Tandutinib (MLN518/CT53518) targeted to stem-like cells by inhibiting the function of ATP-binding cassette subfamily G member 2

Xiao-qin Zhao,1 Chun-ling Dai,2,1 Shinobu Ohnuma, Yong-ju Liang, Wen Deng,2 Jun-Jiang Chen, Mu-Sheng Zeng, Suresh V. Ambudkar,2 Zhe-Sheng Chen2,* Li-Wu Fu*1,2

1 State Key Laboratory of Oncology in South China, Cancer Center, Sun Yat-Sen University, Guangzhou 510060, China 2 Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, St. John’s University, New York, NY 11439, USA

A R T I C L E   I N F O

Article info
Received 10 September 2012
Received in revised form 12 March 2013
Accepted 13 April 2013
Available online 22 April 2013

Keywords:
Tandutinib
Tyrosine kinase inhibitor
Multidrug resistance
ABCG2/BCRP
Stem-like cells

A B S T R A C T
Tandutinib is a novel inhibitor of tyrosine kinases FLT3, PDGFR and KIT. Our study was to explore the capability of tandutinib to reverse ABC transporter-mediated multidrug resistance. Tandutinib reversed ABCG2-mediated drug resistance in ABCG2-482-R2, ABCG2-482-G2, ABCG2-482-T7 and S1-M1-80 cells and increased the accumulation of doxorubicin, rhodamine 123 and [H]3 mitoxantrone in ABCG2-overexpressing cells. Importantly, tandutinib selectively sensitized side population cells to mitoxantrone. Taken together, our results advocate the potency of tandutinib as an ABCG2 modulator and stem-like cells targeted agent to increase efficiency of anticancer drugs.

1. Introduction
The emergence of drug-resistant tumor cells and enrichment of fraction of stem-like cells remain a major impediment to the successful chemotherapy treatment. The development of multidrug resistance (MDR) is characterized by reduced intracellular level of many structurally and functionally unrelated cytotoxic drugs and is frequently associated with overexpression of one or more members of the ATP-binding cassette (ABC) transporters, which transport a wide variety of compounds such as phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile acids, drugs, and other xenobiotics with the energy of ATP hydrolysis (Klein et al., 1999). In the human genome, 49 ABC transporters have been identified and divided into seven subfamilies (A–G) based on sequence similarities (Dean et al., 2001). The P-glycoprotein (P-gp, ABCB1), multidrug resistance protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2) are the major members of the ABC transporters leading to MDR in cancer cells (Dean et al., 2001). In addition to their role in drug resistance, ABCB1, ABCC1 and ABCG2 are expressed in normal tissues and play an important role in detoxification and protection against xenobiotics by their ability to expel drugs from the cells (Goodell et al., 1996). ABCC1 is expressed in most tissues throughout the body with relatively high level in the lung, testis, kidney, skeletal muscle, and peripheral blood mononuclear cells while relatively low level in liver (Cole et al., 1992; Flens et al., 1996). ABCC1 is expressed in most tissues throughout the body with relatively high level in the lung, testis, kidney, skeletal muscle, and peripheral blood mononuclear cells while relatively low level in liver (Cole et al., 1992; Flens et al., 1996). In most tissues, ABCC1 is localized to the basolateral membrane of epithelial cells, which in certain tissues results in the efflux of its substrates into the blood. ABCB1 and ABCG2 have a more limited tissue distribution than ABCC1 and are localized to the apical surface of epithelial cells, generally in sanctuary sites important for xenobiotic protection (Fromm, 2004; Sarkadi et al., 2004).

In recent years, numerous studies have reported the presence of stem cells in solid tumors referred as cancer stem-like cells (Regenbrecht et al., 2008; Yuan et al., 2004). Cancer stem-like cells represent a population of drug-resistant cells that can survive after chemotherapy and repopulate the tumor. These data led to new paradigms of tumorigenesis and chemotherapy focusing on tumor initiation and resistance of cancer stem-like cells to chemotherapeutic agents and radiation (Hambardzumyan et al., 2008). One of the underlying mechanisms for such resistance is the overexpression of ATP-binding cassette (ABC) transporters (Dean et al., 2005). Stem cells are frequently identified as the “side population” (SP) by flow cytometry based on ABCG2-mediated efflux of Hoechst
2.2. Cell lines and cell culture

The following cell lines were cultured in DMEM or RPMI 1640 containing 10% fetal bovine serum at 37°C in the presence of 5% CO₂; the colon carcinoma cell lines S1 and its mitoxantrone-selected derivative ABCG2-overexpressing S1-M1-80 (Litman et al., 2000); KB and KBv200 cells are human epidermoid carcinoma cell lines obtained from Professor Xu-Yi Liu (Cancer Hospital of Beijing, China). KBv200 cells, a classic multidrug resistant cell line expressing high levels of ABCB1, were cloned from drug-sensitive parental KB cells by stepwise exposure to increasing doses of VCR and ethylmethane sulfonate (EMS) mutagenesis (Fu et al., 2004); the human promyelocytic leukemia cell lines HL60 and its doxorubicin-selected derivative ABC11 overexpressing HL60/ADR (Hunakova et al., 1997); the human lung squamous carcinoma cell lines SW1573 and its doxorubicin-selected derivative ABC14 stable overexpressing SW1573/2R120 (Jansen et al., 1994); the murine fibroblasts cell line NIH3T3 and the ABCG4-transfected derivative ABCC4 stable expressing NIH3T3/MRP4-2 (Lee et al., 2000); HEK293/pCDNA3.1 and ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 cells were established by selection with G418 after transfecting HEK293 with either empty pCDNA3.1 vector or pCDNA3.1 vector containing full length ABCG2 coding either arginine (R), glycine (G), or threonine (T) at amino acid 482 position, respectively, and were cultured in medium with 2 mg/mL of G418 (Robey et al., 2003). All cells were grown in drug-free culture media for 2 weeks before assay.

2.3. Cell proliferation assays

Cytotoxicity tests were performed using the MTT assays as described (Shi et al., 2006; Yan et al., 2008). Briefly, cells were distributed evenly into 96-well microtiter plates. For determining the toxicity of tandutinib, various concentrations of tandutinib diluted with medium were added into the wells; for reversal experiments, different concentrations of chemotherapeutic drugs were added into designated wells 1 h after tandutinib, verapamil, fumitremorgin C (FTC) or MK571 was added. After 68 h treatment, 20 μL MTT (5 mg/mL) was added to each well and the plate was further incubated for 4 h. Subsequently, the supernatant was removed, and 100 μL of dimethylsulfoxide (DMSO) were added into each well to dissolve the formazan crystals. Cell viability was measured by Model 550 Microplate reader (BIO-RAD, USA) at 540 nm with 655 nm as reference filter. The concentrations required to inhibit growth by 50% (IC₅₀) were calculated from survival curves using the Bliss method (Shi et al., 2006). Fold of resistance was calculated by dividing the IC₅₀ for the MDR cells by that obtained in the presence of tandutinib.

2.4. Rhodamine123, doxorubicin and [³H]-mitoxantrone accumulation

The intracellular rhodamine123 and doxorubicin accumulation in S1 and S1-M1-80 was examined by flow cytometry (26). Briefly, the logarithmically growing S1 and S1-M1-80 cells (5 × 10⁵ cells each) were incubated in six-well plates, respectively. After incubation overnight, the cells were treated with 0.75, 1.5, or 3.0 μM of tandutinib at 37°C for 3 h, and then 5 μg/mL rhodamine123 was added and further incubated for 30 min or 10 μM of doxorubicin was added and incubated for another 3 h. Subsequently, the cells were collected, centrifuged, and washed twice with cold PBS containing 2.5 μM FTC. Finally, cells were resuspended in 200 μL PBS.
and then subjected to fluorescence analysis by flow cytometry (Beckman Coulter, Cytomics FC500).

The accumulation of mitoxantrone in ABCG2 negative cell line HEK293/pcDNA3.1 and ABCG2-transfected cell lines was measured using \([^{3}H]\)-mitoxantrone. Confluent cells in 24-well plates were preincubated with or without 10 μM tandutinib for 3 h at 37 °C. The cells were then incubated with 0.2 μM \([^{3}H]\)-mitoxantrone for 3 h at 37 °C. To measure ABCG2 function as a drug efflux transporter, HEK293/pcDNA3.1 and ABCG2-482-R2 cells were incubated with \([^{3}H]\)-mitoxantrone containing medium (0.2 μM) for 2 h at 37 °C, then the cells were washed twice with ice-cold DMEM, and then cultured in \([^{3}H]\)-mitoxantrone free medium in the presence or absence of 10 μM tandutinib or 2.5 μM FTC at 37 °C. The cells were harvested at the indicated times and were lysed in 10 mM lysis buffer (pH 7.4, containing 1% Triton X-100 and 0.2% SDS) and were placed in scintillation fluid. Finally, the radioactivity was measured in a Packard Tri-Carb 1900CA liquid scintillation analyzer from Packard Instrument Company, Inc.

2.5. ATPase assay of ABCG2

The Vi-sensitive ATPase activity of ABCG2 in the membrane vesicles of High Five insect cells was measured as previously described (Ambudkar, 1998). The membrane vesicles (10 μg protein) were incubated in ATPase assay buffer [50 mM MES (pH 6.8), 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, 2 mM DTT, 1 mM ouabain, 10 mM MgCl₂] with or without 0.3 mM vanadate at 37 °C for 5 min and then incubated with different concentrations of tandutinib at 37 °C for 3 min. The ATPase reaction was induced by the addition of 5 mM Mg-ATP, and the total volume was 0.1 mL. After incubation at 37 °C for 20 min, the reactions were stopped by loading 0.1 mL of 5% SDS solution. The liberated inorganic phosphate (Pi) was measured as described previously (Ambudkar, 1998).

2.6. Photoaffinity labeling of ABCG2 with \([^{125}I]\)-IAAP

The photoaffinity labeling of ABCG2 with \([^{125}I]\)-IAAP was done as previously described (Dai et al., 2008). The crude membranes from MCF-7/Flv1000 cells expressing R482 ABCG2 was used for photolabeling experiments, and 50 μg of membrane protein was incubated at room temperature with different concentrations of tandutinib in the ATPase assay buffer with \([^{125}I]\)-IAAP (7 nM) for 5 min under subdued light. The samples were photo-crosslinked with 365-nm UV light for 10 min at room temperature. ABCG2 was immunoprecipitated with BXP-21 antibody. The samples were electrophoresed on SDS–PAGE in a 7% Tris–acetate NuPAGE gel; the gels were dried and exposed to Bio-Max MR film (Eastman Kodak Co.) at −70 °C for 12 h. The radioactivity incorporated into ABCG2 band was quantified using the STORM 860 PhosphorImager system and ImageQuaNT (Molecular Dynamics).

2.7. Flow cytometry

The side population (SP) cells were sorted by flow cytometry (Oates et al., 2009). Briefly, human A549 cells (1 × 10⁶ cells/ml) were incubated in RPMI1640 medium with 2% FBS containing freshly added Hoechst 33342 (5 μg/ml final concentration) for 90 min at 37 °C with intermittent mixing. In some experiments, cells were incubated with the Hoechst 33342 dye in the presence of verapamil (50 μM). At the end of incubation, cells were spun down and resuspended in ice-cold PBS. The cells were analyzed on a Coulter Epics flow cytometer. The Hoechst dye was excited with the UV laser at 351–364 nm and its fluorescence measured with a 515-nm side population filter (Hoechst blue) and a 608 EFLP optical filter (Hoechst red). A 540 DSP filter was used to separate the emission wavelengths.

2.8. Reverse transcription-PCR

After treatment with tandutinib, total RNA was isolated from cell cultures with Trizol Reagent (Invitrogen) according to the manufacture instruction. cDNA synthesis reaction was performed with reverse transcriptase (Promega Corp.). Oligonucleotide primers for ABCG2 and GAPDH were synthesized commercially (Invitrogen, Co., China). The PCR assays were performed using primers specific for ABCG2: 5′-TGGCTGTCACTGCTCACTA-3′ (sense) and 5′-GCCACGTGATTTCTTCCAA-3′ (antisense); for GAPDH: 5′-CACC-CTCCTTGCTGTCGCC-3′ (sense) and 5′-CTTGGATCTGGAAGAGA-3′ (antisense). The length of PCR products for ABCG2 and GAPDH are 235 bp and 475 bp, respectively. The GeneAmp PCR system 9700 (PE Applied Biosystem, Foster City, CA) was used and the reactions were carried out at 94 °C for 3 min for initial denaturation, and then at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min. After 35 cycles of amplification, additional extensions were done at 72 °C for 10 min. Products were resolved and examined by 1.5% agarose gel electrophoresis as described (Shi et al., 2006).

2.9. Western blot analysis

To determine whether tandutinib affects the expression of ABCG2, ABCG2-482-R2 cells were incubated with 10 μM tandutinib for 36 and 72 h, respectively, and S1-M1-80 cells were treated with 0.75, 1.5, 3 and 6 μM tandutinib for 48 h or with 3 μM tandutinib for 24, 48 and 72 h, respectively. To test whether tandutinib blocks Akt, Erk1/2, p38 or JNK phosphorylation, S1 and S1-M1-80 cells were incubated with 0.75, 1.5, 3.0 and 12 μM tandutinib for 24 h or with 3 μM tandutinib for 3, 6 and 12 h, respectively. After treatment, the cells were harvested and rinsed twice with ice-cold PBS and total cell lysates were collected with cell lysates buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min with gentle rocking and clarified by centrifugation at 12,000 rpm for 10 min at 4 °C (Yan et al., 2011). Equal amounts (100 μg of protein) of cell lysates were boiled for 20 min and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes. After being incubated in blocking solution containing 5% non-fat milk in TBST buffer (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20) for 2 h at room temperature, membranes were immunoblotted overnight with the appropriately diluted primary monoclonal antibodies against ABCG2, Akt, p-Akt, Erk1/2, p-Erk1/2, p-JNK, JNK, p-p38 or GAPDH at 4 °C. Then the membranes were washed thrice with TBST and incubated at room temperature for 2 h with HRP-conjugated secondary antibody at 1:5000 dilutions. After thrice wash with TBST, the protein-antibody complex were visualized by the enhanced Phototope TM-HRP Detection Kit (Cell Signaling, USA) and exposed to Kodak medical X-ray processor (Kodak, USA) (Dai et al., 2008).

2.9.1. Statistical analysis

All experiments were done at least three. Statistical analysis was done by Student’s t test analyses. The significance was determined at P < 0.05.

3. Results

3.1. Tandutinib reversed ABCG2-mediated MDR in vitro

To evaluate the reversal effect of tandutinib on ABCB1-, ABCG2-, ABCC1-, ABCC4- and LRP-mediated MDR in cancer cells, we first tested the cytotoxicity of tandutinib alone in different cell lines.
with the MTT assay. More than 90% of HEK293/pcDNA3.1 and ABCB1, ABCC1 and ABCG2 transfected cell lines were survived in the present of up to 10 μM of tandutinib, while for other cell lines used in this study, they were viable only up to 3 μM of tandutinib (data not shown). Therefore, we used different concentrations of tandutinib to test its reversal effect in different cell models according to their cytotoxicity in vitro. The IC_{50} values of topotecan to S1 and S1-M1-80 cells were 0.253 ± 0.012 μM and 15.148 ± 1.455 μM, respectively, which indicate that overexpression of ABCG2 resulted in a significant resistance to topotecan (59.9-fold); Similarly, ABCG2 transfected cells ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 also possessed the ability of resistance to SN-38 compared with HEK293/pcDNA3.1 cells (Tables 1 and 2). The sensitivity to topotecan was not altered when S1 cells were coinubated with tandutinib at concentration up to 3.0 μM (Table 1). In contrast, S1-M1-80 cells were sensitized to topotecan by tandutinib in a dose-dependent manner. Similarly, tandutinib restored the sensitivity of ABCG2 transfected cells to SN-38 in a concentration dependent manner but not in HEK293/pcDNA3.1 cells (Table 2). These results suggest that tandutinib potently reverses ABCG2-mediated resistance to topotecan and SN-38 in vitro.

To evaluate the transporter specificity, we also examined the effect of tandutinib on ABCB1-, ABCC1-, ABCC4- and LRP-mediated MDR in cancer cells. As shown in Tables 1 and 2, tandutinib showed no significant effect on ABCB1-, ABCC1-, ABCC4- and LRP-mediated drug resistance. Our data suggest that tandutinib probably specifically reverses ABCG2-mediated MDR.

In addition, the IC_{50} values of tandutinib to S1 and S1-M1-80 cells, HEK293/pcDNA3.1 and ABCG2-transfected cell lines were similar (data not shown), which suggest that overexpression of ABCG2 may not confer significant resistance to tandutinib.

3.2. Tandutinib modulated ABCG2-mediated transport

Our results indicated that tandutinib increased the sensitivity of ABCG2-overexpressing cells to certain chemotherapeutic agents. To elucidate the mechanism, we examined its effect on doxorubicin and rhodamine 123 accumulation in ABCG2-expressing S1-M1-80 cells and parental S1 cells. As shown in Fig. 1A, doxorubicin accumulation was significantly higher (6.7-fold) in S1 cells than in S1-M1-80 cells, and tandutinib increased doxorubicin accumulation in S1-M1-80 cells in a dose dependent manner. The intracellular accumulation of doxorubicin was 1.2-, 1.8- and 2.9-fold higher in S1-M1-80 cells in the presence of 0.75, 1.5 and 3.0 μM of tandutinib than that in the absence of tandutinib, respectively (Fig. 1A). In contrast, tandutinib did not significantly alter doxorubicin accumulation in S1 cells (Fig. 1A). Similarly, rhodamine 123 accumulation was also increased in S1-M1-80 cells by 1.5-, 1.9- and 2.9-fold, respectively (Fig. 1B). However, in the sensitive S1 cells, tandutinib did not significantly alter the intracellular accumulation of rhodamine 123 (Fig. 1B). In addition, tandutinib also increased [\(^3\)H]-mitoxantrone accumulation in wild-type ABCB2-overexpressing cell line ABCG2-482-R2 and mutant ABCG2-overexpressing cell lines ABCG2-482-G2 and ABCG2-482-T7, but did not in HEK293/pcDNA3.1 cells (Fig. 1C).

To determine whether tandutinib inhibits the function of ABCG2 as a drug efflux pump, the efflux of mitoxantrone from HEK293/pcDNA3.1 and ABCG2-482-R2 cells was examined. The time course of release of [\(^3\)H]-mitoxantrone after 2 h incubation at 37 °C was shown (Fig. 1D). ABCG2-482-R2 cells released a higher percentage of accumulated [\(^3\)H]-mitoxantrone than HEK293/pcDNA3.1 cells. At 30 min, 54% of the accumulated [\(^3\)H]-mitoxantrone was released from ABCG2-482-R2 cells, while only 7% was lost from HEK293/pcDNA3.1 cells. Tandutinib at 10 μM significantly inhibited the efflux of [\(^3\)H]-mitoxantrone from ABCG2-482-R2 cells and only about 5% of accumulated [\(^3\)H]-mitoxantrone was pumped out from ABCG2-482-R2 cells, but showed no apparent effect on the HEK293/pcDNA3.1 cells. Taken together, these results suggest that tandutinib inhibits ABCG2-mediated release of established ABCG2 substrates.

3.3. Tandutinib did not affect the ATPase activity of ABCG2

The drug-efflux function of ABCG2 is linked to ATP hydrolysis which is stimulated in the presence of ABCG2 substrates. To assess the effect of tandutinib on the ATPase activity of ABCG2, we measured ABCG2-mediated ATP hydrolysis using various concentrations of tandutinib. As shown in Fig. 2A, tandutinib did not significantly affect the ATPase activity of ABCG2. These data indicate that tandutinib may not be a substrate of ABCG2.

3.4. Tandutinib did not affect the photo-labeling of ABCG2 with [\(^3\)H]-IAAP

ABCG2 can be photo-labeled by a photoaffinity analog of prazosin, [\(^125\)I]-IAAP, and its substrates as well as inhibitors can compete for [\(^125\)I]-IAAP labeling site of ABCG2 (Shukla et al., 2006). Therefore, we examined the photo-labeling of ABCG2 with [\(^125\)I]-IAAP by incubating membrane vesicles in the presence of various concentrations of tandutinib in order to primarily understand the physical interaction of tandutinib with the substrate interaction sites of ABCG2. As indicated in Fig. 2B, tandutinib did not significantly inhibited the photoaffinity labeling of ABCG2 with [\(^125\)I]-IAAP. The results suggest that tandutinib binds to ABCG2 substrate-binding site(s) with low affinity.

### Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC(_{50})SD (μM) (fold-reversal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Topotecan</td>
<td>S1: 0.253 ± 0.012 (10.0) S1-M1-80: 15.148 ± 1.455 (10.0)</td>
</tr>
<tr>
<td>+0.75 μM tandutinib</td>
<td>0.202 ± 0.020 (13.0) 14.704 ± 1.026 (10.0)</td>
</tr>
<tr>
<td>+1.5 μM tandutinib</td>
<td>0.222 ± 0.018 (1.1) 10.545 ± 0.833 (1.4)</td>
</tr>
<tr>
<td>+3.0 μM tandutinib</td>
<td>0.262 ± 0.011 (10.0) 5.175 ± 0.55 (2.9)</td>
</tr>
<tr>
<td>+2.5 μM FTC</td>
<td>0.211 ± 0.016 (12.0) 1.04 ± 0.108 (1.4)</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>S1: 11.881 ± 1.181 (10.0) S1-M1-80: 10.885 ± 1.837 (1.0)</td>
</tr>
<tr>
<td>+3.0 μM FTC</td>
<td>11.187 ± 0.983 (1.1) 11.329 ± 0.822 (1.0)</td>
</tr>
<tr>
<td>+2.5 μM FTC</td>
<td>11.267 ± 1.378 (1.1) 10.902 ± 1.322 (1.1)</td>
</tr>
</tbody>
</table>

#### Data

Cell survival was determined by MTT assay as described in Materials and methods. Data are the means ± SD of at least three independent experiments performed in triplicate. The fold-reversal of MDR was calculated by dividing the IC\(_{50}\) for cells with the anticancer drug in the absence of modulators by that obtained in the presence of modulators.

\( P < 0.05 \) for the values versus that obtained in the absence of modulators.
The fold-reversal of MDR was calculated by dividing the IC50 for cells with the anticancer drug in the absence of modulators by that obtained in the presence of modulators.

Cell survival was determined by MTT assay as described in Materials and methods. Data are the means ± SD of at least three independent experiments performed in triplicate.

ABCG2 with small molecules dramatically lowered the number of SP cells and protects cells them from toxic effects. Blocking in cancer cells (Gagnon et al., 2008; Oh et al., 2006). To determine whether tandutinib could change the drug resistance property of SP cells, SP and non-SP cells were sorted by Hoechst 33342 staining from A549 cells (Fig. 6A). SP and non-SP cells were exposed to 0.2 μM mitoxantrone, a substrate of ABCG2, with or without tandutinib and analyzed for mitoxantrone induced apoptosis using annexin V-FITC/propidium iodide (PI) staining. As shown in Figs. 6B and C, higher apoptosis rate was observed in non-SP cells increased mitoxantrone-induced apoptosis in a dose-dependent manner in SP cells but did not in non-SP cells (Figs. 6B and C). These data suggest that tandutinib treatment maybe represent an effective strategy to conquer the drug resistance of SP cells in chemotherapy.

### 4. Discussion

Tyrosine kinase inhibitors (TKIs) belong to a new generation of chemotherapeutic agents and exert their action through competition with ATP for binding at the catalytic domain of tyrosine kinases to inhibit cellular signaling pathways, thus preventing activation of kinase activity. The ability of gefitinib and CI-1033 to interact with ABCG2 in vitro has been reported (Erl ichman et al., 2001; Nakamura et al., 2005). We also reported that erlotinib, lapatinib, apanitib and sildenafil reversed ABCB1- and ABCG2-mediated MDR in cancer cells through directly inhibiting the drug efflux function of ABCB1 and ABCG2 (Dai et al., 2008; Mi et al., 2010; Shi et al., 2007, 2011), and sunitinib reversed ABCG2 mediated drug resistance in vitro (Dai et al., 2009b).

In this report, we investigated the interaction of tandutinib with the MDR related ABC transporters, and found that tandutinib potentiated the cytotoxicity of ABCG2 substrate drugs such as topotecan in mitoxantrone induced ABCG2-overexpressing cell SP cells and also made them more susceptible to the cancer drug mitoxantrone (Bleau et al., 2009). Our results have shown that tandutinib reversed ABCG2-mediated drug resistance in vitro. To evaluate whether tandutinib could change the drug resistance property of SP cells, SP and non-SP cells were sorted by Hoechst 33342 staining from A549 cells (Fig. 6A). SP and non-SP cells were exposed to 0.2 μM mitoxantrone, a substrate of ABCG2, with or without tandutinib and analyzed for mitoxantrone induced apoptosis using annexin V-FITC/propidium iodide (PI) staining. As shown in Figs. 6B and C, higher apoptosis rate was observed in non-SP cells (13.7%) compared with SP cells (7.6%), which indicated that SP cells were more resistant to mitoxantrone than non-SP cells. Tandutinib increased mitoxantrone-induced apoptosis in a dose-dependent manner in SP cells but did not in non-SP cells (Figs. 6B and C). These data suggest that tandutinib treatment maybe represent an effective strategy to conquer the drug resistance of SP cells in chemotherapy.
line S1-M1-80 and SN-38 in ABCG2 transfected cell lines in a dose-dependent manner (Tables 1 and 2). However, the reversal effect was not observed in the parental S1 and HEK293/pcDNA3.1 cells. Tandutinib did not significantly alter the IC50 values of cisplatin, which is not an ABCG2 substrate, in both ABCG2 overexpressing cell lines and their parental cell lines (Tables 1 and 2). In addition, tandemribin had no significant reversal effect in ABCB1-, ABCC1-, ABCC4- and LRP-mediated MDR in vitro (Tables 1 and 2), which suggests that tandutinib may specifically reverse ABCG2-mediated drug resistance. Interestingly, both ABCG2-transfected and drug-induced ABCG2-overexpressing cells and their parental sensitive cells showed similar cytotoxicity to tandutinib (data not shown), which suggests that tandutinib may not a substrate of ABCG2.

Tandutinib increased doxorubicin and rhodamine 123 accumulation in S1-M1-80 cells in a dose-dependent manner, which was not observed in S1 cells (Fig. 1A and B). In addition, tandutinib increased [3H]-mitoxantrone accumulation in wild-type and mutant ABCG2 transfected cells and significantly inhibited [3H]-mitoxantrone efflux from ABCG2-482-R2 (wild-type) cells (Fig. 1C and D). P-gp knockdown in mouse reveals that tandutinib may be a sub-

---

**Fig. 1.** Effect of tandutinib on the accumulation of doxorubicin, rhodamine 123, mitoxantrone and the efflux of mitoxantrone. Intracellular accumulation of doxorubicin (A) and rhodamine 123 (B) in S1 and S1-M1-80 cells, mitoxantrone in HEK293/pcDNA3.1, ABCG2-482-R2, ABCG2-482-G2 and ABCG2-482-T7 (C), and the efflux of mitoxantrone from ABCG2-482-R2 cells (D) were measured as described in “Materials and Methods”. Data were shown as means ± SD of triplicate determinations. FTC as positive control. "p < 0.01 vs the control.
strate of P-gp (Yang et al., 2010); however, in our experiment model with P-gp overexpressing human cells, we found that all the P-gp overexpressing cells showed no resistance to tandutinib compared with parental or vector cells. Besides, compared with the well-known P-gp inhibitor verapamil, tandutinib did not reverse P-gp mediated multidrug resistance. These indicate that tandutinib is not a substrate of human P-gp. It may be the structural difference between human and mouse P-gp that accounts for the different observation between the former report and ours. Besides, unknown indirect mechanisms may account for the effect of P-gp on the disposition of tandutinib in the P-gp mouse model.

MDR modulators could stimulate, inhibit, or have no effect on ABCB1- or ABCG2-ATPase activity (Doige et al., 1992). As reported previously, lapatinib, erlotinib and apatinib stimulated ATPase activity of ABCB1 and ABCG2 (Dai et al., 2008; Mi et al., 2010; Shi et al., 2007), and FG020326 inhibited ATPase activity of ABCB1 slightly (Dai et al., 2009a). In contrast, LY335979 had no effect on ATPase activity of ABCB1 (Dantzig et al., 1996). As shown in Fig. 2A, tandutinib had significant effect on ATPase activity of ABCG2. Therefore, the reversal activity of tandutinib is independent of effect on the ATPase activity associated with ABCG2. These data, along with the observation that the cytotoxicity of tandutinib by itself is similar for drug-sensitive and drug-resistant cell lines...
suggest that tandutinib may not be a substrate of ABCG2. Interestingly, when the photolabeling of ABCG2 with $^{[125]}$I-IAAP was performed to elucidate interaction of tandutinib with the binding site of ABCG2, we found that tandutinib only slightly inhibited the photoaffinity labeling of ABCG2 with $^{[125]}$I-IAAP (Fig. 2B) indicating that tandutinib may not have similar binding sites as $^{[125]}$I-IAAP. Taken together, these data suggest that tandutinib may not alter the pharmacokinetics of conventional chemotherapeutic agents that are affected by ABC transporters.

Receptor tyrosine kinases such as c-KIT, FLT3, PDGFR and VEGFR play important roles in regulating cell proliferation, differentiation and survival by activating downstream effectors such as signal transducers and activators of transcription (STAT), protein kinase B/AKT and extracellular signal-regulated kinase 1/2 (Erk1/2) (Kessler et al., 2007; Roskoski, 2005). Several recent investigations have shown that P38/Akt or Erk pathway activation is concerned with resistance to conventional chemotherapeutic agents in various cancer cells (Navolanic et al., 2003; West et al., 2002). It has been reported that targeting the p38 MAPK (Barancik et al., 2001; Paillas et al., 2011; Yang et al., 2011) and JNK (Zhang et al., 2013) pathway inhibits the multidrug resistance in some cancer in vivo and in vitro. Tandutinib is a novel quinazoline-based inhibitor of FLT3, PDGFR, and KIT. To address whether the reversal effect of tandutinib is also dependent on the blockade of phosphorylation of AKT, Erk, p38 and JNK, we determined the effect of tandutinib on the AKT, Erk, p38 and JNK phosphorylation. Tandutinib did not inhibit the phosphorylation of AKT, Erk, p38 and JNK at 3 $\mu$M (Figs. 4 and 5). In addition, tandutinib did not downregulate ABCG2 expression at RNA or protein level (Fig. 3). Taken together, our data suggest that tandutinib restore the sensitivity of ABCG2-overexpressing cells to substrate anticancer drugs by inhibiting its function as drug efflux pump.

Our data has demonstrated that ABCG2 effectively increased the intracellular concentrations of anticancer chemotherapeutic agents such as topotecan and SN-38 by directly inhibiting the function of ABCG2. Intriguingly, stem cells are frequently identified as the “side population” (SP) by flow cytometry based on ABCG2-mediated efflux of Hoechst dye 33342 (Goodell et al., 1996). SP cells were first isolated from bone marrow and more recently from various solid tumors and cell lines (Wu and Alman, 2008). SP cells
are pluripotent and have greater growth capacity and resistance to cytotoxic drugs and more tumorigenic after injection into nude mice when isolated from solid tumors as compared with non-SP cells (Bleau et al., 2009; Hirschmann-Jax et al., 2004; Ho et al., 2007; Patrawala et al., 2005). Though the precise physiological function of ABCG2 in SP cells remains unknown; however, the recent studies using ABCG2−/− mice suggested that ABCG2 expression in SP cells is to provide protection from cytotoxic substrates (Zhou et al., 2002). Indeed, The expression of ABCG2 in SP cells selected from saccule indicated that ABCG2 participates in resistance of SP cells to chemotherapeutic drugs such as mitoxantrone (Bleau et al., 2009), and chemotherapy efficacy was limited by ABCG2 expression in the cancer stem-like cells that presumably repopulate the tumor after therapy (Robey et al., 2007). Therefore, the ability of ABCG2 to protect stem cells and as a selectable marker may prove useful for ABCG2 in gene therapy applications (Sarkadi et al., 2004). In order to investigate the role of ABCG2 function in SP cells and evaluate whether tandutinib could inhibit the function of ABCG2 in SP cells, we sorted SP and non-SP cells by Hoechst 33342 staining from A549 cells (Fig. 6A). We found that SP cells were more resistant to mitoxantrone-induced apoptosis than non-SP cells. Importantly, tandutinib increased mitoxantrone-induced apoptosis in SP cells but not in non-SP cells in a dose-dependent manner (Fig. 6B and C). These data suggest that tandutinib inhibits ABCG2-mediated drug efflux in SP cells, which may be useful for overcoming drug resistance in chemotherapy.

In conclusion, tandutinib significantly reverses ABCG2-mediated drug resistance by inhibiting the drug efflux function of ABCG2 and increasing intracellular accumulation of cytotoxic agents in ABCG2-overexpressing cells. Importantly, tandutinib restores the sensitivity of SP cells to chemotherapeutic agents and exerts positive effect on overcoming ABCG2-mediated MDR.

Acknowledgements

This work was supported by the Grants from Chinese National Natural Sciences Foundation Nos. 30672407 and 81072669 (LWF), Chinese Ministry of Education Postdoctor Foundation No. 201004070959 (CLD) and NIH R15 No. 1R15CA143701 (ZSC). We thank Drs S.E. Bates and R.W. Robey (National Cancer Institute, NIH) for the ABCG2 expressing cell line S1-M1-80 and their parental sensitive cell line S1. We thank Gary D. Kruh (Medical Sciences Division, Fox Chase Cancer Center) for murine fibroblasts cell line NIH3T3 and the ABC4-transfected derivative ABC4C stable expressing NIH3T3/MRP4-2.

References

Shukla, S., Robey, R.W., Bates, S.E., Ambudkar, S.V., 2008. Sunitinib (Sutent®), a small-molecule receptor tyrosine kinase inhibitor, blocks function of the ABC transporters, P-glycoprotein (ABCB1) and ABCG2. Drug Metabolism and Disposition: The Biological Fate of Chemicals.