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Lapatinib Antagonizes Multidrug Resistance–Associated Protein 1–Mediated Multidrug Resistance by Inhibiting Its Transport Function

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Lapatinib, a tyrosine kinase inhibitor, is used in the treatment of advanced or metastatic breast cancer overexpressing human epidermal receptor 2 (HER2). Lapatinib can modulate the function of ATP-binding cassette (ABC) transporters (ABCB1 and ABCG2), which are the major mechanism responsible for multidrug resistance (MDR) in cancer. In this study, we investigated the effect of lapatinib on multidrug resistance–associated protein 1 (MRP1 (ABCC1)), MRP2 (ABCC2), MRP4 (ABCC4) and lung relative resistance protein (LRP) drug efflux pumps. We demonstrated that lapatinib could enhance the efficacy of conventional chemotherapeutic agents in MRP1-overexpressing cells *in vitro* and *in vivo*, but no effect in MRP2-, MPR4- and LRP-overexpressing cells. Furthermore, lapatinib significantly increased the accumulation of rhodamine 123 (Rho123) and doxorubicin (DOX) in MRP1-overexpressing cells. However, lapatinib did not alter the protein or mRNA expression levels of MRP1. Further studies showed that the level of phosphorylation of AKT and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) were not altered at the indicated concentrations of lapatinib. In conclusion, lapatinib enhanced the efficacy of conventional chemotherapeutic agents in MRP1-overexpressing cells by inhibiting MRP1 transport function without altering the level of AKT or ERK1/2 phosphorylation. These findings will encourage the clinical research of lapatinib combined with conventional chemotherapeutic drugs in MRP1-overexpressing cancer patients.

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INTRODUCTION

Chemotherapy is the only systemic treatment for many malignant tumors. Unfortunately, the occurrence of multidrug resistance (MDR) limits the application of chemotherapy. The major mechanism responsible for MDR is the overexpression of ATP-binding cassette (ABC) transporters (1). ABC transporters decrease drug intracellular accumulation in cancer cells by pumping lots of anticancer drugs out of the cells with energy derived from ATP hydrolysis, thus resulting in chemotherapy resistance (2). The major members of ABC transporters leading to MDR include ABCB1 (P-glycoprotein [P-gp]), ABCC1 (multidrug resistance–associated protein 1 [MRP1]) and ABCG2 (breast cancer resistant protein [BCRP]) (3,4). These proteins owned the ability of conferring resistance to chemotherapeutic agents.

The ABCC subfamily contains 12 members, and 9 of them are associated with MDR, which is named MRP1–9 (5,6). ABCC7 (CFTR), ABCC8 (SUR1) and ABCC9 (SUR2) are not involved in MDR (6). These nine MRPs were defined ATP-dependent transporters for endogenous substances and xenobiotics on the basis of the functional characterization, localization and cloning studies (7–9).

The main member of the MRP subfamily–associated MDR is MRP1, which was discovered in 1992 (10). MRP1 was confirmed overexpressed in lung carcinoma, chronic lymphocytic leukemia (CLL) and acute lymphocytic leukemia (AML) (10–12). Previous reports indicated that MRP1 is expressed in many normal human tissues including lung, spleen, testis, kidney, placenta, thyroid, bladder and adrenal gland to maintain their functions by transporting various molecules across biological membranes (13). The known substrates of MRP1 range from anticancer drugs to physiological agents. A wide spectrum of anticancer drugs included anthracylines, antifolate neoplastics (MTX, edatrexate), vinca alkaloids, camptothecins, etopo-

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side (VP-16), irinotecan, SN-38, methotrexate and mitoxantrone (5). The organic anion conjugates include glutathione, glucuronides and sulfate conjugates (14). MRP1 also transports endogenous physiological agents such as the signaling molecule leukotriene C4 (LTC4) and metabolites destined for bile, containing bilirubin, glucuronide conjugates and sulfated bile salts (15–18). These transport properties of MRP1 are responsible for the drug resistance in MRP1-overexpressing cancers that do not express ABCB1 or ABCG2.

MRP2 was reported to be expressed in several human tumors, including lung, gastric, renal and colorectal cancers, and shared the similar substrate spectrum with MRP1, such as vincristine, doxorubicin and SN-38 (19,20). Moreover, MRP2 could enhance sensitivity to cisplatin, which is not substrate of MRP1. MRP4 is a potential therapeutic target for MDR. MRP4 was highly expressed in myeloid progenitors and transported a range of endogenous molecules out of cells (6). MRP4 can protect cell function by effluxing 6-mercaptopurine (6-MP) but could confer resistance to anticancer drugs in cancer cells.

Another potential obstacle mechanism for MDR is lung resistance–related protein (LRP). LRP was identical to human major vault protein (MVP) and initially cloned from the non–small cell lung carcinoma (NSCLC) SW1573/2R120 cell line (21). LRP transported a variety of substrates (such as doxorubicin, paclitaxel and cisplatin) between the nucleus and cytoplasm (21). LRP-overexpressing was found in several cancers, including colon carcinoma, leukemias and ovarian cancer (22–24).

Previous reports have demonstrated that tyrosine kinase inhibitors (TKIs) could reverse the ABC transporter–mediated MDR. Lapatinib is an orally active dual TKI that has been used in combination with capecitabine or letrozole for the treatments of patients with advanced breast cancer (25). We previously reported that lapatinib inhibited the function of ABCB1, ABCG2 and MRP10 (26,27). In this study, we explored the effect of lapatinib on the efficacy of conventional chemotherapeutic agents in MRP1-, MRP2-, MRP4- and LRP-overexpressing cancer cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM) and RPMI-1640 were from Gibco BRL (Thermo Fisher Scientific Inc., Waltham, MA, USA). Vincristine, doxorubicin, cisplatin, MK571, rhodamine123, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were all from Sigma-Aldrich (St. Louis, MO, USA). Lapatinib was from LC Laboratories (Woburn, MA, USA). Monoclonal antibodies against MRP1, MRP2, MRP4, LRP, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), p-ERK, AKT and p-AKT were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against glyceraldehyd-3-phosphate dehydrogenase (GAPDH) was from Kangcheng (Shanghai, China).

Cell Culture

Human epidermoid carcinoma cell KB-3-1 and its doxorubicin-selected derivative MRP1-overexprssing C-A120 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (28). Human embryonic kidney cell HEK293 and its pcDNA3.1 and MRP2 stable gene-transfected cells, murine fibroblasts cells NIH3T3 and its MRP4-transfected derivative NIH3T3/MRP4-2 were cultured in DMEM with 10% fetal bovine serum (29,30). All cells were cultured in a humidified atmosphere of 5% CO_2.

Cytotoxicity

We performed an MTT assay to assess cell proliferation activity (31). Briefly, we collected the cells and seeded them at a density of 3.0×10^3 cells per well in 96-well plates. After 24 h, different concentrations of chemotherapeutic agents were added into the wells 1 h after lapatinib was added. After 68 h, MTT (5 mg/mL, 20 μL) was added into each well, and 4 h later, the medium was discarded and 120 μL DMSO was added into the wells. Finally, optical density was measured at 540 nm, with background subtraction at 670 nm by a Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). Experiments were performed at least three times. The Bliss method was used to calculate the half maximal (50%) inhibitory concentration (IC_{50}) values of a substance (32). The fold reversal of MDR was calculated as previously described (33).

Establishment of C-A120 Cell Xenograft Model and Reversal of MDR by Lapatinib *In Vivo*

In this study, we established the C-A120–inoculated nude mice xenograft model. Athymic nude mice (5–6 wks old) were purchased from the center of experimental animals (Sun Yat-sen University). Briefly, each nude mouse had 1.0×10^7 C-A120 cells implanted subcutaneously under the right armpit. Tumor sizes and body weights were measured every 2 d, and when the mean diameter reached 0.5 cm, the animals were divided into four groups: one group received just saline and the other groups received vincristine (every other day [q2d] × 6, intraperitoneally [IP], 0.2 mg/kg) alone, lapatinib (q2d × 6, by mouth [PO], 100 mg/kg) alone and lapatinib (q2d × 6, PO, 100 mg/kg) followed by vincristine (q2d × 6, IP, 0.2 mg/kg), 1 h after lapatinib. The tumor volume (V) was estimated according to the following formula, as previously described (34):

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

A and B represent the two perpendicular diameters of tumors that were recorded every three days.

Intracellular Accumulation of Doxorubicin and Rhodamine 123

The accumulation of doxorubicin and rhodamine 123 in C-A120 and KB-3-1 cells were determined by flow cytometry as previously described (35). The cells (3.0×10^5 /well) were incubated in six-

well plates to allow attachment overnight. Then the cells were exposed to different concentrations of lapatinib (0.625, 1.25 and 2.5 μmol/L). After 3 h, doxorubicin (10 μmol/L) or rhodamine 123 (5 μmol/L) was added to the medium for further incubation for another 3 h or 0.5 h, respectively. The cells were collected, centrifuged and washed twice with ice-cold phosphate-buffered saline (PBS) buffer. Cells were resuspended in 1 mL PBS buffer for flow-cytometric analysis (Cytomics FC500; Beckman Coulter Inc., Brea, CA, USA). MK571 was used as a positive control.

Western Blot Analysis

The C-A120 and KB-3-1 cells were exposed to different concentrations of lapatinib (0.625, 1.25 and 2.5 μmol/L) for different periods (0, 24, 48 and 72 h) to test whether lapatinib affected the expression of MRP1 or the phosphorylation of AKT and ERK1/2. Western blot analysis was conducted as previously described (26). After blocking with 5% nonfat milk, the membranes were immunoblotted by using antibodies including MRP1, ERK1/2, p-ERK, AKT and p-AKT. For loading control, GAPDH was detected (26). Immunoreactive bands were visualized by the Phototope-HRP Western Blot Detection System (Cell Signaling, Danvers, MA, USA) and exposed to Kodak medical X-ray processor (Carestream Health Inc., Rochester, NY, USA). The protein expression level was quantified by using ImageJ software (NIH, Bethesda, MD, USA).

Reverse Transcription–Polymerase Chain Reaction (PCR) and Q-PCR

Total mRNA was isolated by TRI Reagent® according to the manufacturer's instruction (Molecular Research Center Inc., Cincinnati, OH, USA). The cDNA was synthesized by OligodT primers with reverse transcriptase (Promega Corporation, Madison, WI, USA). PCR primers were 5′-CTACCTCCTGTGGCT GAATCTG-3′ (forward) and 5′-CATCA GCTTGATCCGATTGTCT-3′ (reverse) for *MRP1* (151 bp) and 5′-GAGTCAACGG ATTTGGTCGT-3′ (forward) and 5′-GATCT CGCTCCTGGAAGATG-3′ (reverse) for *GAPDH* (224 bp) (36). The reactions were carried out by the GeneAmp PCR system 9700 (Applied Biosystems [Thermo Fisher Scientific]). The reaction condi-

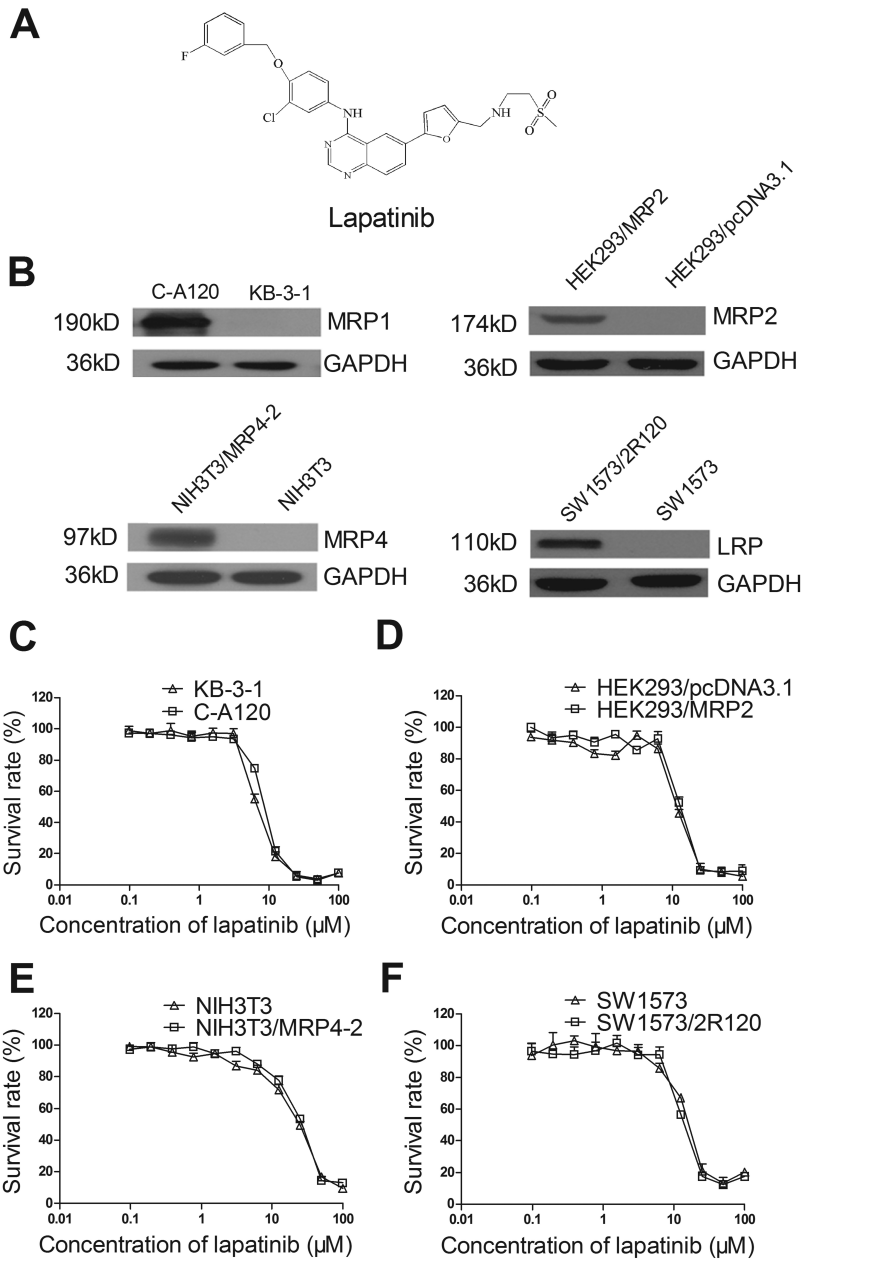

Figure 1. The structure of lapatinib and ABC transporter expression levels in various cell lines as well as cytotoxicity effects of lapatinib. (A) The structure of lapatinib. (B) The protein levels of MRP1, MRP2, MRP4 and LRP in all cell lines. MTT cytotoxicity assay was assessed in pairs of parental and transporter overexpressing cell lines: KB-3-1 and MRP1-overexpressing C-A120 cells (C); HEK293/pcDNA3.1 and MRP2-overexpressing HEK293/MRP2 cells (D); NIH3T3 and MRP4-overexpressing NIH3T3/MRP4-2 cells (E); and SW1573 and LRP-overexpressing SW1573/2R120 cells (F). All the cells were exposed to the full-range concentration of lapatinib for 72 h. Each point represents the means ± SD for three determinations. Each experiment was performed in three replicate wells.

Table 1. Effect of lapatinib on enhancing efficacy of chemotherapeutic agents in ABC transporter-overexpressing cells.

Compounds	IC_{50} ± SD (μmol/L) (fold-reverse)	
	KB-3-1	C-A120 (MRP1)
Doxorubicin	0.1253 ± 0.0106 (1.00)	15.3003 ± 1.1579 (1.00)
+0.625 μmol/L lapatinib	0.1246 ± 0.0013 (1.01)	6.0501 ± 0.0433 (2.53)**
+1.25 μmol/L lapatinib	0.1135 ± 0.0049 (1.10)	4.3649 ± 0.1626 (3.52)**
+2.5 μmol/L lapatinib	0.1180 ± 0.0208 (1.06)	2.5594 ± 0.2003 (5.95)**
+50 μmol/L MK571	0.1069 ± 0.0194 (1.17)	1.0600 ± 0.1645 (14.43)**
Vincristine	0.0036 ± 0.0230 (1.00)	0.5903 ± 0.06570 (1.00)
+0.625 μmol/L lapatinib	0.0035 ± 0.0095 (1.02)	0.2501 ± 0.0458 (2.36)**
+1.25 μmol/L lapatinib	0.0036 ± 0.0125 (0.99)	0.1825 ± 0.0326 (3.23)**
+2.5 μmol/L lapatinib	0.0035 ± 0.0312 (1.01)	0.1171 ± 0.0075 (5.04)**
+50 μmol/L MK571	0.0037 ± 0.0249 (0.98)	0.0962 ± 0.0051 (6.14)**
Cisplatin	0.2590 ± 0.00514 (1.00)	0.40203 ± 0.0142 (1.00)
+0.625 μmol/L lapatinib	0.2480 ± 0.04403 (1.04)	0.44458 ± 0.0692 (0.90)
+1.25 μmol/L lapatinib	0.2165 ± 0.03046 (1.20)	0.45598 ± 0.0530 (0.88)
+2.5 μmol/L lapatinib	0.2625 ± 0.34536 (0.99)	0.51239 ± 0.1803 (0.79)
+50 μmol/L MK571	0.2332 ± 0.08340 (1.11)	0.29262 ± 0.0161 (1.37)
	HEK293/PcDNA3.1	HEK293/MRP2
VP-16	0.1472 ± 0.0378 (1.00)	0.4869 ± 0.1312 (1.00)
+0.625 μmol/L lapatinib	0.1717 ± 0.0867(1.17)	0.4241 ± 0.1223 (0.87)
+1.25 μmol/L lapatinib	0.1311 ± 0.0476 (0.89)	0.3827 ± 0.0986 (0.79)
+2.5 μmol/L lapatinib	0.1500 ± 0.0455 (1.02)	0.4438 ± 0.1136 (0.91)
	NIH3T3	NIH3T3/MRP4-2
6-MP	0.1172 ± 0.0581 (1.00)	34.6526 ± 14.9254 (1.00)
+0.625 μmol/L lapatinib	0.1247 ± 0.0741 (0.92)	33.0321 ± 8.6354 (1.01)
+1.25 μmol/L lapatinib	0.1035 ± 0.0712 (1.10)	28.3643 ± 9.6586 (1.22)
+2.5 μmol/L lapatinib	0.0982 ± 0.0685 (1.23)	29.7465 ± 9.6895 (1.21)
	SW1573	SW1573/2R120 (LRP)
Doxorubicin	0.0983 ± 0.0643 (1.00)	1.5734 ± 0.6325 (1.00)
+0.625 μmol/L lapatinib	0.1002 ± 0.0426 (1.02)	1.5134 ± 0.3728 (1.01)
+1.25 μmol/L lapatinib	0.0856 ± 0.0432 (1.10)	1.5736 ± 0.4222 (1.02)
+2.5 μmol/L lapatinib	0.0863 ± 0.0265 (1.10)	1.4738 ± 0.4488 (1.11)

Cell survival was determined by MTT assays as described in Materials and Methods. Data are the means ± SD of at least three independent experiments. The fold-reversal of MDR was calculated by dividing the IC_{50} for cells with the anticancer drugs in the absence of lapatinib by that obtained in the presence of lapatinib. **$P < 0.01$ for values versus that obtained in the absence of lapatinib.

tions were as follows: 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, for 35 cycles, and then 72°C for 10 min for final extensions. Products were resolved and examined by 1% agarose gel electrophoresis. Q-PCR was performed by Bio-Rad CFX96™ Real-Time (Bio-Rad). The gene encoding GAPDH was used as an internal control. For Q-PCR analysis, the forward primer for *MRP1* was 5′-CCATC CACGACCCTAATCCC-3′; the reverse primer was 5′-ACTTGTTCCGACGTG TCCTC-3′. The forward primer for *GAPDH* was 5′-GAGTCAACGGATTTG GTCGT-3′; the reverse primer was 5′-GATCTCGCTCCTGGAAGATG-3′. The reaction was under the following conditions: 50°C for 2 min, 95°C for 5 min and 40 cycles at 95°C for 15 s, 60°C for 30 s. Relative quantification of *MRP1* was performed using the $2^{-\Delta\Delta Ct}$ method (37). All experiments were repeated three times.

Statistical Analysis

All experiments were repeated at least three times, and the results were depicted as mean values ± standard deviation (SD). The statistical software SPSS16.0 was used in data processing and analyzing. The significant differences of the data were calculated by using the Student *t* test. $P < 0.05$ and $P < 0.01$ were considered significant.

RESULTS

Cytotoxicity Effect of Lapatinib on Sensitive and Resistant Cells

Lapatinib structure and ABC transporter expression levels in various cells are shown in Figures 1A and B. The cytotoxicity of lapatinib in different cells was determined by MTT assay. C-A120, HEK293/MRP2, NIH3T3/MRP4-2 and SW1573/2R120 are drug resistance models with overexpression of MRP1, MRP2, MRP4 and LRP, respectively. MTT assay showed that lapatinib produced the same cytotoxic effects between the parental and resistant cells. The IC_{50} values were 8.54 ± 1.332, 8.28 ± 1.265, 11.07 ± 1.823, 10.76 ± 1.596, 15.75 ± 2.512, 18.31 ± 2.976, 15.98 ± 1.156 and 20.21 ± 2.023 μmol/L for KB-3-1, C-A120, HEK293/pcDNA3.1, HEK293/MRP2, NIH3T3, NIH3T3/MRP4-2, SW1573 and SW1573/2R120, respectively. More than 90% of the cells survived at the concentration of 2.5 μmol/L lapatinib (Figures 1C–F). On the basis of these results, 2.5 μmol/L was chosen as a maximum concentration for combination treatment with known MRP1, MRP2, MRP4 and LRP substrate antineoplastic drugs.

Lapatinib Sensitized MRP1-Overexpressing Cells to Substrate Chemotherapeutic Agents

The IC_{50} values of the substrate chemotherapeutic agents in the parental and MDR cells in the presence or absence of lapatinib are shown in Table 1. As we know, doxorubicin and vincristine are substrates of MRP1; cisplatin is not a substrate of MRP1 and was used as a nega-

tive control (5). VP-16, 6-MP and doxorubicin are the substrates of MRP2, MRP4 and LRP, respectively (6,19–21). MK571, a positive MRP1 inhibitor, was used as a positive control. Lapatinib at 2.5 μmol/L could significantly sensitize MRP1 overexpressing C-A120 cells to its substrate agents such as doxorubicin and vincristine, but not in a non-MRP1 substrate agent such as cisplatin. However, lapatinib did not significantly alter the sensitivity of the drug-sensitive cells KB-3-1 to the antineoplastic drugs used in this study. Furthermore, lapatinib did not enhance the efficacy of chemotherapeutic agents in MRP2-, MRP4-, and LRP-overexpressing MDR cells.

Lapatinib Enhanced the Efficacy of Conventional Chemotherapeutic Agents *In Vivo*

The MRP1-overexpressing C-A120 cell xenograft model in nude mice was established to examine whether lapatinib could enhance the efficacy of vincristine *in vivo*. No significant difference was found in tumor size between groups treated with saline and lapatinib. However, a significant inhibition of tumor growth was observed in the group with a combination of lapatinib and vincristine compared with other groups ($P < 0.05$; Figure 2). Furthermore, we did not observe any death or obvious body weight loss in a combination group, which indicated that the combination regimen developed no additional toxicity.

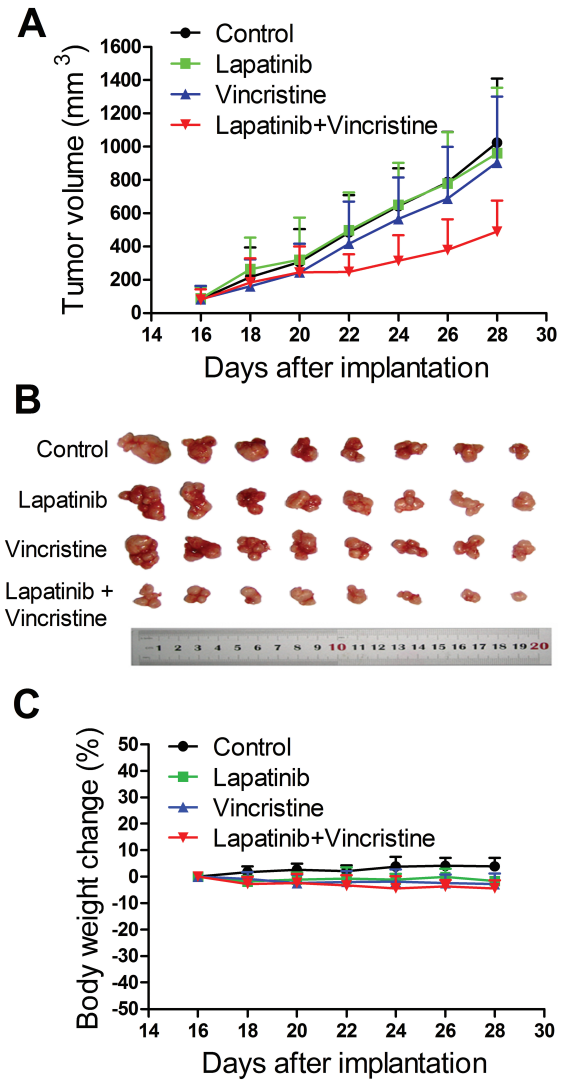

Figure 2. Potentiation of the antitumor effects of vincristine by lapatinib in a C-A120 cell xenograft model in nude mice. (A) Changes in tumor volume with time after tumor cell implantation. Data shown are means ± SD for each group of eight mice after implantation. (B) The photograph of tumor size was taken on the 28th day after implantation. (C) Average percentage of body weight change after treatments. Data shown are means ± SD for each group of eight mice after implantation. The various treatments were as follows: control (vehicle alone); vincristine (q2d × 6, IP, 0.2 mg/kg); lapatinib (q2d × 6, PO, 100 mg/kg); and vincristine (q2d × 6, IP, 0.2 mg/kg) plus lapatinib (q2d × 6, PO, 100 mg/kg, given an hour before vincristine administration).

Lapatinib Enhanced the Accumulation of Doxorubicin and Rhodamine 123 in C-A120 Cells

The above results indicated that lapatinib could enhance the sensitivity of MRP1-overexpressing cells to substrate chemotherapeutic agents. To ascertain the potential mechanisms of reversal MDR by lapatinib, we measured the effect of lapatinib on the accumulation of doxorubicin and rhodamine 123 in KB-3-1 and C-A120 cells. The fluorescence of doxorubicin or rhodamine 123 was significantly higher in KB-3-1 cells than C-A120 cells. Our results showed that the intracellular accumulation of doxorubicin and rhodamine 123 in C-A120 cells was significantly higher compared with that in KB-3-1 cells after treating with lapatinib (Figures 3A, B). In the presence of 0.625, 1.25 and 2.5 μmol/L lapatinib, the fluorescence index of doxorubicin was increased by 2.29-, 3.24- and 3.80-fold and the fluorescence index of rhodamine 123 was increased by 1.95-, 3.37- and 9.02-fold in C-A120 cells, respectively (Figures 3C, D). These results suggested that lapatinib could increase intracellular accumulation of chemotherapeutic agents in MRP1-overexpressing cells.

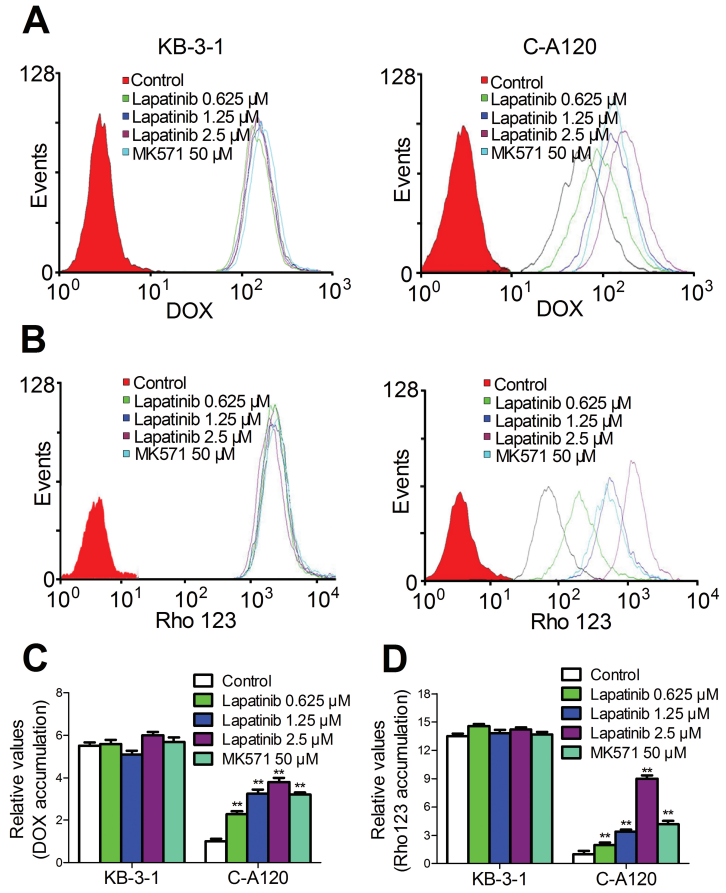

Figure 3. Effect of lapatinib on the intracellular accumulation of doxorubicin and rhodamine 123. The accumulations of doxorubicin (A, C) and rhodamine 123 (B, D) were measured by flow cytometric analysis as described in Materials and Methods. The results are presented as fold-change in fluorescence intensity relative to control MDR cells. Columns, means of triplicate determinations; bars, SDs. *$P < 0.05$, **$P < 0.01$, significantly different from the control group.

Lapatinib Did Not Alter the Expression Level of MRP1

MRP1-mediated MDR could be reversed either by decreasing the protein expression of MRP1 or inhibiting its transport function. We determined the effect of lapatinib on the expression of mRNA levels by using Q-PCR and on protein levels by using Western blot. We incubated C-A120 cells with lapatinib in different concentrations for different times. As shown in Figure 4, lapatinib could not significantly alter the protein levels in C-A120 cells after 24, 48 and 72 h of treatment. Q-PCR showed that the mRNA levels of *MRP1* were not altered after lapatinib treatment. These results indicate that lapatinib antagonized MRP1-mediated MDR by inhibiting its transport function instead of decreasing the expression of MRP1.

Lapatinib Had No Effect on the Blockage of AKT and ERK1/2 Phosphorylation at the Concentration of Reversal MDR

Previous studies have shown that inhibiting AKT and ERK1/2 pathways could block the resistance to antineoplastic drugs in cancer cells (38,39). To determine whether the enhancement effect of lapatinib was related to the phosphorylation change of AKT and ERK1/2 in MRP1-mediated MDR cells, we tested the phosphorylation of AKT and ERK1/2 with various concentrations of lapatinib. As shown in Figure 5, lapatinib (up to 2.5 μmol/L) did not significantly alter the total and phosphorylated forms of AKT or ERK1/2. These results suggested that the enhancement effect of lapatinib on C-A120 cells is independent of the inhibition of AKT and ERK1/2 phosphorylation. Overall, the effect of lapatinib on MRP1-overexpressing cells is mainly through inhibiting its function, but does not involve the change of protein level of MRP1 and the blockage of the AKT and ERK1/2 pathways.

DISCUSSION

Overexpression of ABC transporters is a major obstacle for successful chemotherapy. ABCB1, MRP1 and ABCG2 have been found to be overexpressed in many chemotherapy-resistant tumors such as colon, liver and kidney cancers (40,41). The three proteins transferred chemotherapeutic agents out of cells to protect them from damage. Except the hydrophobic compounds, ABC transporters are also capable to extrude a variety of amphipathic anions and cations. ABCB1 preferentially extrudes large hydrophobic molecules, whereas MRP1 and ABCG2 can transport both hydrophobic drugs and large anionic compounds, for example, drug conjugates (42).

Tyrosine kinases are enzymes that catalyze the transfer of the γ-phosphate of the ATP to the tyrosine hydroxyl groups on target proteins (43). Strict control of the tyrosine kinase activity in the cell regulates important processes such as cell cycle, proliferation and apoptosis (44,45). Some small molecule inhibitors competing with ATP-binding at the tyrosine kinase domain have emerged from oncology clinical trials into mainstream cancer treatment. Several reports have showed that TKIs have an important relationship with ABC transporters, such as ABCG2, ABCB1 and MRP1. For example, EKI-785 and canertinib have been demonstrated to interact with MRP1 and ABCG2, respectively (46,47). Other TKIs including gefitinib, erlotinib and vande-

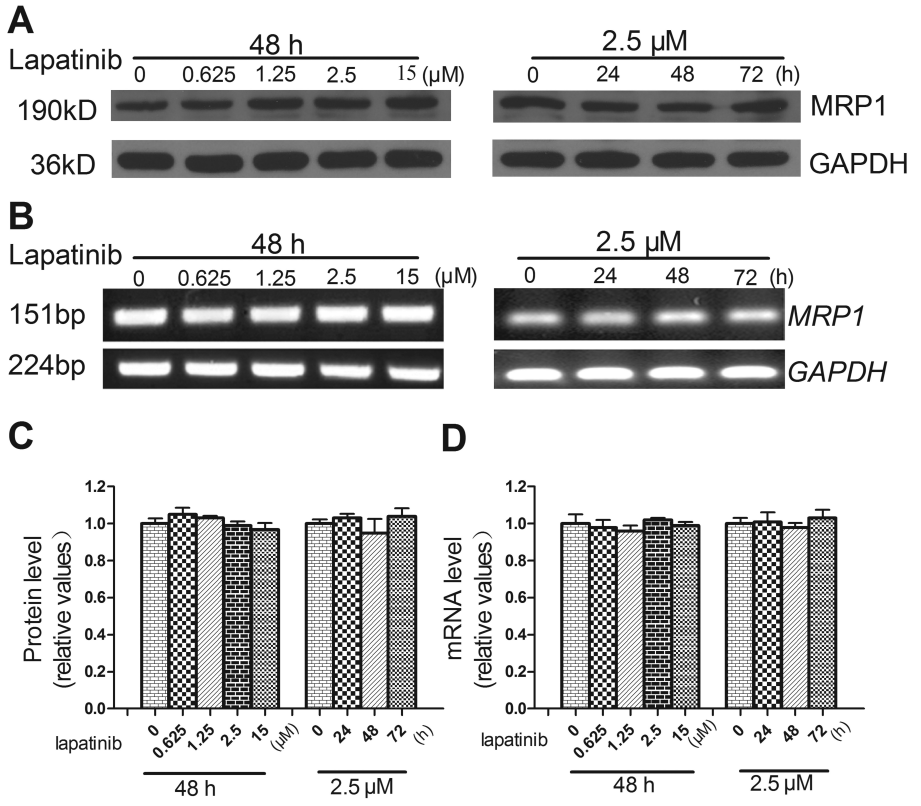

Figure 4. Effect of lapatinib on the expression of MRP1 in C-A120 cells. The protein level of MRP1 was measured by Western blot, and mRNA level was measured by RT-PCR and Q-PCR. (A, B) Lapatinib did not alter the protein levels or mRNA levels in C-A120 cells. (C) Q-PCR was further applied to confirm unchangeable mRNA levels in C-A120 cells. (D) Grayscale ratios of MRP1/GAPDH were analyzed with ImageJ. The grayscale ratios were proportional to the MRP1 protein levels. All these experiments were repeated at least three times, and a representative experiment is shown in each panel.

tanib have shown the ability to antagonize the function of ABCB1 and ABCG2 (36,48,49). Nilotinib is a selective inhibitor of the tyrosine kinase activities of Bcr-Abl and can potentiate the sensitivity of established ABCB1 and ABCG2 substrates. However, nilotinib could not reverse MRP1- or MRP4-mediated MDR (50). Recently, ponatinib, a multitargeted TKI, was reported to potentiate the cytotoxicity of widely used therapeutic substrates of MRP7 (51).

Lapatinib, an inhibitor EGFR and HER-2 receptors, has been used in combination with capecitabine for treatment of advanced or metastatic breast cancer overexpressing HER2. Our previous findings showed lapatinib could inhibit the function of ABCB1, ABCG2 and MRP10 (26,27). To demonstrate the interaction of lapatinib and other MDR proteins, the cell lines C-A120, HEK293/MRP2 and NIH3T3/MRP4-2 as well as SW1573/2R120 were used in our research. We found that lapatinib could sensitize MRP1 overexpressing cells to chemotherapeutic agents, but showed no effect on MRP2-, MRP4-, and LRP-mediated drug resistance (Table 1). These findings suggest that the lapatinib is specific to inhibit the function of ABCB1, ABCG2, MRP10 and MRP1. In the present study, we showed that lapatinib significantly increased the accumulation of doxorubicin and rhodamine 123 in MRP1-overexpressing cells in concentration-dependent manners (Figure 3). The results of flow cytometry studies were consistent with the previous cytotoxic results, suggesting that lapatinib sensitizes the MRP1-mediated MDR cells to anticancer drugs. However, lapatinib did not significantly sensitize the parental sensitive cells to the anticancer agents (Figure 3). Furthermore, lapatinib did not significantly alter the sensitivity of non-MRP1 substrates such as cisplatin in sensitive KB-3-1 cells and resistant C-A120 cells. These findings suggest that lapatinib selectively antagonizes MRP1-mediated MDR in a concentration-dependent manner. In addition, lapatinib could enhance the antitumor activity of vincristine without causing additional toxicity in athymic nude mice bearing the C-A120 xenografts (Figure 2). However, our results showed that lapatinib did not alter the mRNA and protein expressions of MRP1 (Figure 4). Thus, we proposed that the effect of lapatinib on sensitization of MRP1-overexpressing cells to chemotherapeutic agents is due to the inhibition of MRP1 function.

Therefore, the expression levels of ABCB1, ABCG2 and MRP1 would significantly determine the antitumor efficiency of lapatinib in clinics. The *in vivo* experiments could provide suitable concentrations that would better improve the chemotherapeutic effect of the combined use of lapatinib with anticancer drugs. Besides, our data provided a new therapy strategy for treatments of patients overexpressing MRP1. In addition, lapatinib is metabolized by human liver P450s to form *O*- and *N*-dealkylated metabolites (52). Some reports showed that lapatinib was an irreversible inhibitor of CYP3A4 and formed a metabolic intermediate (MI) complex with the latter, and the inhibition of CYP3A4 is a major cause of drug-to-drug interactions (53,54). Consequently, lapatinib could affect the metabolism and elimination of anticancer drugs when these drugs are used in combination.

Previous reports demonstrated that continued activation of AKT was related to the resistance to conventional chemotherapeutic agents (55–58). To determine whether the MDR reversal activity of lapatinib was related to the change of AKT

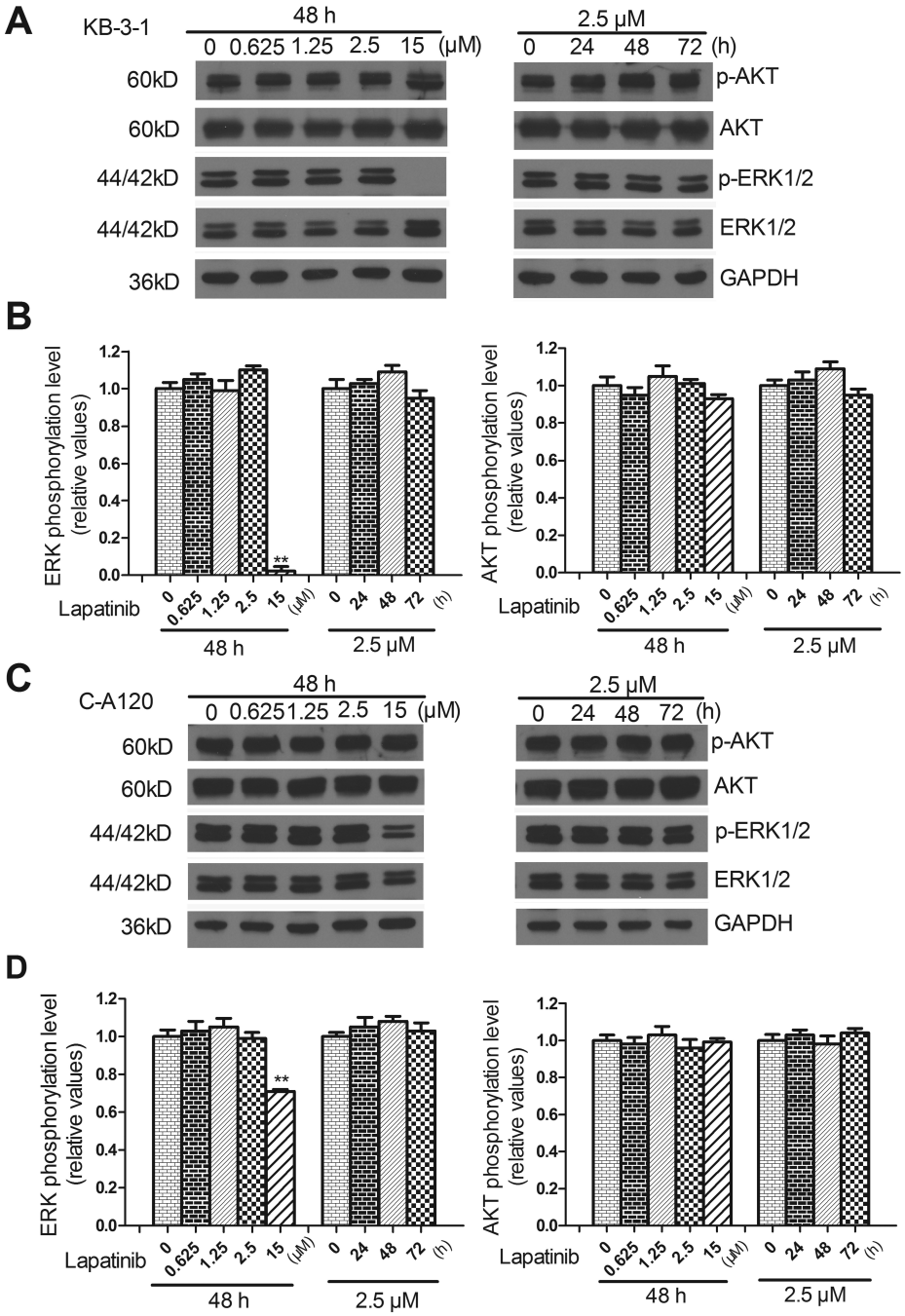

Figure 5. Effect of lapatinib on blockage of AKT and ERK1/2 phosphorylation. KB-3-1 and C-A120 cells were treated with lapatinib at various concentrations for 48 h (A, C). Lapatinib of 15 μmol/L was used as a positive control for blockage of AKT and ERK1/2 phosphorylation. Grayscale ratios of p-ERK/ERK and p-AKT/AKT were analyzed with ImageJ. The grayscale ratios were proportional to the AKT and ERK1/2 phosphorylation levels (B, KB-3-1 cells; D, C-A120 cells). Equal amounts of protein were loaded for Western blot analysis, as described in Materials and Methods. Independent experiments were performed at least three times and results from a representative experiment were shown. $^{**}P < 0.01$ significantly different from control group.

and ERK1/2, we examined the effect of lapatinib on AKT activation. Our data showed that lapatinib (up to 15 μmol/L) could significantly alter the phosphorylation of ERK1/2 without altering the phosphorylation of AKT. However, at the reversal concentration, lapatinib (up to 2.5 μmol/L) did not significantly alter the phosphorylation of AKT and ERK1/2 in MRP1-overexpressing cells. The result revealed that the phosphorylation of AKT and ERK1/2 was not involved in the reversal of MRP1-mediated MDR by lapatinib (Figure 5).

In conclusion, lapatinib enhanced the efficacy of conventional chemotherapeutic agents in MRP1-overexpressing cells by inhibiting MRP1 function at clinically relevant concentrations, but had no enhancement effect on MRP2-, MRP4- and LRP-mediated drug resistance. In addition, the enhancement effect of lapatinib was independent of the blockage of AKT and ERK1/2 phosphorylation. These findings will encourage further study on the combinational therapy of lapatinib with conventional chemotherapeutical drug in MRP1-overexpressing cancer patients.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molec-*

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REFERENCES

1. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. (2006) Targeting multidrug resistance in cancer. *Nat. Rev. Drug. Discov.* 5:219–34.
2. Leonard GD, Fojo T, Bates SE. (2003) The role of ABC transporters in clinical practice. *Oncologist.* 8:411–24.
3. Ozben T. (2006) Mechanisms and strategies to overcome multiple drug resistance in cancer. *FEBS Lett.* 580:2903–9.
4. Perez-Tomas R. (2006) Multidrug resistance: retrospect and prospects in anti-cancer drug treatment. *Curr. Med. Chem.* 13:1859–76.
5. Chen ZS, Tiwari AK. (2011) Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. *FEBS J.* 278:3226–45.
6. Fukuda Y, Schuetz JD. (2012) ABC transporters and their role in nucleoside and nucleotide drug resistance. *Biochem. Pharmacol.* 83:1073–83.
7. Borst P, Evers R, Kool M, Wijnholds J. (2000) A family of drug transporters: the multidrug resistance-associated proteins. *J. Natl. Cancer Inst.* 92:1295–302.
8. Cole SP, Deeley RG. (2006) Transport of glutathione and glutathione conjugates by MRP1. *Trends Pharmacol. Sci.* 27:438–46.
9. Rea PA. (2007) Plant ATP-binding cassette transporters. *Annu. Rev. Plant Biol.* 58:347–75.
10. Cole SP, *et al.* (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science.* 258:1650–4.
11. Kubota T, *et al.* (2001) Resistant mechanisms of anthracyclines: pirarubicin might partly break through the P-glycoprotein-mediated drug-resistance of human breast cancer tissues. *Breast Cancer.* 8:333–8.
12. Nooter K, *et al.* (1995) Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. *Clin. Cancer Res.* 1:1301–10.
13. Flens MJ, *et al.* (1996) Tissue distribution of the multidrug resistance protein. *Am. J. Pathol.* 148:1237–47.
14. Leslie EM, Deeley RG, Cole SP. (2001) Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. *Toxicology.* 167:3–23.
15. Aller SG, *et al.* (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science.* 323:1718–22.
16. Borst P, Elferink RO. (2002) Mammalian ABC transporters in health and disease. *Annu. Rev. Biochem.* 71:537–92.
17. Deeley RG, Westlake C, Cole SP. (2006) Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiol. Rev.* 86:849–99.
18. Homolya L, Varadi A, Sarkadi B. (2003) Multidrug resistance-associated proteins: export pumps for conjugates with glutathione, glucuronate or sulfate. *Biofactors.* 17:103–14.
19. Narasaki F, *et al.* (1997) Human canalicular multispecific organic anion transporter (cMOAT) is expressed in human lung, gastric, and colorectal cancer cells. *Biochem. Biophys. Res. Commun.* 240:606–11.
20. Koike K, *et al.* (1997) A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. *Cancer Res.* 57:5475–9.
21. Laurencot CM, Scheffer GL, Scheper RJ, Shoemaker RH. (1997) Increased LRP mRNA expression is associated with the MDR phenotype in intrinsically resistant human cancer cell lines. *Int. J. Cancer.* 72:1021–6.
22. Dalton WS, Scheper RJ. (1999) Lung resistance-related protein: determining its role in multidrug resistance. *J. Natl. Cancer Inst.* 91:1604–5.
23. Izquierdo MA, *et al.* (1995) Drug resistance-associated marker Lrp for prediction of response to chemotherapy and prognoses in advanced ovarian carcinoma. *J. Natl. Cancer Inst.* 87:1230–7.
24. Volm M, Stammler G, Zintl F, Koomagi R, Sauerbrey A. (1997) Expression of lung resistance-related protein (LRP) in initial and relapsed childhood acute lymphoblastic leukemia. *Anticancer Drugs.* 8:662–5.
25. Jagiello-Gruszfeld A, *et al.* (2010) A single-arm phase II trial of first-line paclitaxel in combination with lapatinib in HER2-overexpressing metastatic breast cancer. *Oncology.* 79:129–35.
26. Dai CL, *et al.* (2008) 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.* 68:7905–14.
27. Kuang YH, *et al.* (2010) Lapatinib and erlotinib are potent reversal agents for MRP7 (ABCC10)-mediated multidrug resistance. *Biochem. Pharmacol.* 79:154–61.
28. Sumizawa T, *et al.* (1994) Non-P-glycoprotein-mediated multidrug-resistant human KB cells selected in medium containing adriamycin, cepharanthine, and mezerein. *Somat. Cell Mol. Genet.* 20:423–35.
29. Cui Y, *et al.* (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol. Pharmacol.* 55:929–37.
30. Lee K, Klein-Szanto AJ, Kruh GD. (2000) Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. *J. Natl. Cancer Inst.* 92:1934–40.
31. Chen LM, *et al.* (2004) Screening novel, potent multidrug-resistant modulators from imidazole derivatives. *Oncol. Res.* 14:355–62.
32. Shi Z, *et al.* (2006) Reversal of MDR1/P-glycoprotein-mediated multidrug resistance by vector-based RNA interference in vitro and in vivo. *Cancer Biol. Ther.* 5:39–47.
33. Mi YJ, *et al.* (2010) Apatinib (YN968D1) reverses multidrug resistance by inhibiting the efflux function of multiple ATP-binding cassette transporters. *Cancer Res.* 70:7981–91.
34. Chen LM, *et al.* (2004) Reversal of P-gp mediated multidrug resistance in-vitro and in-vivo by FG020318. *J. Pharm. Pharmacol.* 56:1061–6.
35. Fu L, *et al.* (2004) Characterization of tetrandrine, a potent inhibitor of P-glycoprotein-mediated multidrug resistance. *Cancer Chemother. Pharmacol.* 53:349–56.
36. Zheng LS, *et al.* (2009) Vandetanib (Zactima, ZD6474) antagonizes ABCC1- and ABCG2-mediated multidrug resistance by inhibition of their transport function. *PLoS One.* 4:e5172.
37. Livak KJ, Schmittgen TD. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402–8.
38. Gagnon V, Van Themsche C, Turner S, Leblanc V, Asselin E. (2008) Akt and XIAP regulate the sensitivity of human uterine cancer cells to cisplatin, doxorubicin and taxol. *Apoptosis.* 13:259–71.
39. Oh SY, *et al.* (2006) ERK activation by thymosin-beta-4 (TB4) overexpression induces paclitaxel-resistance. *Exp. Cell Res.* 312:1651–7.
40. Goldstein LJ, *et al.* (1989) Expression of a multidrug resistance gene in human cancers. *J. Natl. Cancer Inst.* 81:116–24.
41. Diestra JE, *et al.* (2002) Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. *J. Pathol.* 198:213–9.
42. Sarkadi B, Homolya L, Szakacs G, Varadi A. (2006) Human multidrug resistance ABCB and ABCG transporters: participation in a chemoimmunity defense system. *Physiol. Rev.* 86:1179–236.
43. Hanks SK, Quinn AM, Hunter T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science.* 241:42–52.
44. Radha V, Nambirajan S, Swarup G. (1996) Association of Lyn tyrosine kinase with the nuclear matrix and cell-cycle-dependent changes in matrix-associated tyrosine kinase activity. *Eur. J. Biochem.* 236:352–9.
45. Ruetten H, Thiemermann C. (1997) Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat: a possible role for protein tyrosine kinase. *Br. J. Pharmacol.* 122:59–70.
46. Hegedus T, *et al.* (2002) Interaction of tyrosine kinase inhibitors with the human multidrug transporter proteins, MDR1 and MRP1. *Biochim. Biophys. Acta.* 1587:318–25.
47. Erlichman C, *et al.* (2001) 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.* 61:739–48.
48. Kitazaki T, *et al.* (2005) Gefitinib, an EGFR tyrosine kinase inhibitor, directly inhibits the function of P-glycoprotein in multidrug resistant cancer cells. *Lung Cancer.* 49:337–43.

49. Shi Z, *et al.* (2007) 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.* 67:11012–20.
50. Wang F, *et al.* (2014) Nilotinib enhances the efficacy of conventional chemotherapeutic drugs in CD34(+)CD38(-) stem cells and ABC transporter overexpressing leukemia cells. *Molecules.* 19:3356–75.
51. Sun YL, *et al.* (2014) Ponatinib enhances anticancer drug sensitivity in MRP7-overexpressing cells. *Oncol Rep.* 31:1605–12.
52. Castellino S, *et al.* (2012) Human metabolism of lapatinib, a dual kinase inhibitor: implications for hepatotoxicity. *Drug Metab. Dispos.* 40:139–50.
53. Takakusa H, *et al.* (2011) Metabolic intermediate complex formation of human cytochrome P450 3A4 by lapatinib. *Drug Metab. Dispos.* 39:1022–30.
54. Nettleton DO, Einolf HJ. (2011) Assessment of cytochrome p450 enzyme inhibition and inactivation in drug discovery and development. *Curr. Top. Med. Chem.* 11:382–403.
55. Normanno N, *et al.* (2006) Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene.* 366:2–16.
56. Grant S, Qiao L, Dent P. (2002) Roles of ERBB family receptor tyrosine kinases, and downstream signaling pathways, in the control of cell growth and survival. *Front Biosci.* 7:d376–89.
57. Knuefermann C, *et al.* (2003) HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. *Oncogene.* 22:3205–12.
58. Li QQ, *et al.* (2007) Involvement of CD147 in regulation of multidrug resistance to P-gp substrate drugs and in vitro invasion in breast cancer cells. *Cancer Sci.* 98:1064–9.