Inhibiting the Efflux Function of Multiple ATP-Binding Cassette Transporters

Apatinib (YN968D1) Reverses Multidrug Resistance by

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Abstract

Apatinib, a small-molecule multitargeted tyrosine kinase inhibitor, is in phase III clinical trial for the treatment of patients with non–small-cell lung cancer and gastric cancer in China. In this study, we determined the effect of apatinib on the interaction of specific antineoplastic compounds with P-glycoprotein (ABCB1), multidrug resistance protein 1 (MRP1, ABCC1), and breast cancer resistance protein (BCRP, ABCG2). Our results showed that apatinib significantly enhanced the cytotoxicity of ABCB1 or ABCG2 substrate drugs in KBv200, MCF-7/adr, and HEK293/ABC1 cells overexpressing ABCB1 and in S1-M1-80, MCF-7/FLV1000, and HEK293/ABCG2-R2 cells overexpressing ABCG2 (wild-type). In contrast, apatinib did not alter the cytotoxicity of specific substrates in the parental cells and cells overexpressing ABC1. Apatinib significantly increased the intracellular accumulation of rhodamine 123 and doxorubicin in the multidrug resistance (MDR) cells. Furthermore, apatinib significantly inhibited the photoaffinity labeling of both ABCB1 and ABCG2 with [125I]-idoarylazidoprazosin in a concentration-dependent manner. The ATPase activity of both ABCB1 and ABCG2 was significantly increased by apatinib. However, apatinib, at a concentration that produced a reversal of MDR, did not significantly alter the ABCB1 or ABCG2 protein or mRNA expression levels or the phosphorylation of AKT or ERK1/2. Importantly, apatinib significantly enhanced the effect of paclitaxel against the ABCB1-resistant KBv200 cancer cell xenografts in nude mice. In conclusion, apatinib reverses ABCB1- and ABCG2-mediated MDR by inhibiting their transport function, but not by blocking the AKT or ERK1/2 pathway or downregulating ABCB1 or ABCG2 expression. Apatinib may be useful in circumventing MDR to other conventional antineoplastic drugs. Cancer Res; 70(20); OF1–11. ©2010 AACR.

Introduction

Multidrug resistance (MDR) in cancer cells produces resistance to the cytotoxic effects of numerous antineoplastic drugs that are structurally and mechanistically unrelated, and this significantly decreases the efficacy of cancer chemotherapy (1). The most common cause of MDR results from the overexpression of cell membrane–bound ATP-binding cassette (ABC) transporters, which actively extrude a variety of chemotherapeutic drugs out of the cancer cells, thereby attenuating their cytotoxic actions (2). Forty-eight ABC proteins have been identified in the human genome and are divided into seven subfamilies (A–G) based on sequence similarities (3). The ABC transporter subfamily B member 1 (ABCB1/MDR1/P-glycoprotein), subfamily C member 1 (ABCC1/MRP1), and subfamily G member 2 (ABCG2/BCRP) play a major role in producing MDR in tumor cells (4).

ABCB1 was first discovered in drug-resistant Chinese hamster ovarian cells (5). It can transport a wide range of antineoplastic drugs such as the anthracyclines, Vinca alkaloids, taxanes, and epipodophyllotoxins (5). ABCG2 was identified independently from human colon carcinoma cells (S1-M1-80; ref. 6), the placenta (7), and a drug-selected human breast cancer cell line, MCF-7 (8). ABCG2 can actively efflux a wide variety of antineoplastic drugs including mitoxantrone, indolocarbazole, topoisomerase I inhibitors and anthracyclines, as well as fluorescent dyes such as Hoechst 33342 (9). The side population (SP) phenotype cells are present in diverse tumor types and they overexpress ABCG2, producing inherent drug resistance (10, 11). Currently, ABCG2 is considered a molecular marker for the SP cells (12). Thus, targeting ABCG2 in these tumor stem cells represents a

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of cancer Research.
promising and novel strategy to eradicate the entire cancer cell population.

Tyrosine kinase inhibitors (TKIs), a relatively new class of antineoplastic drugs, are believed to exert their mechanism of action by competing with ATP for binding to the ATP site of the catalytic domain of several oncogenic tyrosine kinases. Subsequently, the TKIs can attenuate downstream signaling pathways involved in cancer proliferation, invasion, metastasis, and angiogenesis. Previously, it has been reported that the BCR-Abl TKIs imatinib (Gleevec) and nilotinib (Tasigna) interact with ABCB1 and ABCG2 transporters and significantly inhibit their transport activity (13, 14). In addition, epidermal growth factor receptor (EGFR) TKIs [e.g., lapatinib (15), gefitinib (16), and erlotinib (17)], vascular endothelial growth factor receptor (VEGFR) TKIs [e.g., cediranib (18) and vandetanib (19)], and the multi-kinase TKI sunitinib (20) have been shown to significantly attenuate or reverse ABC transporter–mediated MDR in cancer cells. Thus, it is possible that TKIs could be used as MDR inhibitors. Apatinib (YN968D1) is a small-molecule TKI that inhibits VEGFR-2 (Flk-1/KDR), RET (rearranged during transfection), c-Kit (stem cell factor receptor), and c-Src tyrosine kinases. Apatinib has been used in a phase III clinical trial in China (stem cell factor receptor), and c-Src tyrosine kinases. Apatinib (YN968D1) is a small-molecule TKI that inhibits VEGFR-2 (Flk-1/KDR), RET (rearranged during transfection), c-Kit (stem cell factor receptor), and c-Src tyrosine kinases. Apatinib has been used in a phase III clinical trial in China to determine its efficacy in treating gastric carcinoma and non–small-cell lung cancer. Currently, no studies have examined the effect of apatinib in cell lines or animal models that overexpress ABCB1 or ABCG2 transporters. Therefore, in this study, we conducted experiments to determine if apatinib can potentiate the efficacy of conventional antineoplastic drugs through interaction with ABC transporters in MDR cancer cells.

**Materials and Methods**

**Reagents**

Apatinib was obtained from Jiangsu Hengrui Medicine Co., with a molecular structure as shown in Supplementary Fig. S1A. Monoclonal antibodies against ABCB1 (sc-55510) and ABCC1 (sc-18835) were from Santa Cruz Biotechnology. ABCG2 antibody (MAB4146) was obtained from Chemicon International, Inc. AKT antibody (#4685) was from Cell Signaling Technology, Inc. Monoclonal antibodies C-219 (against ABCB1) and BXP-34 (against ABCG2) were acquired from Signet Laboratories, Inc. Phosphorylated AKT (KC-5A04), phosphorylated extracellular signal–regulated kinase 1/2 (P-ERK1/2; KC-5E04), mitogen-activated protein kinase 1/2 (ERK1/2; KC-5E01), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Kangchen Co. N-iodoarylazidoprazosin (IAAP; 2,200 Ci/mmol) was obtained from Perkin-Elmer Life Sciences. DMEM and RPMI 1640 were from Life Technologies, Inc. Rhodamine 123 (Rho 123), MTT, paclitaxel, doxorubicin (DOX), vincristine, verapamil, topotecan, and other chemicals were purchased from Sigma Chemical Co.

**Cell lines**

The following cell lines were cultured in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO₂ the human breast carcinoma cell line MCF-7, its DOX-selected ABCB1-overexpressing derivative MCF-7/adr (21), and flavopiridol-resistant ABCG2–overexpressing MCF-7/FLV1000 sublines (22) were kindly provided by Dr. S.E. Bates (National Cancer Institute, NIH, Bethesda, MD). The human oral epidermoid carcinoma cell line KB and its vincristine-selected ABCB1-overexpressing derivative KBv200 were a gift from Dr. Xu-Yi Liu (Cancer Hospital of Beijing, Beijing, China; ref. 23). KB-3-1 and KB/ ABC1 transfectant cells were kindly provided by Dr. S. Akiyama (Kagoshima University, Kagoshima, Japan; ref. 24). The human colon carcinoma cell line S1 and its mitoxantrone-selected ABCG2–overexpressing derivative S1-M1-80 (25) and the human primary embryonic kidney cell line HEK293 and its pcDNA3.1, ABCB1, ABCG2, and ABCC1 stable gene-transfected cell lines HEK293/pcDNA3.1, HEK293/ABC1 (26), HEK293/ ABCG2-R2 (27), and HEK293/ABCC1 (28) 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 (except HEK293/ABCC1 cell line, which was cultured with 800 μg/mL G418; ref. 27). All resistant cells were authenticated by comparing their fold resistance with that of the parental drug-sensitive cells and examining the expression levels of ABC transporters. All cells were grown in drug-free culture medium for >2 weeks before assay.

**Animals**

Athymic nude mice (BALB/c-nu/nu), 5 to 6 weeks old and weighing 18 to 24 g, were obtained from the Center of Experimental Animals, Sun Yat-Sen University (China), and used for the KBv200 cell xenografts. All animals received sterilized food and water. All experiments were carried out in accordance with the guidelines on animal care and experiments of laboratory animals (Center of Experimental Animals, Sun Yat-Sen University, China), which was approved by the ethics committee for animal experiments.

**Cytotoxicity test**

The MTT assay was done as previously described to assess the sensitivity of cells to drugs (29). The IC₅₀ was calculated from survival curves using the Bliss method (30). The degree of resistance was estimated by dividing the IC₅₀ for the MDR cells by that of the parental sensitive cells; and the fold-reversal factor of MDR was calculated by dividing the IC₅₀ of the anticancer drug in the absence of apatinib by that obtained in the presence of apatinib.

**Nude mouse xenograft model**

The KBv200-inoculated nude xenograft model previously established by Chen and colleagues was used in this study (31). The xenograft was found to maintain the MDR phenotype in vivo and was extremely resistant to paclitaxel treatment. Briefly, KBv200 cells grown in vitro were harvested and implanted s.c. under the shoulder in the nude mice. When the tumors reached a mean diameter of 0.5 cm, the mice were randomized into four groups and treated with various regimens: (a) saline (q3d × 4); (b) paclitaxel (18 mg/kg, i.p., q3d × 4); (c) apatinib (70 mg/kg, p.o., q3d × 4); and (d) paclitaxel (18 mg/kg,
i.p., q3d × 4) + apatinib (70 mg/kg, p.o., q3d × 4 given 1 hour before injecting paclitaxel). The body weights of the animals and the two perpendicular diameters (A and B) were recorded every 3 days, and tumor volume (V) was estimated according to the following formula (31):

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

The curve of tumor growth was drawn according to tumor volume and time of implantation. The mice were anesthetized and sacrificed when the mean tumor weight was more than 1 g in the control group. Tumor tissues were excised from the mice and their weights were measured. The ratio of growth inhibition (IR) was calculated according to the following formula (31):

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

**DOX and Rho 123 accumulation**

The effect of apatinib on the intracellular accumulation of DOX and Rho 123 was performed as previously described (21). Verapamil, an ABCB1 inhibitor, was used as a positive control for KB, KBv200, MCF-7, and MCF-7/adr cells, and fumitremorgin C was used as a positive control for ABCG2 in S1 and S1-M1-80 cells (32, 33).

**In vitro transport assays**

DOX was added to the medium to obtain final concentrations of 2.5 to 20 μmol/L in the absence or presence of apatinib, and cells were incubated at 37°C for 3 hours. The cells were collected, centrifuged, washed once with cold PBS, and resuspended in medium with free DOX in the absence or presence of apatinib. Subsequently, cells were incubated for 5 minutes at 37°C, centrifuged, and washed three times with cold PBS. In the control experiments, the apical uptake reaction was kept at 0°C. Finally, the intracellular concentration of DOX was determined by flow cytometric analysis (Cytomics FC500, Beckman Coulter; ref. 34). The quantity of DOX efflux by ABC transporter was calculated by subtracting the values obtained at 37°C from those at 0°C. The inhibitory effect of apatinib was analyzed using Lineweaver-Burk plots as previously described (35).

**Reverse transcription-PCR**

ABCB1 and ABCG2 expression were assayed as described (15). Total RNA was isolated using the Trizol reagent RNA extraction kit (Molecular Research Center) and subject to reverse transcription-PCR (Promega Corp.). The PCR primers were ABCB1, 5′-ccatgcatctgcctcagc-3′ (forward) and 5′-gtaaccttgctgtctgga-3′ (reverse); ABCG2, 5′-ttggtctcgattgccagt-3′ (forward) and 5′-gggaacggtctctctcaca-3′ (reverse); and GAPDH, 5′-cctttggtatcgtggaagga-3′ (forward) and 5′-gccacgtgattcttccacaa-3′ (reverse). The products were resolved using gel electrophoresis (1.5% agarose gel).

**Western blot analysis**

Cells were lysed after washing two times with ice-cold PBS. The protein concentration was quantified using the Bradford method (36). Equal amounts of protein were resolved by SDS-PAGE and transferred onto nitrocellulose membranes; chemoluminescence was used to detect the protein.

**ATPase assay of ABCB1 and ABCG2**

The ATPase activity of vanadate (V)-sensitive ABCB1 and beryllium fluoride (BeFx)-sensitive ABCG2 in the membrane vesicles of High Five insect cells was measured as previously described (37). Apatinib was added and the cells were incubated at 37°C for the duration of the experiment. The ATPase reaction was initiated by adding 5 mmol/L Mg-ATP into a total reaction mixture of 0.1 mL. After incubation at 37°C for 20 minutes, the reactions were terminated by the addition of 0.1 mL of 5% SDS solution. The liberated inorganic phosphate (P) was measured as previously described (15, 37).

**Photoaffinity labeling of ABCB1 and ABCG2 with [125I]IAAP**

The photoaffinity labeling of ABCB1 or ABCG2 with [125I]IAAP was performed as previously described (15, 37). ABCB1 was immunoprecipitated with the C219 antibody, whereas ABCG2 was immunoprecipitated with the BXP-21 antibody (38). The samples were subjected to SDS-PAGE using a 7% Tris-acetate NuPAGE gel, which was dried and exposed to Bio-Max MR film (Eastman Kodak Co.) at −80°C for 3 to 5 hours. The radioactivity incorporated into the ABCB1 or ABCG2 band was quantified using the Storm 860 PhosphorImager system and ImageQuant (Molecular Dynamics).

**Statistical analysis**

All experiments were repeated at least three times and the differences were determined by using Student’s t test. Statistical significance was set at P < 0.05.

**Results**

Apatinib reverses MDR in cells overexpressing ABCB1 and ABCG2

The cytotoxicity of apatinib in different cell lines was determined by the MTT assay. The IC50 values were 15.18 ± 0.63, 11.95 ± 0.69, 17.16 ± 0.25, 14.54 ± 0.26, 9.30 ± 0.72, 11.91 ± 0.32, and 19.13 ± 1.13 μmol/L for KB, KBv200, MCF-7, MCF-7/adr, S1, S1-M1-80, and MCF-7/FLV1000 cells, respectively (Supplementary Fig. S1). For HEK293/pcDNA3.1, HEK/ABC1, HEK/ABCG2-R2, and HEK293/ABCC1 cells, the IC50 values of apatinib were >30 μmol/L (data not shown). Based on the cytotoxicity curves, apatinib was used at a maximum concentration of 3.0 μmol/L, a concentration at which more than 90% of the cells were viable in all cell lines used in the MDR reversal study. The IC50 values of the antineoplastic drugs in sensitive and resistant cells at different concentrations of apatinib are shown in Table 1. Apatinib produced a concentration-dependent decrease in the IC50 values of (a) DOX and paclitaxel in the KBv200 cells, DOX in MCF-7/adr cells; (b) mitoxantrone and topotecan...
Table 1. Effect of apatinib on reversing ABCB1- and ABCG2-mediated MDR in drug selected cell lines

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC_{50} ± SD (μmol/L; fold-reversal)</th>
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<tbody>
<tr>
<td></td>
<td>KB</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.029 ± 0.002 (1.00)</td>
</tr>
<tr>
<td>+0.75 μmol/L apatinib</td>
<td>0.029 ± 0.002 (1.00)</td>
</tr>
<tr>
<td>+1.5 μmol/L apatinib</td>
<td>0.030 ± 0.001 (0.98)</td>
</tr>
<tr>
<td>+3.0 μmol/L apatinib</td>
<td>0.028 ± 0.003 (1.04)</td>
</tr>
<tr>
<td>+10 μmol/L verapamil</td>
<td>0.029 ± 0.003 (1.00)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.0018 ± 0.0002 (1.00)</td>
</tr>
<tr>
<td>+0.75 μmol/L apatinib</td>
<td>0.0018 ± 0.0003 (1.00)</td>
</tr>
<tr>
<td>+1.5 μmol/L apatinib</td>
<td>0.0019 ± 0.0002 (0.95)</td>
</tr>
<tr>
<td>+3.0 μmol/L apatinib</td>
<td>0.0018 ± 0.0002 (1.00)</td>
</tr>
<tr>
<td>+10 μmol/L verapamil</td>
<td>0.0019 ± 0.0002 (0.95)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.726 ± 0.055 (1.00)</td>
</tr>
<tr>
<td>+3.0 μmol/L apatinib</td>
<td>0.714 ± 0.057 (1.01)</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.344 ± 0.037 (1.00)</td>
</tr>
<tr>
<td>+0.75 μmol/L apatinib</td>
<td>0.349 ± 0.011 (0.99)</td>
</tr>
<tr>
<td>+1.5 μmol/L apatinib</td>
<td>0.331 ± 0.019 (1.04)</td>
</tr>
<tr>
<td>+3.0 μmol/L apatinib</td>
<td>0.350 ± 0.036 (0.98)</td>
</tr>
<tr>
<td>+10 μmol/L verapamil</td>
<td>0.340 ± 0.038 (1.01)</td>
</tr>
<tr>
<td>S1</td>
<td>5.811 ± 0.533 (1.00)</td>
</tr>
<tr>
<td>+2.5 μmol/L FTC</td>
<td>5.624 ± 0.211 (1.03)</td>
</tr>
<tr>
<td>Topotecan</td>
<td>0.194 ± 0.027 (1.00)</td>
</tr>
<tr>
<td>+0.75 μmol/L apatinib</td>
<td>0.196 ± 0.041 (0.98)</td>
</tr>
<tr>
<td>+1.5 μmol/L apatinib</td>
<td>0.185 ± 0.058 (1.05)</td>
</tr>
<tr>
<td>+3.0 μmol/L apatinib</td>
<td>0.136 ± 0.067 (1.42)</td>
</tr>
<tr>
<td>+2.5 μmol/L FTC</td>
<td>0.188 ± 0.011 (1.03)</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.262 ± 0.042 (1.00)</td>
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<tr>
<td>+0.75 μmol/L apatinib</td>
<td>0.264 ± 0.022 (0.99)</td>
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<tr>
<td>+1.5 μmol/L apatinib</td>
<td>0.247 ± 0.017 (1.05)</td>
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<tr>
<td>+3.0 μmol/L apatinib</td>
<td>0.196 ± 0.055 (1.33)</td>
</tr>
<tr>
<td>+2.5 μmol/L FTC</td>
<td>0.254 ± 0.016 (1.02)</td>
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<tr>
<td>Cisplatin</td>
<td>12.811 ± 1.181 (1.00)</td>
</tr>
<tr>
<td>+3.0 μmol/L apatinib</td>
<td>12.280 ± 1.990 (1.04)</td>
</tr>
<tr>
<td>S1-M1-80 (ABCG2)</td>
<td>12.98 ± 0.455 (1.00)</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.015 ± 0.001(1.00)</td>
</tr>
<tr>
<td>+0.75 μmol/L apatinib</td>
<td>0.014 ± 0.004 (1.07)</td>
</tr>
<tr>
<td>+1.5 μmol/L apatinib</td>
<td>0.014 ± 0.003 (1.06)</td>
</tr>
<tr>
<td>+3.0 μmol/L apatinib</td>
<td>0.011 ± 0.002 (1.37)</td>
</tr>
<tr>
<td>+2.5 μmol/L FTC</td>
<td>0.015 ± 0.004 (1.03)</td>
</tr>
</tbody>
</table>

NOTE: Cell survival was determined by MTT assays as described in Materials and Methods. Data are the mean ± SD 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 in the absence of apatinib, verapamil or FTC by that obtained in the presence of apatinib, verapamil or FTC.

Abbreviation: FTC, fumitremorgin C.

*P < 0.01, versus the values obtained in the absence of apatinib, verapamil or FTC.
in S1-M1-80 cells; and (c) mitoxantrone in MCF-7/FLY1000 cells. In addition, 3 μmol/L apatinib completely reversed ABCG2-mediated resistance to mitoxantrone and SN-38 in wild-type HEK293/ABCG2-R2 cells transfected with ABCG2 (Table 2). Furthermore, 3 μmol/L apatinib significantly decreased the IC₅₀ values of mitoxantrone, vincristine, and DOX in stably transfected HEK293/ABC2B1 cells (Table 2). However, apatinib did not significantly alter the cytotoxicity of the antineoplastic drugs in the parental cells (Tables 1 and 2). Furthermore, apatinib did not significantly alter the cytotoxicity of non-ABCB1 or non-ABCG2 substrates (cisplatin) in either MDR cells or their parental sensitive cells (Tables 1 and 2). Apatinib significantly decreased the IC₅₀ values of DOX and paclitaxel compared with the ABCB1 inhibitor verapamil in KBv200 and MCF-7/adr cells. Similarly, apatinib significantly decreased the IC₅₀ of topotecan (from 10.3 to 0.7 μmol/L) compared with the ABCG2 inhibitor fumitremorgin C (positive control) in S1-M1-80 cells. In contrast, apatinib did not significantly alter the sensitivity of the drug-sensitive parental cells to the antineoplastic drugs used in this study. In addition, apatinib had no significant reversal effect on ABC1-mediated drug resistance in ABC1 gene transfectant cell lines such as KB/ABCC1 and HEK293/ABCC1 (data not shown). Therefore, our results suggest that apatinib significantly sensitizes cells overexpressing ABCB1 or ABCG2 to antineoplastic drugs that are substrates of ABCB1 or ABCG2.

Apatinib significantly increases the accumulation of DOX and Rho 123 in cells overexpressing ABCB1 and ABCG2

To ascertain the potential mechanism by which apatinib sensitizes the MDR cells to antineoplastic drugs, we examined the effect of apatinib on the accumulation of DOX and Rho 123 in cells overexpressing ABCB1 or ABCG2. In the absence of apatinib, the intracellular levels of DOX and Rho 123 were low in MDR cells, whereas apatinib significantly increased the intracellular accumulation of DOX and Rho 123 in a concentration-dependent manner (Fig. 1; Supplementary Figs. S2 and S3). The fluorescence index of DOX in the presence of 0.75, 1.5, and 3 μmol/L apatinib was increased by 1.21-, 1.72-, and 2.19-fold, respectively, in KBv200 cells; 1.31-, 1.86-, and 2.44-fold, respectively, in MCF-7/adr cells; and 1.37-, 1.71-, and 2.11-fold, respectively, in S1-M1-80 cells (Fig. 1A). As shown in Fig. 1B, apatinib, at 0.75, 1.50, and 3 μmol/L, increased the intracellular accumulation of Rho 123 by 1.91-, 3.43-, and 5.17-fold, respectively, in KBv200 cells; 1.92-, 2.83-, and 3.59-fold, respectively, in MCF-7/adr cells; and 2.13-, 3.42-, and 4.16-fold, respectively, in S1-M1-80 cells. However, apatinib did not significantly alter the intracellular accumulation of DOX and Rho 123 in the parental sensitive KB, MCF-7, and S1 cells. It should be noted that S1-M1-80, but not the wild-type ABCG2, overexpressed the R482G variant, which can transport Rho 123 (39). Taken together, these results suggest that apatinib significantly inhibits ABCB1- and ABCG2-mediated transport in MDR cells.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ ± SD (μmol/L: fold-reversal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEK293/pcDNA3.1</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.0569 ± 0.0035 (1.00)</td>
</tr>
<tr>
<td>+3 μmol/L apatinib</td>
<td>0.0349 ± 0.0097 (1.63)</td>
</tr>
<tr>
<td>+3 μmol/L FTC</td>
<td>0.0528 ± 0.0093 (1.08)</td>
</tr>
<tr>
<td>+3 μmol/L PSC83</td>
<td>0.0543 ± 0.0069 (1.04)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.0724 ± 0.0054 (1.00)</td>
</tr>
<tr>
<td>+3 μmol/L apatinib</td>
<td>0.0512 ± 0.0033 (1.41)</td>
</tr>
<tr>
<td>+10 μmol/L verapamil</td>
<td>0.0957 ± 0.0142 (0.77)</td>
</tr>
<tr>
<td>+3 μmol/L FTC</td>
<td>0.0528 ± 0.0093 (1.08)</td>
</tr>
<tr>
<td>SN-38</td>
<td>0.0073 ± 0.0003 (1.00)</td>
</tr>
<tr>
<td>+3 μmol/L apatinib</td>
<td>0.0045 ± 0.0009 (1.62)</td>
</tr>
<tr>
<td>+3 μmol/L FTC</td>
<td>0.0050 ± 0.0003 (1.46)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.0437 ± 0.0022 (1.00)</td>
</tr>
<tr>
<td>+3 μmol/L apatinib</td>
<td>0.0335 ± 0.0039 (1.30)</td>
</tr>
<tr>
<td>+10 μmol/L verapamil</td>
<td>0.0450 ± 0.0003 (0.97)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.8240 ± 0.4728 (1.00)</td>
</tr>
<tr>
<td>+3 μmol/L apatinib</td>
<td>1.5256 ± 0.3717 (1.19)</td>
</tr>
</tbody>
</table>

NOTE: Cell survival was determined by MTT assays as described in Materials and Methods. Data are the mean ± SD of at least three independent experiments performed in triplicate. The fold-reversal of MDR (values given in parentheses) was calculated by dividing the IC₅₀ for cells with the anticancer drugs in the absence of inhibitor by that obtained in the presence of inhibitor.

*P < 0.01, versus the values obtained in the absence of inhibitor.
Inhibition kinetics of apatinib on intracellular DOX efflux by ABCB1 or ABCG2

To obtain information about the mechanism of transport inhibition of ABCB1 and ABCG2 by apatinib, we determined the effect of apatinib on the kinetics of the intracellular DOX efflux by ABCB1 or ABCG2 transporter using KBv200 and S1-M1-80 cells, respectively. The inhibitory effect of apatinib was analyzed using Lineweaver-Burk plots in the presence or absence of apatinib. Subsequent analysis indicated that apatinib was a competitive inhibitor of DOX efflux (Fig. 2E and F). The $K_i$ values of apatinib for DOX transport by ABCB1 and ABCG2 were $1.98 \pm 0.21$ and $1.37 \pm 0.17 \mu\text{mol/L}$, respectively.

Apatinib stimulates the ATPase activity of ABCB1 and ABCG2

To assess the effect of apatinib on the ATPase activity of ABCB1 and ABCG2, we evaluated the effect of apatinib on both ABCB1 and ABCG2 ATPase activities. Apatinib produced a 3-fold stimulation of ABCB1 ATPase activity in a concentration-dependent manner, and the concentration required for 50% stimulation was $\approx 950 \mu\text{mol/L}$ (Fig. 2A). In contrast, apatinib had a biphasic effect on ABCG2 ATP hydrolysis, as it stimulated the ATPase activity of ABCG2 at lower concentrations but produced inhibition at higher concentrations (Fig. 2B). The ATPase data suggest that apatinib has a higher affinity for ABCG2 compared with ABCB1 and that it is likely a substrate for both ABCG2 and ABCB1.

Apatinib inhibits the photoaffinity labeling of ABCB1 and ABCG2 with $[^{125}\text{I}]\text{IAAP}$

$[^{125}\text{I}]\text{IAAP}$ photoaffinity labeling of both ABCB1 and ABCG2 and their binding can be competitively inhibited by substrates or inhibitors of the respective transporters (38). Therefore, to further investigate the interaction of apatinib with the substrate-binding sites of ABCB1 and ABCG2, the membrane vesicles of these transporters were incubated with $[^{125}\text{I}]\text{IAAP}$ in the absence or presence of apatinib. Apatinib produced a concentration-dependent inhibition of $[^{125}\text{I}]\text{IAAP}$ photoaffinity labeling of both ABCB1 (Fig. 2C) and ABCG2 (Fig. 2D), with $IC_{50}$ values of $2.9 \pm 0.4 \mu\text{mol/L}$ and $11 \pm 4 \mu\text{mol/L}$, respectively. These results suggest that apatinib binds with higher affinity to ABCG2 substrate-binding site(s) than to ABCB1 substrate-binding site(s).

Apatinib does not significantly alter the mRNA or protein levels of ABCB1 and ABCG2

The reversal of ABCB1- and ABCG2-mediated MDR can be achieved either by inhibiting their function or by lowering
their expression. Therefore, we determined the effect of apatinib on the protein levels and mRNA content of ABCB1 and ABCG2. Apatinib (Fig. 3), at 0.75, 1.5, or 3 μmol/L, did not significantly alter the expression of protein or mRNA for ABCB1 or ABCG2 transporters in KBv200, MCF-7/adr, or S1-M1-80 cells. In addition, quantitative real-time PCR results indicated that there was no significant difference in the expression of mRNA in the MDR cells (data not shown). These data suggest that the reversal of MDR is most likely obtained by direct inhibition of the efflux function of ABCB1 and ABCG2 as opposed to the downregulation of their mRNA or protein levels.

Figure 2. Effect of apatinib on Vi-sensitive ABCB1 and BeFx-sensitive ABCG2 ATPase activity, photoaffinity labeling of ABCB1 and ABCG2 with [125I]IAAP, and the transport kinetics of DOX. A, ATPase activity of Vi-sensitive ABCB1. B, ATPase activity of BeFx-sensitive ABCG2. B, inset, effect of lower concentrations of apatinib on ABCG2 ATPase activity. The amount of inorganic phosphate released was quantitated using a colorimetric method. The photoaffinity labeling of ABCB1 (C) and ABCG2 (D) with [125I]IAAP was performed using the indicated concentrations of apatinib as described in Materials and Methods. The amount of inorganic phosphate released was quantitated using a colorimetric method. Crude membranes from High Five insect cells expressing ABCB1 (C) and from MCF-7/FLV1000 cells expressing ABCG2 (D) were incubated with various concentrations of apatinib for 10 min at room temperature, and 3 to 6 nmol/L [125I]IAAP (2,200 Ci/mmol) were then added before illuminating with a UV lamp (365 nm) as described in Materials and Methods. In all three blots, lane 1 is control without apatinib. E and F, effect of apatinib on the transport kinetics of intracellular DOX efflux mediated by the ABCB1 (E) and ABCG2 (F) transporters. The quantity of efflux DOX in MDR cells was measured for 5 min at 37°C at various DOX concentrations (2.5–20 μmol/L) in the presence or absence of apatinib by flow cytometry. Points, mean of at least three different experiments; bars, SD. The Ki values were determined from the double reciprocal Lineweaver-Burk plots in the absence (○) or presence of 3 μmol/L apatinib (▲).
Apatinib does not block the phosphorylation of AKT and ERK1/2 at MDR reversal concentration

Previous studies have shown that the inhibition of the AKT and ERK1/2 pathways may decrease the resistance to anti-neoplastic drugs in cancer cells (40, 41). Consequently, we determined the effect of apatinib on the levels of total and phosphorylated forms of AKT and ERK1/2 in all cell lines. As shown in Fig. 4, the incubation of cells with apatinib (0.75–3 μmol/L) for 48 hours did not significantly alter the total and phosphorylated forms of AKT and ERK1/2. This suggests that the MDR reversal effect of apatinib in KBv200, MCF-7/adr, and S1-M1-80 cells is independent of the inhibition of AKT and ERK1/2 phosphorylation.

Apatinib reverses ABCB1-mediated MDR in the nude mouse xenograft model

An established KBv200 cell xenograft model in nude mice was used to evaluate the efficacy of apatinib to reverse the resistance to paclitaxel in vivo. There was no significant difference in tumor size between animals treated with saline, apatinib, or paclitaxel, indicating the in vivo resistance to paclitaxel. However, the combination of apatinib and paclitaxel produced a significant inhibition of tumor growth compared with animals treated with saline, paclitaxel, or apatinib alone ($P < 0.05$; Fig. 5). The ratio of tumor growth inhibition by the combination was 52.7%. Furthermore, at the doses tested, no mortality or apparent decrease in body weight was observed in the combination treatment groups, suggesting that the combination regimen did not increase the incidence of toxic side effects.

Discussion

Molecular targeted therapy for various types of cancer has become an active field of basic science and clinical research ever since imatinib (Gleevec, STI-571) was approved by the Food and Drug Administration in 2001 as a first-line drug...
to treat chronic myeloid leukemia. Cytokine receptor signal transduction pathways are pivotal mediators of cancer oncogenesis, proliferation, invasion, metastasis, and angiogenesis. Particularly, the EGFR and VEGFR-2 pathways are vital in cancer cells and cancer-associated endothelial cells and, hence, are one of the most extensively studied pathways (42, 43). In recent few years, many compounds have been developed to block these two pathways including receptor TKIs and monoclonal antibodies targeting EGFR, VEGFR, and VEGF Trap (44).

Interestingly, several TKIs were found to interact with the major MDR transporters, such as ABCB1, ABCC1, and ABCG2. Initially, imatinib (STI-571) was found to be an ABCB1 substrate, and EKI-785 was shown to interact with ABCC1 (13). More recently, CI1033 was reported to be a substrate and inhibitor of ABCG2 (45). Other TKIs such as gefitinib (16, 46), erlotinib (17), vandetanib (19, 33), and lapatinib (15) have also been shown to inhibit ABCB1 and ABCG2 function. Apatinib is a promising multi-tyrosine kinase inhibitor and is in phase III clinical development. However, little is known to date about the interaction between apatinib and ABC transporters.

In the present study, we showed that apatinib significantly potentiated the cytotoxicity of established ABCB1 and ABCG2 substrates and increased the accumulation of DOX and Rho 123 in ABCB1- or ABCG2-overexpressing cells. However, apatinib at 3.0 μmol/L did not significantly sensitize the parental sensitive KB, MCF-7, S1, or HEK293/pcDNA3.1 cells to the anticancer agents used in this study (Tables 1 and 2). These findings suggest that the sensitization of the resistant cells by apatinib is specific to overexpression of ABCB1 or ABCG2. Furthermore, apatinib significantly enhanced the intracellular accumulation of DOX and Rho 123 in MDR cells. The results of the fluorescent drug accumulation studies were consistent with our cytotoxic results, suggesting that apatinib sensitizes the ABCB1- and ABCG2-mediated MDR cells to anticancer drugs. The downregulation of ABCB1 and ABCG2 expression on treatment with apatinib could have potentiated the reversal effect of apatinib on ABCB1- and ABCG2-mediated MDR. However, protein expression of ABCB1 or ABCG2 in the corresponding resistant cells was not affected by a 48-h treatment with 0.75, 1.5, or 3.0 μmol/L apatinib (Fig. 3). We thus proposed that the MDR reversal effect of apatinib is due to the inhibition of the efflux function of the ABC transporters as revealed in the drug accumulation assay (Fig. 1). To examine whether apatinib can also reverse MDR in vivo, we investigated the effect of apatinib on the anticancer activity of paclitaxel in the nude mouse xenograft model. We found that the combination of paclitaxel with apatinib remarkably enhanced the anticancer activity of paclitaxel in our ABCB1-overexpressing xenograft model (Fig. 5). However, there was no substantial increased loss of body weight in mice treated with the drug combination compared with the individual drug treatment alone.

ABC transporters move substrates out of cells using ATP as the energy source. Therefore, the rate of ATP hydrolysis...
(ATPase activity) is directly proportional to the transport activity of the transporters (47). We have previously reported that some TKIs such as lapatinib, sunitinib, and erlotinib, even at low concentrations, can stimulate the ATPase activities of the transporters (15, 17, 48). In fact, lapatinib stimulated both Vi-sensitive ABCB1 and BeF-sensitive ABCG2 ATPase at lower concentrations, as seen with the aforementioned TKIs (Fig. 2A and B), but inhibited BeF-sensitive ABCG2 ATPase at higher concentrations (Fig. 2B). These results suggest that lapatinib is likely to be a substrate of both ABCB1 and ABCG2. In addition, we speculate, based on these findings, that lapatinib has a direct interaction with these transporters. Further experiments showed that lapatinib inhibited the photoaffinity labeling of ABCB1 and ABCG2 with [125I]IAAP, definitively illustrating the direct interaction between lapatinib and these transporters. Taken together, these data suggest that lapatinib reverses MDR by directly inhibiting the function of ABC drug transporters.

Receptor tyrosine kinases (RTK) such as VEGFR, platelet-derived growth factor receptor, and FLT3 play crucial role in modulating cell proliferation, differentiation, and survival by activating downstream signal molecules such as signal transducers and activators of transcription, protein kinase B/AKT, and ERK1/2 (49). Aberrant activation of different RTKs is believed to be associated with cancer growth, angiogenesis, and metastasis. Moreover, it has been reported that activation of the phosphatidylinositol 3-kinase/AKT and/or ERK pathways is related to resistance to conventional anticancer drugs (50). To rule out the involvement of the AKT and ERK1/2 signaling pathways in the MDR reversal of lapatinib, activation of AKT and ERK1/2 was examined. Our data showed that lapatinib (up to 3.0 μmol/L) did not block the phosphorylation of AKT and ERK1/2 in all the cell lines tested (Fig. 4). Therefore, blockade of AKT and ERK1/2 activation is not involved in the reversal of ABCB1- or ABCG2-mediated MDR by lapatinib.

In conclusion, lapatinib reverses ABCB1- and ABCG2-mediated MDR by directly inhibiting ABCB1 and ABCG2 function, resulting in elevated intracellular concentrations of substrate chemotherapeutic drugs. Also, the reversal of MDR is not associated with the blockade of tyrosine kinases. Confirmation of MDR reversal by lapatinib in tumor xenograft model further supports the potential usefulness of combining lapatinib with other conventional anticancer drugs in overcoming clinical resistance in cancer chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Apatinib (YN968D1) Reverses Multidrug Resistance by Inhibiting the Efflux Function of Multiple ATP-Binding Cassette Transporters

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