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ORIGINAL ARTICLE

Cediranib (recentin, AZD2171) reverses ABCB1- and ABCC1-mediated multidrug resistance by inhibition of their transport function

Li-yang Tao · Yong-ju Liang · Fang Wang · Li-ming Chen · Yan-yan Yan · Chun-ling Dai · Li-wu Fu

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Abstract
Purpose Cediranib (recentin, AZD2171) is an oral small-molecule multiple receptor tyrosine kinases inhibitor. Here we investigate the ability of cediranib to reverse tumor multidrug resistance (MDR) due to overexpression of ABCB1 (P-glycoprotein) and ABCC1 (MRP1) transporters.
Methods KBv200,MCF-7/adr, C-A120 and their parental sensitive cell lines KB, MCF-7 and KB-3-1 were used for reversal study. The intracellular accumulations of doxorubicin and rhodamine 123 were determined by flow cytometry. The expressions levels of ABCB1 and ABCC1 were investigated by Western blot and RT-PCR analyses. ATPase activity assay were performed by Luminescence. The functions of ERK in MCF-7/adr were investigated by RNA interference.
Results Cediranib significantly enhanced the sensitivity of ABCB1 or ABCC1 substrates in MDR cells, with no effect found on sensitive cells. However, the expressions of these transporters were not affected and the reversal activity of cediranib was not related to the phosphorylation of AKT or ERK1/2. Further studies showed that cediranib inhibited ATPase activity of ABCB1 (P-glycoprotein) in a dose-dependent manner.
Conclusions Cediranib reverses ABCB1- and ABCC1-mediated MDR by directly inhibiting their drug efflux function. These findings may be useful for cancer combinational therapy with cediranib in the clinic.

L. Tao · Y. Liang · F. Wang · L. Chen · Y. Yan · C. Dai · L. Fu (✉)
State Key Laboratory of Oncology in South China, Cancer Center, Sun Yat-Sen University, 651 Dongfeng East Road, 510060 Guangzhou, China
e-mail: fulw@mail.sysu.edu.cn; sohutly@163.com

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Introduction

Multidrug resistance (MDR) is a major problem to successful chemotherapy treatment with numerous and complex mechanisms. One of the best known mechanisms is the overexpression of ATP-binding cassette (ABC) transporters which are able to efflux drugs out of tumor cells. In the human genome, 48 different ABC transporters have been identified and divided into seven subfamilies (A–G) based on sequence similarities [1]. So far, 14 ABC transporters (ABCA2, ABCB1, ABCB4, ABCB11, ABCC1-9, and ABCG2) have been associated with drug resistance and drug transport [2]. ABCB1 (P-glycoprotein, Pgp, MDR1), the first cloned human ABC transporter[3], has been demonstrated to be a promiscuous transporter of hydrophobic substrates, either uncharged or slightly positively charged, including most chemotherapeutic agents such as colchicine, Vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes [4]. ABCC1 (MRP1) was identified in a multidrug resistant small-cell lung carcinoma cell line (NCI-H69) that did not overexpress ABCB1 [5]. The structural similarities between ABCB1 and ABCC1 confer to some overlap in their drug resistance spectra; however, nonanionic compounds may be transported by ABCC1 as glutathione, glucuronide, or sulfate conjugates, or cotransported with glutathione without conjugation [6].

One of the effective ways to overcome ABCB1- and ABCC1-mediated MDR is to develop inhibitors of transporter pumps. Up to date, three generations of MDR inhibitors have been developed. Some compounds have shown

effective activity to reverse MDR, but clinical trials are still in the beginning stages. Recent studies showed that tyrosine kinase inhibitors, STI-571 and AG1393, interacted with ABCB1 and ABCC1 and significantly inhibited the function of ATP-binding cassette (ABC) transporters [7]. Following that, many other tyrosine kinase inhibitors, such as gefitinib [8], imatinib [9], erlotinib [10] and lapatinib [11], were discovered to reverse chemotherapy resistance in multidrug-resistant cancer cell expressing ABC family proteins. Another oral small molecular tyrosine kinase inhibitor of VEGFRs, ZD6474 (Vandetanib), also showed reversal activity by inhibiting the function of ABCB1 [12]. These reports collectively suggest that tyrosine kinase inhibitor may be promising MDR inhibitors. Cediranib (recentin, AZD2171) is an oral small-molecule tyrosine kinase inhibitor that targets vascular endothelial growth factor receptor-2 (VEGFR-2) with a median inhibition concentration value (IC_{50}) of less than 1 nmol/L, and also has activity against VEGFR-1, VEGFR-3, platelet-derived growth factor receptors (PDGFR) and stem cell factor receptor (c-KIT) [13]. So it is conceivable that cediranib could reverse ABC transporters mediated MDR by inhibiting the functions of ABC transporters. These have spurred on efforts to investigate whether cediranib can enhance the efficacy of conventional chemotherapeutic drugs via interaction with ABC transporters in MDR cancer cells.

Methods

Chemicals and reagents

Cediranib was obtained from Astra-Zeneca Pharmaceuticals. Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 were products of Gibco BRL. Monoclonal antibodies to ABCB1, ABCC1 and HSP70 were products of Santa Cruz Biotechnology, Inc. Extracellular signal-regulated kinase (ERK1/2), p-ERK1/2, p-AKT and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Kangchen Co. (Shanghai, China). Akt antibody was a product of Cell Signaling Technology, Inc. (Danvers, MA, USA). Doxorubicin (DOX), vincristine, 3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide (MTT), rhodamine 123 and other chemicals were obtained from Sigma Chemical Co. (San Diego, CA, USA).

Cell lines and cell culture

The following cell lines were cultured in DMEM or RPMI 1640 containing 10% fetal bovine serum at 37°C in the presence of 5% CO_2: the human oral epidermoid carcinoma cell line KB and its vincristine-selected derivative ABCB1 overexpressing cell line KBv200 [14]; the human breast carcinoma cell line MCF-7 and its doxorubicin-selected derivative ABCB1 overexpressing cell line MCF-7/adr [15]; the human epidermoid carcinoma cell line KB-3-1 and its doxorubicin-selected derivative ABCC1 overexpressing cell line C-A120 [16].

Cell proliferation assays

The MTT assay was used to examine cytotoxicity, which was previously described [17]. The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from survival curves using 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} for cells with the anticancer drug in the absence of cediranib by that obtained in the presence of cediranib.

Flow cytometric analyses with the accumulations of DOX and rhodamine 123

The effects of cediranib on the cellular accumulations of DOX and rhodamine 123 were measured by flow cytometry

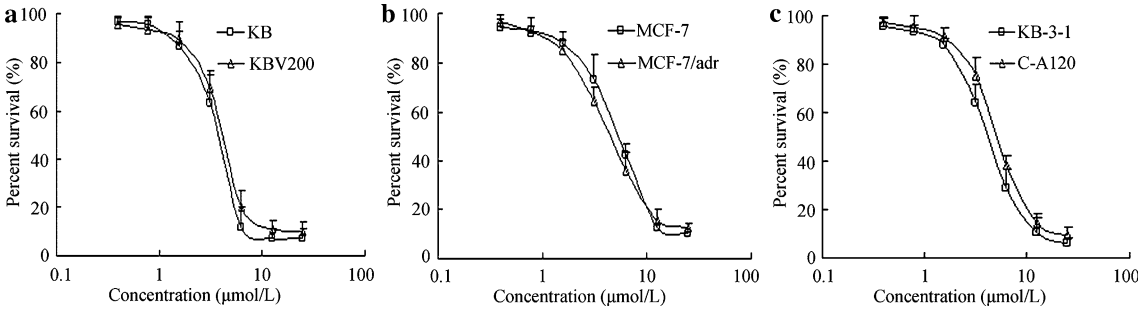

Fig. 1 Cediranib inhibition of tumor cell proliferation. ABCB1-negative KB and ABCB1-positive KBv200 cells (**a**), ABCB1-negative MCF-7 and ABCB1-positive MCF-7/adr cells (**b**), and ABCC1-negative KB-3-1 and ABCC1-positive C-A120 cells(**c**) were exposed to the indicated concentrations of cediranib for 72 h. Each *point* represents the mean ± standard deviations (SDs) for three determinations. Each experiment was performed in three replicate wells

as previously described [15]. Briefly, 5×10^5 cells were incubated in 6-well plates and allowed to attach overnight. The cells were then treated with cediranib of desired concentration and vehicle at 37°C for 3 h. Then 10 μmol/L DOX or 5 μmol/L rhodamine 123 was added and further incubated for another 3 h or 0.5 h, respectively. Following this incubation, the cells were collected and washed twice with ice-cold PBS buffer. Finally, the cells were resuspended in PBS buffer for flow cytometric analysis (Beckman Coulter, Cytomics FC500, USA). Verapamil (VPL) was used as a positive control [12, 18]. The relative values were identified by dividing the fluorescence intensity of each measurement by that of control cells.

Western blot analysis

After drug treatment for 48 h, the cells were harvested and rinsed twice with ice-cold PBS buffer. Western blot was performed as previously described [14].

Reverse transcription-PCR

The cells were treated with cediranib for 48 h. Total cellular RNA was isolated by Trizol Reagent (Molecular Research Center, USA) RNA extraction kit following manufacturer's instruction. The first strand 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, 5′-cta cct cct gtg gct gaa tct g-3′ (forward) and 5′-cat cag ctt gat ccg att gtc t-3′ (reverse) for ABCC1, and 5′-ctt tgg tat cgt gga agg a-3′ (forward) and 5′-cac cct gtt gct gta gcc-3′ (reverse) for GAPDH. 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. Products were resolved and examined by 1.5% agarose gel electrophoresis. Expected reverse transcription-PCR (RT-PCR) products were 157 bp for ABCB1, 151 bp for ABCC1, and 475 bp for GAPDH, respectively.

RNA interference

siRNAs specific for human p44/42 ERK and GAPDH, and the nonspecific negative control siRNA with or without FAM-labeled were purchased from Shanghai GenePharma Co., Ltd. MCF-7/adr cells were seeded in 60-mm dishes to reach 30–50% confluent and transfected with siRNA using Lipofectamine 2000 (Invitrogen) in antibiotics and serum free media, according to the manufacturer's instructions. The efficiency was observed using fluorescence microscopy and quantified by flow cytometry after 6–8 h, and total proteins were extracted for Western blot analysis after 48, 72 and 96 h to assay for gene knockdown.

Table 1 Effects of cediranib on reversing ABCB1- and ABCC1-mediated drug resistance

Compounds	$IC_{50} \pm$ SDs μmol/L (fold-reversal)	
	KB	KBv200 (ABCB1)
Doxorubicin	0.050 ± 0.004	3.20 ± 0.15
+0.5 μmol/L cediranib	0.049 ± 0.003 (1.02)	1.16 ± 0.10 (2.76)**
+1.0 μmol/L cediranib	0.048 ± 0.005 (1.05)	0.70 ± 0.03 (4.59)**
+1.5 μmol/L cediranib	0.049 ± 0.003 (1.01)	0.33 ± 0.04 (9.70)**
Cisplatin	2.18 ± 0.16	3.28 ± 0.32
+1.5 μmol/L cediranib	2.29 ± 0.25 (0.95)	3.56 ± 0.24 (0.92)
	MCF-7	MCF-7/adr (ABCB1)
Doxorubicin	0.39 ± 0.02	22.33 ± 2.12
+0.5 μmol/L cediranib	0.40 ± 0.01 (0.98)	10.64 ± 0.75 (2.10)**
+1.0 μmol/L cediranib	0.38 ± 0.03 (1.03)	6.31 ± 0.54 (3.54)**
+1.5 μmol/L cediranib	0.40 ± 0.06 (0.99)	2.53 ± 0.34 (8.82)**
Cisplatin	16.87 ± 0.63	13.93 ± 0.95
+1.5 μmol/L cediranib	17.43 ± 1.60 (0.97)	13.64 ± 1.45 (1.02)
	KB-3-1	C-A120 (ABCC1)
Vincristine	0.0017 ± 0.0002	0.237 ± 0.024
+0.5 μmol/L cediranib	0.0018 ± 0.0003 (0.94)	0.175 ± 0.026 (1.35)*
+1.0 μmol/L cediranib	0.0017 ± 0.0003 (0.99)	0.095 ± 0.016 (2.49)**
+1.5 μmol/L cediranib	0.0016 ± 0.0002 (1.03)	0.051 ± 0.007 (4.64)**
Cisplatin	1.43 ± 0.15	4.13 ± 0.15
+1.5 μmol/L cediranib	1.49 ± 0.16 (0.96)	4.06 ± 0.645 (1.02)

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

* and ** represent $P < 0.05$ and $P < 0.01$, respectively, for values versus that obtained in the absence of cediranib

ABCB1 ATPase activity assay

The changes of ATPase activity were estimated by Pgp-Glo™ assay systems (Promega, USA). The inhibitory effects of cediranib were examined against a verapamil-stimulated Pgp ATPase activity. Sodium orthovanadate (Na_3VO_4) was used as a Pgp ATPase inhibitor. Various concentrations of cediranib diluted with assay buffer were incubated in 0.1 mmol/L verapamil, 5 mmol/L MgATP 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, USA) was read on luminometer (spectraMax M5, molecular devices, USA). The changes of relative light units (ΔRLU) were determined by comparing Na_3VO_4-treated samples with cediranib and verapamil combination-treated samples.

All experiments were done at least thrice. Statistical analysis was done by Student's *t*-test analyses. The significance was determined at $P < 0.05$.

Results

Effects of cediranib and chemotherapeutic agents in various MDR cells and their parental cells

We examined the cytotoxic effects of cediranib alone in different cell lines using the MTT assay. The IC_{50} values were 3.62 ± 0.16, 3.80 ± 0.38, 5.11 ± 0.29, 4.50 ± 0.28, 4.15 ± 0.23 and 5.05 ± 0.37 μmol/L for KB, KBv200, MCF-7, MCF-7/adr, KB-3-1, C-A120 cells, respectively (Fig. 1). More than 90% of cells were viable at the concentrations of cediranib up to 1.5 μmol/L in all cells used in the

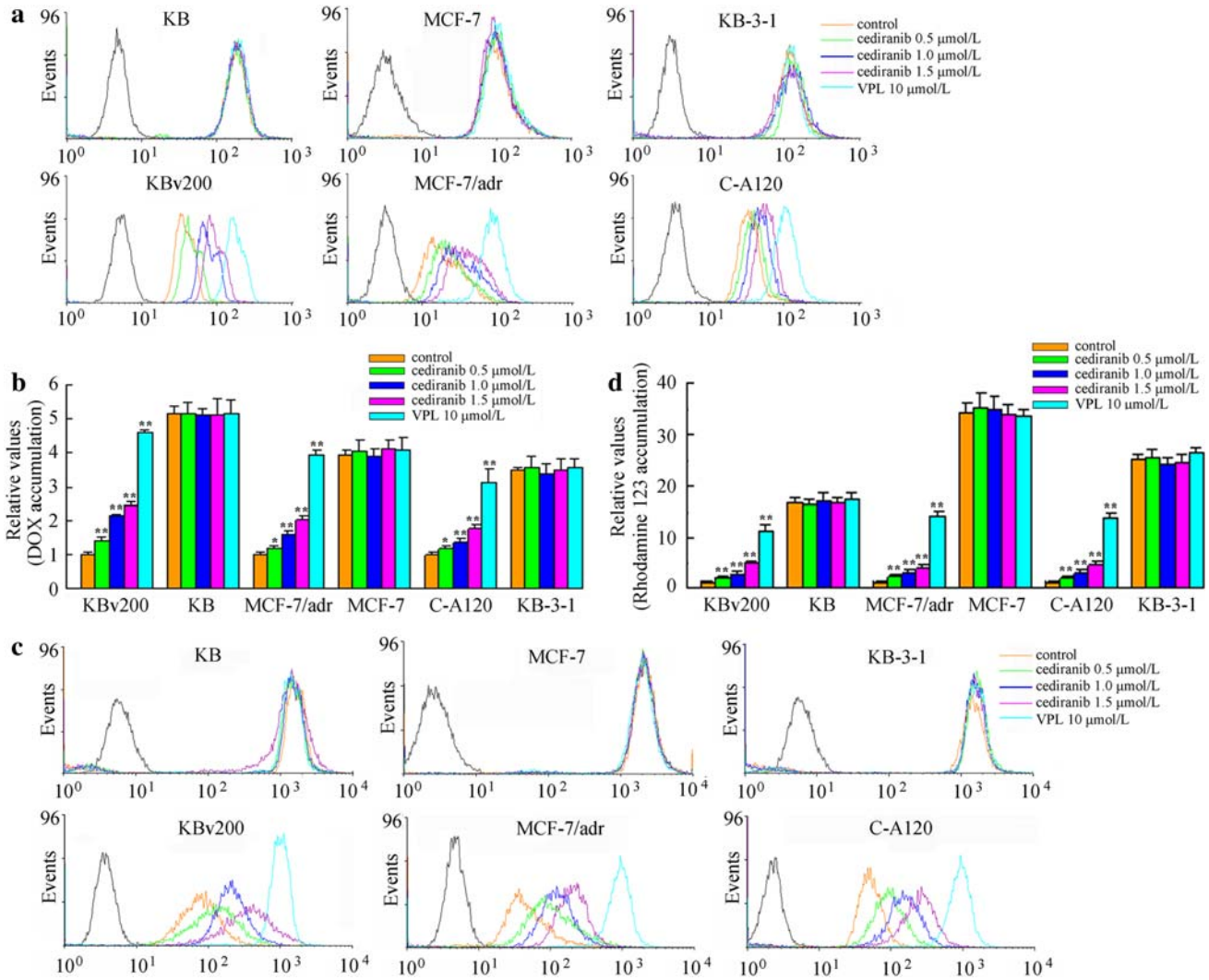

Fig. 2 Effects of cediranib on the accumulation of doxorubicin and rhodamine 123. **a**, **b** Effects of cediranib on the accumulation of doxorubicin (DOX). Cells were incubated with cediranib (0, 0.5, 1.0 and 1.5 μmol/L) at 37°C for 3 h, then 10 μmol/L DOX was added for another 3 h incubation. **c**, **d** Effects of cediranib on the accumulation of rhodamine 123. After incubated with cediranib for 3 h, 5 μmol/L rhodamine 123 was added for another 0.5 h incubation. Intracellular fluorescence was analyzed by flow cytometry with an excitation wavelength of 488 nm. Control cells did not receive any cediranib treatment, and VPL (10 μmol/L) was used as a positive control. All these experiments were repeated at least thrice, and a representative experiment is shown. *Columns* means of triplicate determinations; *bars* SDs. $^*P < 0.05$; $^{**}P < 0.01$ versus control group

experiments. So we selected cediranib of 0.5, 1.0, and 1.5 μmol/L to reverse MDR in vitro. The mean IC_{50} values of chemotherapeutic agents in various pairs of sensitive and resistant cells in different concentrations of cediranib are shown in Table 1. In ABCB1-overexpressing KBv200, MCF-7/adr and ABCC1-overexpressing C-A120 cells, cediranib produced a significant dose-dependent increase in the cytotoxicity of chemotherapeutic agents. In contrast, cediranib did not increase chemosensitivity in their parental sensitive cells. To evaluate substrate specificity of transporter, cisplatin which is not a substrate of ABCB1 or ABCC1 was selected as a control. Cediranib did not significantly alter the IC_{50} values of cisplatin in MDR and drug-sensitive cells. These results suggest that cediranib strongly enhances the sensitivity of ABCB1 and ABCC1 overexpressing MDR cells to conventional chemotherapeutic agents, but has no significant effects on their parental cells.

Cediranib enhances the accumulation of DOX and rhodamine 123 in MDR cells overexpressing ABCB1 and ABCC1

The results above indicated that cediranib could enhance the sensitivity of MDR cells to certain chemotherapeutic agents. The mechanism by which this occurs is unknown. Therefore, we examined its effects on DOX and rhodamine 123 accumulation in ABCB1 and ABCC1 expressing MDR cells and their parental cells. The fluorescence of DOX or rhodamine 123 was significantly higher in the sensitive cancer cells than in the MDR cells. Cediranib treatment enhanced the intracellular accumulation of DOX and rhodamine 123 in MDR cells in a dose-dependent manner, but showed no significant effects on parental sensitive cells. Figure 2a illustrates the effects of cediranib on the accumulation of DOX. The intracellular accumulations of DOX were enhanced 1.39, 2.12 and 2.48-fold in KBv200 cells, 1.18, 1.56 and 2.02-fold in MCF-7/adr cells, 1.17, 1.35 and 1.76-fold in C-A120 cells in the presence of 0.5, 1.0 or 1.5 μmol/L of cediranib, respectively (Fig. 2b). As depicted in Fig. 2d, the rhodamine 123 fluorescence was enhanced 1.80, 2.48 and 4.73-fold in KBv200 cells, 2.31, 2.80 and 3.77-fold in MCF-7/adr cells, 1.90, 2.48 and 4.52-fold in C-A120 cells at cediranib varying from 0.5 to 1.5 μmol/L. However, fluorescence in sensitive cells had no significant changes (Fig. 2a, c). These results demonstrate that cediranib was able to interfere with ABCB1 and ABCC1-mediated transport.

Effects of cediranib on the blockade of AKT and ERK1/2 phosphorylation

The phosphorylation of AKT and ERK1/2, the downstream markers, were always used to assess targeted activity of cediranib. Studies have been successful in attenuating chemotherapeutic resistance by inhibiting AKT and ERK pathways [19, 20]. To determine whether the reversal activity is related to the change of phosphorylation, we tested phosphorylation of AKT or ERK1/2 at varying concentrations of cediranib. As shown in Fig. 3, after exposed to 0.5–1.5 μmol/L of cediranib for 48 h, the phosphorylation of AKT was not significantly blocked. Except for MCF-7/adr cells, the concentrations of cediranib varied from 0.5 to 1.5 μmol/L did not significantly block the phosphorylation of ERK1/2. So it is conceivable that the decreasing phosphorylation of ERK1/2 may contribute to the sensitization of MCF-7/adr cells to chemotherapeutic agents.

The reversal activity of cediranib was independent of blocking ERK1/2 signal transduction

To investigate further whether the enhancement efficacy of chemotherapeutic agents by cediranib is related to the blockade of ERK1/2 phosphorylation, we designed siRNA to silence ERK target gene and observed the cytotoxicity alteration of DOX in absence or presence of cediranib. As shown in Fig. 4a, the transfection efficiency reached to at least 80% quantified using flow cytometry. The Western blot analysis revealed targeted siRNA could downregulate

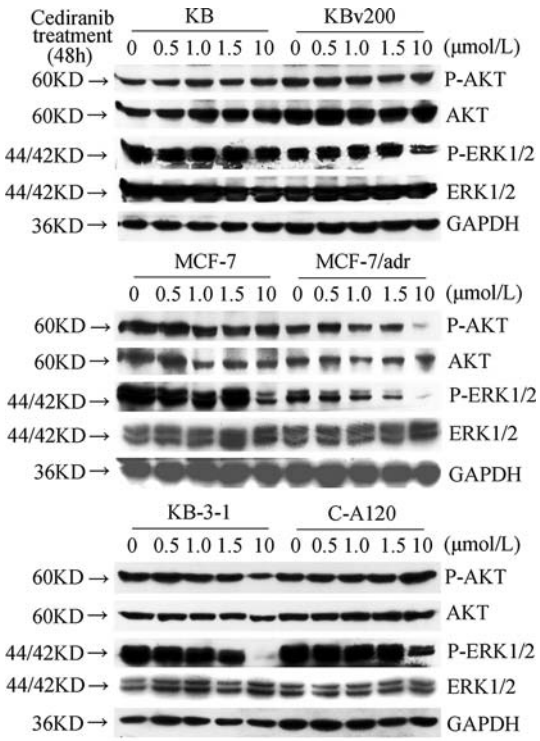

Fig. 3 Effects of cediranib on blockade of Akt and Erk1/2 phosphorylation. Equal amount of protein was loaded for Western blot as described in Methods. All these experiments were repeated at least thrice, and a representative experiment is shown in each *panel*

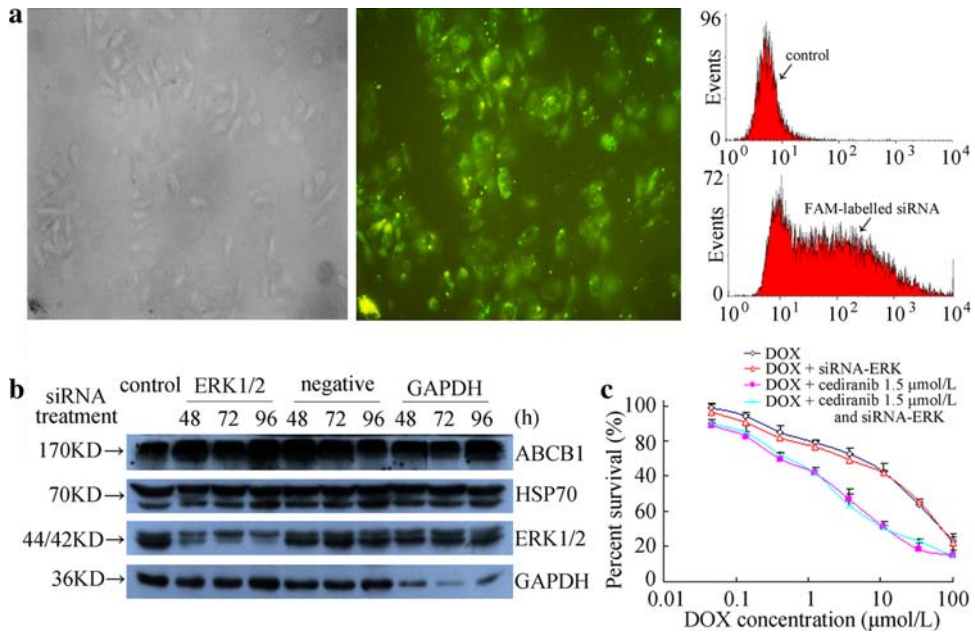

Fig. 4 The Effect of the silence of ERK1/2 by siRNA on the sensitization of MCF-7/adr cells to DOX. **a** siRNA p44/42 ERK transfectional efficiency. The negative control siRNA-FAM was used to indirectly determine the transfectional efficiency according to manufacturer's recommendations. The *left* was on the light and the *middle* was on the fluorescence in random microscopic fields at 400× magnification. The *right* was fluorescence counting by flow cytometry. **b** The effect of siRNA on protein expression in MCF-7/adr cells with Western blot analysis. The total proteins were extracted for Western blot analysis at 48, 72 or 96 h after transfection. The expressions of GAPDH and HSP70 were used as a positive and loading control, respectively. The experiment was performed at least three times. **c** The sensitization of MCF-7/adr cells to DOX was independent of ERK1/2 signal blockade. 8,000 cells were seeded in 96-well plates. 50 nmol/L RNAi duplex was selected to transfect cells. Incubate the cells for 6–8 h at 37°C in a CO_2 incubator and change the culture medium. After 24 h interfering, cytotoxicity was measured using MTT assay. Data represented means ± SDs of at least triplated determinations. Each experiment was performed in three replicate wells

ERK1/2 and GAPDH after transfection for 48 h (Fig. 4b). However, nonspecific negative control siRNA had no effect on protein expression. When ERK1/2 was interfered, the protein lever of ABCB1 was not changed. Furthermore, the MTT cytotoxicity assay was performed in the presence of ERK1/2 RNAi duplex. Interestingly, the results showed that the downregulation of ERK1/2 by siRNA did not inhibit cell growth and alter IC_{50} values of DOX in MCF-7/adr cells. The similar reversal activity of cediranib was exhibited with or without ERK1/2 RNAi duplex treatment (Fig. 4c). This demonstrates that the enhancing efficacy of chemotherapeutic in MCF-7/adr cells is independent of the state of ERK1/2 phosphorylation.

Effects of cediranib on the expression of mRNA and protein levels of ABCB1 and ABCC1

The reversal of ABC transporter-mediated MDR can usually be achieved either by decreasing transporter expression or inhibiting function. Therefore, we determined the effects of cediranib on the expression of ABCB1 and ABCC1. Our results showed that the expression in protein (Fig. 5a) or mRNA level (Fig. 5b) was not affected by the indicated concentrations of cediranib treatment. These results provide evidence that cediranib does not affect the expression of ABCB1 and ABCC1. Thus, it suggests that the reversal of MDR may be obtained by inhibiting the function of ABCB1 and ABCC1.

Cediranib inhibits the ATPase activity of ABCB1

The drug-efflux functions of ABCB1 and ABCC1 are linked to ATP hydrolysis. ATP consumption reflects ATPase activity. To assess the effects of cediranib on the ATPase activity of MDR proteins, we measured ABCB1-mediated ATP hydrolysis with various concentrations of cediranib. We found cediranib was an inhibitor of ABCB1 ATPase. As shown in Fig. 5c, cediranib reduced verapamil-stimulated ATPase activity in a dose-dependent manner. Cediranib at 1.5 μmol/L inhibited about 20% of 100 μmol/L verapamil-stimulated ABCB1 ATPase activity.

Discussion

Cediranib is a highly potent inhibitor of the tyrosine kinase activity associated with all three VEGFRs, PDGFR, c-KIT and FGFR2. Preclinical studies have shown that cediranib

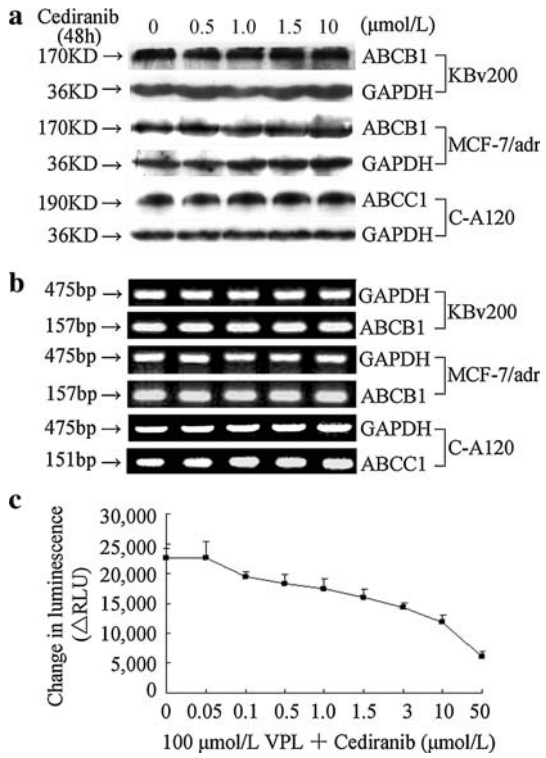

Fig. 5 Cediranib inhibition of verapamil-stimulated ABCB1 ATPase activity, and no effects on ABCB1 and ABCC1 expression. **a** KBv200, MCF-7/adr and C-A120 cells were treated with cediranib of various concentrations for 48 h. Equal amounts of total cell lysates were used for loading and detected with Western blotting. **b** The mRNA levels of ABCB1 and ABCC1 were determined by RT-PCR as described in Methods. All these experiments were repeated at least thrice, and a representative experiment is shown in each *panel*. **c** Luminescent ABCB1 ATPase assays were performed according to Pgp-Glo™ Assay Systems instruction. Each *point* represents the mean ± SDs for triplated independent determinations

has broad antitumor activity, including those refractory to previous treatment, and could impair the clearance of paclitaxel [21–25]. Cediranib is now being extensively investigated as monotherapy or in combination with chemotherapy for a range of tumor types [21–24]. A series of randomized, double-blind phase II and phase II/III trials are ongoing. In vitro studies using biochemical and cell assays showed that tyrosine kinase inhibitors modulated the function of ABC transporters such as ABCG2, ABCB1 and ABCC1 [7, 23, 26]. The present study shows that cediranib displays almost equal activity in ABCB1 or ABCC1-negative and -positive cancer cells (Fig. 1). This suggests that cediranib may be a good option for patients already developing MDR. Our finding showed that cediranib dose-dependently enhanced cytotoxicity of established ABCB1 and ABCC1 substrates in MDR cells. However, in drug-sensitive cells, the cytotoxicity generated by the ABCB1 and ABCC1 substrates was unaffected in the presence of cediranib (Table 1). Furthermore, cediranib did not significantly alter the sensitivity of non-ABCB1 and ABCC1 substrate such as cisplatin in sensitive and resistant cells, similar to trends observed with other TKIs including imatinib mesylate, erlotinib and GW282974A [10, 27, 28].

Cediranib was first developed as a VEGFR tyrosine kinase inhibitor, but the following tests showed that cediranib inhibited many other receptor tyrosine kinases [13, 25]. Their downstream signaling pathways, such as PI3K/AKT and ERK were always accordingly inhibited. However, PI3K/AKT and ERK are constitutively activated in cancer cells and are the potential targets for enhancing the cytotoxicity of chemotherapeutic agents in treatment of cancer [29, 30]. There are data implicate PI3K/Akt or ERK pathway activation is related with resistance to conventional chemotherapeutic agents [19, 20, 31, 32]. Our results showed that treatment with 1.5 μmol/L cediranib did not affect the phosphorylation of AKT, and excepting in MCF-7/adr cells, the phosphorylation of ERK was not affected, either. It is possible that decreasing ERK1/2 phosphorylation confers to the sensitization of MCF-7/adr cells to chemotherapeutic drug. To explore this issue, we knocked down ERK1/2 by a siRNA, and MTT assays were done in the presence of ERK1/2 RNAi duplex. ERK1/2 down-regulation altered neither the cell proliferation nor the sensitivity of chemotherapeutic agent. In addition, the cytotoxic effect of DOX only enhanced in the presence of cediranib. These observations suggest that ERK1/2 phosphorylation blockade by cediranib does not confer to the sensitization of MCF-7/adr cells to chemotherapeutic drug. Recent researches had shown a link between MDR transporter ABCB1 and Raf1/MEK/ERK signaling [32, 33]. However, in our experiment, silencing ERK1/2 in MCF-7/adr cells did not decrease the expression of ABCB1. The results were coincident to another research which examined the potential role of MAPK activation in TPA-mediated MDR1 induction in human leukemia K562 cells [34]. These data suggest that the mechanisms of ABCB1 induction by chemotherapeutic stimuli are independent of ERK pathway.

The profile of the drug-stimulated ATPase activity is thought to reflect the nature of interaction of transporter pumps with drug substrates. Based on their effects on ATPase activity, compounds could be categorized into three distinct classes. The first class MDR modulators stimulate ATPase activity at low concentrations but inhibit the activity at high concentrations, the second class MDR modulators enhance ATPase activity in a dose-dependent manner without any inhibition, whereas the third class MDR modulators inhibit both basal-stimulated and verapamil-stimulated ATPase activity [35]. Our results show that cediranib belongs to the third class MDR modulators. Because ATP hydrolysis is required for transport, compounds that inhibit ATPase activity are unlikely to be transported by ABC drug transporters. We speculated that cediranib might not be a substrate of these transporters. ABCB1- and ABCC1-over-

expressing MDR cells were not resistant to cediranib, which provided evidence to support our speculation. Cediranib significantly inhibited the efflux of DOX and rhodamine 123 in MDR cells and reversed ABCB1- and ABCC1-mediated MDR. However, the mRNA and protein expressions of ABCB1 and ABCC1 were not affected. It is believed that cediranib reversed MDR by directly inhibiting the function of ABC drug transporters.

In conclusion, cediranib strongly enhances the efficacy of chemotherapeutic drugs in ABCB1- and ABCC1-overexpressing MDR cells, and this is achieved by inhibiting ATPase activity. In addition, the reversal of MDR by cediranib is not associated with the blockade of tyrosine kinase. These findings indicate that combination of cediranib with other anticancer drugs may be important in surmounting clinical resistance in cancer chemotherapy.

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