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# Euphorbia Factor L1 Reverses ABCB1-Mediated Multidrug Resistance Involving Interaction With ABCB1 Independent of ABCB1 Downregualtion

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## ABSTRACT

Euphorbia factor L1 (EFL1) belongs to diterpenoids of genus *Euphorbia*. In this article, its reversal activity against ABCB1-mediated MDR in KBv200 and MCF-7/adr cells was reported. However, EFL1 did not alter the sensitivity of KB and MCF-7 cells to chemotherapeutic agents. Meanwhile, EFL1 significantly increased accumulation of doxorubicin and rhodamine 123 in KBv200 and MCF-7/adr cells, showing no siginficant influence on that of KB and MCF-7 cells. Furthermore, EFL1 could enhance the ATP hydrolysis activity of ABCB1 stimulated by verapamil. At the same time, EFL1 inhibited the efflux of ABCB1 in KBv200 and MCF-7/adr cells. In addition, EFL1 did not downregulate expression of ABCB1 in KBv200 and MCF-7/adr cells either in mRNA or protein level. J. Cell. Biochem. 112: 1076–1083, 2011. 

**KEY WORDS:** MULTIDRUG RESISTANCE; EUPHORBIA FACTOR L1; ABCB1

Multidrug resistance (MDR) enabling cancer cells to acquire resistance to a broad spectrum of structurally and functionally different anticancer drugs is thought to be the major obstacle of the successful cancer chemotherapy [Cornwell et al., 1986; Glavinas et al., 2004]. MDR is usually mediated by overexpression of ATP-binding cassette (ABC) transporters, which transport chemotherapeutic drugs out of tumor cells against a concentration gradient with the use of energy supplied by ATP hydrolysis [Litman et al., 2001; Mitomo et al., 2003]. In the human genome, 48 different ABC transporters have been identified and divided into seven subfamilies (A–G) based on sequence similarities [Dean et al., 2001; Glavinas et al., 2004]. Thus far, the major members of the ABC transporters leading to MDR include ABC subfamily B member 1 (ABCB1; also called P-glycoprotein), ABC subfamily C members (ABCCs, MDR-associated proteins), and ABC subfamily G member 2 (ABCG2; also called breast cancer resistance protein, mitoxantrone resistance protein, and placenta-specific ABC transporter) [Dai et al., 2008]. These proteins are highly varied transporters sharing the ability to recognize and efflux a large number of structurally diverse, mainly hydrophobic compounds out of cancer cells. Usually, each transporter translocates its unique drugs, in addition to the overlapping substrate specificity of several transporters [Dai et al., 2009; Zheng et al., 2009]. Drugs transported by ABCB1 comprise hydrophobic compounds, either uncharged or slightly positively charged, including most chemotherapeutic agents such as vinca alkaloids, anthracyclines, epipodophyllotoxins, and

Abbreviations: MDRmultidrug resistanceABCATP-binding cassetteGAPDHglyceraldehyde glyceraldehyde-3-phosphate dehydrogenaseDOXdoxorubicinMTT3-(4,5-dimethylthiazol-yl)-2,5-diphenyllapatinibrazolium bromideVCR-vincristineRh123rhodamine 123R-VRPverapamil R-enantiomerFBSfetal bovine serum

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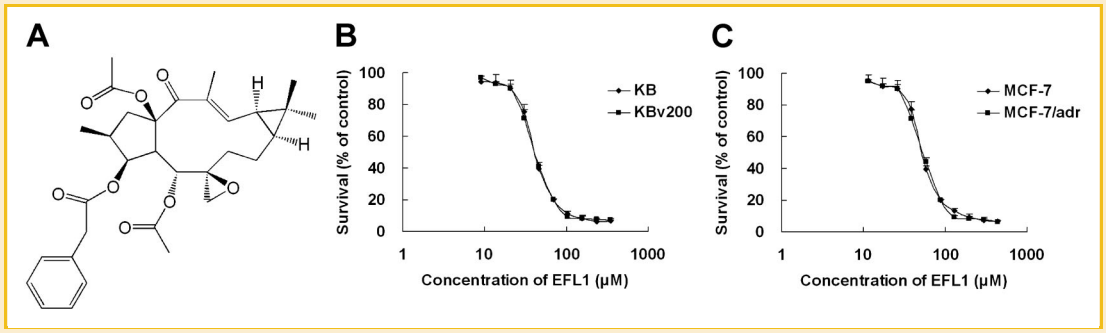


Fig. 1. Chemical structure of EFL1 (A) and its $IC_{50}$ curves of KB, KBv200, MCF-7, MCF-7/adr cells, respectively. ABCB1-negative KB and ABCB1-positive KBv200 cells (B), ABCB1-negative MCF-7 and ABCB1-positive MCF-7/adr cells (C), were treated with indicated concentrations of EFL1 for 72 h. Each point represents the means ± standard deviations (SDs) out of three determinations. Each experiment was performed in three replicate wells.

taxanes. ABCB1 (P-glycoprotein, Pgp, and MDR1), the first cloned human ABC transporter, has been demonstrated to take the leading importance of mediating MDR [Dai et al., 2007; Tao et al., 2009].

Enormous effort has been devoted to development of inhibitors of ABC transporters to overcome MDR. Three generations of MDR inhibitors have been exploited, some of which have showed potent ability of reversing MDR [Aoki et al., 2001; Shi et al., 2006; Dai et al., 2008]. Clinical trials evaluating the potential reversal of MDR by several inhibitors are still underway. In addition to the reversal potency, the acting mechanisms of reversal agents are also interesting. The drug efflux function of ABCB1 is coupled ATP hydrolysis stimulated by ABCB1 substrates. According to the effect on ATPase activity of ABC transporters, agents are categorized into three distinct types. The first type of compounds stimulates ATPase activity at low concentrations while inhibits the activity at high concentrations, the second enhances ATPase activity showing dose-dependent manner without any inhibition, whereas the third inhibits both basal and stimulated ATPase activity [Ambudkar et al., 1999]. Lapatinib was demonstrated to reverse ABCB1-mediated MDR and can increase the ATPase activities of the transporters at low concentrations [Dai et al., 2008]. It has been reported that vandetanib and verapamil can increase the ATPase activity of ABCB1 in a dose-dependent manner [Hamada and Tsuruo, 1988; Mi and Lou, 2007].

Recently, we found Euphorbia factor L1 (EFL1, Fig. 1A) belonging to diterpenoid showed the potent ability of reversing MDR mediated by ABCB1. It has been reported that this type of compounds exhibited potential of reversing MDR [Ferreira et al., 2005; Duarte et al., 2006; Engi et al., 2007; Choi et al., 2009]. However, the reversal potency and the detailed mechanisms involving remained unclear. In this article, we reported the reversal of MDR reversed by EFL1 and the involving mechanisms for the first time, during which KBv200 and MCF-7/adr cells overexpressing ABCB1 were concerned.

## MATERIALS AND METHODS

### CHEMICALS AND REAGENTS

EFL1 (Fig. 1A) was isolated from Caper Euphorbia Seed and identified as purity of more than 98% [Zhang et al., 2010]. Dulbecco's modifed Eagle's medium (DMEM) and RPMI 1640 were products of Gibco BRL. Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-mouse, and anti-rabbit IgG-HRP were products of Kangchen Co. (Shanghai, China). Monoclonal antibody to ABCB1 was purchased from Santa Cruz Biotechnology, Inc. Vincristine (VCR), doxorubicin (DOX), 3-(4,5-dimethylthiazol-yl)-2,5-diphenyllapatinibrazolium bromide (MTT), rhodamine 123 (Rh123), and verapamil R-enantiomer (R-VRP) were products of Sigma Chemical Co.

### CELL LINES AND CELL CULTURE

The human oral epidermoid carcinoma cell line KB and its VCR-selected derivative ABCB1 overexpressing cell line KBv200 were maintained in RPMI 1640 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). The human breast carcinoma cell line MCF-7 and its DOX-selected derivative ABCB1 overexpressing cell line MCF-7/adr were cultured in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. All cells were cultured in a humidified atmosphere incubator containing 5% $CO_2$ and 95% air at 37°C [Zhang et al., 2007].

### CELL VIABILITY ASSAY

The MTT assay was applied to examine cytotoxicity as described before [Zhang et al., 2009]. The concentrations required to inhibit growth by 50% ($IC_{50}$) were calculated from survival curves with the Bliss method. The degree of resistance was calculated by dividing the $IC_{50}$ for the MDR cells by that of the parental sensitive cells, and the degree of the reversal of MDR was calculated by dividing the $IC_{50}$ of the anticancer drug in the absence of EFL1 by that obtained in the presence of EFL1 [Chen et al., 2004].

### DOX AND Rh 123 ACCUMULATION

The effect of EFL1 on the accumulation of DOX and Rh 123 was measured by flow cytometry as previously described. Briefly, $5 \times 10^5$ cells of KB, KBv200, MCF-7, and MCF-7/adr were incubated in six-well plates and allowed to attach overnight, respectively. The cells were treated with indicated concentrations of EFL1 at 37°C for 3 h. Then 10 μM DOX or 5 μM Rh 123 of final concentration was added and the cells were further cultured for another 3 or 0.5 h,

respectively. Cells were then collected and washed twice with ice-cold PBS buffer. Finally, cells were resuspended in PBS buffer for flow cytometric analysis (Beckman Coulter, Cytomics FC500) and $1 \times 10^4$ cells were counted for the fluorescence intensity. R-VRP was used as the control inhibitor in the experiments [Zheng et al., 2009].

### PREPARATION OF CELL LYSATES AND WESTERN BLOT ANALYSIS

After EFL1 treatment, cells were harvested and washed twice with ice-cold PBS buffer. Cell extracts were collected in cell lysis buffer ($1 \times$ PBS, 1% Nonidet P-40, 0.1% SDS, 100 mg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) for 30 min with occasional rocking and clarified by centrifugation at $12{,}000 \times g$ for 15 min at 4°C. Equal amount of lysate protein was separated on 8–12% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membrane (Pall). The nonspecific binding sites were blocked by TBST buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.4, and 0.4% (v/v) Tween 20) containing 5% nonfat dry milk for 2 h at room temperature. The membranes were then incubated overnight at 4°C with specific primary antibodies. Thereafter, the membranes were washed thrice with TBST buffer for 15 min and incubated at room temperature for 2 h with HRP-conjugated secondary antibody. After washed thrice with TBST buffer for 15 min, the immunoblots were visualized by the enhanced Phototope™-HRP Detection Kit purchased from Cell Signaling Technology (Beverly, MA) and exposed to Kodak medical X-ray processor (Kodak). GAPDH or HSP70 were used as the loading control [Tao et al., 2009].

### REVERSE TRANSCRIPTION-PCR

After cells were treated with EFL1 for 48 h, total cellular RNA was isolated by Trizol Reagent RNA extraction kit (Molecular Research Center) following the manufacturer's instruction. The first strand of cDNA was synthesized by Oligo dT primers. PCR primers were 5′-ccc atc att gca ata gca gg-3′ (forward) and 5′ -gtt caa act tct gct cct ga-3′(reverse) for ABCB1 and 5′-ctt tgg tat cgt gga agg a-3′ (forward) and 5′ -cac cct gtt gct gta gcc-3′ (reverse) for GAPDH. With the GeneAmp PCR system 9700 (PE Applied Biosystems), reactions were carried out at 94°C for 2 min for initial denaturation, and then at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min. After 35 cycles of amplification, additional extensions were perfromed at 72°C for 10 min. Products were resolved and examined by 1.5% agarose gel electrophoresis. Expected reverse transcription-PCR (RT-PCR) products were 157 bp for ABCB1 and 475 bp for GAPDH, respectively [Zheng et al., 2009].

### ABCB1 ATPASE ACTIVITY ASSAY

Changes of ATPase activity were estimated by Pgp-Glo™ assay systems (Promega). The inhibitory effects of EFL1 were examined against a verapamil-stimulated Pgp ATPase activity. Sodium orthovanadate ($Na_3VO_4$) was used as the Pgp ATPase inhibitor. Series concentrations of EFL1 diluted by assay buffer were incubated in solution containing 0.1 mmol/L verapamil, 5 mmol/L $Mg^{2+}$ATP, and 25 μg recombinant human Pgp membranes at 37°C for 40 min. Luminescence was initiated by ATP detection buffer. After incubated at room temperature for 20 min to allow luminescent signal to develop, the untreated white opaque 96-well plate (corning) was read on luminometer (spectraMax M5, molecular devices). The changes of relative light units (ΔRLU) were determined by comparing $Na_3VO_4$-treated samples with EFL1 and verapamil combination-treated samples [Dai et al., 2008].

### EXPERIMENTS OF Rh123 EFFLUX

Experiments of Rh123 efflux were carried out following modified methods as described before [Dai et al., 2009]. After KB, KBv200, MCF-7, and MCF-7/adr cells were treated with 5 μM Rh123 for 30 min at 37°C, the cells were washed twice with ice-cold PBS and

TABLE I. Effects of EFL1 on Reversing ABCB1-Mediated Drug Resistance

| | $IC_{50} \pm SD$ (μM) (fold-reversal) | | | |
|---|---|---|---|---|
| | KB | | KBv200 (ABCB1) | |
| VCR | 0.0083 ± 0.0007 | | 1.2644 ± 0.0303 | |
| +2.5 μM EFL1 | 0.0078 ± 0.0009 | (1.06) | 0.3602 ± 0.0214** | (3.51) |
| +5.0 μM EFL1 | 0.0089 ± 0.0012 | (0.93) | 0.1877 ± 0.0041** | (6.74) |
| +10.0 μM EFL1 | 0.0074 ± 0.0014 | (1.12) | 0.0502 ± 0.0011** | (25.19) |
| Cisplatin | 2.98 ± 0.21 | | 3.67 ± 0.29 | |
| +10.0 μM EFL1 | 3.14 ± 0.27 | (0.95) | 3.54 ± 0.32 | (1.04) |
| DOX | 0.0257 ± 0.0033 | | 1.5674 ± 0.0409 | |
| +2.5 μM EFL1 | 0.0279 ± 0.0044 | (0.92) | 1.1774 ± 0.0167* | (1.33) |
| +5.0 μM EFL1 | 0.0243 ± 0.0039 | (1.06) | 0.4251 ± 0.0435** | (3.69) |
| +10.0 μM EFL1 | 0.0256 ± 0.0027 | (1.00) | 0.1436 ± 0.0115** | (10.92) |
| | MCF-7 | | MCF-7/adr (ABCB1) | |
| DOX | 0.639 ± 0.042 | | 35.83 ± 2.54 | |
| +2.5 μM EFL1 | 0.627 ± 0.068 | (1.02) | 17.60 ± 2.08** | (2.04) |
| +5.0 μM EFL1 | 0.655 ± 0.092 | (0.98) | 9.93 ± 0.94** | (3.61) |
| +10.0 μM EFL1 | 0.621 ± 0.083 | (1.03) | 3.54 ± 0.78** | (10.12) |
| Cisplatin | 22.06 ± 1.48 | | 19.22 ± 1.73 | |
| +10.0 μM EFL1 | 20.61 ± 1.53 | (1.07) | 21.27 ± 2.01 | (0.90) |

Cell survival was measured by MTT assay as described in "Materilas and Methods Section". Data are the means ± standard deviations (SDs) of at least three independent experiments. Each experiment was carried out for three replicate wells. The reversal fold of MDR was calculated by dividing the $IC_{50}$ values of corresponding cells exposed to anticancer agents in the absence of EFL1 by that obtained in the presence of EFL1.
*$P < 0.05$ indicates significance.
**$P < 0.01$ indicates very significance.

subsequently maintained at 37°C and absence of Rh123 with culture media containing 10 μM EFL1 or not. At time of 0, 15, 30, 60, and 120 min, cells were gathered and washed twice with ice-cold PBS. Finally, cells were resuspended in ice-cold PBS buffer for flow cytometric analysis immediately (Beckman Coulter) and the fluorescence intensity was determined.

### STATISTICAL ANALYSIS

All experiments were carried out at least thrice. Statistical analysis was done by Student's *t*-test analyses. $^{*}P < 0.05$ was indicative of significant difference and $^{**}P < 0.01$ was indicative of very significant difference.

## RESULTS

### EFFECTS OF EFL1 IN VARIOUS MDR CELLS AND THEIR PARENTAL CELLS

We examined the cytotoxicity of EFL1 alone in different cell lines by the MTT assay. The $IC_{50}$ values were $50.05 \pm 4.31$, $52.49 \pm 3.58$, $65.31 \pm 4.29$, and $62.34 \pm 4.74$ μM to KB, KBv200, MCF-7, MCF-7/adr cells, respectively (Fig. 1B,C). More than 90% of cells were viable at the concentrations of EFL1 up to 10.0 μM in all cells under experiments. We selected EFL1 of 2.5, 5.0, and 10.0μM to assess reversal of MDR in vitro.

### EFL1 REVERSED ABCB1-MEDIATED MDR In Vitro

The indicated concentrations of EFL1 were chosen for combination treatment with known anticancer drugs acting as substrates of ABCB1, such as VCR and DOX. The results (Table I) showed that EFL1 led to increase of cytoxicity of VCR and DOX to ABCB1-overexpressing KBv200 and MCF-7/adr cells. However, EFL1 did not show the effect on the cytoxicity of VCR and DOX to parental sensitive KB and MCF-7 cells. To evaluate substrate specificity of the transporter, cisplatin which is not the substrate of ABCB1 was selected as the control. Intriguingly EFL1 did not significantly alter the $IC_{50}$ values of cisplatin in parental sensitive and ABCB1-mediated MDR cells. These results suggest that EFL1 strongly enhances the sensitivity of ABCB1-overexpressiong MDR cells to conventional chemotherapeutic agents. Meanwhile, EFL1 do not affect the sensitivity of parental sensitive cells.

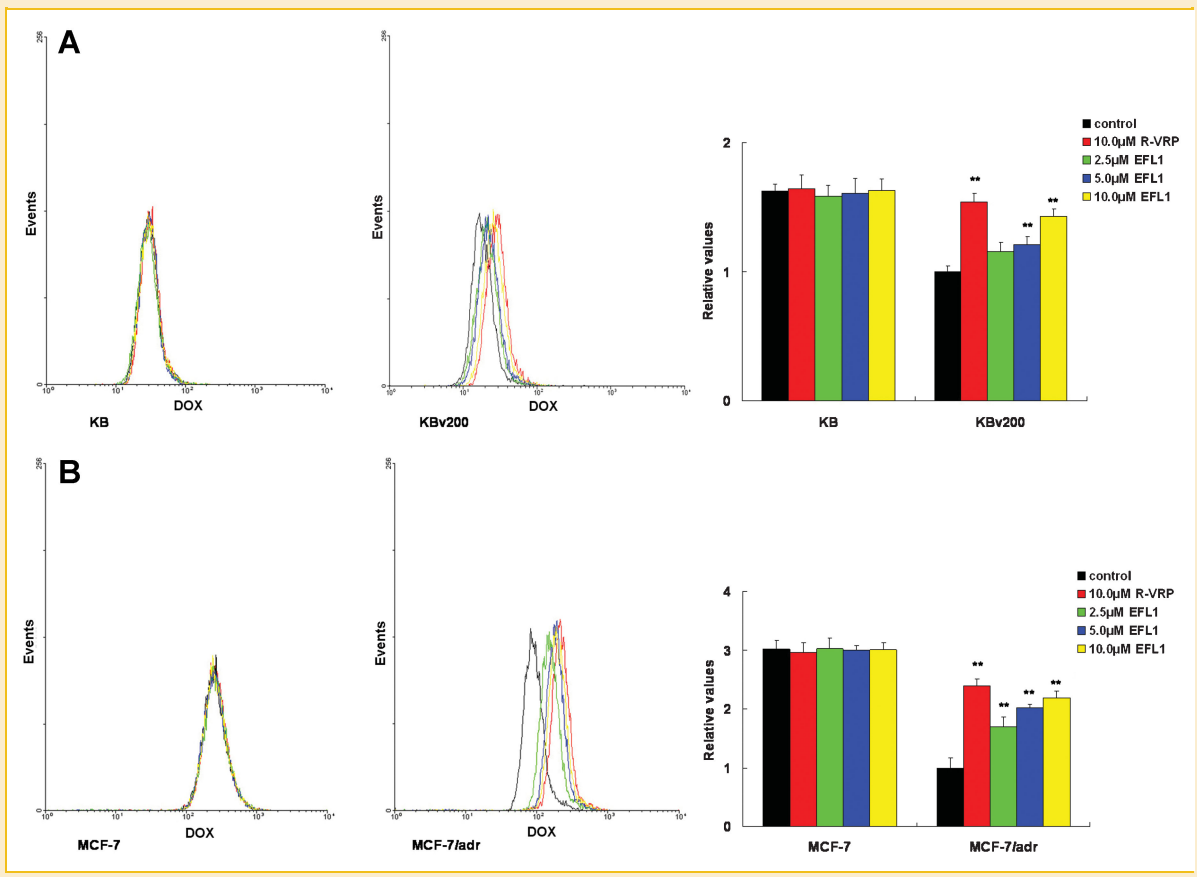


Fig. 2. Effects of EFL1 on the accumulation of DOX. KB, KBv200, MCF-7, and MCF-7/adr cells were incubated with 0, 2.5, 5.0, and 10.0 μM EFL1 at 37°C for 3 h. Then 10 μM DOX of final concentration was added for another 3 h incubation. Intracellular fluorescence was analyzed by flow cytometry with an excitation wavelength of 488 nm. VRP of 10.0 μM of final concentration was used as the positive control. A: accumulation of DOX in KB, KBv200 cells, and the column analysis. B: Accumulation of DOX in MCF-7, MCF-7/adr cells, and the column analysis. All experiments were repeated at least thrice and the representative experiment result is shown. The relative value was calculated by dividing the fluorescence intensity of sensitive or corresponding drug resistance cells by that of the drug resistance cells without treatment of verapamil or EFL1, respectively. Columns, means of triplicate determinations; *bars* SDs. $^{*}P < 0.05$; $^{**}P < 0.01$ versus the control group.

### EFL1 ELEVATED INTRACELLULAR ACCUMULATION OF DOX IN MDR KBv200 AND MCF-7/adr CELLS OVEREXPRESSING ABCB1

The above results exhibited the reversal activity of EFL1. To investigate the related mechanisms involving, we examined whether EFL1 affect the accumulation of DOX in parental sensitive and ABCB1-mediated MDR cells. The results (Fig. 2) showed that EFL1 increased the accumulation of DOX in KBv200 and MCF-7/adr cells showing the significantly higher fluorescence of DOX assayed by flow cytometry. In KBv200 cells, the intracellular accumulation of DOX was enhanced to 1.54, 1.15, 1.21, and 1.43 fold for 10.0 μM R-VRP, 2.5, 5.0, and 10.0 μM EFL1, respectively. In MCF-7/adr cells, the intracellular accumulation of DOX were enhanced to 2.39, 1.70, 2.02, and 2.18 fold for 10.0 μM R-VRP, 2.5, 5.0, and 10.0 μM EFL1, respectively. However, EFL1 did not increase the intracellular accumulation of DOX in KB and MCF-7 cells. These results demonstrated that EFL1was able to interfere with ABCB1-mediated transport.

### EFL1 INCREASED INTRACELLULAR ACCUMULATION OF Rh123 IN MDR KBv200 AND MCF-7/adr CELLS OVEREXPRESSING ABCB1

DOX accumulation experiment indicated that EFL1 was able to increase intracellular accumulation of DOX in KBv200 and MCF-7/adr cells. To make further clarification that EFL1 could increase intracellular accumulation of chemotherapeutic agents, we carried out the experiment of Rh123 accumulation. The results (Fig. 3) exhibited that EFL1 notably enhanced the accumulation of Rh123 in KBv200 and MCF-7/adr cells. Meanwhile, the intracellular accumulation of Rh123 in KB and MCF-7 did not change after treatment of EFL1. In KBv200 cells, the intracellular accumulation of Rh123 were increased to 29.65, 1.97, 2.91, and 5.63 fold for 10.0 μM R-VRP, 2.5, 5.0, and 10.0 μM EFL1, respectively. In MCF-7/adr cells, the intracellular accumulation of Rh123 were raised to 38.19, 5.38, 7.76, and 10.18 fold for 10.0 μM R-VRP, 2.5, 5.0, and 10.0 μM EFL1, respectively. These results supplied confirmations that EFL1 enhanced the intracellular

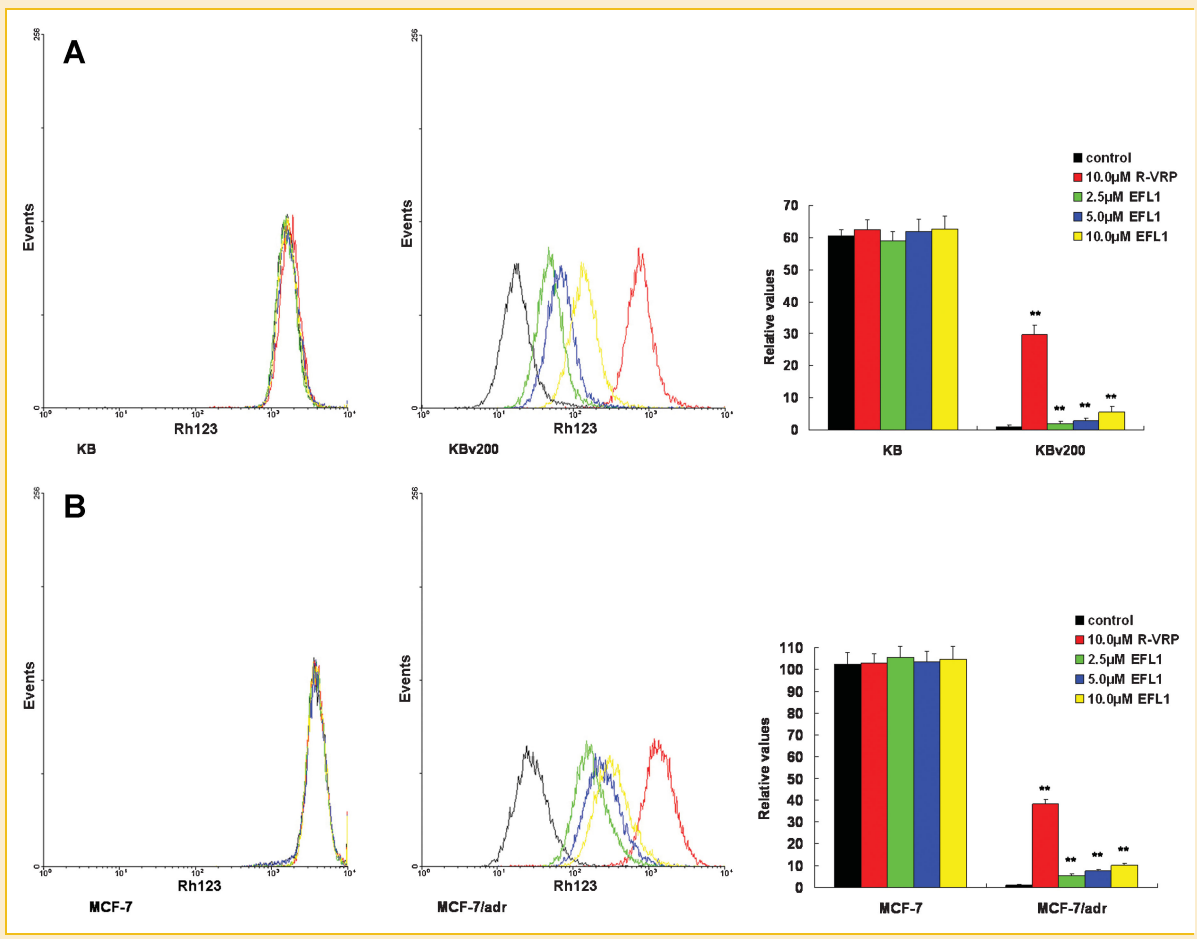


Fig. 3. Effects of EFL1 on the accumulation of Rh123. Indicated cells were incubated with 0, 2.5, 5.0, and 10.0 μM EFL1 at 37°C for 3 h. Then 5 μM Rh123 of final concentration was added for another 0.5 h incubation. Intracellular fluorescence was determined by flow cytometry with the excitation wavelength of 488 nm. VRP of 10.0 μM of final concentration was added as the positive control. A: Accumulation of Rh123 in KB, KBv200 cells, and the column analysis. B: Accumulation of Rh123 in MCF-7, MCF-7/adr cells, and the column analysis. All these experiments were carried out at thrice and the representative experiment result is shown here. The relative value was calculated by dividing the fluorescence intensity of sensitive or corresponding drug resistance cells by that of the drug resistance cells without treatment of verapamil or EFL1, respectively. Columns, means of triplicate determinations; *bars* SDs. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group.

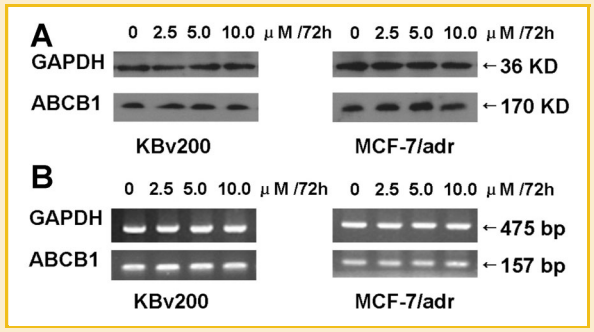


Fig. 4. EFL1 did not altered ABCB1 expression of KBv200 and MCF-7/adr cells. A: KBv200 and MCF-7/adr cells were treated with EFL1 of various concentrations for 72 h. Equal amounts of total cell lysates were applied for with Western blotting. B: The mRNA levels of ABCB1 of KBv200 and MCF-7/adr cells were determined by RT-PCR as described in "Materials and Methods Section." All these experiments were carried out at least three times and the representative experiment result is shown in each panel.

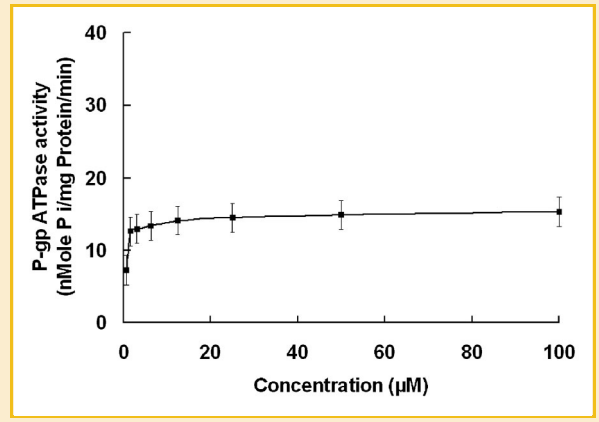


Fig. 5. Luminescent ABCB1 ATPase assays were performed according to Pgp-Glo™ Assay Systems instruction to illustrate how EFL1 affected ABCB1 ATPase activity. Each point represents the mean ± SDs out of triplicate independent determinations.

accumulation of chemotherapeutic agents in ABCB1-overexpressing MDR cells.

### EFL1 DID NOT INFLUENCE THE EXPRESSION OF ABCB1 EITHER IN mRNA OR PROTEIN LEVEL

The reversal of ABCB1-mediated MDR can be achieved by decreasing its expression or inhibiting its function. Therefore, we determined the expression of ABCB1 in KBv200 and MCF-7/adr cells after exposure to EFL1 of indicated concentrations for 72 h (Fig. 4A,B). The results shown in Fig. 4 demonstrated that EFL1 did not alter expression of ABCB1 in either mRNA or protein levels. As such, these results suggested that reversal of ABCB1-mediated MDR by EFL1 was not related to decrease of ABCB1 expression.

### EFL1 STIMULATED THE ATPase ACTIVITY OF ABCB1

As mentioned above, the drug-effux function of ABCB1 is linked to ATP hydrolysis and ATP consumption reflects ATPase activity. To assess the effects of EFL1 on the ATPase activity of ABCB1, we measured ABCB1-mediated ATP hydrolysis with various concentrations of EFL1. We found that EFL1 was an activator of ABCB1 ATPase. As shown in Fig. 5, EFL1 increased VRP-stimulated ATPase activity in a dose-dependent manner.

### EFL1 INHIBITED THE EFFLUX OF Rh123 IN KBv200 AND MCF-7/adr CELLS WITHOUT INHIBITING THE EFFLUX IN KB AND MCF-7 CELLS

EFL1 has showed to increase intracellular accumulation of anticancer agents such as DOX and Rh123 in ABCB1 MDR cells. Also, EFL1 could enhance ATPase activity of ABCB1. It should be clarified whether the increased accumulation of anticancer agents was due to inhibition of efflux. The time course of Rh123 efflux during 2 h after accumulation was shown in Fig. 6. Our results displayed that EFL1 inhibited drug efflux of ABCB1 in KBv200 and MCF-7/adr cells and did not influence drug efflux in sensitive KB and MCF-7 cells. For example, at time of 120 min, 36.7% of accumulated Rh123 was effluxed out of KBv200 cells under presence of 10 μM EFL1, while 59.4% of accumulated Rh123 was effluxed out of KBv200 cells at the absence of EFL1 ($P < 0.05$). In KB cells, 16.8% of accumulated Rh123 was effluxed out of KB cells at 120 min under the presence of 10 μM EFL1, while 18.3% of accumulated Rh123 was effluxed out of KB cells at the absence of EFL1 ($P > 0.05$). As far as MCF-7/adr cells were concerned, 39.2% of accumulated Rh123 was effluxed from MCF-7/adr cells under the presence of 10 μM EFL1, while 62.2% of accumulated Rh123 was effluxed from MCF-7/adr cells at

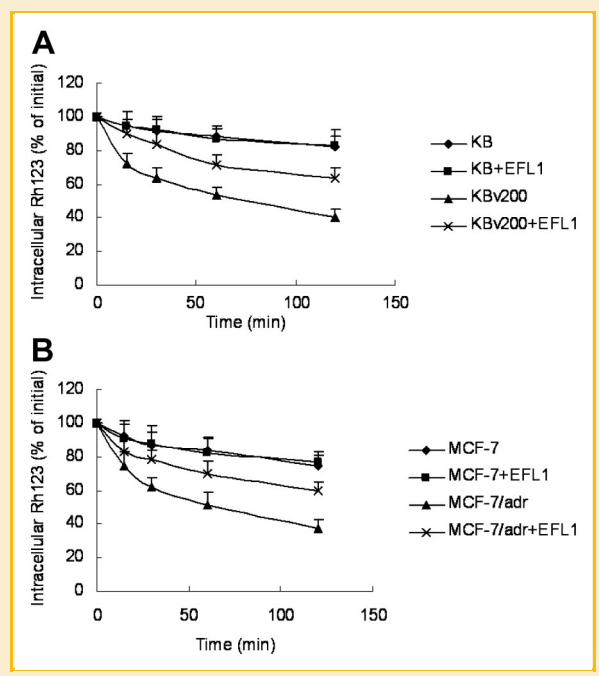


Fig. 6. EFL1 inhibited the efflux of ABCB1 in KBv200 and MCF-7/adr cells and did not influence efflux of KB and MCF-7 cells. A: Time course of Rh123 efflux was described in KB and KBv200 cells at the presence of 10.0 μM EFL1 or at the absence of EFL1, respectively. B: Time course of Rh123 efflux was described in MCF-7 and MCF-7/adr cells at the presence of 10.0 μM EFL1 or at the absence of EFL1, respectively.

the absence of EFL1 ($P < 0.05$). In MCF-7 cells, 25.6% of accumulated Rh123 was effluxed from MCF-7 cells at 120 min under the presence of 10 μM EFL1, while 23.5% of accumulated Rh123 was effluxed from MCF-7 cells at the absence of EFL1 ($P > 0.05$). These results indicated that EFL1 could effectively inhibit drug efflux of ABCB1.

## DISCUSSION

ABC transporters-mediated MDR is a serious obstacle of cancer chemotherapy, among which ABCB1-mediated MDR is the major cause of most MDR cases [Dean et al., 2001]. Much work has been done to reveal the mechanisms involved, among which the efflux function of ABC transporters is thought to be very important [Sauna et al., 2007; Chang, 2010]. In this study, our data showed that overexpression of ABCB1 confered drug resistance to the drug-selected resistant sublines KBv200 and MCF-7/adr. KBv200 cells were about 150-fold resistant to VCR and 60-fold resistant to DOX compared with its parental KB cells while MCF-7/adr cells were about 55-fold resistant to DOX compared with its parental MCF-7 cells (Table I). One of the effective ways to overcome ABCB1-mediated MDR is to develop inhibitors of transporter pumps. Up till now, three generations of MDR inhibitors have been developed and some of them have shown potent activity to reverse MDR [Deng et al., 2007; Shi et al., 2007]. However, clinical trials are still in the beginning stages.

Qiterpenoids isolated from geunus *Euphorbia* are the big family containing hundreds of compounds performing various biological activities such as antiproliferation, modulability of MDR, cytotoxic activity, antimicrobial, and antiinflammatory activity [Shi et al., 2008; Choi et al., 2009]. Recently, evidence proves that some of them can modulate ABCB1 [Duarte et al., 2007; Duarte et al., 2008; Jiao et al., 2009]. Herein EFL1 showed potent ability of reversing ABCB1-mediated MDR (Table I). At concentration of 10 μM EFL1, the most reversal fold is 25.19 to KBv200 cells with chemotherapeutic agent VCR. Meanwhile, under 10 μM EFL1 the survival rate of tested cancer cells including KB, KBv200, MCF-7, and MCF-7/adr was more than 90% (Fig. 1). These results indicated that EFL1 was a potent reversal agent to ABCB1-mediated MDR. The reversal ability of EFL1 has not been reported before.

The mechanism of ABCB1 reversal has been widely explored. MDR modulators are likely to reverse MDR via multiple pathways, such as inhibiting ABCB1 function or its expression. Most modulators can enhance the intracellular accumulation of anticancer drugs through inhibiting ABCB1 function [Fu et al., 2002; Chen et al., 2004]. In this article, ABCB1 expression and transport activity were determined to investigate the mechanisms of ABCB1-mediated MDR reversal by EFL1. EFL1 significantly increased intracellular accumulation of DOX in KBv200 and MCF-7/adr cells in a concentration-dependent manner while it performed no significant effect in the drug-sensitive KB and MCF-7 cells (Fig. 2). Similarly, accumulation of Rh123 in KBv200 and MCF-7/adr cells were enhanced by EFL1. However, intracellular Rh123 accumulation in KB and MC7–7 cells did not siginficantly alter after exposure to EFL1. These results strongly implied that EFL1 could increase intracellular accumulation of chemotherapeutic agents. Probably it's one of the mechanisms through which EFL1 reverse ABCB1-mediated MDR in KBv200 and MCF-7/adr cells. It is well known that the drug-effux function of ABCB1 is linked to ATP hydrolysis and ATP consumption reflects ATPase activity [Shi et al., 2007]. Herein, EFL1 increased the ATPase activity of ABCB1 stimulated by VRP showing the concentration-dependent manner. The majority of substrates interacting with the ABC drug transporters promote ATP hydrolysis [Ernst et al., 2010; Nervi et al., 2010]. The fact that presence of EFL1 stimulated the ATP hydrolysis by ABCB1 suggested that it behaved similarly to the known substrates of these transporters. As described before, MDR reversal agents against ABCB1 could be divided into three classes. Compounds belonging to the second class such as vandetanib and verapamil increasing the ATPase activity of ABCB1 in a dose-dependent manner could inhibit the efflux of ABCB1 [Hamada and Tsuruo, 1988; Mi and Lou, 2007]. Herein, EFL1 stimulated the ATPase activity, increased intracellular accumulation of anticancer drugs and inhibited drug efflux of ABCB1. Thus, it is likely that EFL1 belongs to the second class of ABCB1 reversal agents.

It is attractive whether EFL1 influence experssion of ABCB1. Thus, the protein and mRNA expression of ABCB1 of KBv200 and MCF-7/adr cells were determined after treatment of EFL1 of indicated concentrations for 72 h. The results exhibited that EFL1 did not downregulate expression of ABCB1 either in mRNA or protein level. Actually, it is rarely reported that ABCB1 modulator altered ABCB1 expression [Chen et al., 2009; Padowski and Pollack, 2010]. Our results suggested that downregulation of ABCB1 was not included in the reversal by EFL1.

In summary, we showed for the first time that EFL1 potentiated the sensitivity of established ABCB1 substrates and increased accumulation of DOX and Rh123 in ABCB1-mediated MDR KBv200 and MCF-7/adr cells. The reversal of EFL1 is related to its being a stimulator of ABCB1 ATPase activity and inhibiting efflux of ABCB1 in KBv200 and MCF-7/adr cells. Meanwhile, EFL1 did not downregulate the expression of ABCB1 either in mRNA or protein level.

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