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Nilotinib (AMN107, Tasigna®) reverses multidrug resistance by inhibiting the activity of the ABCB1/Pgp and ABCG2/BCRP/MXR transporters

Amit K. Tiwari [a], Kamlesh Sodani [a], Si-Rong Wang [a], Ye-Hong Kuang [a,b], Charles R. Ashby Jr.[a], Xiang Chen [b], Zhe-Sheng Chen [a,1,*]

[a] Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Jamaica, NY 11439, USA
[b] Department of Dermatology, Xiang Ya Hospital, Central South University, 87 Xiang Ya Road, Changsha, Hunan 410008, China

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ABSTRACT

Nilotinib, a BCR-Abl tyrosine kinase inhibitor (TKI), was developed to surmount resistance or intolerance to imatinib in patients with Philadelphia positive chronic myelogenous leukemia. Recently, it was shown that several human multidrug resistance (MDR) ATP-binding cassette (ABC) proteins could be modulated by specific TKIs. MDR can produce cancer chemotherapy failure, typically due to overexpression of ABC transporters, which are involved in the extrusion of therapeutic drugs. Here, we report for the first time that nilotinib potentiates the cytotoxicity of widely used therapeutic substrates of ABCG2, such as mitoxantrone, doxorubicin, and ABCB1 substrates including colchicine, vincristine, and paclitaxel. Nilotinib also significantly enhances the accumulation of paclitaxel in cell lines overexpressing ABCB1. Similarly, nilotinib significantly increases the intracellular accumulation of mitoxantrone in cells transfected with ABCG2. Furthermore, nilotinib produces a concentration-dependent inhibition of the ABCG2-mediated transport of methotrexate (MTX), as well as $E_2$17βG a physiological substrate of ABCG2. Uptake studies in membrane vesicles overexpressing ABCG2 have indicated that nilotinib inhibits ABCG2 similar to other established TKIs as well as fumitremorgin C. Nilotinib is a potent competitive inhibitor of MTX transport by ABCG2 with a K_i value of 0.69 ± 0.083 μM as demonstrated by kinetic analysis of nilotinib. Overall, our results indicate that nilotinib could reverse ABCB1- and ABCG2-mediated MDR by blocking the efflux function of these transporters. These findings may be used to guide the design of present and future clinical trials with nilotinib, elucidating potential pharmacokinetic interactions. Also, these findings may be useful in clinical practice for cancer combination therapy with nilotinib.

1. Introduction

One of the primary impediments to the successful treatment of cancer is the presence of cells that have acquired the multidrug resistance (MDR) genotype [1,2]. MDR is often a result of the overexpression of some ATP-dependent transporters that are known as ATP-binding cassette (ABC) transporters. ABC transporters are ubiquitous, in which the most diverse and the largest superfamily of transporters are present on plasma membranes. A majority of the 49 ABC transporters are classified on the basis of two highly conserved nucleotide-binding domains (NBDs), which are located on the cytosolic site [3]. These NBDs have conserved Walker A and B consensus sequences of 90–110 amino acids, where ATP binds and is hydrolyzed via an ATPase enzyme [4]. The energy derived from ATP regulates the transport of various organic molecules, such as vitamins, saccharides, proteins, and several hydrophobic drugs, both intracellularly and extracellularly [5]. It is well established that these ABC transporters, particularly the ABC transporter-subfamily B member 1 (ABCB1/P-gp/MDR1), -subfamily G member 2 (ABCG2/BCRP/MXR/ABCP), and -subfamily C member 1 (ABCC1/MRP1), play a crucial role in the efflux of important chemotherapeutic agents from cells, thereby, inducing MDR [1–7]. ABCB1 and ABCC1 can also transport various hydrophobic drugs, and ABCC1 can transport some anionic drugs or drug conjugates, including antifolates, certain nucleotides, and also vinca alkaloids [1,7]. The substrate profile of ABCG2 partially overlaps with that of ABCC1 and ABCB1 tansporters, whose substrates include mitoxantrone (MX), methotrexate (MTX), indolocarbazole topoisomerase I inhibitors, anthracyclines, flavopir-

Abbreviations: MDR, multidrug resistance; ABC, ATP-binding cassette; ABCB1 (P-gp), P-glycoprotein; ABCG2, also called BCRP (breast cancer resistance protein)/MXR (mitoxantrone resistance protein); ABCC1 (MRP1), multidrug resistance protein 1; EGFR, epidermal growth factor receptor; HER, human epidermal receptor; TKI, tyrosine kinase inhibitor; PBS, phosphate-buffered saline; FTC, fumitremorgin C; $E_2$17βG, estradiol 17-β-D-glucuronide; MX, mitoxantrone; MTX, methotrexate.

* Corresponding author. Tel.: +1 718 990 1432; fax: +1 718 990 1877.
E-mail address: chenz@stjohns.edu (Z.-S. Chen).

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idols, daunorubicin, and some camptothecin-derived inhibitors [8]. Also, ABC transporters affect drug disposition by altering absorption, distribution, metabolism, and excretion, as well as their toxicity [9–11].

In the human genome, more than 500 protein kinases have been identified as molecular switches that catalyze the transition between active and inactive states [12]. Most of these receptors and cytoplasmic protein kinases belong to the serine/threonine and tyrosine kinase families, which play an important role in cellular signaling. Of the 90 genes that code for tyrosine kinases, 32 genes encode non-receptor entities, while the remaining 58 genes encode receptor protein tyrosine kinases (PTKs) [12,13]. The deregulation of PTKs has been implicated in the development and progression of various solid tumors and hematological malignancies [14]. Recently, a number of tyrosine kinase inhibitors (TKIs) have received FDA approval for the treatment of various types of cancer. Mechanistically, ATP-competitive TKIs directly inhibit autophosphorylation of key tyrosine residues located in the protein kinase activation-loop domain, with subsequent inhibition of substrate phosphorylation and signal transduction. Through binding within a deep cleft between the C-terminal and N-terminal lobes of the conserved kinase domains, TKIs inhibit the access of MgATP to its binding site, thereby preventing the transfer of a phosphate group to a substrate tyrosine residue subsequently abrogating the enzyme's catalytic function in mediating intracellular signaling [15]. There are a significant number of studies indicating that the currently approved TKIs act as anticancer drugs by inhibiting cancer development, proliferation, invasion, metastasis, angiogenesis, and the induction of apoptosis [16,17].

Currently, no drug has been clinically approved for the reversal of MDR due to detrimental pharmacokinetic interactions, including toxicity issues. Recently, it has been reported that certain clinically used TKIs interact with ABCB1 and ABCG2 transporters [18–25]. Since hydrophobic TKIs target the intracellular PTK domain, it was hypothesized that some transmembrane transporters, such as ABC transporters, might also modulate the pharmacological activity of specific TKIs [18]. Interestingly, *in vitro* studies have shown that submicromolar concentrations of several 4-anilinoquinazoline-derived TKIs, such as canertinib, EKI-485, gefitinib, tyrphostin AG1478, erlotinib, lapatinib, and a phenylamino-pyrimidine derivative imatinib (Fig. 1) can selectively modulate MDR protein–ATPase activity, inhibit MDR-dependent active drug efflux, and significantly affect the drug resistance patterns in cells with MDR mediated by ABCB1 and ABCG2 transporters [18–25]. *In vivo* studies have suggested that the co-administration of gefitinib enhances oral bioavailability and hence antitumor activity of irinotecan [26]. Gefitinib also decreases the clearance and increases the oral absorption of topotecan by modulating ABCB1 and ABCG2 function in mice [27].

Previously, we have shown that the response to paclitaxel is augmented by lapatinib in ABCB1-overexpressing KBv200 cell xenografts in nude mice [24]. Imatinib has been reported to inhibit ABCG2, enhancing the efficacy of photodynamic therapy by increasing the intracellular accumulation of photosensitizers [28]. In addition, imatinib can significantly reverse topotecan and SN-38 resistance *in vitro* [29]. The inhibition of ABCB1 and ABCG2 improves the pharmacokinetic delivery of imatinib in brain tumors of mice [30].

Nilotinib (AMN107, Tasigna®) is a phenyl-amino-pyrimidine derivative (Fig. 1), which was designed based on the crystal structure of imatinib–Abl complex. Nilotinib is a potent, relatively selective inhibitor of the tyrosine kinase activities of BCR-Abl, platelet-derived growth factors (PDGFR) and mast/stem-cell growth factor (c-KIT) [31]. A previous study suggested that nilotinib has a high affinity for ABCG2 and that nilotinib is likely to be a substrate of ABCG2 [32]. In addition, a recent study has suggested that nilotinib may also be a substrate of ABCB1 [33]. However, there are no published data indicating if nilotinib can be used to modulate and reverse ABCG2- and ABCB1-mediated MDR. Therefore, in this study, we have sought to determine if nilotinib could modulate ABCB1- and ABCG2-mediated resistance.

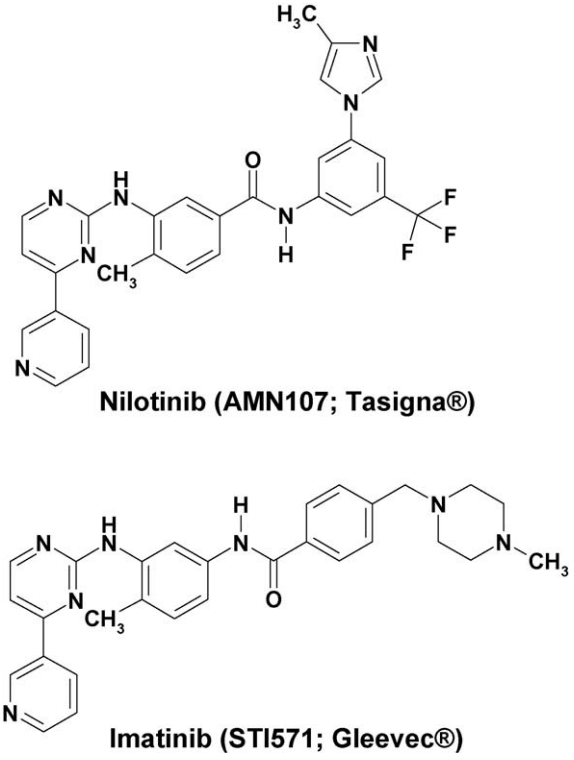

Fig. 1. Chemical structures of nilotinib and imatinib.

2. Materials and methods

2.1. Materials

[^{3}H]-MTX (23 Ci/mmol), [^{3}H]-paclitaxel (37.9 Ci/mmol), and [^{3}H]-MX (4 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Monoclonal antibodies BXP-34 (against ABCG2) and C-219 (against ABCB1) were acquired from Signet Laboratories, Inc. (Dedham, MA). Anti-actin monoclonal antibody (sc-8432) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Nilotinib was obtained as a gift from Novartis pharmaceuticals (Basel, Switzerland). Fumitremorgin C (FTC) was synthesized by Thomas McCloud Developmental Therapeutics Program, Natural Products Extraction Laboratory, NCI, NIH (Bethesda, MD). MX, paclitaxel, doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell lines and cell culture

HEK293/pcDNA3.1, wild-type ABCG2-482-R2, mutant ABCG2-482-G2, and another mutant ABCG2-482-T7 cells were established by transfecting HEK293 with either the empty pcDNA3.1 vector or pcDNA3.1 vector containing the full length *ABCG2*, coding either arginine (R), glycine (G), or threonine (T) at amino acid 482, respectively, and were cultured in medium with 2 mg/ml of G418 [34]. The parental human epidermoid carcinoma cell line KB-3-1 was selected in a stepwise manner using increasing concentrations of colchicine to establish the ABCB1/P-gp-overexpressing drug-resistant cell line, KB-C2 [35]. Another ABCB1-overexpressing cell line KB-V1 was cultured in medium containing 1 μg/ml vinblas-

tine. All of the cell lines were grown as adherent monolayers in flasks with DMEM culture medium (Hyclone Co., UT) supplemented with 10% fetal bovine serum in a humidified incubator containing of 5% CO_2 at 37 °C.

2.3. Preparation of total cell lysates and membrane vesicles

Total cell lysates were prepared by harvesting the cells and rinsing twice with PBS. Cell extracts were prepared by incubating cells for 30 min with radioimmunoprecipitation assay (RIPA) buffer [1× PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 μg/ml leupeptin, 100 μg/ml p-aminophenylmethylsulfonyl fluoride (p-APMSF), and 10 μg/ml aprotinin] with occasional rocking followed by centrifugation at 12,000 × *g* at 4 °C for 15 min. The supernatant containing total cell lysates was stored at −80 °C until needed for experiments. Membrane vesicles were prepared by using the nitrogen cavitation method [36]. Cells were rinsed twice with PBS and then scraped into PBS containing 1% aprotinin. Cells were then washed at 4 °C in PBS, collected by centrifugation (4000 × *g* for 10 min), suspended in buffer A (10 mM Tris–HCl, pH 7.4, 0.25 M sucrose, 1 mM p-APMSF, and 0.2 mM $CaCl_2$), and then equilibrated at 4 °C for 15 min under a nitrogen pressure of 500 psi. EDTA was added to the suspension of lysed cells to a final concentration of 1 mM. The suspension was then diluted 1:4 with buffer B (10 mM Tris–HCl, pH 7.4, 0.25 M sucrose, and 1 mM p-APMSF) and centrifuged at 4000 × *g* for 10 min at 4 °C to remove nuclei and unlysed cells. The supernatant was layered onto a sucrose cushion (35% sucrose, 10 mM Tris–HCl, pH 7.4, and 1 mM EDTA) and centrifuged for 30 min at 16,000 × *g* at 4 °C. The interface was collected and centrifuged for 45 min at 100,000 × *g* for 4 °C. The pellet was resuspended in buffer B by repeated passage through a 25-gauge needle. The protein concentration was determined by bicinchoninic acid (BCA™)-based protein assay (Thermo Scientific, Rockford, IL).

2.4. Cell cytotoxicity by MTT assay

The MTT colorimetric assay with slight modifications from that previously described [37] was used to detect the sensitivity of cells to anticancer drugs. Cells were harvested with trypsin and resuspended in a final concentration of 4×10^4 cells/well for KB-3-1, 7.5×10^3 cells/well for KB-C2 and 8×10^3/well for all the other cell lines. Cells were seeded evenly into (180 μl/well) 96-well multiplates. For the reversal experiments, nilotinib, verapamil, or FTC (10 μl/well) was added followed by different concentrations of chemotherapeutic drugs (10 μl/well) into designated wells. After 72 h of incubation, 20 μl of MTT solution (4 mg/ml) was added to each well, and the plate was further incubated for 4 h, allowing viable cells to convert the yellow-colored MTT into dark-blue formazan crystals. Subsequently, the medium was discarded, and 100 μl of dimethylsulfoxide (DMSO) was added into each well to dissolve the formazan crystals. The absorbance was determined at 570 nm by an OPSYS Microplate Reader from DYNEX Technologies, Inc. (Chantilly, VA). The degree of resistance was calculated by dividing the IC_{50} (concentrations required to inhibit growth by 50%) for the MDR cells by that of the parental sensitive cells. The degree of the reversal of MDR was calculated by dividing the IC_{50} for cells with the anticancer drug in the absence of nilotinib or other reversal agents by that obtained in the presence of nilotinib/reversal agent. The IC_{50} were calculated from survival curves using the Bliss method [38].

2.5. Western blot analysis

Equal amounts of total cell lysates (80 μg protein) were resolved by sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. After incubation in a blocking solution in TBST buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, the membranes were immunoblotted overnight with primary monoclonal antibodies against either ABCB1 or actin at 1:200 dilution or ABCG2 at 1:500 dilution at 4 °C, and were then incubated for 3 h at room temperature with horseradish peroxide (HRP)-conjugated secondary antibody (1:1000 dilution). The protein–antibody complex was detected by enhanced chemiluminescence detection system (Amersham, NJ). The protein expression was quantified by Scion Image Software (Scion Co., MD) [22].

2.6. Paclitaxel and MX accumulation

The effect of nilotinib on the intracellular accumulation of paclitaxel in KB-3-1 and KB-C2 cells was determined as previously described [23,39]. The intracellular accumulation of MX in ABCG2 was determined by measuring the intracellular accumulation of [^{3}H]-MX in ABCG2 related cells. Confluent cells in 24-well plates were preincubated with or without the putative MDR reversing compounds for 1 h at 37 °C. Intracellular drug accumulation was measured by incubating cells with 0.1 μM [^{3}H]-paclitaxel or 0.2 μM [^{3}H]-MX for 2 h in the presence or absence of the putative MDR-reversing compounds at 37 °C. The cells were washed three times with ice-cold PBS, typsinized and lysed in 10 mM lysis buffer (pH 7.4, containing 1% Triton X-100 and 0.2% SDS). Each sample was placed in scintillation fluid and radioactivity was measured in a Packard TRI-CARB® 1900CA liquid scintillation analyzer from Packard Instrument Company, Inc. (Downers Grove, IL).

2.7. In vitro transport assays

Transport assays were performed using the rapid filtration method as previously described [36]. Membrane vesicles were incubated with various concentrations of inhibitors for 1 h on ice, transport experiments were carried out at 37 °C for 10 min in a total volume of 50 μl medium (10 μg membrane vesicles, 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 4 mM ATP or 4 mM AMP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and 0.25 μM [^{3}H]-$E_2$17βG or 0.5 μM [^{3}H]-MTX). Reactions were stopped by the addition of 3 ml of ice-cold stop solution (0.25 M sucrose, 100 mM NaCl, and 10 mM Tris–HCl, pH 7.4). During the rapid filtration step, samples were passed through 0.22 μM GVWP filters (Millipore Corporation, Billerica, MA) that were presoaked in the stop solution. The filters were washed three times with 3 ml of ice-cold stop solution. Radioactivity was measured by the use of a liquid scintillation analyzer from Packard Instrument Company, Inc. (Downers Grove, IL). Rates of net ATP-dependent transport were determined by subtracting the values obtained in the presence of 4 mM AMP from those obtained in the presence of 4 mM ATP.

2.8. Statistical analysis

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

3. Results

3.1. Nilotinib significantly enhances the sensitivity of ABCB1- and ABCG2-overexpressing cells to antineoplastic drugs and does not effect the expression of both ABCB1 and ABCG2

Prior to determining the effects of nilotinib to reverse MDR, we examined its effect on the cell viability. The IC_{50} values of nilotinib alone in ABCG2- and ABCB1-overexpressing cells were ~10 and

Table 1
The effect of nilotinib and verapamil on the response of MDR cells overexpressing ABCB1/P-gp to colchicine, paclitaxel, vinblastine and cisplatin.

Compounds	IC50 ± SD[a] (nM)		
	KB-3-1	KB-C2	KB-V1
Colchicine	7.6 ± 2.1 (1.0)[b]	5292.1 ± 640.2 (696.3)	247.0 ± 48.2 (32.5)
+Nilotinib 1 μM	6.9 ± 12.4 (0.9)	2438.8 ± 180.4 (320.9)	191.4 ± 28.4 (25.2)
+Nilotinib 2.5 μM	6.2 ± 2.8 (0.8)	828.9 ± 98.3 (109.1)	122.9 ± 72.1 (16.2)
+Verapamil 5 μM	6.4 ± 1.3 (0.8)	247.1 ± 82.2 (32.5)	98.0 ± 64.2 (12.9)
Paclitaxel	8.6 ± 1.4 (1.0)[b]	4252.1 ± 348.0 (494.4)	5359.5 ± 825.5 (623.2)
+Nilotinib 1 μM	7.4 ± 12.1 (0.9)	2421.8 ± 245.8 (281.6)	3354.0 ± 495.2 (390.0)
+Nilotinib 2.5 μM	6.9 ± 3.2 (0.8)	780.3 ± 153.1 (90.7)	1018.2 ± 189.8 (118.4)
+Verapamil 5 μM	6.8 ± 2.8 (0.8)	224.3 ± 18.8 (26.1)	0720.7 ± 94.1 (83.8)
Vinblastine	52.3 ± 1.6 (1.0)[b]	394.2 ± 194.2 (7.5)	4952.0 ± 725.2 (94.7)
+Nilotinib 1 μM	49.0 ± 12.5 (0.9)	244.0 ± 105.6 (4.7)	2955.0 ± 364.3 (56.5)
+Nilotinib 2.5 μM	43.1 ± 22.7 (0.8)	138.2 ± 85.4 (2.6)	1412.1 ± 192.6 (27)
+Verapamil 5 μM	34.2 ± 12.2 (0.5)	92.4 ± 51.3 (1.8)	831.6 ± 95.1 (15.9)
Cisplatin	1915.2 ± 120.4 (1.0)[b]	1795.1 ± 143.7 (0.9)	1726.0 ± 298.6 (0.9)
+Nilotinib 1 μM	1801.2 ± 215.6 (0.9)	1635.0 ± 215.4 (0.9)	1628.7 ± 595.2 (0.9)
+Nilotinib 2.5 μM	1789.6 ± 172.4 (0.9)	1584.3 ± 235.5 (0.8)	1584.6 ± 459.5 (0.8)
+Verapamil 5 μM	1728.2 ± 116.2 (0.9)	1705.3 ± 218.2 (0.9)	1726.5 ± 265.3 (0.9)

[a] Values in table represent the mean ± SD of at least three independent experiments performed in triplicate.
[b] Fold resistance was the values of IC_{50} for colchicine, paclitaxel, vinblastine, and cisplatin of KB-3-1 cells in the presence of either nilotinib or verapamil, or KB-C2 or KB-V1 cells with or without the reversing agents, divided by the IC_{50} values for colchicines, vinblastine, paclitaxel, and cisplatin of KB-3-1 cells without the reversing agents. Cell survival was determined by the MTT assay as described in "Section 2.4".

~30 μM, respectively (data not shown). However, to avoid toxicity in subsequent experiments, the highest concentration of nilotinib used in the reversal experiments was 2.5 μM, a concentration that caused <10% inhibition of growth in all the cell lines used in this study.

Subsequently, we determined whether nilotinib could sensitize the MDR cells to certain chemotherapeutic drugs. Nilotinib at a concentration of 2.5 μM, significantly decreased the IC_{50} values for colchicine, vinblastine and paclitaxel (Table 1), all of which are substrates of ABCB1 in the KB-C2 and KB-V1 overexpressing ABCB1 cells (Fig. 2A). However, nilotinib did not significantly alter the cytotoxicity effects of the aforementioned chemotherapeutic drugs in parental KB-3-1 cells. Similarly, the ABCB1 inhibitor verapamil at 5 μM significantly decreased the IC_{50} values of colchicine, vinblastine, and paclitaxel in KB-V1 cells. Verapamil almost completely reversed the resistance of KB-C2 cells to vinblastine, whereas it only partially reversed the resistance to colchicine, and paclitaxel (Table 1). As shown in Table 2, 1 μM of nilotinib significantly decreased the IC_{50} values of ABCG2 substrates MX and doxorubicin while it partially reversed resistance to these drugs in both transfected-wild-type or -mutant ABCG2-overexpressing cells (Fig. 2B). Furthermore, 2.5 μM of nilotinib produced a significantly greater degree of sensitization to ABCG2-overexpressing cells with the aforementioned drugs MX and doxorubicin, when compared to nilotinib at 1 μM. The magnitude of the sensitization produced by 2.5 μM nilotinib was similar to that induced by the known specific ABCG2 inhibitor FTC at 2.5 μM. However, in the parental HEK293/pcDNA3.1 cells, the growth inhibitory effects of either doxorubicin or MX, in the presence or absence of nilotinib, showed no significant change (Table 2). Similarly, there was no significant effect in the IC_{50} values for the drugs in all of the cell lines when cisplatin, which is not a substrate of ABCG2, was incubated with nilotinib or FTC (Table 2). Therefore, our results suggest that nilotinib selectively sensitizes ABCB1- or ABCG2-overexpressing cells to chemotherapeutic drugs that are the substrates of ABCB1 or ABCG2. Nilotinib appeared to be more potent in inhibiting the ABCG2-mediated versus ABCB1-mediated resistance.

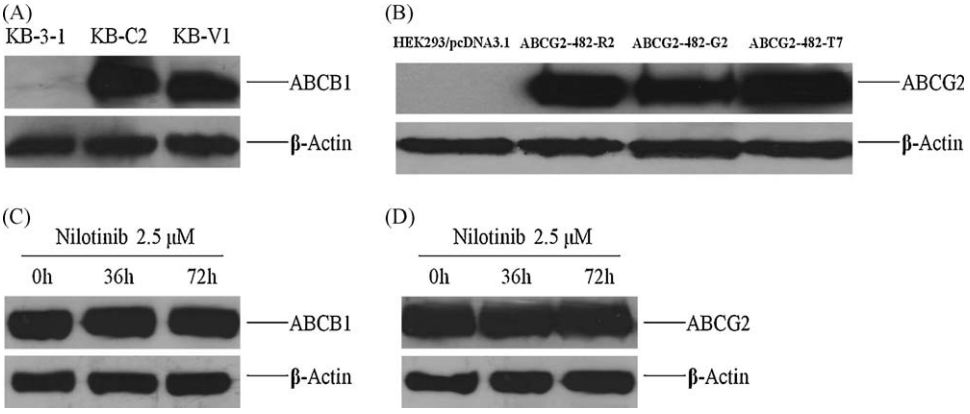

Fig. 2. Immunoblot detection of ABCB1 and ABCG2 and the effect of nilotinib on ABCB1 and ABCG2 expression. (A) Expression of ABCB1 in KB-3-1, KB-C2 and KB-V1 cells. (B) Expression of ABCG2 in HEK293/pcDNA3.1, ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 cells. (C) Effect of Nilotinib at 2.5 μM on the expression level of ABCB1 (KB-C2) for 36 and 72 h. (D) Effect of Nilotinib 2.5 μM on the expression level of ABCG2 (ABCG2-482-T7) for 36 and 72 h. Equal amounts (80 μg protein) of total cell lysate were used for each sample. The membranes were immunoblotted with primary antibody against ABCB1 or actin at 1:200 dilution or ABCG2 at 1:500 dilutions at 4 °C overnight, and then incubated with HRP-conjugated secondary antibody at 1:1000 dilutions at room temperature for 3 h. Protein–antibody complex was detected by chemiluminescence as described in "Section 2". Representative result is shown here and similar results were obtained in two other trials.

Table 2
The effect of nilotinib and FTC on the response of MDR cells overexpressing ABCG2 cells to mitoxantrone, doxorubicin and cisplatin.

Compounds	IC50 ± SD[a] (nM)			
	HEK 293/pcDNA-3	ABCG2-482-R2	ABCG2-482-T7	ABCG2-482-G2
Mitoxantrone	41.50 ± 2.3 (1.0)[b]	592.3 ± 12.4 (14.3)	1103.0 ± 215.6 (26.5)	1504.7 ± 354.0 (36.3)
+Nilotinib 1 μM	39.2 ± 11.2 (0.9)	298.8 ± 95.7 (7.2)	444.1 ± 130.1 (10.7)	473.1 ± 124.5 (11.4)
+Nilotinib 2.5 μM	37.9 ± 5.6 (0.9)	128.9 ± 45.3 (3.1)	147.2 ± 49.3 (3.6)	139.3 ± 3.7 (3.4)
+FTC 2.5 μM	35.3 ± 8.2 (0.9)	57.0 ± 6.5 (1.4)	91.4 ± 18.5 (1.9)	109.3 ± 52.1 (2.0)
Doxorubicin	97.8 ± 3.3 (1.0)[b]	485.2 ± 86.4 (5.0)	1734.1 ± 180.1 (17.7)	1435.1 ± 98.1 (14.7)
+Nilotinib 1 μM	95.6 ± 9.7 (1.0)	352.8 ± 82.5 (3.6)	997.6 ± 268.3 (10.2)	811.7 ± 95.6 (8.3)
+Nilotinib 2.5 μM	90.5 ± 12.7 (0.9)	180.3 ± 12.1 (1.8)	197.1 ± 42.5 (2.0)	179.2 ± 82.2 (1.8)
+FTC 2.5 μM	89.1 ± 2.4 (0.9)	124.1 ± 17.3 (1.3)	110.6 ± 84.2 (1.1)	136.9 ± 71.4 (1.4)
Cisplatin	1743.2 ± 219.6 (1.0)[b]	1415.2 ± 158.1 (0.8)	1982.0 ± 214.1 (1.1)	1384.6 ± 125.2 (0.8)
+Nilotinib 1 μM	1658.2 ± 321.2 (1.0)	1409.0 ± 236.2 (0.8)	1689.2 ± 131.8 (1.0)	1559.7 ± 172.8 (0.9)
+Nilotinib 2.5 μM	1521.5 ± 134.8 (0.9)	1225.4 ± 120.8 (0.7)	1485.1 ± 425.9 (0.9)	1321.5 ± 156.1 (0.8)
+FTC 2.5 μM	1253.0 ± 177.1 (0.7)	1186.5 ± 183.1 (0.7)	1359.1 ± 258.2 (0.8)	1166.1 ± 56.3 (0.7)

[a] Values in table represent the mean ± SD of at least three independent experiments performed in triplicate.
[b] Fold resistance was the IC_{50} values for mitoxantrone, doxorubicin, and cisplatin of HEK293/pcDNA3 cells in the presence of either nilotinib or FTC, or the transfectant cells wild-type ABCG2-482-R2, mutant ABCG2-482-T7 and mutant ABCG2-482-G2) with or without the reversing agents, divided by the IC50 values for mitoxantrone, doxorubicin, and cisplatin of HEK293/pcDNA3 cells without the reversing agents. Cell survival was determined by the MTT assay as described in "Section 2.4".

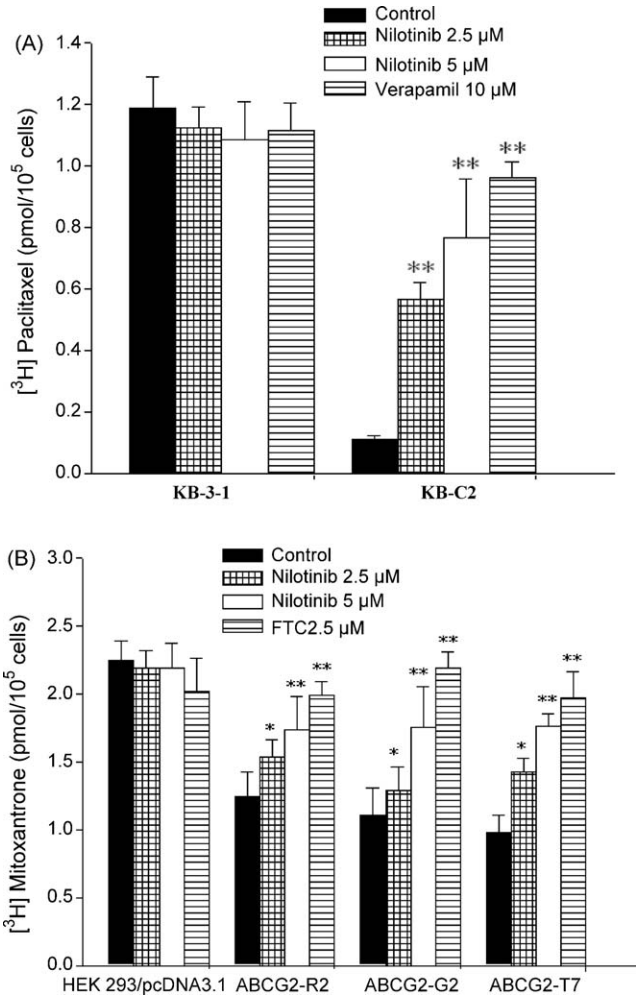

Fig. 3. The effect of nilotinib on the accumulation of [^{3}H]-paclitaxel and [^{3}H]-MX. The accumulation of [^{3}H]-paclitaxel (A) or [^{3}H]-MX (B) was measured after cells were preincubated with or without nilotinib, verapamil, or fumitremorgin C (FTC) for 1 h at 37 °C and then incubated with 0.1 μM [^{3}H]-paclitaxel or 0.02 μM [^{3}H]-MX for another 2 h at 37 °C. Columns, mean of triplicate determinations; bars, SD. *$P < 0.05$; **$P < 0.01$, versus the control group. Experiments were repeated at least three times and a representative experiment is shown.

ABCB1- and ABCG2-mediated MDR can be reversed either by decreasing protein expression level of ABCB1 and ABCG2 or by inhibiting their transport function. To ascertain the reversal cause of nilotinib, we incubated ABCB1 (KB-C2) and ABCG2 (ABCG2-482-T7) overexpressing cells with nilotinib at 2.5 μM for 36 and 72 h. The results shown in Fig. 2C and D indicate that nilotinib does not significantly alter the protein expression levels in both ABCB1 and ABCG2, respectively, at 2.5 μM concentration.

3.2. Accumulation of [^{3}H]-paclitaxel in cells overexpressing ABCB1 and [^{3}H]-MX in cells overexpressing ABCG2

In order to ascertain the mechanism of nilotinib on the function of ABCB1 and ABCG2, we determined the effect of nilotinib on the accumulation of known chemotherapeutic substrates of ABCB1 and ABCG2 transporters in cells overexpressing ABCB1 and ABCG2. The intracellular levels of [^{3}H]-paclitaxel, a known substrate of ABCB1, were measured in the presence or absence of nilotinib in both KB-3-1 and KB-C2 cells. The intracellular levels of [^{3}H]-paclitaxel in ABCB1-overexpressing KB-C2 cells were significantly lower compared to KB-3-1 cells after 2 h of incubation. The results of the accumulation of [^{3}H]-paclitaxel are shown in Fig. 3A. Nilotinib at 2.5 and 5 μM, produced a concentration-dependent increase in the intracellular levels of [^{3}H]-paclitaxel in KB-C2 cells. Furthermore, the increase in the intracellular levels of [^{3}H]-paclitaxel in KB-C2 cells produced by nilotinib at 5 μM was comparable to that of 10 μM of verapamil. However, the intracellular level of [^{3}H]-paclitaxel in KB-3-1 cells was not altered by either nilotinib or verapamil (Fig. 3A). Additionally, the intracellular level of [^{3}H]-MX, a known substrate of ABCG2, was measured in cells overexpressing ABCG2 in the presence or absence of nilotinib (Fig. 3B). Nilotinib produced a significant concentration-dependent increase in the intracellular levels of [^{3}H]-MX in all the ABCG2-transfected cell lines (Fig. 3B). However, there was no significant change in the intracellular levels of [^{3}H]-MX in parental HEK293/pcDNA3.1 cells incubated with nilotinib or FTC (Fig. 3B). Consistent with our cytotoxicity analysis, these data suggest that nilotinib inhibits the efflux function of both ABCB1 and ABCG2 in a concentration-dependent manner, thus producing an increase in the intracellular concentration of [^{3}H]-paclitaxel and [^{3}H]-MX in cells overexpressing in ABCB1 and ABCG2, respectively.

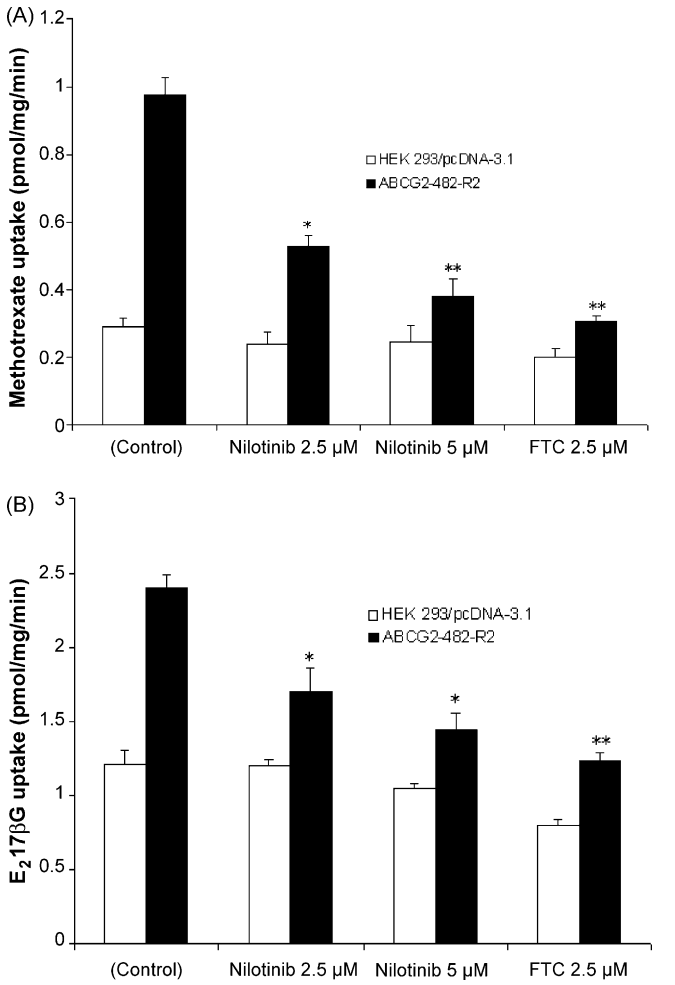

Fig. 4. The effect of nilotinib on the ABCG2-mediated transport of MTX and $E_2$17βG, Membrane vesicles (10 μg) were prepared from HEK293/pcDNA3.1 and ABCG2-482-R2 cells. The rate of uptake of [^{3}H]-MTX (A) and [^{3}H]-$E_2$17βG (B) into membrane vesicles was measured for 10 min at 37 °C in uptake medium containing 4 mM of ATP or AMP. For inhibition experiments, membrane vesicles from HEK293/pcDNA3.1 and ABCG2-482-R2 cells were incubated with various concentrations of nilotinib or FTC for 1 h on ice, and then transport reactions were carried out for 10 min at 37 °C in an uptake medium containing 4 mM ATP. Each column represent the mean of triplicate determinations and the bars represent SD. $^*P < 0.05$; $^{**}P < 0.01$, versus the control group. Experiments were repeated at least three times and a representative experiment is shown.

3.3. Inhibition of [^{3}H]-MTX and [^{3}H]-$E_2$17βG transport in membrane vesicles by nilotinib and its comparison with other TKIs

In the present study, the potency of nilotinib as an inhibitor of ABCG2 was assessed by analyzing the ability of nilotinib to inhibit the transport activity of ABCG2 by using the chemotherapeutic drug substrate [^{3}H]-MTX and the physiological substrate [^{3}H]-$E_2$17βG. Previously, we have reported that only wild-type ABCG2 was able to robustly transport MTX and $E_2$17βG in the *in vitro* transport system [22,36]. Thus, we only used the membrane vesicles prepared from HEK293/pcDNA3.1 and ABCG2-482-R2 cell lines for this study. Nilotinib produced a significant concentration-dependent inhibition of the ATP-energized uptake rate of both MTX and $E_2$17βG in ABCG2-482-R2 membrane vesicles, although its inhibitory effect was weaker than that of FTC at the same concentration (Fig. 4A and B). These *in vitro* transport results suggest that nilotinib is able to directly inhibit the transport of MTX and $E_2$17βG by the wild-type ABCG2.

Previously, we have shown that other quinazoline-derived HER family of TKIs strongly inhibit [^{3}H]-MTX and [^{3}H]-$E_2$17βG uptake by ABCG2 transporter [22,24]. Therefore, to gain insight into the interactions of ABCG2 with MTX and $E_2$17βG in comparison with nilotinib, several TKIs of HER family such as lapatinib, erlotinib and sunitinib, were screened for their capacity to inhibit MTX and $E_2$17βG transport. As shown in Table 3, nilotinib produced a significant concentration-dependent inhibition of ATP-dependent uptake of MTX. The percentage inhibition of MTX uptake brought by nilotinib at 5 μM was 24.9 ± 4.7, which was significantly greater than erlotinib at 5 μM and sunitinib at 5 μM being 36.4 ± 12 and 54.5 ± 9.4, respectively. However, significant percentage uptake inhibition of MTX by 5 μM lapatinib was seen as compared to nilotinib at a similar concentration i.e. 16.8 ± 4.3 which was very close to 15.2 ± 8.1 brought by positive control FTC at 5 μM. A similar trend was seen among these inhibitors while reversing the uptake of yet another ABCG2 substrate, $E_2$17βG, in the plasma membrane vesicles (Table 3). These findings suggest that nilotinib potently inhibits the uptake of both MTX and $E_2$17βG by ABCG2.

Table 3
The effect of tyrosine kinase inhibitors on the ABCG2-mediated transport of MTX and $E_2$17βG.

Inhibitors	Concentration (μM)	Uptake rate (% of control)
[^{3}H]-MTX		
No inhibitor		100
Nilotinib	1	60.6 ± 8.9
	2.5	36.1 ± 7.1
	5	24.9 ± 4.7
Lapatinib	5	16.8 ± 4.3
Erlotinib	5	36.4 ± 12
Sunitinib	5	54.5 ± 9.4
FTC	2.5	21.2 ± 6.7
	5	15.2 ± 8.1
[^{3}H]-$E_2$17βG		
No inhibitor		100
Nilotinib	1	78.6 ± 11.9
	2.5	56.1 ± 9.1
	5	32.6 ± 16.4
Lapatinib	5	23.2 ± 14.6
Erlotinib	5	48.6 ± 13.5
Sunitinib	5	50.5 ± 16.9
FTC	2.5	26.3 ± 5.9
	5	19.1 ± 12.1

Membrane vesicles prepared from HEK293/ABCG2-482-R2 were incubated at 37 °C for 10 min in medium containing $E_2$17βG or MTX in the presence or absence of the indicated compounds. ATP-dependent uptake was calculated by subtracting values obtained in the presence of 4 mM ATP from those in the presence of 4 mM AMP. Transport is expressed as the percentage of uptake in the presence of test compounds. The values shown are the mean ± SD of at least three measurements performed at least in duplicate.

3.4. Inhibition kinetics of nilotinib on MTX transport by ABCG2

We have previously shown that anti-metabolite MTX is a high capacity low affinity chemotherapeutic substrate of ABCG2 [36]. The ATP-dependent uptake of MTX was measured in HEK293 cells transfected with wild-type ABCG2 in order to obtain an approximate measure of the Michaelis Menten kinetics of MTX. The K_m value for the ATP-dependent MTX transport mediated by ABCG2 in three independent sets of experiments was 1.41 ± 0.2 mM, when measured over a wide range of MTX concentrations. This finding was congruent with that of a previous study from our laboratory [36]. The mechanism of the inhibitory effect of nilotinib was analyzed using Lineweaver–Burk plots of MTX uptake in both the presence or absence of nilotinib. Subsequent analysis indicated that nilotinib behaved as a competitive inhibitor of MTX uptake (Fig. 5).

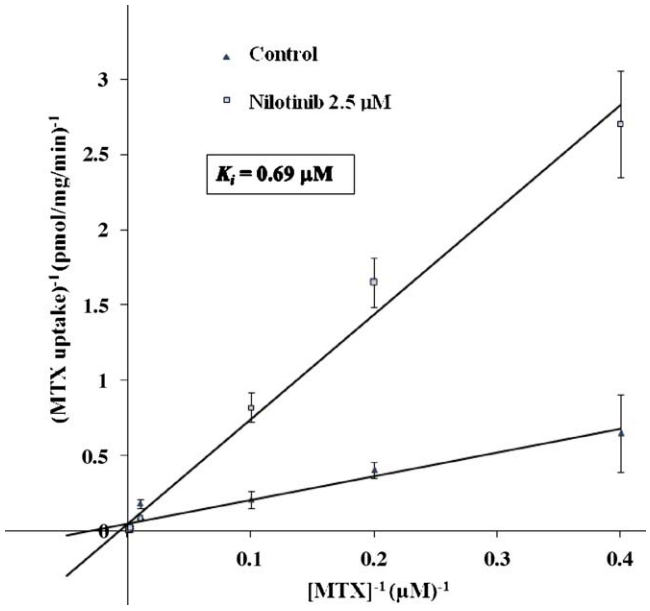

Fig. 5. The effect of nilotinib on the transport kinetics of [^{3}H]-MTX mediated by the ABCG2 transporter. The rate of ATP-dependent uptake of [^{3}H]-MTX into membrane vesicles (10 μg) prepared from ABCG2-enriched R2 cells was measured for 10 min at 37 °C in uptake medium containing 4 mM ATP or 4 mM AMP at various substrate concentrations (0.6–2500 μM), in the presence or absence of nilotinib. The values shown (means ± SD) are the rates measured in the presence of ATP minus rates measured in the presence of AMP with duplicate determinations. The bars represent the SD value. The K_i values were determined from the double reciprocal Lineweaver–Burk plots in the absence (▲) or presence of 2.5 μM nilotinib (□). The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis [53]. The experiments were repeated at least three times and a representative experiment is shown.

The K_i value for nilotinib at 2.5 μM for MTX transport by ABCG2 was 0.69 ± 0.083 μM.

4. Discussion

Previously, it has been shown that intracellular accumulation of imatinib in the tumor cells was significantly reduced on chronic exposure due to the induction of ABCB1 and ABCG2 transporters, thus leading to pharmacokinetic resistance by decreasing the oral bioavailability of imatinib [40,41]. In fact, it was seen that the distribution of imatinib in the brain is limited by ABCB1 mediated efflux [30], and that ABCB1 function is responsible for CML resistance on chronic treatment with imatinib [40,41]. The functional ABCG2 transporter is highly expressed in multipotent human hematopoietic stem cells (HSCs), which are the precursor of CML cells [42,43]. ABCG2 normally protects HSCs from xenobiotics/cytotoxic agents, but it may also lead to imatinib resistance [30]. Studies have reported that imatinib is a substrate of ABCG2 [20,32,44], as well as ABCB1 [10]. Similarly, nilotinib was suggested to be a substrate of ABCG2 [32]. A recent study suggested that nilotinib resistance may be due to BCR-Abl, Src kinase, or ABCB1 overexpression and that nilotinib might be a substrate of ABCB1 [33].

One of the major findings of this study was that nilotinib significantly potentiated the toxicity of established ABCG2 substrates in both transfected-wild-type (ABCG2-482-R2) and -mutant (ABCG2-482-G2 and ABCG2-482-T7) MDR cells (Table 2). In addition, nilotinib significantly enhanced the cytotoxicity of the ABCB1 substrates colchicine, vinblastine and paclitaxel in KB-C2 and KB-V1 cells (Table 1). However, nilotinib (2.5 μM) did not significantly sensitize the parental HEK293/pcDNA3.1 and KB-3-1 cells to the anticancer drugs used in this study (Tables 1 and 2). In addition, there was no significant alteration in sensitivity of cancer cells to compounds that were not ABCB1 or ABCG2 substrates following incubation with nilotinib. These findings suggest that the efficacy of nilotinib to reverse MDR is specific to ABCB1- and ABCG2-mediated drug resistance. Furthermore, nilotinib significantly enhanced the intracellular accumulation of [^{3}H]-MX in both ABCG2 expressing wild-type and mutant cells. Similarly, nilotinib, in a concentration-dependent manner, significantly enhanced the intracellular accumulation of [^{3}H]-paclitaxel in ABCB1 overexpressing KB-C2 cells (Fig. 3). In addition, nilotinib inhibited the ABCG2-mediated membrane vesicle transport of the well-established ABCG2 substrates, MTX and $E_2$17βG in a concentration-dependent manner (Fig. 4). The results of the accumulation and transport experiments were consistent with our cytotoxic results, suggesting that nilotinib interacts synergistically with both ABCB1 and ABCG2 substrates and sensitizes the ABCB1- and ABCG2-mediated MDR cells to anticancer drugs. Also, the down-regulation of ABCB1 and ABCG2 expression on the treatment with nilotinib could have potentiated the reversal effect of nilotinib on ABCB1- and ABCG2-mediated MDR. However, when ABCB1- and ABCG2-overexpressing cells were treated with nilotinib at 2.5 μM for 36 and 72 h, respectively, there was no change in protein expression of both ABCB1 and ABCG2 (Fig. 2C and D). Thus, the reversal effect of nilotinib on ABCB1 and ABCG2 in MDR cells is not due to its effect on expression but most likely related to its inhibition of efflux and transport function of these ABC transporters. However, we cannot rule out the possibility that part of the reversing effect of nilotinib could result from its effect on some proteins and their cross-talk with other proteins which may regulate the transport function of ABCB1 and ABCG2. To exclude this possibility, further experiments are mandated. In addition, signal transducers protein kinase A and protein kinase C are known to phosphorylate human ABCB1/P-gp and the phosphorylation sites are S661, S667, S671 and S683. It has been shown that by replacing S661, S667, S671 and S683 with either A or D, which could result in lack of phosphorylation of ABCB1 and leads to inactivation of drug transport function of this transporter [45]. Also, Nakanishi et al. [46] suggested that imatinib downregulated ABCG2 expression in K562/BCRP-MX10 cells by inhibition of BCR-Abl, leading to the decreased phosphorylation of Akt, however, there was no downregulation of ABCG2 in other cells lacking BCR-Abl overexpression. It is possible that phosphorylation may have some effect on the trafficking or stabilization of ABCB1 in mammalian cells but this needs to be studied further.

Previous studies have shown that 482 position in ABCG2 is hot spot for mutation and wild-type Arg^{482} mutation to Gly^{482} and Thr^{482} mutant variants have shown significant difference in determining the effectiveness of ABCG2 antagonists as well as the ability to transport selected chemotherapeutic drugs [8,34]. Robey et al. [34] have shown that the efficacy of ABCG2 transporters are also altered in these mutant cell lines, for e.g. novobiocin can only block wild-type ABCG2 and is nearly ineffective against mutant variants, however, FTC was shown to block both wild-type as well as mutant ABCG2. Our result shows that like FTC, nilotinib significantly enhanced the sensitivity of ABCG2 substrates not only in cells overexpressing wild-type R482 but also in the R482G/T variants of ABCG2.

Our results, which indicate that specific TKIs interact with the ABCG2 and ABCB1 transporters, support the findings previously published by us and other groups. We have reported earlier that quinazoline-derived HER kinase family inhibitors, such as erlotinib, lapatinib, and AG1478, were able to significantly antagonize ABCB1- and ABCG2-mediated MDR, and thus these drugs may be substrates of these ABC transporters [22–24]. Previously, it has been shown that other quinazoline-derived compounds, such as the EGFR/HER1 inhibitor gefitinib and the pan-HER inhibitor CI-1033, can reverse topotecan resistance by inhibiting the function of

ABCG2 [21,27]. In addition, imatinib a phenyl-amino-pyrimidine derivative is also a potent inhibitor of ABCG2, and reverses the resistance to topotecan and SN-38 *in vitro* [29]. Nilotinib is a potent inhibitor of MTX and $E_2$17βG uptake by ABCG2 transporter in addition to lapatinib, erlotinib, or a mycotoxin FTC (Table 3). Furthermore, kinetic analysis of the inhibition type with the Lineweaver–Burk plot suggests that nilotinib and MTX compete for the same binding site on ABCG2 (Fig. 5). This, combined with our other results, suggests that nilotinib competitively inhibits the efflux function of ABC transporters, and thus increases the intracellular accumulation of certain substrates into the MDR cells.

Nilotinib inhibits autophosphorylation intracellularly by binding to the inactive conformation of the Abl tyrosine kinase, in which the activation-loop of SH1-domain disrupts the substrate binding site [47], and the P-loop folds over the ATP-binding site to block the ATP-phosphate-binding site, thereby abrogating enzyme's catalytic function [15,48]. It was hypothesized that the inhibitory effect of imatinib on the ABC transporter could be due to its interaction with the ATP-binding pocket in NBD, similar to its binding to the ATP-binding pocket on the BCR-Abl kinase [49]. Presumably, since nilotinib is structurally similar to imatinib, it is possible that nilotinib may exert its inhibitory action via the aforementioned mechanism. Nilotinib has several additional disease targets, including myeloproliferative diseases characterized by kinase fusions such as FIP1-like-1-platelet-derived growth factor receptor-alpha (FIP1-like-1-PDGFRalpha), which causes hypereosinophilic syndrome, and TEL-PDGFRbeta, which causes chronic myelomonocytic leukaemia [31,50], as well as mastocytosis and GIST, as a consequence of c-KIT inhibition [31,51,52].

In conclusion, nilotinib, at clinically relevant concentrations, effectively inhibits intracellular ABCB1 and ABCG2 efflux function, without affecting their expression, and reverses ABCB1- and ABCG2-mediated MDR. Nilotinib potently inhibits the ABCG2-mediated MTX and $E_2$17βG transport in the membrane vesicles, suggesting that it interacts directly with ABCG2 transporter. These results tentatively suggest that nilotinib could be used to augment the clinical response to conventional chemotherapeutic agents that are substrates of ABCB1 and ABCG2 as well as other BCR-Abl TKIs, in patients with MDR.

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References

[1] Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med 2002;53:615–27.
[2] Borst P, Elferink RO. Mammalian ABC transporters in health and disease. Annu Rev Biochem 2002;71:537–92.
[3] Dean M, Annilo T. Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. Annu Rev Genomics Hum Genet 2005;6:123–42.
[4] Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. J Bioenerg Biomembr 2001;33:453–8.
[5] Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002;2:48–58.
[6] Fojo T, Coley HM. The role of efflux pumps in drug-resistant metastatic breast cancer: new insights and treatment strategies. Clin Breast Cancer 2007;7:749–56.
[7] Kruh GD, Belinsky MG. The MRP family of drug efflux pumps. Oncogene 2003;22:7537–52.
[8] Mao Q, Unadkat JD. Role of the breast cancer resistance protein (ABCG2) in drug transport. AAPS J 2005;7:E118–33.
[9] Polli JW, Humphreys JE, Harmon KA, Castellino S, O'Mara MJ, Olson KL, et al. The role of efflux and uptake transporters in [N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]amino}methyl)-2-furyl]-4-quinazolinamine (GW572016, lapatinib) disposition and drug interactions. Drug Metab Dispos 2008;36:695–701.
[10] Shukla S, Sauna ZE, Ambudkar SV. Evidence for the interaction of imatinib at the transport-substrate site(s) of the multidrug-resistance-linked ABC drug transporters ABCB1 (P-glycoprotein) and ABCG2. Leukemia 2008;22:445–7.
[11] Shukla S, Robey RW, Bates SE, Ambudkar SV. Sunitinib (Sutent(R), SU11248), a small-molecule receptor tyrosine kinase inhibitor, blocks function of the ABC transporters, P-glycoprotein (ABCB1) and ABCG2. Drug Metab Dispos 2008.
[12] Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. Science 2002;298:1912–34.
[13] Robinson DR, Wu YM, Lin SF. The protein tyrosine kinase family of the human genome. Oncogene 2000;19:5548–57.
[14] Quintas-Cardama A, Kantarjian H, Cortes J. Flying under the radar: the new wave of BCR-ABL inhibitors. Nat Rev Drug Discov 2007;6:834–48.
[15] Mamot C, Rochlitz C. Targeting the epidermal growth factor receptor (EGFR)—a new therapeutic option in oncology? Swiss Med Wkly 2006;136:4–12.
[16] Harari PM, Huang SM. Combining EGFR inhibitors with radiation or chemotherapy: will preclinical studies predict clinical results? Int J Radiat Oncol Biol Phys 2004;58:976–83.
[17] Harari PM. Anti-EGFR therapy update: clinical experience and adverse event insights. Oncology (Williston Park) 2006;20:3–4.
[18] Hegedus T, Orfi L, Seprodi A, Varadi A, Sarkadi B, Keri G. Interaction of tyrosine kinase inhibitors with the human multidrug transporter proteins, MDR1 and MRP1. Biochim Biophys Acta 2002;1587:318–25.
[19] Ozvegy-Laczka C, Cserepes J, Elkind NB, Sarkadi B. Tyrosine kinase inhibitor resistance in cancer: role of ABC multidrug transporters. Drug Resist Updat 2005;8:15–26.
[20] Ozvegy-Laczka C, Hegedus T, Varady G, Ujhelly O, Schuetz JD, Varadi A, et al. High-affinity interaction of tyrosine kinase inhibitors with the ABCG2 multidrug transporter. Mol Pharmacol 2004;65:1485–95.
[21] Erlichman C, Boerner SA, Hallgren CG, Spieker R, Wang XY, James CD, et al. The HER tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. Cancer Res 2001;61:739–48.
[22] Shi Z, Peng XX, Kim IW, Shukla S, Si QS, Robey RW, et al. Erlotinib (Tarceva, OSI-774) antagonizes ATP-binding cassette subfamily B member 1 and ATP-binding cassette subfamily G member 2-mediated drug resistance. Cancer Res 2007;67:11012–20.
[23] Shi Z, Tiwari AK, Shukla S, Robey RW, Kim IW, Parmar S, et al. Inhibiting the function of ABCB1 and ABCG2 by the EGFR tyrosine kinase inhibitor AG1478. Biochem Pharmacol 2008.
[24] Dai CL, Tiwari AK, Wu CP, Su XD, Wang SR, Liu DG, et al. Lapatinib (Tykerb, GW572016) reverses multidrug resistance in cancer cells by inhibiting the activity of ATP-binding cassette subfamily B member 1 and G member 2. Cancer Res 2008;68:7905–14.
[25] Shi Z, Parmar S, Peng XX, Shen T, Robey RW, Bates SE, et al. The epidermal growth factor tyrosine kinase inhibitor AG1478 and erlotinib reverse ABCG2-mediated drug resistance. Oncol Rep 2009;21:483–9.
[26] Stewart CF, Leggas M, Schuetz JD, Panetta JC, Cheshire PJ, Peterson J, et al. Gefitinib enhances the antitumor activity and oral bioavailability of irinotecan in mice. Cancer Res 2004;64:7491–9.
[27] Leggas M, Panetta JC, Zhuang Y, Schuetz JD, Johnston B, Bai F, et al. Gefitinib modulates the function of multiple ATP-binding cassette transporters in vivo. Cancer Res 2006;66:4802–7.
[28] Liu W, Baer MR, Bowman MJ, Pera P, Zheng X, Morgan J, et al. The tyrosine kinase inhibitor imatinib mesylate enhances the efficacy of photodynamic therapy by inhibiting ABCG2. Clin Cancer Res 2007;13:2463–70.
[29] Houghton PJ, Germain GS, Harwood FC, Schuetz JD, Stewart CF, Buchdunger E, et al. Imatinib mesylate is a potent inhibitor of the ABCG2 (BCRP) transporter and reverses resistance to topotecan and SN-38 in vitro. Cancer Res 2004;64:2333–7.
[30] Breedveld P, Pluim D, Cipriani G, Wielinga P, van Tellingen O, Schinkel AH, et al. The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. Cancer Res 2005;65:2577–82.
[31] Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW, Ray A, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell 2005;7:129–41.
[32] Brendel C, Scharenberg C, Dohse M, Robey RW, Bates SE, Shukla S, et al. Imatinib mesylate and nilotinib (AMN107) exhibit high-affinity interaction with ABCG2 on primitive hematopoietic stem cells. Leukemia 2007;21:1267–75.
[33] Mahon FX, Hayette S, Lagarde V, Belloc F, Turcq B, Nicolini F, et al. Evidence that resistance to nilotinib may be due to BCR-ABL, Pgp, or Src kinase overexpression. Cancer Res 2008;68:9809–16.
[34] Robey RW, Honjo Y, Morisaki K, Nadjem TA, Runge S, Risbood M, et al. Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. Br J Cancer 2003;89:1971–8.
[35] Akiyama S, Fojo A, Hanover JA, Pastan I, Gottesman MM. Isolation and genetic characterization of human KB cell lines resistant to multiple drugs. Somat Cell Mol Genet 1985;11:117–26.
[36] Chen ZS, Robey RW, Belinsky MG, Shchaveleva I, Ren XQ, Sugimoto Y, et al. Transport of methotrexate, methotrexate polyglutamates, and 17-beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. Cancer Res 2003;63:4048–54.

[37] Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 1987;47:936–42.
[38] Shi Z, Liang YJ, Chen ZS, Wang XW, Wang XH, Ding Y, et al. Reversal of MDR1/P-glycoprotein-mediated multidrug resistance by vector-based RNA interference in vitro and in vivo. Cancer Biol Ther 2006;5:39–47.
[39] Aoki S, Chen ZS, Higasiyama K, Setiawan A, Akiyama S, Kobayashi M. Reversing effect of agosterol A, a spongean sterol acetate, on multidrug resistance in human carcinoma cells. Jpn J Cancer Res 2001;92:886–95.
[40] Burger H, Nooter K. Pharmacokinetic resistance to imatinib mesylate: role of the ABC drug pumps ABCG2 (BCRP) and ABCB1 (MDR1) in the oral bioavailability of imatinib. Cell Cycle 2004;3:1502–5.
[41] Burger H, van Tol H, Brok M, Wiemer EA, de Bruijn EA, Guetens G, et al. Chronic imatinib mesylate exposure leads to reduced intracellular drug accumulation by induction of the ABCG2 (BCRP) and ABCB1 (MDR1) drug transport pumps. Cancer Biol Ther 2005;4:747–52.
[42] Jordanides NE, Jorgensen HG, Holyoake TL, Mountford JC. Functional ABCG2 is overexpressed on primary CML CD34+ cells and is inhibited by imatinib mesylate. Blood 2006;108:1370–3.
[43] Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 2001;7:1028–34.
[44] Burger H, van Tol H, Boersma AW, Brok M, Wiemer EA, Stoter G, et al. Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein (BCRP)/ABCG2 drug pump. Blood 2004;104:2940–2.
[45] Germann UA, Chambers TC, Ambudkar SV, Licht T, Cardarelli CO, Pastan I, et al. Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. J Biol Chem 1996;271:1708–16.
[46] Nakanishi T, Shiozawa K, Hassel BA, Ross DD. Complex interaction of BCRP/ABCG2 and imatinib in BCR-ABL-expressing cells: BCRP-mediated resistance to imatinib is attenuated by imatinib-induced reduction of BCRP expression. Blood 2006;108:678–84.
[47] Schlessinger J. A solid base for assaying protein kinase activity. Nat Biotechnol 2002;20:232–3.
[48] Manley PW, Cowan-Jacob SW, Mestan J. Advances in the structural biology, design and clinical development of Bcr-Abl kinase inhibitors for the treatment of chronic myeloid leukaemia. Biochim Biophys Acta 2005;1754:3–13.
[49] Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. Science 2000;289:1938–42.
[50] Stover EH, Chen J, Lee BH, Cools J, McDowell E, Adelsperger J, et al. The small molecule tyrosine kinase inhibitor AMN107 inhibits TEL-PDGFRbeta and FIP1L1-PDGFRalpha in vitro and in vivo. Blood 2005;106:3206–13.
[51] von Bubnoff N, Gorantla SH, Kancha RK, Lordick F, Peschel C, Duyster J. The systemic mastocytosis-specific activating cKit mutation D816V can be inhibited by the tyrosine kinase inhibitor AMN107. Leukemia 2005;19:1670–1.
[52] Gleixner KV, Mayerhofer M, Aichberger KJ, Derdak S, Sonneck K, Bohm A, et al. PKC412 inhibits in vitro growth of neoplastic human mast cells expressing the D816V-mutated variant of KIT: comparison with AMN107, imatinib, and cladribine (2CdA) and evaluation of cooperative drug effects. Blood 2006;107:752–9.
[53] Marquardt DW. An algorithm for nonlinear estimation of nonlinear parameters. J Soc Ind Appl Math 1963;11:431–41.