeutic accumulation.

Previous studies have indicated that the PI3K/Akt pathway is excessively activated in a wide variety of hematologic malignancies including CML, diffuse large B-cell lymphoma, acute myeloid leukemia, T-cell acute lymphoblastic leukemia and chronic lymphoblastic leukemia; it is considered to be responsible for tumorigenesis by simultaneously promoting proliferation and inhibiting apoptosis (39). Morishita *et al* proposed that Akt phosphorylation can induce chemotherapeutic resistance in B-pre-acute lymphoblastic leukemia (40). Related studies suggest that Akt is involved in the regulation of the MDR1 gene and that P-gp is one of the key factors downstream of the PI3K/Akt pathway (41). To understand why osthole decreased the expression level of MDR1, we further detected the phosphorylation of Akt following treatment of osthole. Interestingly, we found a parallel relationship between the activity of Akt and expression of P-gp. In other words, the expression level of p-Akt and P-gp were both decreased by addition of osthole, but without a change in the levels of total Akt kinase. Our findings are consistent with previous investigations in other MDR tumor cells such as breast cancer cells, and gastric carcinoma cells (19,42), which illustrate that excessive activation of PI3K/Akt has an intimate correlation with P-gp expression. Importantly, specific PI3K/Akt signaling pathway inhibitor LY294002 and stimulator IGF-1 were used. In the LY294002 stimulating experiment, the inhibitory effect of osthole on p-Akt and P-gp demonstrated in the LY294002 addition group was enhanced in contrast with the combination of LY294002 and osthole group. Moreover, IGF-1 was capable of escalating MDR1 expression and promoting phosphorylation of Akt, while the trend was mostly reversed by osthole. Thus, osthole reversed P-gp-mediated MDR by inhibiting the PI3K/Akt signaling cascade. Unfortunately, it should be noted that various alterations in the PI3K/Akt pathway provide multiple molecular targets for treatment and compounds the difficulty to identify the key hub or hubs where osthole or other targeted therapeutic interventions would be most efficacious.

Overall, we first revealed that osthole is a novel and potent modulator which opens the way for reversing MDR in human myelogenous leukemia K562/ADM cells. At the same time, the potential association of the reversal of P-gp-mediated MDR and the PI3K/Akt signaling cascade was identified, which may be the major mechanism involved in the reversal of MDR by osthole in K562/ADM cells.

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