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Apatinib (YN968D1) enhances the efficacy of conventional chemotherapeutical drugs in side population cells and ABCB1-overexpressing leukemia cells

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ABSTRACT

P-glycoprotein (P-gp, ABCB1) overexpression and enrichment of stem-like cells are linked to poor prognosis in tumor patients. In this study, we investigated the effect of apatinib, an oral multi-targeted tyrosine kinase inhibitor (TKI) on enhancing the efficacy of conventional anticancer drugs in side population (SP) cells and ABCB1-overexpressing leukemia cells *in vitro*, *in vivo* and *ex vivo*. Our results showed that apatinib significantly enhanced the cytotoxicity and cell apoptosis induced by doxorubicin in SP cells sorted from K562 cells. Furthermore, apatinib also strongly reversed multidrug resistance (MDR) in K562/ADR cells, and the primary leukemia blasts overexpressing ABCB1 while showed no synergistic interactions with chemotherapeutic agents in MRP1-, MRP4-, MRP7- and LRP-overexpressing cells. Apatinib treatment markedly increased the intracellular accumulation of doxorubicin and rhodamine 123 in K562/ADR cells and the accumulation of rhodamine 123 in the primary leukemia blasts with ABCB1 overexpression. Apatinib stimulated the ATPase activity of P-gp in a dose-dependent manner but did not alter the expression of ABCB1 at both mRNA and protein levels. The phosphorylation level of AKT and ERK1/2 remained unchanged after apatinib treatment in both sensitive and MDR cells. Importantly, apatinib significantly enhanced the antitumor activity of doxorubicin in nude mice bearing K562/ADR xenografts. Taken together, our results suggest that apatinib could target to SP cells and ABCB1-overexpressing leukemia cells to enhance the efficacy of chemotherapeutic drugs. These findings should be useful for the combination of apatinib and chemotherapeutic agents in the clinic.

1. Introduction

Leukemia is known as a clonal expansion and arrest at a specific stage of normal myeloid and lymphoid hematopoiesis. Over the past two decades, major advances have been achieved in the treatment of cancers thanks to the highly potent antineoplastic drugs. However, chemo-resistance is still a significant obstacle for successful chemotherapy of leukemia patients. Multidrug resistance (MDR), a major obstacle towards curative cancer chemotherapy, is a well-defined phenomenon of cross-resistance of cancer cells to a variety of anticancer drugs with different structures and mechanisms. One of the most frequently studied mechanisms responsible for MDR phenotype of cancer cells is overexpression of membrane-bound ATP-dependent drug efflux pumps from the ABC transporter family. Current studies have identified at least 49 ABC transporters of seven subfamilies (A–G) based on sequence similarities [1]. Among them, ABCB1 (MDR1, P-glycoprotein, P-gp), ABCC1 (MRP1) and ABCG2 (BCRP/MXR/ABCP) are considered to be the most important transporters contributing to MDR in tumor cells. Many studies have indicated that overexpression of P-glycoprotein/ABCB1 encoded by the MDR1 gene is associated with poor response to chemotherapy and low overall survival in cancer patients [2–5]. The multidrug resistance protein 1 (MRP1)/ABCC1 was shown to be expressed at higher levels in patients with relapsed acute myeloid leukemia (AML) compared with primary patients [6,7]. Sauerbrey et al. [8] showed that poor prognosis of children with AML was associated with the high expression level of ABCG2. Besides the ABC membrane transporters, other proteins are also associated with MDR phenotype, such as the lung resistance protein (LRP) which has been found to be overexpressed in adult acute T lymphoblastic leukemia patients (T-ALL) [9,10]. Furthermore, higher expression of LRP was observed in T-ALL patients compared with B cell-derived leukemia patients [11]. It was also reported that LRP might contribute to drug resistance in children with ALL [12]. In this

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respect, tremendous efforts have been made to discover and synthesize MDR reversal agents, which re-sensitize MDR cancer cells to anticancer drugs [13–16]. Many inhibitors of MDR transporters have been identified over the years and some of them are currently under clinical trials for specific forms of advanced cancers [17,18].

The side population (SP) phenotype cells, believed as cancer stem-like cells, are present in diverse tumor types and overexpress ABC transporters such as ABCB1 and ABCG2, leading to inherent drug resistance [19,20]. Some features of leukemic stem-like cells (LSCs) can be reflected through the studies on SP cells. Like normal stem cells, LSCs are able to self-renew, differentiate, and extensively proliferate. The myeloid leukemia originating from rare stem-like cells can transfer the disease to immunodeficient mice suggesting that these LSCs are responsible for relapse of leukemia following conventional or targeted cancer therapy and that eradication of LSCs might be necessary to cure the disease permanently. However, current therapeutic strategies may not effectively ablate the LSCs which result in the potential for disease progression or relapse. Several recent studies have provided insight into the signaling pathways underlying the LSCs phenotype and have also described approaches to eliminate LSCs [21,22].

Other reports collectively suggest that tyrosine kinase inhibitors (TKIs) may be promising to target LSCs and reverse MDR. For example, imatinib, nilotinib and AG1393, could inhibit the function of ABCB1 and ABCG2 transporters [23]; ZD6474 (vandetanib), another oral small molecule tyrosine kinase inhibitor of VEGFR-2, also showed reversal activity by inhibiting the function of ABCB1 [24]. Apatinib (YN968D1) is an oral small-molecule receptor tyrosine kinase inhibitor which targets VEGFR-2 (FIK-1/KDR, vascular endothelial growth factor receptor 2), RET (rearranged during transfection) and stem cell factor receptor (c-Kit), and has been used to treat gastric carcinoma and lung cancer in phase III clinical trial in China. Our earlier studies also showed that apatinib could reverse drug resistance in drug-resistant solid tumor cells, which spurs on effort to investigate whether apatinib can target LSCs and enhance the efficacy of conventional chemotherapeutic drugs in MDR leukemia cells [25].

2. Materials and methods

2.1. Reagents

Apatinib was obtained from Jiangsu Hengrui Medicine Co. (Jiangsu, China), of which the molecular structure was shown in Fig. 1A. Anti-P-gp monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (California, USA). Anti-AKT antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies against glyceraldehyd-3-phosphate dehydrogenase (GAPDH), phosphorylated AKT and phosphorylated extracellular signal-regulated kinase (MAPK1/2, ERK1/2) were purchased from Kangcheng Co. (Shanghai, China). Rhodamine 123 (Rho 123), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazoliumbromide (MTT), doxorubicin (Dox), vincristine (VCR), verapamil (VRP), MK571 and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell lines and cell culture

The following cell lines were cultured in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C in the presence of 5% CO_2: the human lung squamous carcinoma cell line SW1573 and its doxorubicin-selected LRP overexpressing derivative cell line SW1573/2R120 [26,27]; the human leukemia cell line K562 and its doxorubicin-selected ABCB1 overexpressing derivative cell line K562/ADR [28]; the human leukemia cell line HL60 and its doxorubicin-selected ABCC1 overexpressing derivative cell line HL60/ADR [29]; the human colon carcinoma cell line S1 and its mitoxantrone-selected ABCG2-overexpressing derivative S1-M1-80 [30]; murine fibroblasts cell line NIH3T3 and the ABCC4-transfected derivative ABCC4 stable expressing NIH3T3/MRP4-2 [31]; HEK293 and its ABCB1, MRP7 and ABCG2 stable gene-transfected cell lines HEK293/ABCB1 [32], HEK293/MRP7-2 [33] and HEK293/R2 [34] were obtained from Dr. S.E. Bates (National Cancer Institute, NIH). All of the transfected cells were cultured in medium with 2 mg/ml G418.

2.3. Patient samples

Bone marrow samples from diagnosed AML or chronic myelogenous leukemia (CML) patients according to the French–American–British (FAB) classification were obtained after their informed consent, and this study was approved by the Ethics Review Committee at Sun Yat-Sen University. Leukemic blasts were isolated using Ficoll-Hypaque density gradient by centrifugation and cultured in RPMI 1640 medium containing 10% fetal calf serum, penicillin 100 U/ml, streptomycin 100 U/ml and incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.4. Cell cytotoxicity assay

The MTT assay was performed for the analysis of the cytotoxicity as previously described. The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from survival curves using the Bliss method [35]. The degree of resistance was calculated by dividing the IC_{50} for the MDR cells by that of the parental sensitive cells. The fold-reversal factor of MDR was calculated by dividing the IC_{50} of the chemotherapeutical drugs in the absence of apatinib by that obtained in the presence of apatinib.

2.5. Animals and tumor xenograft experiments

Male athymic nude mice (BALB/c-nu/nu) of 5–6 weeks, 20–25 g, were purchased from Shanghai Slike Experimental Animals Co. (animal experimental license no. SCXKhu2007-0005, Shanghai, China) and raised under specific-pathogen-free conditions. Approximately 1×10^7 K562/ADR cells were injected subcutaneously into the posterior flanks region of the nude mice. Mice bearing tumors about 100 mm^3 in volume (15 days after tumor inoculation) were randomized into nine mice per group. Total four groups of mice bearing K562/ADR xenografts were treated with: (a) saline (q3d × 7, i.p.); (b) apatinib (70 mg/kg, q3d × 7, p.o.); (c) doxorubicin (2 mg/kg, q3d × 7, i.p.); (d) doxorubicin (2 mg/kg, q3d × 7, i.p.) plus apatinib (70 mg/kg, q3d × 7, p.o.) (apatinib was given 1 h before doxorubicin administration). The body weight of the animals and the two perpendicular diameters (A and B) were recorded every 3 days. Tumor volume (V) was calculated as:

$$V = \frac{\pi}{6}\left(\frac{A+B}{2}\right)^3$$

Mice were sacrificed when the mean of tumor weights was over 1 g in the control group and tumor tissue was excised from the mice and weighted. The rate of inhibition (IR) was calculated as:

$$\mathrm{IR}(\%) = 1 - \frac{\text{Mean tumor weight of experimental group}}{\text{Mean tumor weight of control group}} \times 100\%$$

2.6. SP analysis and sorting

The cell suspensions were labeled with Hoechst 33342 dye using the methods described by Goodell et al. [36] with modifications. Briefly, K562 cells were resuspended at 1×10^6/ml in

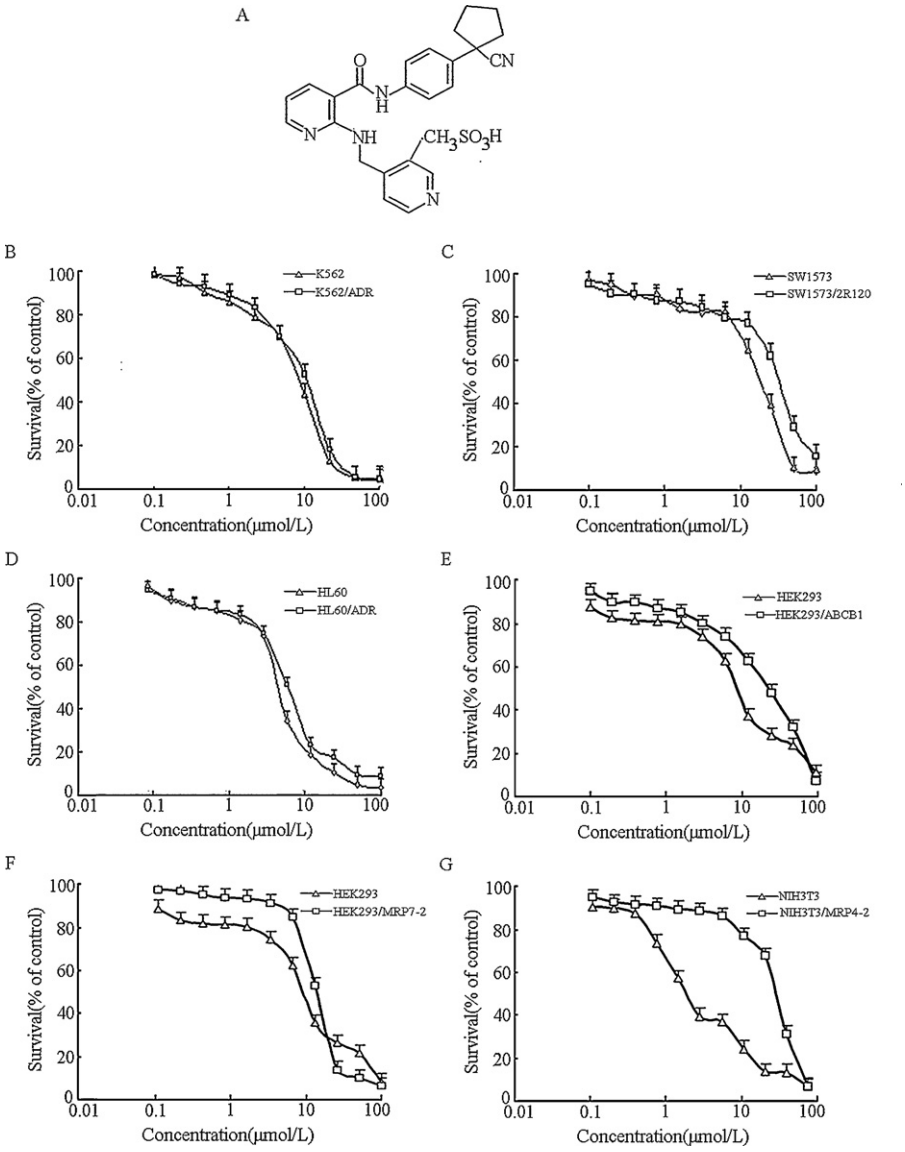

Fig. 1. The structure of apatinib and cytotoxicity effects of apatinib. (A) The structure of apatinib. MTT cytotoxicity assay was assessed in pairs of parental and transporter-overexpressing cell lines: (B) K562 and ABCB1-overexpressing K562/ADR cells. (C) SW1573 and LRP-overexpressing SW1573/2R120 cells. (D) HL60 and ABCC1-overexpressing HL60/ADR cells. (E) HEK293 and HEK293/ABCB1 cells. (F) HEK293 and HEK293/MRP7-2 cells. (G) NIH3T3 and MRP4-overexpressing NIH3T3/MRP4-2 cells. All the cells were exposed to the full-range concentration of apatinib for 68 h. Each point represents the mean ± standard deviations (SDs) for three determinations. Each experiment was performed in three replicate wells.

prewarmed 1640 with 2% FCS and 10 mmol/L HEPES buffer. Hoechst 33342 dye was added at a final concentration of 5 μg/ml in the presence or absence of VRP (100 μmol/L) and the cells were incubated at 37 °C for 90 min with intermittent shaking. At the end of the incubation, the cells were washed with ice-cold PBS, centrifuged down at 4 °C, and resuspended in ice-cold PBS. Propidium iodide at a final concentration of 2 μg/ml was added to the cells to gate viable cells. The cells were filtered through a 40-μm cell strainer to obtain single cell suspension before sorting. Analyses and sorting were done on a FACS. The Hoechst 33342 dye was excited at 357 nm and its fluorescence was dual-wavelength analyzed (blue, 402–446 nm; red, 650–670 nm).

2.7. Expression of ABCB1 and ABCG2 examined by flow cytometry

Expression of ABCB1 in the primary leukemia blasts was assessed by flow cytometry. Briefly, single cell suspensions were prepared and washed three times with an isotonic PBS buffer (supplemented with 0.5% BSA). Then, 10 μl of PE-conjugated anti-human ABCB1 antibody or APC-conjugated anti-human Bcrp1/ABCG2 (R&D Systems, Minneapolis, USA) antibody was mixed with 25 μl of cells (4×10^6 cells/ml). After incubating for 45 min at 4 °C in the dark, the cells were washed twice with PBS buffer (supplemented with 0.5% BSA) and resuspended in 400 μl PBS buffer for flow cytometric analysis. Isotype control samples were treated in an identical manner with PE-conjugated mouse IgG_{2a} for ABCB1 and APC-labeled mouse IgG_{2b} antibody for ABCG2.

2.8. Apoptosis assay

Cells were seeded onto a 6-well plate at a density of about 2×10^5 cells/well. After treated with different concentrations of apatinib in the presence of 0.01 μmol/L Dox for 48 h, both floated and attached cells were collected, washed with ice-cold PBS twice. Cells were resuspended in 100 μl of 1× binding buffer, and the Alexa Fluoro 488 Annexin V (5 μl) and PI (1 μl) were added before incubation at room temperature (RT) for 15 min. After the incubation

period, added 400 μl 1× binding buffer, mixed gently and analyzed via FACS.

2.9. Dox and rhodamine 123 accumulation

The effect of apatinib on the intracellular accumulation of Dox and rhodamine 123 in K562 and K562/ADR cell lines and primary leukemia blasts was measured by flow cytometry as previously described [37]. Briefly, K562 and K562/ADR cells (5×10^5 cells/well) or primary leukemia blasts (1×10^6 cells/well) were incubated in 6-well plates overnight. The cells were treated with 0.5, 1.0 and 2.0 μmol/L apatinib, verapamil (10 μmol/L) or the vehicle at 37 °C for 3 h. Then doxorubicin (10 μmol/L) or rhodamine 123 (5 μg/ml) was added and further incubated for another 3 h or 0.5 h, respectively. Finally, the cells were washed with ice-cold PBS three times and resuspended in 500 μl PBS for flow cytometric analysis (Beckman Coulter, Cytomics FC500, USA). A minimum of 10,000 cells were analyzed for each histogram generated. Verapamil, an ABCB1 inhibitor, was used as a positive control [38].

2.10. ABCB1 ATPase activity assay

The Pgp-Glo™ Assay Systems (Promega Co., Madison, USA) were used to estimate the changes of ATPase activity. The effects of apatinib on a verapamil-stimulated P-gp ATPase activity were examined. Sodium orthovanadate (Na_3VO_4) was used as a P-gp ATPase inhibitor. Various concentrations of apatinib, diluted with assay buffer were incubated in 0.1 mmol/L verapamil, 5 mmol/L Mg^{2+}-ATP and 25 μg recombinant human P-gp 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, Inc., Corning, USA) was read on luminometer (SpectraMax M5, Molecular Devices, USA). The liberated inorganic phosphate (Pi) was determined by comparing Na_3VO_4-treated samples with apatinib and verapamil combination-treated samples.

2.11. Western blot analysis

To identify whether apatinib affects the expressions of ABC transporters and blocks the phosphorylations of AKT and ERK1/2, the cells were incubated with different concentrations of apatinib for 24 h or 48 h. Then, the cells were harvested and rinsed twice with PBS. Cell extracts were prepared by incubating with the buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Identical amounts (100 μg protein) of cell lysates were resolved by SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes. After being incubated in blocking solution containing 5% nonfat milk in TBST buffer [10 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Tween 20] for 2 h at room temperature, membranes were incubated with the appropriately diluted primary antibody overnight at 4 °C. The membranes were washed thrice with TBST buffer and incubated with HRP-conjugated secondary antibody at 1:5000 dilution for 2 h at room temperature. Then, the membranes were washed thrice with TBST buffer, and the protein–antibody complex was visualized by the enhanced phototope TM-HRP Detection Kit (Cell Signaling Technology, Inc., Danvers, USA). At last, the membranes were exposed to Kodak medical X-ray processor (Carestream Health, Inc., Rochester, USA). The expression of GAPDH was used as a loading control.

2.12. Reverse transcription-PCR

The cells were treated with apatinib for 48 h. Total cellular RNA was isolated by Trizol Reagent (Molecular Research Center, Inc., Cincinnati, USA) RNA extraction kit following the manufacturer's instruction. The first strand cDNA was synthesized by Oligo dT primers. PCR was carried out using the following primers: ABCB1-fw 5′-ccc atc att gca ata gca gg-3′, ABCB1-rev 5′-gtt caa act tct gct cct ga-3′, GAPDH-fw 5′-ctt tgg tat cgt gga agg a-3′, and GAPDH-rev 5′-cac cct gtt gct gta gcc-3′. Using the GeneAmp PCR system 9700 (PE Applied Biosystems, USA), reactions were carried out at 94 °C for 2 min for initial denaturation, and then at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. After 35 cycles of amplification, additional extensions were done at 72 °C for 10 min. And then resolve the products and examine them by 1.5% agarose gel electrophoresis. The expected reverse transcription-PCR (RT-PCR) products were 157 bp for ABCB1, and 475 bp for GAPDH, respectively.

2.13. Statistical analysis

All experiments were repeated at least thrice and the differences were determined by using the Student's *t*-test. The statistical significance was determined at $P < 0.05$.

3. Results

3.1. In vitro cytotoxicity of apatinib

We examined the cytotoxic effects of apatinib on cell lines by MTT assay. K562/ADR, SW1573/2R120 and HL60/ADR are drug resistance models with overexpression of ABCB1, LRP and ABCC1, respectively. No significant difference in the cytotoxicity of apatinib was observed between the parental and resistant cells. As shown in Fig. 1, the IC_{50} values were 5.14 ± 0.18, 5.41 ± 0.44, 10.215 ± 1.060, 21.447 ± 1.246, 4.84 ± 0.40, 5.45 ± 0.43 μmol/L for K562, K562/ADR, SW1573, SW1573/2R120, HL60, HL60/ADR cells, respectively. More than 90% of cells were viable under the concentration of 2.0 μmol/L of apatinib. Thereafter, apatinib at concentrations of 0.5, 1.0 and 2.0 μmol/L were used in the MDR reversal study. In addition, the IC_{50} values for transfected MDR cells and their parental sensitive cells were also detected. Apatinib showed weaker cytotoxicity to transfected resistant cells than to the sensitive cells. The IC_{50} values were 13.58 ± 1.45, 19.73 ± 1.96, 15.28 ± 1.83, 2.66 ± 0.21, and 31.54 ± 3.12 μmol/L for HEK293, HEK293/ABCB1, HEK293/MRP7-2, NIH3T3 and NIH3T3/MRP4-2 cells, respectively. So the optimal concentration (under which 90% of cells were viable) for apatinib to reverse drug resistance was chosen according to its cytotoxic effect on the parental sensitive cells. Thus apatinib at concentrations of 0.75, 1.5, and 3.0 μmol/L were used to investigate their reversal effects on the efficacy of chemotherapeutic drugs in HEK293, HEK293/ABCB1 and HEK293/MRP7-2 cells, while 0.125, 0.25, and 0.5 μmol/L were used to NIH3T3 and NIH3T3/MRP4-2 cells.

3.2. Apatinib reversed ABCB1-mediated MDR in vitro

The IC_{50} values of chemotherapeutical agents in the MDR cells and their parental sensitive cells treated alone or in combination with various concentrations of apatinib were shown in Tables 1 and 2. Verapamil and MK571, the specific inhibitors for ABCB1 and ABCC1, were used as positive control, respectively. The overexpression of ABCB1, ABCC1, MRP4, MRP7 and LRP resulted in significant high IC_{50} values of substrate drugs. Apatinib strongly reduced the IC_{50} values of conventional chemotherapeutic agents in K562/ADR and HEK293/ABCB1 cells. Apatinib at 2.0 μmol/L has 14.85-, 18.41- and 16.90-fold for the reversal of resistance to Dox, daunorubicin and vincristine in K562/ADR cells. And 3.0 μmol/L apatinib has 12.04-, 13.44-fold for the reversal of resistance to paclitaxel and vincristine in HEK293/ABCB1 cells. However, the

Table 1
Effect of apatinib on reversing ABCB1-, LRP- and ABCC1-mediated drug resistance.

Compounds	$IC_{50} \pm SDs$ μmol/L (fold-reversal)	
	K562	K562/ADR (ABCB1)
Doxorubicin	0.129 ± 0.015 (1.00)	7.512 ± 0.120 (1.00)
+0.5 μM apatinib	0.108 ± 0.013 (1.19)	1.971 ± 0.029** (3.81)
+1.0 μM apatinib	0.116 ± 0.018 (1.11)	0.873 ± 0.016** (8.60)
+2.0 μM apatinib	0.119 ± 0.023 (1.08)	0.506 ± 0.076** (14.85)
+10 μM verapamil	0.113 ± 0.021 (1.14)	0.366 ± 0.032** (20.52)
Daunorubicin	0.0074 ± 0.0005 (1.00)	1.491 ± 0.026 (1.00)
+0.5 μM apatinib	0.0075 ± 0.0003 (0.99)	0.417 ± 0.019** (3.58)
+1.0 μM apatinib	0.0073 ± 0.0002 (1.01)	0.182 ± 0.015** (8.19)
+2.0 μM apatinib	0.0073 ± 0.0003 (1.01)	0.081 ± 0.003** (18.41)
+10 μM verapamil	0.0072 ± 0.0002 (1.02)	0.074 ± 0.007** (20.27)
Vincristine	0.0005 ± 0.0001 (1.00)	1.673 ± 0.032 (1.00)
+0.5 μM apatinib	0.0004 ± 0.0001 (1.04)	0.733 ± 0.028** (2.28)
+1.0 μM apatinib	0.0004 ± 0.0001 (1.07)	0.461 ± 0.048** (3.63)
+2.0 μM apatinib	0.0004 ± 0.0001 (1.06)	0.099 ± 0.013** (16.90)
+10 μM verapamil	0.0004 ± 0.0001 (1.05)	0.078 ± 0.017** (21.52)
Cisplatin	2.90 ± 0.019 (1.00)	1.812 ± 0.026 (1.00)
+2.0 μM apatinib	2.28 ± 0.148 (1.27)	1.834 ± 0.025 (0.99)
+10 μM verapamil	2.27 ± 0.034 (1.28)	1.801 ± 0.021 (1.01)
Compounds	$IC_{50} \pm SDs$ μmol/L (fold-reversal)	
	SW1573	SW1573/2R120 (LRP)
Doxorubicin	0.15 ± 0.013 (1.00)	1.763 ± 0.052 (1.00)
+0.5 μM apatinib	0.12 ± 0.021 (1.19)	1.623 ± 0.031 (1.09)
+1.0 μM apatinib	0.13 ± 0.018 (1.16)	1.496 ± 0.033 (1.18)
+2.0 μM apatinib	0.14 ± 0.021 (1.04)	1.443 ± 0.045 (1.22)
Compounds	$IC_{50} \pm SDs$ μmol/L (fold-reversal)	
	HL60	HL60/ADR (ABCC1)
Doxorubicin	0.036 ± 0.002 (1.00)	5.704 ± 0.378 (1.00)
+0.5 μM apatinib	0.035 ± 0.002 (1.04)	5.493 ± 0.289 (1.03)
+1.0 μM apatinib	0.037 ± 0.002 (0.97)	5.528 ± 0.515 (1.02)
+2.0 μM apatinib	0.033 ± 0.004 (1.09)	5.719 ± 0.594 (1.00)
+5 0 μM MK571	0.034 ± 0.002 (1.06)	0.955 ± 0.026** (5.98)

Cell survival was determined by MTT assay as described in Section 2. Data are the means ± standard deviations (SDs) of at least three independent experiments performed in triplicate. The fold reversal of MDR was calculated by dividing the IC_{50} for cells with the anticancer drug in the absence of inhibitor by that obtained in the presence of inhibitor.

** $P < 0.01$ *versus* that obtained in the absence of inhibitor.

sensitivity to these chemotherapeutic agents was not altered when K562 and HEK293 cells were treated concomitantly with apatinib. Apatinib did not alter the IC_{50} value of non-ABCB1 substrate (cisplatin) in neither MDR K562/ADR nor sensitive K562 cells. Moreover, apatinib showed no significant effect on ABCC1-, MRP4-, MRP7- and LRP-mediated drug resistance in HL60/ADR, NIH3T3/MRP4-2, HEK293/MRP7-2 and SW1573/2R120 cells, respectively.

3.3. Apatinib reversed ABCB1-mediated MDR in vivo

The *in vivo* reversal activity of apatinib for drug resistance to doxorubicin was evaluated using an established K562/ADR cell xenograft model in nude mice. As shown in Fig. 2, neither doxorubicin nor apatinib significantly inhibited the growth of xenograft. However, the combination of doxorubicin and apatinib drastically inhibited the growth of K562/ADR xenografts in nude mice. The weight of tumors excised from the mice were 1.277 ± 0.426 g, 1.384 ± 0.645 g, 1.190 ± 0.570 g and 0.640 ± 0.356 g for saline, doxorubicin, apatinib and combination groups, respectively. The inhibition rate of the combination group was 49.8%. Neither significant body weight loss nor treatment-related deaths was observed in the combination group, indicating that apatinib effectively enhanced the antitumor activity of doxorubicin without causing additional toxicity.

3.4. Apatinib enhanced the efficacy of chemotherapeutic drug in SP cells

K562 cells contained 3.01% of SP cells which significantly decreased in the presence of verapamil (Fig. 3A). SP cells expressed much higher levels of ABCG2 than non-SP cells while no significant expression of ABCB1 was detected in different K562 cell subsets (Fig. 3B). Compared with non-SP cells, SP cells exhibited about a 2-fold resistance to Dox. Apatinib significantly decreased the IC_{50} value of Dox in SP cells in a dose-dependent manner (Fig. 3D). But the IC_{50} value of Dox was not altered in the absence or presence of apatinib in non-SP cells. Furthermore, apatinib significantly increased the percentage of apoptosis induced by Dox in a

Table 2
Effect of apatinib on reversing ABCB1- MRP7- and MRP4-mediated MDR in transfected cell lines.

Compounds	IC50 ± SDs nmol/L (fold-reversal)		
	HEK293	HEK293/ABCB1	HEK293/MRP7-2
Paclitaxel	23.04 ± 5.16 (1.00)	636.81 ± 27.42 (1.00)	210.04 ± 15.16 (1.00)
+0.75 μM apatinib	22.74 ± 4.04 (1.01)	192.33 ± 17.23** (3.31)	207.78 ± 14.04 (1.01)
+1.50 μM apatinib	20.98 ± 1.13 (1.10)	84.41 ± 9.82** (7.54)	209.35 ± 20.13 (1.00)
+3.00 μM apatinib	21.54 ± 1.02 (1.02)	52.91 ± 8.02** (12.04)	215.02 ± 21.02 (0.98)
+10.0 μM verapamil	22.68 ± 3.15 (1.01)	89.78 ± 8.69** (7.09)	–
Vincristine	0.86 ± 0.16 (1.00)	88.02 ± 5.16 (1.00)	–
+0.75 μM apatinib	0.84 ± 0.24 (1.04)	60.74 ± 4.04* (1.45)	–
+1.50 μM apatinib	0.81 ± 0.13 (1.11)	7.31 ± 1.13** (12.03)	–
+3.00 μM apatinib	0.80 ± 0.09 (1.12)	6.54 ± 1.02** (13.44)	–
+10.0 μM verapamil	0.81 ± 0.15 (1.11)	6.07 ± 3.15** (14.50)	–
Compounds	IC50 ± SDs μmol/L (fold-reversal)		
	NIH3T3	NIH3T3/MRP4-2	HEK293/MRP7-2
6-MP	0.95 ± 1.04 (1.00)	18.95 ± 1.04 (1.00)	–
+0.125 μM apatinib	0.90 ± 1.16 (1.05)	17.34 ± 1.23 (1.15)	–
+0.25 μM apatinib	0.84 ± 1.23 (1.13)	17.49 ± 0.94 (1.14)	–
+0.50 μM apatinib	0.73 ± 0.94 (1.79)	16.95 ± 1.42 (1.17)	–

Cell survival was determined by MTT assays as described in Section 2. Data are the mean ± SDs of at least three independent experiments performed in triplicate. The fold-reversal of MDR (values given in parentheses) was calculated by dividing the IC_{50} for cells with the anticancer drugs in the absence of inhibitor by that obtained in the presence of inhibitor.

* $P < 0.05$ *versus* the values obtained in the absence of inhibitor.

** $P < 0.01$ *versus* the values obtained in the absence of inhibitor.

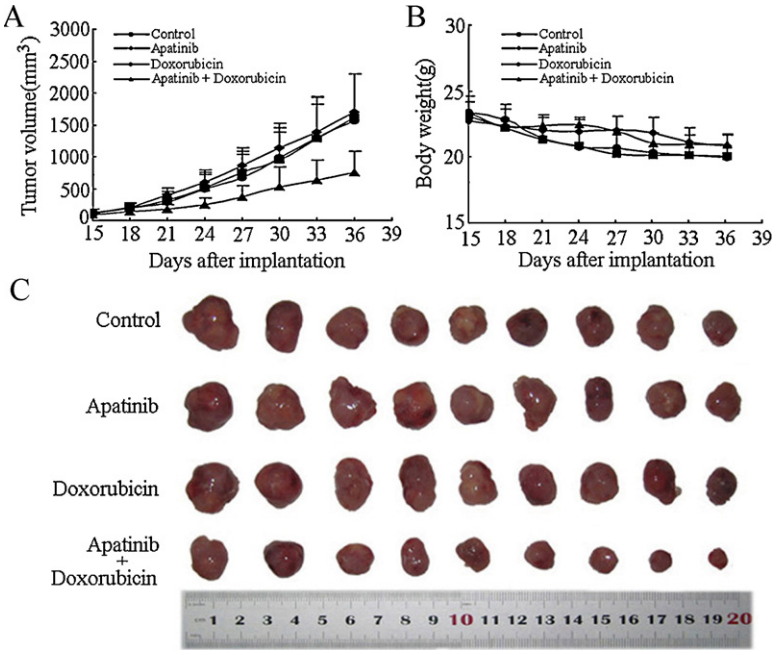

Fig. 2. Potentiation of the antitumor effects of doxorubicin by apatinib in a K562/ADR cell xenograft model in athymic nude mice. Potentiation of antitumor effects of doxorubicin by apatinib in K562/ADR xenograft model in nude mice. (A) Changes in tumor volume with time. Each point represents the mean ± SDs of tumor volumes from nine mice in the group. (B) Changes in body weight. Each point represents the mean ± SDs of body weight from nine mice in the group. Mice were treated with saline (control, ◆); apatinib alone at 70 mg/kg (■); doxorubicin alone at 2 mg/kg (●); doxorubicin at 2 mg/kg plus apatinib 70 mg/kg (▲) (apatinib was given 1 h before doxorubicin administration). (C) Tumor size. The picture was taken on the 36th day after implantation.

dose-dependent manner in SP cells, but did not alter the percentage of apoptosis in non-SP cells (Fig. 3C and E).

3.5. Apatinib increased the accumulation of Dox and rhodamine 123 in K562/ADR cells and stimulated the ATPase activity of ABCB1

Our results showed apatinib could enhance the sensitivity of MDR cells to certain chemotherapeutic agents. To elucidate the underlying mechanism, we measured the accumulation of doxorubicin and rhodamine 123 in the presence or absence of apatinib in K562 and K562/ADR cells. The fluorescence of doxorubicin and rhodamine 123 were much higher in K562 cells than in K562/ADR cells. The intracellular accumulation of doxorubicin was increased by 1.54-, 1.82- and 2.03-fold in K562/ADR cells in the presence of 0.5, 1.0 or 2.0 μmol/L of apatinib, respectively (Fig. 4A and C). Similarly, the fluorescence value of rhodamine 123 was enhanced by 1.37-, 2.16- and 2.64-fold in K562/ADR cells, in the presence of 0.5, 1.0 or 2.0 μmol/L of apatinib, respectively (Fig. 4B and D). However, the intracellular accumulations of doxorubicin and rhodamine 123 were not altered in K562 cells in the presence of apatinib.

The drug-efflux function of ABCB1 is linked to ATPase activity. To determine the effect of apatinib on the ATPase activity of ABCB1, we measured the ABCB1-mediated ATP hydrolysis with various concentrations of apatinib (Fig. 4E). The results showed that apatinib increased verapamil-stimulated ATPase activity in a dose-dependent manner.

3.6. Effect of apatinib on the blockade of AKT and ERK1/2 phosphorylation and the expression of ABCB1

To understand whether the blockade of VEGFR downstream pathways confer to the reversal of MDR mediated by apatinib, the phosphorylation of AKT and ERK1/2, two VEGFR downstream signal markers, were examined. Lapatinib at 10 μmol/L was used as a positive control. As shown in Fig. 5A, there were no significant blockade effect on the phosphorylation levels of AKT and ERK1/2 in all the cells tested after treating with 0.5, 1.0, 2.0 or 10 μmol/L of apatinib for 24 h.

The reversal of ABC transporter-mediated MDR can usually be obtained either by cutting down expression or inhibiting function of the transporters. Therefore, we detected the effect of apatinib on the expression of ABCB1 at mRNA and protein levels. Our results showed that the protein and mRNA expression levels of ABCB1 (Fig. 5B) were not affected by the treatment of apatinib with indicated concentrations.

3.7. Apatinib enhanced the intracellular accumulation of rhodamine 123 in primary leukemic blasts with ABCB1 overexpression and sensitized the cells to ABCB1 substrate anticancer drugs

To investigate whether apatinib could reverse ABCB1-mediated MDR in *ex vivo*, clinical samples of ABCB1-overexpressing leukemia cells were collected from the bone marrow of four patients (two males and two females) with leukemia. Two of them were diagnosed with acute myeloid leukemia (AML) and two were CML. All of them were primary patients with WBC (white blood cells) $> 100 \times 10^9$/L. ABCB1 was found to be overexpressed in all the primary leukemia blast cells (Fig. 6A). We then examined the effect of apatinib on intracellular accumulation of rhodamine 123 in these ABCB1-overexpressing primary leukemia blasts using flow cytometric analysis. Our results showed that apatinib enhanced the intracellular accumulation of rhodamine 123 in a dose-dependent manner (0.5–2.0 μmol/L). Verapamil (10 μmol/L), a known ABCB1 inhibitor, also significantly increased the fluorescence value of rhodamine 123 in the leukemia blasts (Fig. 6B and C). Furthermore, the sensitization effect of apatinib on other anticancer drugs in the *ex-vivo* model was also examined. As shown in Fig. 6D, the IC_{50} values of doxorubicin in the four leukemia blast samples were 0.87 ± 0.11, 0.92 ± 0.07, 0.65 ± 0.15 and 1.19 ± 0.06 μmol/L, respectively. Apatinib at 2.0 μmol/L significantly sensitized all four samples to doxorubicin treatment as compared to the control ($P < 0.05$) and the

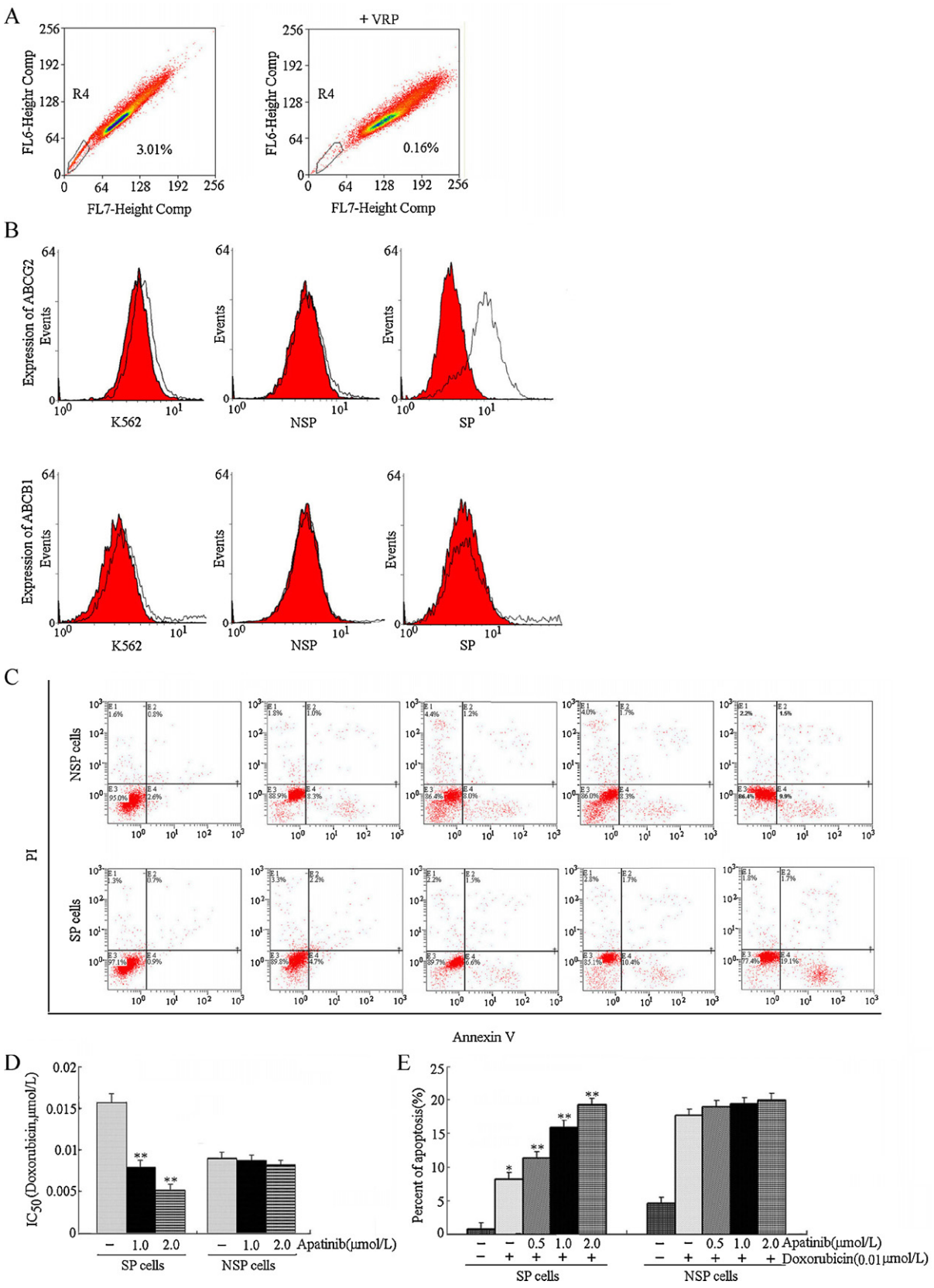

Fig. 3. Apatinib targeted to SP cells and enhanced the effect of chemotherapeutical agents on the inhibition of cell proliferation and the induction of apoptosis. (A) The K562 cells were sorted by Hoechst 33342. (B) Expression of ABCB1 and ABCG2 in K562 cell subsets. (C) Sorted SP and non-SP cells treated with doxorubicin and apatinib in the indicated concentrations for 48 h, respectively. Apoptosis was analyzed by flow cytometry as the percentage of cells labeled by Annexin V and PI. (D) Growth inhibition was determined by the MTT assay according to the protocol described in Section 2. Induction of 50% cell death in SP and non-SP cells by doxorubicin in the presence of apatinib at indicated concentrations. (E) Apatinib markedly increased cell apoptosis induced by doxorubicin in dose-dependent manner in K562 derivative SP cells but no in non-SP cells. All these experiments were repeated at least thrice, and a representative experiment was shown. Columns, means of triplicate determinations; bars, SDs. *, $P < 0.05$; **, $P < 0.01$, compared with doxorubicin treatment.

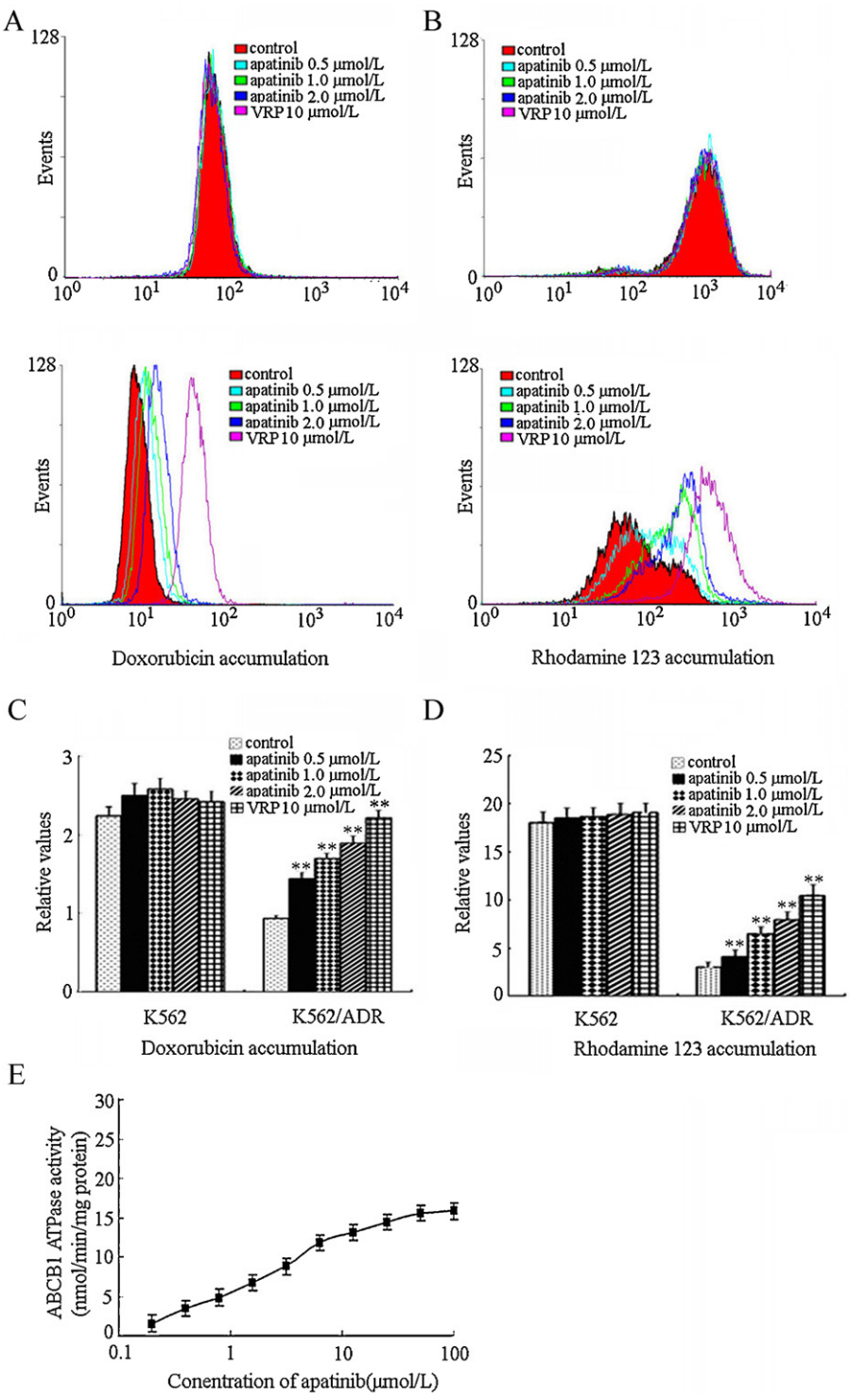

Fig. 4. Effects of apatinib on the accumulation of doxorubicin (Dox) and rhodamine 123 and on the ATPase activity of ABCB1. (A) and (B) The accumulation of doxorubicin and rhodamine 123 was measured by flow cytometric analysis as described in Section 2. (C) and (D) The results are presented as fold change in fluorescence intensity relative to control MDR cells. They are calculated by dividing the fluorescence intensity of each sample with that of MDR cells treated with doxorubicin or rhodamine 123 alone. (E) Luminescent ABCB1 ATPase assays were performed according to Pgp-Glo™ Assay Systems instruction. Each point represents the mean ± SDs for triplated independent determinations. Columns, means of triplicate determinations; bars, SDs. $^{**}P < 0.01$ *versus* control group.

fold-reversals were 1.80, 1.77, 2.12 and 1.58, respectively. The sensitization effect of apatinib at 2.0 μmol/L was found to be similar to that of verapamil at 10 μmol/L. These results suggest that apatinib may be useful in sensitizing leukemia cells to conventional anticancer drugs and could be used to circumvent MDR in ABCB1-overexpressing leukemia patients.

4. Discussion

One of the most important causes of treatment failure in leukemia patients is the overexpression of transmembrane transport proteins, such as P-gp, MRP1, BCRP and LRP. The clinical relevance and prognostic significance of MDR-related efflux pumps

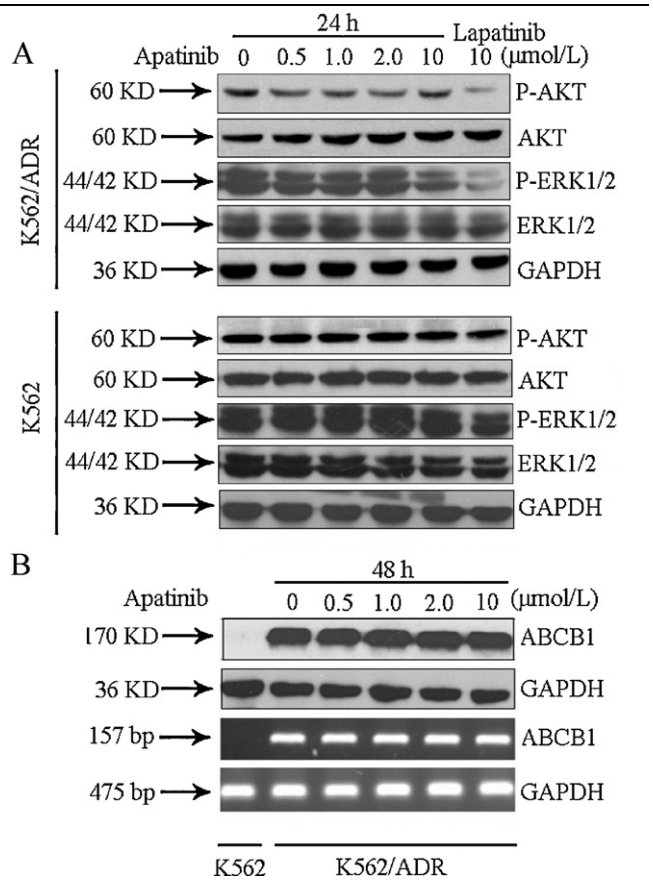

Fig. 5. Effects of apatinib on blockade of AKT and ERK1/2 phosphorylation and the expression of ABCB1 in MDR cells. (A) The K562 and K562/ADR cells were treated with apatinib at 0.5, 1.0, 2.0 and 10.0 μmol/L for 24 h. Equal amount of protein was loaded for Western blot analysis as described in Section 2. (B) K562/ADR cells were treated with apatinib at 0.5, 1.0, 2.0 and 10.0 μmol/L for 48 h. The expression of ABCB1 at both protein and mRNA levels were detected as described in Section 2. The K562 cells were used as negative control for the detection. Independent experiments were performed at least three times and result from a representative experiment is shown.

in leukemia patients have been well established in literature [5,39]. Most human leukemic cells have limited proliferative capacity [40]. Only a small population of them, currently referred to as LSCs, possess the capacity for self-renewal, which are essential for the expansion of malignant disorder. These cells are characterized by a high expression of a number of ABC efflux transporters, which are involved in protecting LSCs from a broad range of chemotherapeutics [41–44]. The LSCs with high ABC transporters expression are also likely to be the most drug-resistant population in the tumor and current therapies are unable to eradicate LSCs thus leading to disease relapse.

In recent years, many studies have focused on the interactions of TKIs with the major MDR transporters such as ABCB1, ABCC1 and ABCG2. Initially, transporter-mediated TKI efflux has been implicated as a possible mechanism for resistance to imatinib [45,46]. Mahon et al. have demonstrated that ABCB1 can confer nilotinib resistance [47]. Dasatinib, is also a substrate for both ABCB1 and ABCG2 [48]. On the other hand, the inhibitory effects of TKIs on MDR transporters were also observed, both nilotinib and dasatinib were found to act as inhibitors of ABCB1 and ABCG2, EKI-785 was able to interact with ABCC1 [23]. More recently, CI1033 was reported to be a substrate and inhibitor of ABCG2 [49]. Other TKIs such as gefitinib [50,51], erlotinib [52], vandetanib [24,53] and lapatinib [54] have also been shown to inhibit the function of ABCB1 and ABCG2. The pattern of MDR-ABC transporter-TKI interactions may help to understand the anti-cancer effect and the pharmacokinetics of new TKIs.

Apatinib is a potent and selective inhibitor of VEGFR-2, RET, c-Kit and c-Src tyrosine kinases [55]. It effectively inhibited the proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVEC), blocked the microvessel budding of rat aortic ring and showed antitumor efficacy *in vivo* against a variety of established tumor xenografts with good tolerance [56]. Preliminary investigation of apatinib in patients with advanced solid malignancies in a phase I study has shown encouraging antitumor activity and a manageable toxicity (MTD 850 mg/day) [57]. Indeed, the inhibitor has demonstrated a wide range of activity against various tumors in phase II studies, including metastatic gastric adenocarcinoma, advanced non-squamous and non-small cell lung cancer, advanced hepatocellular carcinoma and metastatic triple-negative breast cancer. Our earlier study also has demonstrated that apatinib could reverse ABCB1- and ABCG2-mediated MDR in solid tumor cell lines [25].

In the present study, our data showed that apatinib dose-dependently enhanced the cytotoxicity of established ABCB1 substrates in MDR cells. However, in drug sensitive cells, the cytotoxicity generated by the ABCB1 substrates was unaffected in the presence of apatinib. The K562/ADR and HEK293/ABCB1 cells are drug resistance models with overexpression of ABCB1 and show no detectable expression of ABCG2 (Fig. S1). Apatinib at 2.0 μmol/L has 14.85-, 18.41- and 16.90-fold for the reversal of resistance to Dox, daunorubicin and vincristine in K562/ADR cells. And 3.0 μmol/L of apatinib has 12.04-, 13.44-fold for the reversal of resistance to paclitaxel and vincristine in HEK293/ABCB1 cells (Tables 1 and 2). These suggest apatinib may be promising in combination with chemotherapeutical drugs. Furthermore, apatinib did not significantly alter the sensitivity of non-ABCB1 substrates such as cisplatin in sensitive K562 cells and resistant K562/ADR cells. In addition, apatinib could not alter the sensitivity of MRP4-, MRP7- and LRP-overexpressing cells to chemotherapeutic agents. These suggest that apatinib selectively reverses ABCB1 mediated MDR in a dose-dependent manner. In athymic nude mice bearing the MDR K562/ADR xenografts, apatinib drastically enhanced the antitumor activity of doxorubicin without causing additional toxicity (Fig. 2). Our data also showed that apatinib was able to enhance the chemotherapeutic sensitivity of doxorubicin and increase apoptosis induction of doxorubicin on SP cells (Fig. 3). The results were in accordance with our previous study that apatinib was able to inhibit ABCG2 transport activity and thus it could be used in conjunction with other conventional anticancer drugs to eradicate the cancer stem cells (Table S1). Importantly, in an *ex-vivo* model of ABCB1-overexpressing primary leukemia blast cells obtained from four newly diagnosed acute leukemia patients, we demonstrated that apatinib could increase the accumulation of rhodamine 123 and enhance the cytotoxicity of doxorubicin (Fig. 6) in those leukemia blast cells. Besides that, apatinib of 6 μmol/L in the plasma concentration may be achieved in humans (phase I data, not published).

Supplementary material related to this article found, in the online version, at doi:10.1016/j.bcp.2011.12.007.

Our data indicate that apatinib increases intracellular accumulation of Dox and rhodamine 123 (Fig. 4A–D). We also found that apatinib was able to stimulate the ATPase activity in a dose-dependent manner (Fig. 4E), indicating that apatinib may directly interact with the drug-substrate binding sites on transporters. However, apatinib did not affect the mRNA or protein expression levels of ABCB1 (Fig. 5B).

Receptor tyrosine kinases (RTKs) such as VEGFR, PDGFR and FLT3 play a crucial role in modulating cell proliferation, differentiation and survival by activating downstream signal molecules such as

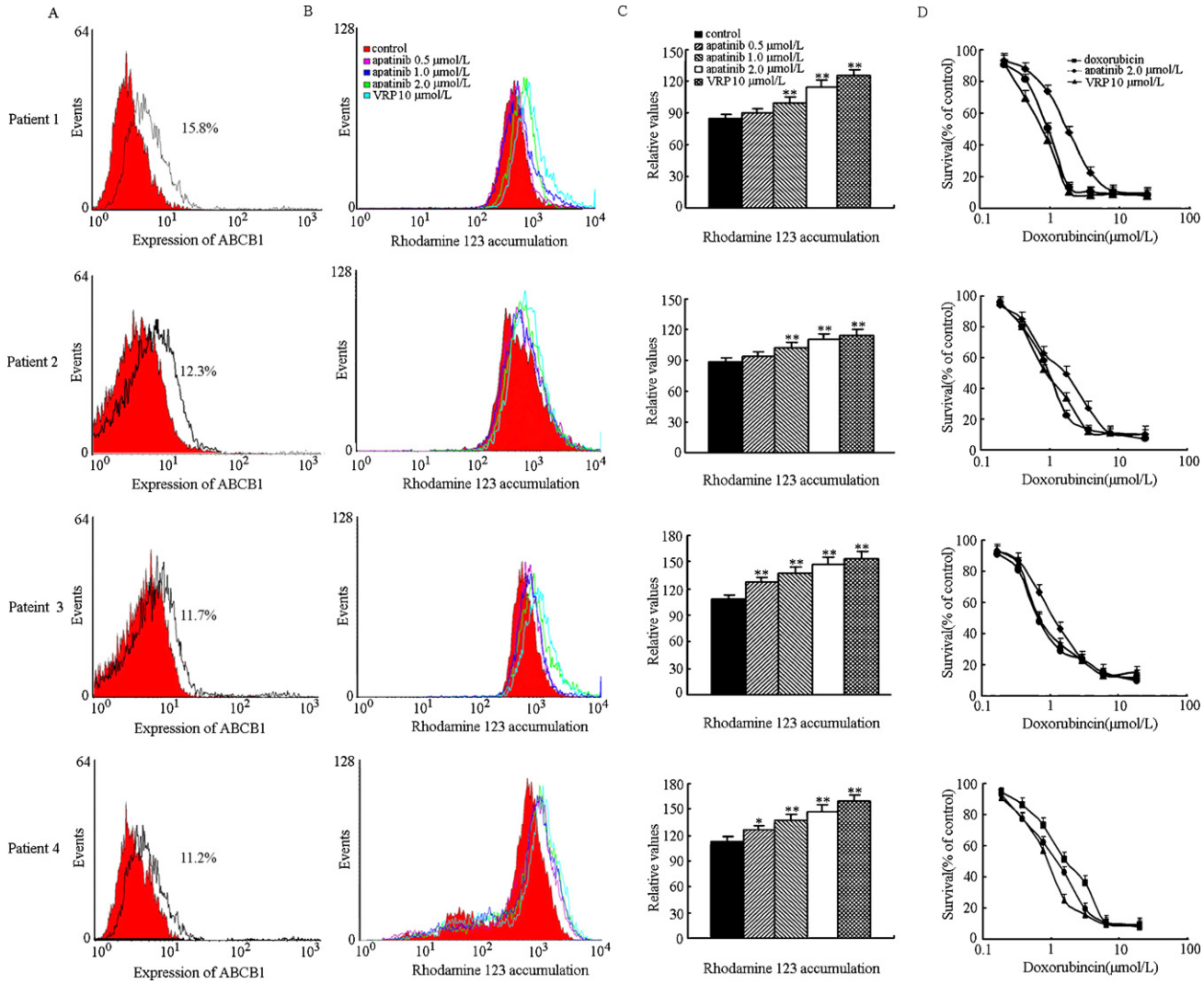

Fig. 6. Apatinib increased the intracellular accumulation of rhodamine 123 and enhanced the cytotoxicity of doxorubicin in primary leukemia blasts with ABCB1 overexpression. Four newly diagnosed patients' bone marrows were collected. Mononuclear cells were isolated as described in Section 2. (A) The expression of ABCB1 in primary leukemia blasts was determined by flow cytometry. Red and black histograms represent the isotype control and ABCB1 expression. (B) Intracellular accumulation of rhodamine123 in primary leukemia blasts with or without apatinib treatment was determined by flow cytometry. (C) The relative levels of intracellular accumulation of rhodamine 123 (expressed in mean fluorescent intensity unit) as determined in above (B) were plotted in bar graphs. (D) Enhancement of doxorubicin cytotoxicity in primary leukemia blasts with ABCB1-overexpression by apatinib. Cytotoxicity was determined by MTT assay as described in Section 2. Data represent the means ± SDs from at least three independent experiments performed in triplicate. $^{*}P < 0.05$.

signal transducers and activators of transcription (STAT), protein kinase B/AKT and extracellular signal-regulated kinase 1/2 (ERK1/2) [58]. In many tumors, continued activation of AKT has been implicated as a mechanism of resistance to cytotoxic chemotherapy agents [59,60]. Therefore, the blockade of the AKT phosphorylation could enhance the efficacy of chemotherapeutical agents. Our data showed that apatinib (up to 10.0 μmol/L) did not block the phosphorylation of AKT and ERK1/2 in all the tested cell lines (Fig. 5A). Therefore, the blockade of AKT and ERK1/2 activation is not involved in the reversal of ABCB1-mediated MDR by apatinib. Lapatinib, a potent reversible, dual inhibitor of the tyrosine kinase domains of both EGFR and erbB2 was used as positive control [61].

In conclusion, apatinib enhances the efficacy of conventional chemotherapeutic drugs in SP cells and ABCB1-overexpressing MDR leukemia cells via directly inhibiting the drug transport function of ABCB1 and increasing the intracellular concentrations of substrate chemotherapeutic drugs. In addition, the reversal of MDR is not associated with the blockade of the phosphorylation of tyrosine kinases. Our results suggest that apatinib may be used in combination with conventional ABCB1 substrate chemotherapeutic drugs to overcome multidrug resistance in the clinic.

Acknowledgments

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References

[1] Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006;5:219–34.

[2] Guerci A, Merlin JL, Missoum N, Feldmann L, Marchal S, Witz F, et al. Predictive value for treatment outcome in acute myeloid leukemia of cellular daunorubicin accumulation and P-glycoprotein expression simultaneously determined by flow cytometry. Blood 1995;85:2147–53.
[3] Lamy T, Goasguen JE, Mordelet E, Grulois I, Dauriac C, Drenou B, et al. P-glycoprotein (P-170) and CD34 expression in adult acute myeloid leukemia (AML). Leukemia 1994;8:1879–83.
[4] Wood P, Burgess R, MacGregor A, Yin JA. P-glycoprotein expression on acute myeloid leukaemia blast cells at diagnosis predicts response to chemotherapy and survival. Br J Haematol 1994;87:509–14.
[5] Leith CP, Kopecky KJ, Chen IM, Eijdems L, Slovak ML, McConnell TS, et al. Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia: a Southwest Oncology Group Study. Blood 1999;94:1086–99.
[6] Schneider E, Cowan KH, Bader H, Toomey S, Schwartz GN, Karp JE, et al. Increased expression of the multidrug resistance-associated protein gene in relapsed acute leukemia. Blood 1995;85:186–93.
[7] Zhou DC, Zittoun R, Marie JP. Expression of multidrug resistance-associated protein (MRP) and multidrug resistance (MDR1) genes in acute myeloid leukemia. Leukemia 1995;9:1661–6.
[8] Sauerbrey A, Zintl F, Hermann J, Volm M. Multiple resistance mechanisms in acute nonlymphoblastic leukemia (ANLL). Anticancer Res 1998;18:1231–6.
[9] Ikeda K, Oka M, Yamada Y, Soda H, Fukuda M, Kinoshita A, et al. Adult T-cell leukemia cells over-express the multidrug-resistance-protein (MRP) and lung-resistance-protein (LRP) genes. Int J Cancer 1999;82:599–604.
[10] Yasunami T, Wang YH, Tsuji K, Takanashi M, Yamada Y, Motoji T. Multidrug resistance protein expression of adult T-cell leukemia/lymphoma. Leuk Res 2007;31:465–70.
[11] Cortez MA, Scrideli CA, Yunes JA, Valera ET, Toledo SR, Pavoni-Ferreira PC, et al. mRNA expression profile of multidrug resistance genes in childhood acute lymphoblastic leukemia. Low expression levels associated with a higher risk of toxic death. Pediatr Blood Cancer 2009;53:996–1004.
[12] Chen ZS, Furukawa T, Sumizawa T, Ono K, Ueda K, Seto K, et al. ATP-dependent efflux of CPT-11 and SN-38 by the multidrug resistance protein (MRP) and its inhibition by PAK-104P. Mol Pharmacol 1999;55:921–8.
[13] Hyafil F, Vergely C, Du Vignaud P, Grand-Perret T. In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. Cancer Res 1993;53:4595–602.
[14] Mistry P, Stewart AJ, Dangerfield W, Okiji S, Liddle C, Bootle D, et al. In vitro and in vivo reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. Cancer Res 2001;61:749–58.
[15] Zhou Y, Hopper-Borge E, Shen T, Huang XC, Shi Z, Kuang YH, et al. Cepharanthine is a potent reversal agent for MRP7(ABCC10)-mediated multidrug resistance. Biochem Pharmacol 2009;77:993–1001.
[16] Shukla S, Wu CP, Ambudkar SV. Development of inhibitors of ATP-binding cassette drug transporters: present status and challenges. Expert Opin Drug Metab Toxicol 2008;4:205–23.
[17] Swerts K, De Moerloose B, Dhooge C, Laureys G, Benoit Y, Philippe J. Prognostic significance of multidrug resistance-related proteins in childhood acute lymphoblastic leukaemia. Eur J Cancer 2006;42:295–309.
[18] Klepsch F, Chiba P, Ecker GF. Exhaustive sampling of docking poses reveals binding hypotheses for propafenone type inhibitors of P-glycoprotein. PLoS Comput Biol 2011;7:e1002036.
[19] Kim M, Turnquist H, Jackson J, Sgagias M, Yan Y, Gong M, et al. The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. Clin Cancer Res 2002;8:22–8.
[20] Rizzo S, Hersey JM, Mellor P, Dai W, Santos-Silva A, Liber D, et al. Ovarian cancer stem cell-like side populations are enriched following chemotherapy and overexpress EZH2. Mol Cancer Ther 2011;10:325–35.
[21] Cheng L, Bao S, Rich JN. Potential therapeutic implications of cancer stem cells in glioblastoma. Biochem Pharmacol 2010;80:654–65.
[22] Subramaniam D, Ramalingam S, Houchen CW, Anant S. Cancer stem cells: a novel paradigm for cancer prevention and treatment. Mini Rev Med Chem 2010;10:359–71.
[23] Hegedus T, Orfi L, Seprodi A, Varadi A, Sarkadi B, Keri G. Interaction of tyrosine kinase inhibitors with the human multidrug transporter proteins, MDR1 and MRP1. Biochim Biophys Acta 2002;1587:318–25.
[24] Mi Y, Lou L. ZD6474 reverses multidrug resistance by directly inhibiting the function of P-glycoprotein. Br J Cancer 2007;97:934–40.
[25] Mi YJ, Liang YJ, Huang HB, Zhao HY, Wu CP, Wang F, et al. Apatinib (YN968D1) reverses multidrug resistance by inhibiting the efflux function of multiple ATP-binding cassette transporters. Cancer Res 2010;70:7981–91.
[26] Kubota T, Furukawa T, Tanino H, Suto A, Otan Y, Watanabe M, et al. Resistant mechanisms of anthracyclines—pirarubicin might partly break through the P-glycoprotein-mediated drug-resistance of human breast cancer tissues. Breast Cancer 2001;8:333–8.
[27] Slovak ML, Ho JP, Cole SP, Deeley RG, Greenberger L, de Vries EG, et al. The LRP gene encoding a major vault protein associated with drug resistance maps proximal to MRP on chromosome 16: evidence that chromosome breakage plays a key role in MRP or LRP gene amplification. Cancer Res 1995;55:4214–9.
[28] Ichikawa Y, Hirokawa M, Aiba N, Fujishima N, Komatsuda A, Saitoh H, et al. Monitoring the expression profiles of doxorubicin-resistant K562 human leukemia cells by serial analysis of gene expression. Int J Hematol 2004; 79:276–82.
[29] Tang R, Faussat AM, Majdak P, Perrot JY, Chaoui D, Legrand O, et al. Valproic acid inhibits proliferation and induces apoptosis in acute myeloid leukemia cells expressing P-gp and MRP1. Leukemia 2004;18:1246–51.
[30] Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, et al. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci 2000;113:2011–21.
[31] Lee K, Klein-Szanto AJ, Kruh GD. Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. J Natl Cancer Inst 2000;92:1934–40.
[32] Robey RW, Shukla S, Finley EM, Oldham RK, Barnett D, Ambudkar SV, et al. Inhibition of P-glycoprotein (ABCB1)- and multidrug resistance-associated protein 1 (ABCC1)-mediated transport by the orally administered inhibitor, CBT-1((R)). Biochem Pharmacol 2008;75:1302–12.
[33] Shen T, Kuang YH, Ashby CR, Lei Y, Chen A, Zhou Y, et al. Imatinib and nilotinib reverse multidrug resistance in cancer cells by inhibiting the efflux activity of the MRP7 (ABCC10). PLoS ONE 2009;4:e7520.
[34] Robey RW, Honjo Y, Morisaki K, Nadjem TA, Runge S, Risbood M, et al. Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. Br J Cancer 2003;89:1971–8.
[35] Shi Z, Liang YJ, Chen ZS, Wang XW, Wang XH, Ding Y, et al. Reversal of MDR1/P-glycoprotein-mediated multidrug resistance by vector-based RNA interference in vitro and in vivo. Cancer Biol Ther 2006;5:39–47.
[36] Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 1997;3: 1337–45.
[37] Venne A, Li S, Mandeville R, Kabanov A, Alakhov V. Hypersensitizing effect of pluronic L61 on cytotoxic activity, transport, and subcellular distribution of doxorubicin in multiple drug-resistant cells. Cancer Res 1996;56: 3626–9.
[38] Baer MR, George SL, Dodge RK, O'Loughlin KL, Minderman H, Caligiuri MA, et al. Phase 3 study of the multidrug resistance modulator PSC-833 in previously untreated patients 60 years of age and older with acute myeloid leukemia: Cancer and Leukemia Group B Study 9720. Blood 2002;100: 1224–32.
[39] Benderra Z, Faussat AM, Sayada L, Perrot JY, Tang R, Chaoui D, et al. MRP3, BCRP, and P-glycoprotein activities are prognostic factors in adult acute myeloid leukemia. Clin Cancer Res 2005;11:7764–72.
[40] Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature 1994;367:645–8.
[41] Schaich M, Koch R, Soucek S, Repp R, Ehninger G, Illmer T. A sensitive model for prediction of relapse in adult acute myeloid leukaemia with t(8;21) using white blood cell count, CD56 and MDR1 gene expression at diagnosis. Br J Haematol 2004;125:477–9.
[42] van der Kolk DM, de Vries EG, van Putten WJ, Verdonck LF, Ossenkoppele GJ, Verhoef GE, et al. P-glycoprotein and multidrug resistance protein activities in relation to treatment outcome in acute myeloid leukemia. Clin Cancer Res 2000;6:3205–14.
[43] van der Kolk DM, de Vries EG, Noordhoek L, van den Berg E, van der Pol MA, Muller M, et al. Activity and expression of the multidrug resistance proteins P-glycoprotein, MRP1, MRP2, MRP3 and MRP5 in de novo and relapsed acute myeloid leukemia. Leukemia 2001;15:1544–53.
[44] van der Kolk DM, Vellenga E, Scheffer GL, Muller M, Bates SE, Scheper RJ, et al. Expression and activity of breast cancer resistance protein (BCRP) in de novo and relapsed acute myeloid leukemia. Blood 2002;99:3763–70.
[45] Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood 2004;104:3739–45.
[46] Jiang X, Zhao Y, Smith C, Gasparetto M, Turhan A, Eaves A, et al. Chronic myeloid leukemia stem cells possess multiple unique features of resistance to BCR-ABL targeted therapies. Leukemia 2007;21:926–35.
[47] Mahon FX, Hayette S, Lagarde V, Belloc F, Turcq B, Nicolini F, et al. Evidence that resistance to nilotinib may be due to BCR-ABL, Pgp, or Src kinase overexpression. Cancer Res 2008;68:9809–16.
[48] Hiwase DK, Saunders V, Hewett D, Frede A, Zrim S, Dang P, et al. Dasatinib cellular uptake and efflux in chronic myeloid leukemia cells: therapeutic implications. Clin Cancer Res 2008;14:3881–8.
[49] Erlichman C, Boerner SA, Hallgren CG, Spieker R, Wang XY, James CD, et al. The HER tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. Cancer Res 2001;61:739–48.
[50] Elkind NB, Szentpetery Z, Apati A, Ozvegy-Laczka C, Varady G, Ujhelly O, et al. Multidrug transporter ABCG2 prevents tumor cell death induced by the epidermal growth factor receptor inhibitor Iressa (ZD1839, Gefitinib). Cancer Res 2005;65:1770–7.
[51] Kitazaki T, Oka M, Nakamura Y, Tsurutani J, Doi S, Yasunaga M, et al. Gefitinib, an EGFR tyrosine kinase inhibitor, directly inhibits the function of P-glycoprotein in multidrug resistant cancer cells. Lung Cancer 2005;49:337–43.
[52] Shi Z, Peng XX, Kim IW, Shukla S, Si QS, Robey RW, et al. Erlotinib (Tarceva, OSI-774) antagonizes ATP-binding cassette subfamily B member 1 and ATP-binding cassette subfamily G member 2-mediated drug resistance. Cancer Res 2007;67:11012–20.
[53] Zheng LS, Wang F, Li YH, Zhang X, Chen LM, Liang YJ, et al. Vandetanib (Zactima, ZD6474) antagonizes ABCC1- and ABCG2-mediated multidrug resistance by inhibition of their transport function. PLoS ONE 2009;4:e5172.
[54] Dai CL, Tiwari AK, Wu CP, Su XD, Wang SR, Liu DG, et al. Lapatinib (Tykerb, GW572016) reverses multidrug resistance in cancer cells by inhibiting the

activity of ATP-binding cassette subfamily B member 1 and G member 2. Cancer Res 2008;68:7905–14.
[55] Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, et al. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. Clin Cancer Res 2003;9:327–37.
[56] Tian S, Quan H, Xie C, Guo H, Lu F, Xu Y, et al. YN968D1 is a novel and selective inhibitor of vascular endothelial growth factor receptor-2 tyrosine kinase with potent activity in vitro and in vivo. Cancer Sci 2011;102:1374–80.
[57] Li J, Zhao X, Chen L, Guo H, Lv F, Jia K, et al. Safety and pharmacokinetics of novel selective vascular endothelial growth factor receptor-2 inhibitor YN968D1 in patients with advanced malignancies. BMC Cancer 2010;10:529.
[58] Kessler T, Fehrmann F, Bieker R, Berdel WE, Mesters RM. Vascular endothelial growth factor and its receptor as drug targets in hematological malignancies. Curr Drug Targets 2007;8:257–68.
[59] Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001;61:3986–97.
[60] Tenzer A, Zingg D, Rocha S, Hemmings B, Fabbro D, Glanzmann C, et al. The phosphatidylinositide 3′-kinase/Akt survival pathway is a target for the anticancer and radiosensitizing agent PKC412, an inhibitor of protein kinase C. Cancer Res 2001;61:8203–10.
[61] Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. Oncogene 2002;21:6255–63.