Masitinib Antagonizes ATP-Binding Cassette Subfamily C Member 10–Mediated Paclitaxel Resistance: A Preclinical Study

Rishil J. Kathawala1, Kamlesh Sodani1, Kang Chen2,3,4,5, Atish Patel1, Alaa H. Abuznait6, Nagaraju Anreddy1, Yue-Li Sun1, Amal Kaddoumi6, Charles R. Ashby Jr1, and Zhe-Sheng Chen1

Abstract
Paclitaxel displays clinical activity against a wide variety of solid tumors. However, resistance to paclitaxel significantly attenuates the response to chemotherapy. The ABC transporter subfamily C member 10 (ABCC10), also known as multidrug resistance protein 7 (MRP7) efflux transporter, is a major mediator of paclitaxel resistance. In this study, we show that masitinib, a small molecule stem-cell growth factor receptor (c-Kit) tyrosine kinase inhibitor, at nontoxic concentrations, significantly attenuates paclitaxel resistance in HEK293 cells transfected with ABCC10. Our in vitro studies indicated that masitinib (2.5 µmol/L) enhanced the intracellular accumulation and decreased the efflux of paclitaxel by inhibiting the ABCC10 transport activity without altering the expression level of ABCC10 protein. Furthermore, masitinib, in combination with paclitaxel, significantly inhibited the growth of ABCC10-expressing tumors in nude athymic mice in vivo. Masitinib administration also resulted in a significant increase in the levels of paclitaxel in the plasma, tumors, and lungs compared with paclitaxel alone. In conclusion, the combination of paclitaxel and masitinib could serve as a novel and useful therapeutic strategy to reverse paclitaxel resistance mediated by ABCC10. Mol Cancer Ther; 13(3); 714–23. ©2014 AACR.

Introduction
Paclitaxel alone or in combination with other antineoplastic agents has been the first-line drug for the treatment of non–small cell lung cancer (1). Paclitaxel is also used to treat a variety of other metastatic or advanced cancers, including metastatic ovarian, breast, head and neck cancer, and advanced Kaposi sarcoma (2). The novel mechanism of action of paclitaxel suggested that it would demonstrate antineoplastic efficacy in multidrug resistant tumor cells (3). Unfortunately, as with other chemotherapeutic drugs, resistance is commonly seen with paclitaxel treatment. One emerging and recently identified cause for paclitaxel resistance is its active efflux from the cells due to the expression of the ATP-binding cassette subfamily C member 10 (ABCC10), also known as multidrug resistant protein 7 (MRP7), located on the basolateral cell surface (4–7). The human ABCC10 transporter is a 171-kDa protein containing three membrane spanning domains (MSD1, MSD2, and MSD3) and two nucleotide binding domains (NBD1 and NBD2), and belongs to the class of long ABCCs, such as ABCC1, ABCC2, ABCC3, and ABCC6. Apart from paclitaxel, ABCC10 is reported to mediate the cellular efflux of several other antineoplastic drugs, including docetaxel, vincristine, vinblastine, vinorelbine, cytarabine, gemcitabine, 2',3'-dideoxyctydidine, 9-(2-phosphonyl methoxyethyl)adenine (PMEA), and epothilone B, and endogenous substances such as estra
diol-17β-3-gluconuride (E2;17BG) and leukotriene C4 (5, 8, 9).

Masitinib, a novel phenylaminothiazole-type derivative (Fig. 1A), is an inhibitor of the class III receptor tyrosine kinase stem-cell growth factor receptor (c-Kit), platelet-derived growth factor receptor α and β and the nonreceptor tyrosine kinase Lyn (10, 11). Masitinib is not active against kinases whose inhibition has been associated with significant toxic effects, such as breakpoint cluster region-ABL, vascular endothelial growth factor, and endothelial growth factor (10, 12). In a phase II trial, masitinib has been reported to significantly increase the overall survival rate and progression-free survival in...
patients with locally advanced or metastatic gastrointestinal stromal tumor (13–16).

We have previously established that mechanistically unrelated compounds, such as cepharanthine (17), nilotinib (18), erlotinib (19), tariquidar (20), and tandutinib (21), can sensitize ABCC10-transfected HEK293/ABCC10 cells to paclitaxel. However, these compounds also inhibited ABCB1-mediated paclitaxel resistance, rendering cells to paclitaxel. However, these compounds also inhibited ABCB1-mediated paclitaxel resistance, rendering them nonspecific and toxic (22–24). Here, we discover that masitinib, an inhibitor of receptor tyrosine kinases (25), thereby increasing the intracellular concentration of paclitaxel. We further demonstrate that masitinib enhanced paclitaxel-mediated inhibition of the growth of ABCC10-expressing tumor in a tumor xenograft mouse model in vivo and report the pharmacokinetics of paclitaxel in combination with masitinib.

**Materials and Methods**

**Materials**

[^3H]-Paclitaxel (37.9 Ci/mmol) was purchased from Moravek Biochemicals, Inc. Dulbecco’s Modified Eagle Medium (DMEM), Iscove’s DMEM, FBS, PBS, 10,000 IU/mL penicillin, and 10,000 μg/mL streptomycin, and trypsin (0.25%) were purchased from Hyclone. Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Cell Signaling Technology. Antibody D-19 against ABCC10 was obtained from Santa Cruz Biotechnology, Inc. Monoclonal antibody Ab81 against c-Kit and monoclonal antibody D12E12 against phospho-c-Kit (p-c-Kit) were obtained from Cell Signaling Technology. Masitinib was a gift from AB Science. Cepharanthine was generously given by Kakusohyaku Co. PAK-104P was a gift from Nissan Chemical Industries. Paclitaxel, docetaxel, vincristine, vinblastine, and cisplatin were purchased from Tocris Bioscience. Boron-dipyrromethene (BODIPY) FL (fluorescent) paclitaxel was purchased from Life Technologies, Invitrogen. 3-(4,5-Dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and verapamil were obtained from Sigma-Aldrich.

**Cell lines and cell culture**

The parental empty pcDNA3.1 plasmid-transfected cell line, HEK293/pcDNA3.1, and the cell line stably transfected with pcDNA3.1 containing an expression construct encoding ABCB1 (HEK293/ABCB1) were used in all experiments as previously reported (8, 20). The HEK293/ABCB1 and HEK293/ABCC1 cell lines were kindly provided by Dr. Suresh V. Ambudkar (NCI, NIH, Bethesda, MD) in 2012. HEK293/pcDNA3.1, HEK293/ABCB1, HEK293/ABCC1, and HEK293/ABCC1 cell lines were cultured in DMEM, supplemented with 10% heat-inactivated FBS and 1% of 100 times diluted 10,000 IU/mL penicillin-10,000 μg/mL streptomycin (25). All in vitro experiments were conducted at 60% to 80% cell confluency. The human mast cell leukemia cell line HMC-1 was obtained from Dr. Andrea Cerutti (Icahn School of Medicine at Mount Sinai, NY) in 2012 and cultured in Iscove’s DMEM, supplemented with 10% heat-inactivated FBS and 1% of 100 times diluted 10,000 IU/mL penicillin-10,000 μg/mL streptomycin (26, 27). Cells used in the in vitro experiments were trypsinized and centrifuged at 2,000 rpm for 2 minutes at 25°C, washed twice with PBS, and reconstituted in DMEM at a concentration of 1 × 10^7 cells. HEK293/pcDNA3.1 and HEK293/ABCC10 cell lines were authenticated using short tandem repeat analysis by American Type Culture Collection. HMC-1, HEK293/ABCB1, and HEK293/ABCC1 cell lines were not authenticated.

**Cell viability assay**

A modified MTT assay was performed to detect the sensitivity of the cells to the anticancer drugs in vitro (28). The cell numbers seeded into 96-well plates were 5,000 per well for HEK293/pcDNA3.1 and HEK293/ABCC10. Every MTT assay was run in triplicate and drugs tested included paclitaxel (0.001 to 1 μmol/L), docetaxel (0.001 to 1 μmol/L), vincristine (0.001 to 1 μmol/L), vinblastine (0.001 to 1 μmol/L), masitinib (0.625, 1.25, and 2.5 μmol/L), cepharanthine (2.5 μmol/L), verapamil (10 μmol/L), and PAK-104P.
(5 μmol/L). After seeding cells in 180 μL medium in 96-well plates and incubating for 24 hours at 37°C, 20 μL of the appropriate anticancer drug at various concentrations was added (20 μL of fixed concentration of test compound for reversal were added 1 hour prior to adding anticancer drugs). Subsequently, the anticancer drugs, in DMEM supplemented with 10% FBS, were incubated at 37°C for 72 hours. After 72 hours, 20 μL MTT (4 mg/mL) was added to each well. The plates were incubated at 37°C for another 4 hours. The MTT with medium was removed from each well, and 100 μL of DMSO was added to each well. The absorbance was read at 570 nm by an Opsys microplate reader (Dynex Technologies). The degree of resistance was calculated by dividing the IC50 (calculated using Bliss method) for resistant cells by that of the parental sensitive cells (29).

The degree of the reversal of resistance was calculated by dividing the IC50 for cells with the anticancer drug resistant cells by that of the parental sensitive cells (29).

**[3H]-Paclitaxel accumulation assay**

HEK293/pcDNA3.1 and HEK293/ABCC10 cells were harvested and transferred to DMEM supplemented with 10% FBS in the presence or absence of inhibitor (masitinib or cepharanthine at 2.5 μmol/L). After 2 hours at 37°C, cells were centrifuged at 4,000 rpm for 3 minutes and resuspended in medium with or without the inhibitor, in addition to 40 nmol/L BODIPY-paclitaxel. One hour later, cells were centrifuged at 4,000 rpm for 3 minutes, resuspended, and immediately analyzed in an LSRFortessa flow cytometer (BD Biosciences). 7-Aminoactinomycin D (BD Biosciences) was used to exclude nonviable cells from the analysis. The amount of BODIPY-paclitaxel that accumulated inside the cells was determined by overlaying histograms of cells that were incubated with masitinib. The distance between the two peaks indicated the amount of BODIPY-paclitaxel accumulation (30).

**[3H]-Paclitaxel efflux assay**

Cells were exposed to the same procedure as stated in the drug accumulation experiment and then incubated in fresh medium at 37°C at various times (0, 30, 60, and 120 minutes) in the presence or absence of the inhibitor (masitinib or cepharanthine at 2.5 μmol/L). After washing three times with ice-cold PBS, the cells were lysed using 200 μL lysis buffer and transferred to scintillation vials. Each sample was placed in scintillation fluid and radioactivity was measured in a liquid scintillation counter (Packard Instrument).

**Immunoblot analysis**

To determine whether masitinib affects the expression of ABCC10, and is able to block c-Kit and p-c-Kit, cells were incubated with masitinib at 2.5 μmol/L for different time periods (0, 24, 48, and 72 hours). Then, approximately 6 × 10^5 cells were harvested and suspended in PBS, and centrifuged at 2,000 rpm for 2 minutes, followed by two washings with PBS. The lysis buffer (1X PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 100 mg/mL p-aminophenylmethylsulfonyl fluoride) and 1% aprotinin were added to the suspension followed by vortexing. The resuspended cells were kept on ice for 30 minutes, followed by centrifugation at 12,000 rpm for 20 minutes. The supernatant was separated and stored in −80°C for the experiment. Protein concentrations in the lysates were determined by the bicinchoninic acid–based protein assay. Equal amounts of total cell lysates (40 μg protein) were resolved by SDS–PAGE electrophoresis and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. After incubation in a blocking solution in TBS + Tween 20 (TBST) buffer (10 μmol/L Tris-HCl, pH 8.0, 150 μmol/L NaCl, and 0.1% Tween 20) for 1 hour at room temperature, the membranes were immunoblotted overnight with primary monoclonal antibodies against ABCC10 at 1:200 dilution, c-Kit, p-c-Kit, or GAPDH at 1:1,000 dilution. The protein–antibody complex was detected using chemiluminescence.

**Methodology for the tumor xenograft model**

Male athymic NCR (nu/nu) nude mice (NCRI NU M, homozygous, albino; 18–25 g; 6–10 week; Taconic Farms) were used for the tumor xenograft experiments. All animals were maintained on an alternating 12 hours light/dark cycle with ad libitum water and rodent chow. The ABCC10-expressing HEK293/ABCC10 model was designed for the first time with a slight modification of the KBv200 cell xenograft model previously established by Chen and colleagues (31, 32). Briefly, HEK293/pcDNA3.1 and HEK/ABCC10 (1.0 × 10^7) cells were injected subcutaneously under the armpits. When the tumors reached a mean diameter of 0.5 cm (day 0), the mice were randomized into four groups (n = 8) and treated with one of the following regimens: (i) vehicle
(autoclaved water; q3d × 6), (ii) paclitaxel [15 mg/kg, intraperitoneally (i.p.), q3d × 6], (iii) masitinib diluted in autoclaved water [12.5 mg/kg, per os (p.o.), q3d × 6], and (iv) masitinib (12.5 mg/kg, p.o., q3d × 6, given 1 hour before giving paclitaxel) + paclitaxel (15 mg/kg, i.p., q3d × 6). Paclitaxel was prepared by dissolving 6 mg paclitaxel in 50% of dehydrated alcohol (EMD) and 50% of Cremophor ELP (BASF). The tumor sizes were measured using calipers and body weights were recorded (29). The body weight of the animals was monitored every third day to adjust the drug dosage and to determine treatment-related toxicities as well as disease progression. The two perpendicular diameters of tumors were recorded every third day and the tumor volume was estimated (29, 31). All the animals were killed by terminal bleeding through cardiac puncture under isofluorane anesthesia, and plasma, various organs, and tumor tissue were excised and stored at −80°C. All mice were maintained at the St. John’s University Animal Facility. The IACUC at St. John’s University approved this project, and the research was conducted in compliance with the Animal Welfare Act and other federal statutes. Animals were treated humanely and cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals, and all studies were approved and supervised by the IACUC at St. John’s University (Queens, NY).

Collection of plasma and tissues

In a separate group of experiments, mice bearing HEK293/ABCC10 tumors were divided into two groups: (i) vehicle pretreatment (given 1 hour before paclitaxel; 15 mg/kg); (ii) masitinib pretreatment (12.5 mg/kg p.o.) and paclitaxel (n = 7). After treatment, animals were anesthetized and blood was obtained using supraorbital puncture and placed in heparinized tubes and plasma was harvested at 10, 30, 60, 120, or 240 minutes after paclitaxel administration in both groups. In addition, the tumors and lungs were removed, weighed, snap-frozen in liquid nitrogen, and stored at −80°C until analysis (32).

High-performance liquid chromatography conditions

Quantification of paclitaxel was conducted using an isocratic Shimadzu LC-20AB liquid chromatograph equipped with an SIL-20A HT autosampler and LC-20AB pump connected to a Dgu-20A3 degasser (Shimadzu), according to the method described by Gill and colleagues (33). The column used was a reversed-phase, Phenomenex Luna C18 column (250 × 4.6 mm i.d., 5 μm; Phenomenex) with an octadecyl silane guard column (4 mm × 3 mm; Phenomenex). The injection volume was 20 μL, and the mobile phase used for the separation of paclitaxel in plasma and tissue homogenate samples consisted of acetonitrile and water (53:47, v/v) delivered at 1.0 mL/min flow rate. For paclitaxel detection, the UV SPD-20A (Shimadzu)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>HEK293/pcDNA3.1</th>
<th>HEK293/ABCC10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 ± SD (nmol/L)</td>
<td>FR</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>8.86 ± 0.9</td>
<td>[1.0]</td>
</tr>
<tr>
<td>Paclitaxel + masitinib 0.625 μmol/L</td>
<td>8.53 ± 1.1</td>
<td>[1.0]</td>
</tr>
<tr>
<td>Paclitaxel + masitinib 1.25 μmol/L</td>
<td>7.67 ± 1.5</td>
<td>[0.9]</td>
</tr>
<tr>
<td>Paclitaxel + masitinib 2.5 μmol/L</td>
<td>6.69 ± 0.7</td>
<td>[0.7]</td>
</tr>
<tr>
<td>Paclitaxel + cepharanthine 2.5 μmol/L</td>
<td>5.65 ± 0.4b</td>
<td>[0.6]</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>8.69 ± 1.2</td>
<td>[0.9]</td>
</tr>
<tr>
<td>Docetaxel + masitinib 0.625 μmol/L</td>
<td>7.44 ± 1.6</td>
<td>[0.8]</td>
</tr>
<tr>
<td>Docetaxel + masitinib 1.25 μmol/L</td>
<td>6.81 ± 0.8</td>
<td>[0.8]</td>
</tr>
<tr>
<td>Docetaxel + masitinib 2.5 μmol/L</td>
<td>5.62 ± 0.9b</td>
<td>[0.6]</td>
</tr>
<tr>
<td>Docetaxel + cepharanthine 2.5 μmol/L</td>
<td>7.22 ± 0.5</td>
<td>[0.8]</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>5,244.6 ± 426.3</td>
<td>[1.0]</td>
</tr>
<tr>
<td>Cisplatin + masitinib 2.5 μmol/L</td>
<td>5,523.0 ± 159.3</td>
<td>[1.0]</td>
</tr>
<tr>
<td>Cisplatin + cepharanthine 2.5 μmol/L</td>
<td>4,532.1 ± 181.8</td>
<td>[0.9]</td>
</tr>
</tbody>
</table>

Table 1. Masitinib significantly sensitizes HEK293/ABCC10 cells to paclitaxel and docetaxel, but not cisplatin, with no or minimal effect on HEK293/pcDNA3.1 cells

-aIC50, the drug concentration that inhibited cell survival by 50% (means ± SD).
-bFold resistance (FR) was determined by dividing the IC50 values of substrate in HEK293/ABCC10 cells by the IC50 of substrate in HEK293/pcDNA3.1 cells in the absence of masitinib, or the IC50 of substrate in HEK293/pcDNA3.1 cells in the presence of masitinib divided by the IC50 of substrate in HEK293/pcDNA3.1 cells in the absence of masitinib. Values in the table are representative of at least three independent experiments, each performed in triplicate.
-cSignificant statistical difference from the IC50 values of HEK293/pcDNA3.1 without reversal drug; P < 0.05.
-dSignificant statistical difference from the IC50 values of HEK293/ABCC10 without reversal drug; P < 0.01.
detector set at 227 nm was used. Data acquisition and analysis was achieved using LC Solution software version 1.22 SP1 (Shimadzu). All samples were analyzed in duplicate. Under these chromatographic conditions, the total run time was 15 minutes, with a retention time of 12 minutes for paclitaxel. Standard curves for paclitaxel in plasma and tissue homogenates were prepared in the ranges of 25 to 5,000 ng/mL. The analytical method described in this work has been already established and validated previously (32, 33).

**Extraction of paclitaxel from plasma and tissue homogenate samples**

A simple, one-step protein precipitation with acetonitrile was used for sample preparation. Tumor and lung tissues were homogenized in saline (1:2, v/v). Paclitaxel was extracted from plasma and tissue homogenate samples by precipitation with acetonitrile in 1:1 and 1:2 ratios (v/v), respectively. Samples were vortexed for 1 minute, followed by centrifugation for 10 minutes at 10,000 rpm. The supernatant was transferred to insert vials from which 20 µL was injected onto the high-performance liquid chromatography (HPLC) column. Samples with concentrations higher than the calibration range limit were appropriately diluted to fit within the working calibration curve.

**Statistical analyses**

All experiments were repeated at least three times and the differences were determined using the two-tailed Student t test and statistical significance was determined at \( P < 0.05 \).

**Results**

**Masitinib significantly enhances the sensitivity of HEK293/ABCC10 cells to paclitaxel**

Before determining the effect of masitinib on paclitaxel resistance, we examined its effect on the growth of the cell lines used in our study using MTT assay. On the basis of the cytotoxicity assays, we chose to use masitinib (Fig. 1B) at concentrations of 0.625, 1.25, and 2.5 µmol/L, because at these concentrations, at least 80% to 90% of the cells survived. Masitinib, at 0.625, 1.25, and 2.5 µmol/L, significantly decreased the resistance to paclitaxel and docetaxel in the HEK293/ABCC10 cell line as compared with the control HEK293/pcDNA3.1 cells (Table 1). Cepharanthine (2.5 µmol/L), which has been shown to inhibit ABCC10 function, significantly decreased the resistance of HEK293/ABCC10 to paclitaxel as compared with the parental HEK293/pcDNA3.1 (17). The incubation of 2.5 µmol/L of masitinib or cepharanthine did not significantly alter the IC\(_{50}\) values of cisplatin, which is not a substrate for ABCC10 in HEK293/pcDNA3.1 and HEK293/ABCC10 cells (34). Masitinib also significantly increased the response of HEK293/ABCC10 cells to vincristine and vincristine, which are substrates of ABCC10 (Supplemental Table 1). To determine the effect of masitinib on the ABCB1 and ABCC1 transporters, we chose the HEK293/ABCB1 and HEK293/ABCC1 cells lines, which express the ABCB1 and ABCC1 transporters, respectively. We used paclitaxel and vincristine as substrates for ABCB1 and ABCC1, respectively. Masitinib did not significantly reverse ABCB1- and ABCC1-mediated resistance to paclitaxel or vincristine (Supplemental Tables 2 and 3).

**Masitinib increases the cellular accumulation of BODIPY-paclitaxel in HEK293/ABCC10 cells**

The accumulation of BODIPY-paclitaxel, a substrate for ABCC10, was determined in HEK293/ABCC10 cells using flow cytometry. The incubation of HEK293/ABCC10 cells with masitinib (2.5 µmol/L) significantly increased the accumulation of BODIPY-paclitaxel in the cells expressing ABCC10 compared with HEK293/pcDNA3.1 cells (Fig. 2).

![Figure 2. Masitinib increases the cellular accumulation of BODIPY-paclitaxel in HEK293/ABCC10 cells. The accumulation of BODIPY-paclitaxel alone or with masitinib was measured after the cells (HEK293/pcDNA3.1 and HEK293/ABCC10) were preincubated with or without masitinib (2.5 µmol/L) for 2 hours at 37°C and then incubated with 40 nmol/L BODIPY-paclitaxel for another 60 minutes at 37°C. All samples were analyzed in a flow cytometer.](mct.aacrjournals.org)
Masitinib increases the cellular accumulation of [\(^3\)H]-paclitaxel in HEK293/ABCC10 cells

To determine the mechanism responsible for the reversal action of masitinib at the ABCC10 transporter, we examined the effect of masitinib on the intracellular accumulation of [\(^3\)H]-paclitaxel in HEK293/ABCC10 cells. Masitinib produced a significant, concentration-dependent increase in the intracellular accumulation of [\(^3\)H]-paclitaxel compared with cells incubated with the solvent buffer for masitinib (Fig. 3A). In addition, the results obtained with masitinib were comparable to those obtained using the known ABCC10 transport inhibitor cepharanthine.

Masitinib decreases the cellular efflux of [\(^3\)H]-paclitaxel in HEK293/ABCC10 cells

We further determined the amount of [\(^3\)H]-paclitaxel present in the cells following incubation with masitinib. The amount of [\(^3\)H]-paclitaxel present in the intracellular medium of HEK293/ABCC10 cells was significantly lower compared with that of HEK293/pcDNA3.1 cells, due to the active efflux of the [\(^3\)H]-paclitaxel via ABCC10. However, over a period of time (0, 30, 60, and 120 minutes), masitinib (2.5 \(\mu\)mol/L) significantly reduced the efflux of [\(^3\)H]-paclitaxel in HEK293/ABCC10 (Fig. 3B).

Masitinib has no effect on the expression levels of ABCC10, c-Kit, and p-c-Kit

Immunoblot analysis of ABCC10 indicated a band with a molecular weight of about 171 kDa in the HEK293/ABCC10 cell lysates, suggesting the presence of ABCC10. In contrast, this band was not present in HEK293/pcDNA3.1, indicating the absence of the ABCC10 protein (Fig. 4A). To confirm that the paclitaxel resistance modulation by masitinib was not due to a decrease in the expression of the ABCC10 protein, we incubated the cells with masitinib (2.5 \(\mu\)mol/L) for 0, 24, 48, and 72 hours. Masitinib did not significantly alter the expression levels of the ABCC10 transporter in the HEK293/ABCC10 cells (Fig. 4B). In a different set of experiment, we found that expressions of c-Kit (145-kDa) and p-c-Kit (145-kDa) proteins were not detected in HEK293/pcDNA3.1 or HEK293/ABCC10 cell lysates where HMC-1 cell lysate was used as positive control (Supplemental Fig. 1). These findings suggested that the reversal of paclitaxel resistance by masitinib did not result from the alteration in ABCC10, c-Kit, or p-c-Kit protein expression.
Masitinib significantly potentiates the anticancer activity of paclitaxel in an ABCC10-expressing tumor xenograft model

The i.p. dose of paclitaxel (15 mg/kg) used in this study was determined after a series of pilot experiments, which indicated that it produced significant resistance in HEK293/ABCC10 tumor xenograft model compared with the HEK293/pcDNA3.1 tumor xenograft model (32). Masitinib, alone (12.5 mg/kg p.o.) or in combination with paclitaxel, did not produce any visible toxicity or phenotypic changes in the male athymic NCR nude mice. The tumors expressing the ABCC10 transporter showed significant resistance to a 15 mg/kg i.p. dose of paclitaxel (Fig. 5A–C). This is in contrast with the HEK293/pcDNA3.1 tumor, which was almost completely eliminated by 15 mg/kg of paclitaxel (Supplemental Fig. 2A and B). No apparent weight loss was observed among the treatment groups compared with animals treated with vehicle (Fig. 5D). Masitinib (12.5 mg/kg, p.o.), in combination with paclitaxel (15 mg/kg, i.p.), significantly decreased the sizes, weights, and tumor volumes of the tumors expressing the ABCC10 transporter (HEK293/ABCC10).

Figure 4. Immunoblot analysis of the expression of ABCC10 transporter. A, the expression of ABCC10 in HEK293/pcDNA3.1 and HEK293/ABCC10 cells. B, the expression of the ABCC10 protein in HEK293/ABCC10 cells treated with masitinib (2.5 \( \mu \)mol/L) for the indicated period of time, determined by immunoblot. GAPDH was used as a loading control. Data presented are representative of three independent experiments.

Figure 5. The effect of masitinib on the growth of ABCC10-expressing tumors in nude athymic mice. A, images of excised HEK293/ABCC10 tumors implanted subcutaneously in athymic NCR nude mice (n = 8) that were treated with vehicle, paclitaxel, masitinib, or the combination of masitinib and paclitaxel, at the end of the 18-day treatment period. Results are representative of three independent experiments performed. B, the changes in tumor volume over time following the implantation. Data points represent the mean tumor volume for each treatment group (n = 8). Error bars, SEM. *, P < 0.01 versus the vehicle group; **, P < 0.01 versus the paclitaxel-alone group. C, mean weight (n = 8) of the excised HEK293/ABCC10 tumors from the mice treated with vehicle, paclitaxel, masitinib, or the combination of masitinib and paclitaxel, at the end of the 18-day treatment period. Error bars, SEM. †, P < 0.05 versus vehicle group; ††, P < 0.05 versus the paclitaxel group. D, the changes in mean body weight of mice (n = 8) before and after the treatment. NS, not statistically significant (P > 0.05).
ABCC10) over a period of 18 days, compared with animals treated with vehicle, masitinib alone, or paclitaxel alone (*P < 0.01; Fig. 5A–C, respectively). These results suggest that masitinib significantly attenuates paclitaxel resistance in tumors expressing the ABCC10 transporter.

Masitinib increases the plasma and tumor paclitaxel concentration in ABCC10-expressing tumor xenograft model

In a separate study, we measured the plasma, tumor, and lung concentrations of paclitaxel in animals pretreated with vehicle or masitinib (12.5 mg/kg, p.o.) before the administration of paclitaxel (15 mg/kg, i.v.). The pharmacokinetic data showed that coadministration of masitinib and paclitaxel produced a transient increase in the plasma levels of paclitaxel after 10 minutes of administration, but not at subsequent time points (Fig. 6A). The combination of masitinib and paclitaxel significantly increased the intratumoral concentration of paclitaxel (69.93 ± 14.15 ng/mL) as compared with paclitaxel administration alone (16.31 ± 6.45 ng/mL, *P < 0.05; Fig. 6B) after 240 minutes following the administration. However, the coadministration of masitinib and paclitaxel also did not significantly increase the concentration of paclitaxel in the lungs as compared with paclitaxel administration alone (Fig. 6C) after 240 minutes following the administration. These data suggest that masitinib-induced paclitaxel sensitivity in ABCC10-expressing tumors is due at least in part to its direct inhibition of the transporter activity of ABCC10, thereby increasing the intracellular accumulation of paclitaxel.

Discussion

One of the major findings of this study was that masitinib significantly enhanced the sensitivity of HEK293/ABCC10 cells to paclitaxel and docetaxel, and to other substrates, such as vinblastine and vincristine. In addition, masitinib did not significantly potentiate the toxic effect of cisplatin, a drug that is not a substrate for the ABCC10 transporter, further supporting the specificity of masitinib. To our knowledge, this is the first study demonstrating that masitinib potentiates the cytotoxic effects of paclitaxel in cells expressing ABCC10 transporter. Masitinib did not significantly alter the IC50 values of paclitaxel or vincristine in HEK293/ABCB1 and HEK293/ABCC1 cell lines, respectively (35, 36). These results suggest that the masitinib-induced potentiation of the toxic effects of paclitaxel and vincristine in ABCC10 expressing cells is not due to its interaction with the ABCB1 or ABCC1 transporters.

Our results also indicated that masitinib (1.25 or 2.5 μmol/L) produced a significant concentration-dependent increase in the intracellular accumulation of [3H]-paclitaxel in HEK293 cells that expressed the ABCC10 transporter. In addition, consistent with the aforementioned results, masitinib (2.5 μmol/L) significantly increased the intracellular accumulation of BODIPY-paclitaxel and significantly decreased the efflux of [3H]-paclitaxel from cells.
expressing the ABCC10 transporter. These findings tentatively suggest that masitinib increases the sensitivity of ABCC10-expressing cells to paclitaxel by inhibiting its efflux from the cells. This could be due to the direct interaction of masitinib with the ABCC10 transporter, although this remains to be proven. Masitinib (2.5 μmol/L) did not significantly alter the expression of the ABCC10 protein (171-kDa protein in the immunoblot assay in HEK293/ABCC10 cells, Fig. 4). This finding suggests that masitinib re-sensitizes HEK293/ABCC10 cells to paclitaxel without significantly altering their expression levels of ABCC10.

Our preclinical studies indicated that masitinib, in combination with paclitaxel, significantly attenuated tumor growth in athymic nude mice implanted with HEK293 cells expressing the ABC10 transporter in a tumor xenograft model (Fig. 5A–C). Masitinib essentially restored the sensitivity of tumors expressing ABC10 transporter to paclitaxel without eliciting visible changes in phenotype. In addition, pharmacokinetic data indicated that masitinib significantly increased the levels of paclitaxel in ABC10-expressing tumors in mice compared with paclitaxel alone. Pharmacokinetic data from clinical studies have reported that masitinib, at doses ≤12 mg/kg/d, can produce plasma concentrations of 2 μmol/L (37). Therefore, the dose and concentrations of masitinib used in our in vitro and in vivo experiments were consistent with these data (37). However, additional studies must be conducted to determine whether the combination of masitinib and paclitaxel produces significant effects on CYP450 enzymes involved in drug metabolism.

Collectively, our results show that masitinib, a receptor tyrosine kinase inhibitor, has previously unknown function in sensitizing ABC10-expressing tumor cells to the ABC10 substrate paclitaxel in vitro and in vivo (Supplemental Fig. 3). This effect was likely due in part to a blockade of paclitaxel efflux by ABC10, as opposed to

the alteration of the expression of the ABCC10 protein. In addition, masitinib potentiates the antitumor efficacy of paclitaxel in vivo. Thus, the combination therapy of masitinib and paclitaxel could represent an efficient strategy to treat patients with cancers that are resistant to paclitaxel as a result of the expression of ABCC10.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R.J. Kathawala, A. Patel, C.R. Ashby Jr, Z.-S. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.J. Kathawala, K. Sodani, K. Chen, A.H. Abuznait, N. Anreddy, Y.-L. Sun, A. Kaddoumi, C.R. Ashby Jr, Z.-S. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatis-
Writing, review, and/or revision of the manuscript: R.J. Kathawala, K. Sodani, K. Chen, A. Patel, A.H. Abuznait, N. Anreddy, Y.-L. Sun, A. Kaddoumi, C.R. Ashby Jr, Z.-S. Chan
Administrative, technical, or material support (i.e., reporting or organ-

Acknowledgments
The authors thank AB Science for giving us masitinib, the late Dr. Gary D. Kruh (University of Illinois at Chicago, IL) for the ABC10 plasmid, Dr. Shin-ichi Akiyama (Kagoshima University, Japan) for PAK-104P, and Dr. Suresh V. Ambudkar (NIH, MD) for the HEK293/ABCB1 and HEK293/ABCC1 cell lines.

Grant Support
This work was supported by funds from the NIH (1R15CA147071) and St. John’s University Research Seed Grant (579-1110-7002) to Z.-S. Chen.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 4, 2013; revised December 16, 2013; accepted January 6, 2014; published OnlineFirst January 15, 2014.

References
8. Chen ZS, Hopper-Borge E, Belinsky MG, Shchaveleva I, Kotova E, Kruh GD. Characterization of the transport properties of human multi-
12. D’Allard D, Gay J, Descarpentries C, Frisan E, Adam K, Verdier F, et al. Tyrosine kinase inhibitors induce down-regulation of c-Kit by tar-
getting the ATP pocket. PLoS ONE 2013;8:e60961.
14. Campanella NC, de Oliveira AT, Scapulastempo-Neto C, Guimarães DP, Reis RM. Biomarkers and novel therapeutic targets in
gastrointestinal stromal tumors (GISTs). Recent Pat Anticancer Drug Discov 2013;8:288–97.
Masitinib Antagonizes ATP-Binding Cassette Subfamily C Member 10–Mediated Paclitaxel Resistance: A Preclinical Study


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0743

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/01/15/1535-7163.MCT-13-0743.DC1

Cited articles
This article cites 35 articles, 12 of which you can access for free at:
http://mct.aacrjournals.org/content/13/3/714.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/13/3/714.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.