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

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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 multi-drug 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 non-toxic concentrations, significantly attenuates paclitaxel resistance in HEK293 cells transfected with *ABCC10*. Our *in vitro* studies indicated that masitinib (2.5 μ M) 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 to 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.

Keywords

Masitinib; ABCC10; ABC transporters; Paclitaxel; Tyrosine kinase inhibitors

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Introduction

Paclitaxel alone or in combination with other anti-neoplastic 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 cancer, including metastatic ovarian, breast, head and neck cancer and advanced Kaposi's sarcoma (2). The novel mechanism of action of paclitaxel provided the hope that it would demonstrate anti-neoplastic 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 anti-neoplastic drugs, including docetaxel, vincristine, vinblastine, vinorelbine, cytarabine, gemcitabine, 2', 3'-dideoxycytidine, 9-(2-phosphonyl methoxyethyl)adenine (PMEA), epothilone B and endogenous substances like estradiol-17 β -D-glucuronide (E₂17 β G) 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 non-receptor 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 them non-specific and toxic (22–24). Here we discover that masitinib, an inhibitor of receptor tyrosine kinases (10), at non-toxic concentrations, show previously unknown activity in antagonizing the efflux function of ABCC10 *in vitro*, 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 (25.7 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Dulbecco modified Eagle medium (DMEM), Iscove's DMEM, fetal bovine serum (FBS), phosphate buffer saline (PBS), 10,000 IU/ml penicillin and 10,000 μ g/ml streptomycin, and trypsin 0.25% were purchased from Hyclone (Waltham, MA). Monoclonal antibody against GAPDH was purchased from Cell Signaling Technologies (Beverly, MA). Antibody D-19 against ABCC10 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody Ab81 against c-Kit and monoclonal antibody D12E12 against phospho-c-Kit (p-c-Kit) were obtained from Cell Signaling Technologies (Beverly, MA). Masitinib was a gift from AB Science (Paris, France). Cepharanthine was generously given

by Kakenshoyaku Co. (Tokyo, Japan). PAK-104P was a gift from Nissan Chemical Industries (Tokyo, Japan). Paclitaxel, docetaxel, vincristine, vinblastine and cisplatin were purchased from Tocris Bioscience (Ellisville, MO). Boron-dipyrromethene (BODIPY) FL (fluorescent) paclitaxel was purchased from Life technologies, Invitrogen (New York, NY). 3-(4, 5-Dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) and verapamil were obtained from Sigma-Aldrich Co. (St. Louis, MO).

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 ABCC10 (HEK293/ABCC10) were used in all experiments as previously reported (8, 20). The HEK293/ABCB1 and HEK293/ABCC1 cells were kindly provided by Dr. Suresh V. Ambudkar (NCI, NIH, MD) in 2012. HEK293/pcDNA3.1, HEK293/ABCC10, HEK293/ABCB1 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 vivo* experiments were trypsinized and centrifuged at 2000 rpm for 2 min 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/well for HEK293/pcDNA3.1 and HEK293/ABCC10. Every MTT assay was run in triplicate and drugs tested included paclitaxel (0.001 to 1 µM), docetaxel (0.001 to 1 µM), vinblastine (0.001 to 1 µM), vincristine (0.001 to 1 µM), cisplatin (0.1 to 100 µM), masitinib (0.625 µM, 1.25 µM and 2.5 µM), cepharanthine (2.5 µM), verapamil (10 µM), and PAK-104P (5 µM). After seeding cells in 180 µl medium in 96-well plates and incubating for 24 h 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 h prior to adding anticancer drugs). Subsequently, the anticancer drugs, in DMEM supplemented with 10% FBS, were incubated at 37°C for 72 h. After 72 h, 20 µl MTT (4 mg/ml) was added to each well. The plates were incubated at 37°C for another 4 h. 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 Opsy microplate reader (Dynex Technologies, VA). 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 in the absence of masitinib or other reversal compounds by that obtained in the presence of masitinib or cepharanthine.

Flow cytometry

HEK293/pcDNA3.1 and HEK293/ABCC10 cells were harvested and transferred to DMEM supplemented with 10% FBS in presence or absence of inhibitor (masitinib or cepharanthine at 2.5 µM). After 2 h at 37°C, cells were centrifuged at 4000 rpm for 3 min and resuspended in medium with or without the inhibitor, in addition to 40 nM BODIPY-paclitaxel. One hour

later, cells were centrifuged at 4000 rpm for 3 min, resuspended and immediately analyzed in a LSRFortessa flow cytometer (BD Biosciences, CA). 7-aminoactinomycin D (BD Biosciences, CA) was used to exclude non-viable 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 accumulation assay

The HEK293/pcDNA3.1 and HEK293/ABCC10 cell lines were harvested at 80% confluency. All cell lines were trypsinized and cell count was done using a hemocytometer. Approximately 7×10^6 cells were incubated at 37°C in DMEM, supplemented with 10% FBS with and without masitinib (1.25 or 2.5 μ M) for 2 h. Subsequently, HEK293/pcDNA3.1 and HEK293/ABCC10 cells were incubated with 0.01 μ M [³H]-paclitaxel in presence or absence of masitinib (1.25 or 2.5 μ M) for 2 h. Following incubation, the medium was removed and the cells were rinsed three times with cold PBS. The cells were lysed using 200 μ l lysis buffer and transferred to scintillation vials. Each sample was placed in scintillation fluid and radioactivity was measured using a liquid scintillation counter (Packard Instrument, IL).

[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 min) in the presence or absence of the inhibitor (masitinib or cepharanthine at 2.5 μ M). After washing three times with ice-cold PBS, the cells were lysed using 200 μ l 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, IL).

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 μ M for different time periods (0, 24, 48, and 72 h). Then, approximately 6×10^5 cells were harvested and suspended in PBS, centrifuged at 2000 rpm for 2 min, 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 was added to the suspension followed by vortexing. The resuspended cells were kept on ice for 30 min, followed by centrifugation at 12,000 rpm for 20 min. The supernatant was separated and stored in -80° C for the experiment. Protein concentrations in the lysates were determined by the bicinchonic acid based protein assay. Equal amounts of total cell lysates (40 μ g protein) were resolved by SDS-PAGE electrophoresis and electrophoretically transferred onto 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 ABCC10 at 1:200 dilution, c-Kit, p-c-Kit or GAPDH at 1:1000 dilution at 4°C and were then incubated for 3 h at room temperature with HRP-conjugated secondary antibody (1:1000 dilution). The protein-antibody complex was detected using chemiluminescence.

Methodology for the tumor-xenograft model

Male athymic NCR (nu/nu) nude mice (NCRNU M, homozygous, albino; 18–25 g; 10–15 week; Taconic Farms, NY) were used for the tumor-xenograft experiments. All animals were maintained on an alternating 12 h 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 s.c. 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: (a) vehicle (autoclaved water) (q3d \times 6), (b) paclitaxel (15 mg/kg, i.p., q3d \times 6), (c) masitinib diluted in autoclaved water (12.5 mg/kg, p.o., q3d \times 6), and (d) masitinib (12.5 mg/kg, p.o., q3d \times 6, given 1 h before giving paclitaxel) + paclitaxel (15 mg/kg, i.p., q3d \times 6). Paclitaxel was prepared by dissolving 6 mg paclitaxel in 50% of dehydrated alcohol (EMD, MA) and 50% of Cremophor ELP (BASF, NJ). The tumor sizes were measured using calipers and body weights were recorded (29). The body weight of the animals was monitored every 3rd 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 3rd day and tumor volume was estimated (29, 31). All the animals were killed by terminal bleeding through cardiac puncture under isoflurane 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 US 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.

Collection of plasma and tissues

In a separate group of experiments, mice bearing HEK293/ABCC10 tumors were divided into two groups: 1) vehicle pretreatment (given 1 h before paclitaxel) and (15 mg/kg); 2) 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 min 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).

HPLC conditions

Quantification of paclitaxel was conducted using an isocratic Shimadzu LC-20AB liquid chromatograph equipped with an Shimadzu SIL-20A HT autosampler and LC-20AB pump connected to a Dgu-20A3 degasser (Shimadzu, OR), according to the method described by Gill et al. (33). The column used was a reversed-phase, Phenomenex Luna C18 column (250×4.6 mm i.d., $5 \mu\text{m}$; Phenomenex, CA) with an ODS guard column ($4 \text{ mm} \times 3 \text{ mm}$; Phenomenex, CA). The injection volume was $20 \mu\text{l}$, 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 Shimadzu UV SPD-20A (Shimadzu, OR) detector set at 227 nm was used. Data acquisition and analysis was achieved using LC Solution software version 1.22 SP1 (Shimadzu, OR). All samples were analyzed in duplicate. Under these chromatographic conditions, the total run time was 15 min with a retention time of 12 min for paclitaxel. Standard curves for paclitaxel in plasma and tissue homogenates were prepared in the ranges of 25–5000 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 min, followed by centrifugation for 10 min at 10,000 rpm. The supernatant was transferred to insert vials from which 20 μ l was injected onto the 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's *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. Based on the cytotoxicity assays, we chose to use masitinib (Fig. 1B) at concentrations of 0.625 μ M, 1.25 μ M and 2.5 μ M because at these concentrations, at least 80–90% of the cells survived. Masitinib, at 0.625 μ M, 1.25 μ M and 2.5 μ M, significantly decreased the resistance to paclitaxel and docetaxel in the HEK293/ABCC10 cell line as compared to the control HEK293/pcDNA3.1 cells (Table 1). Cepharanthine (2.5 μ M), which has been shown to inhibit ABCC10 function, significantly decreased the resistance of HEK293/ABCC10 to paclitaxel as compared to the parental HEK293/pcDNA3.1 (17). The incubation of 2.5 μ M of masitinib or cepharanthine did not significantly alter the IC₅₀ 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 vinblastine, which are substrates of ABCC10 (Supplemental Table 1). In order 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 Table 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 μ M) significantly increased the accumulation of BODIPY-paclitaxel in the cells expressing ABCC10 compared to HEK293/pcDNA3.1 cells (Fig. 2).

Masitinib increases the cellular accumulation of [³H]-paclitaxel in HEK293/ABCC10 cells

In order 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 [³H]-paclitaxel in HEK293/ABCC10 cells. Masitinib produced a significant, concentration-dependent increase in the intracellular accumulation of [³H]-paclitaxel compared to 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 [³H]-paclitaxel in HEK293/ABCC10 cells

We further determined the amount of [³H]-paclitaxel present in the cells following incubation with masitinib. The amount of [³H]-paclitaxel present in the intracellular medium of HEK293/ABCC10 cells was significantly lower compared to that of HEK293/pcDNA3.1 cells due to active efflux of the [³H]-paclitaxel via ABCC10. However, over a period of time (0, 30, 60, 120 min) masitinib (2.5 μM) significantly reduced the efflux of [³H]-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). In order 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 μM) for 0, 24, 48 and 72 h. 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 expression 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 to 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 15 mg/kg i.p. dose of paclitaxel (Fig. 5A, B and C). This is in contrast to HEK293/pcDNA3.1 tumor, which were 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 to 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) over a period of 18 days, compared to animals treated with vehicle, masitinib alone or paclitaxel alone ($p < 0.01$; Fig. 5A, B and 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.) prior to the administration of paclitaxel (15 mg/kg, i.v.). The pharmacokinetic data showed that co-administration of masitinib and paclitaxel produced a transient increase in the plasma levels of paclitaxel after 10 min 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 to paclitaxel administration alone (16.31 ± 6.45 ng/ml, $p < 0.05$) (Fig. 6B) after 240 min following administration. However, the coadministration of masitinib and paclitaxel also did not significantly increase the concentration of paclitaxel in

the lungs as compared to paclitaxel administration alone (Fig. 6C) after 240 min following 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 μ M) 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 μ M) 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 the direct interaction of masitinib with the ABCC10 transporter, although this remains to be proven. Masitinib (2.5 μ M) 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 its expression.

Our preclinical studies indicated that masitinib, in combination with paclitaxel, significantly attenuated tumor growth in athymic nude mice implanted with HEK293 cells expressing the ABCC10 transporter in a tumor-xenograft model (Fig. 5A, B and C). Masitinib essentially restored the sensitivity of tumors expressing ABCC10 transporter to paclitaxel without eliciting visible changes in phenotype. In addition, pharmacokinetic data indicated that masitinib significantly increased the levels of paclitaxel in ABCC10-expressing tumors in mice compared to paclitaxel alone. Pharmacokinetic data from clinical studies have reported that masitinib, at doses 12 mg/kg/day, can produce plasma concentrations of 2 μ M (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 in order to determine if 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 ABCC10-expressing tumor cells to the ABCC10 substrate paclitaxel *in vitro* and *in vivo* (Supplemental Fig. 3). This effect was likely due in part to a blockade of paclitaxel efflux by ABCC10 as opposed to the alteration of the expression of the ABCC10 protein. In addition, masitinib potentiates the anti-tumor 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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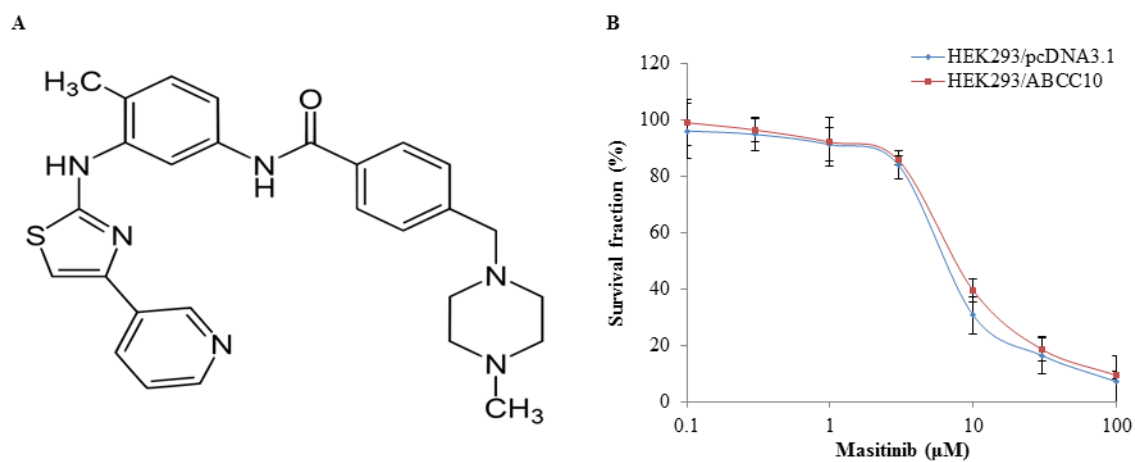


Figure 1. Cytotoxicity of masitinib. A, the chemical structure of masitinib [(4-[(4-methylpiperazin-1-yl) methyl]-N-(4-methyl-3-[[4-(pyridin-3-yl)-1, 3-thiazol-2-yl] amino} phenyl) benzamide mesylate)]. B, cytotoxicity of masitinib was determined by the MTT assay in HEK293/pcDNA3.1 and HEK/ABCC10 cells. Error bars indicate SD.

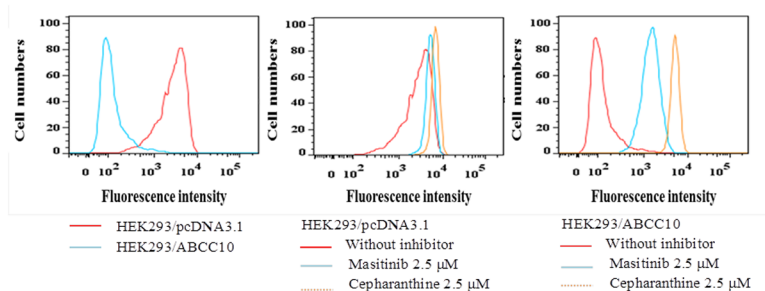


Figure 2.

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 pre-incubated with or without masitinib (2.5 μM) for 2 h at 37°C and then incubated with 40 nM BODIPY-paclitaxel for another 60 min. at 37°C. All samples were analysed in a flow cytometer.

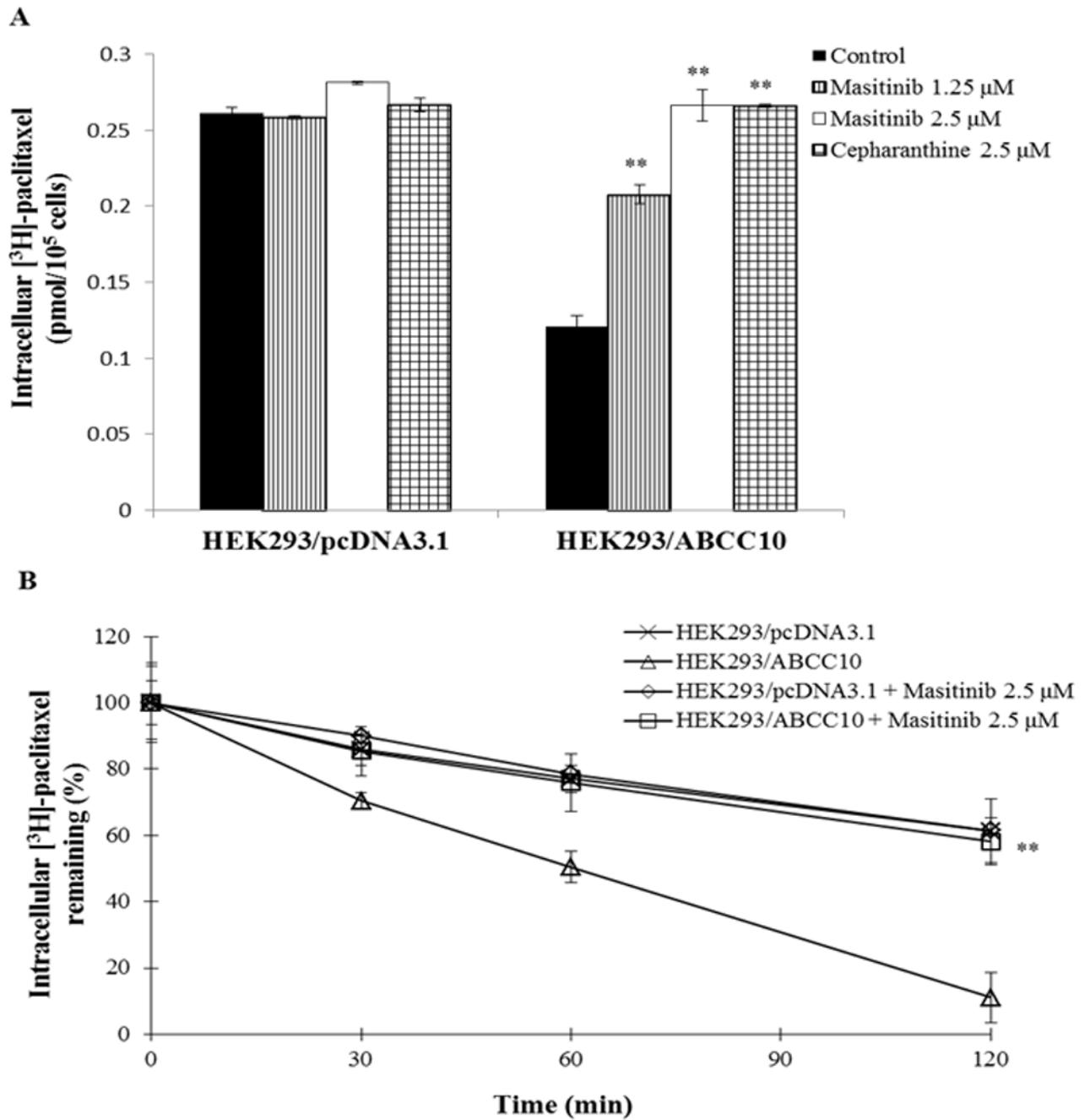


Figure 3. Masitinib increases the cellular accumulation of [³H]-paclitaxel and decreases the cellular efflux of [³H]-paclitaxel in HEK293/ABCC10 cells. A, the accumulation of [³H]-paclitaxel was measured after the cells (HEK293/pcDNA3.1 and HEK293/ABCC10) were pre-incubated with or without masitinib or cepharanthine for 2 h at 37°C and then incubated with 0.01 μM [³H]-paclitaxel for another 2 h at 37°C. Error bars indicate SD. **: *p* < 0.01 versus the control group. B, a time course versus the percentage of intracellular [³H]-paclitaxel remaining (%) was plotted (0, 30, 60, 120 min). Error bars indicate SD. **: *p* < 0.01 versus the control group.

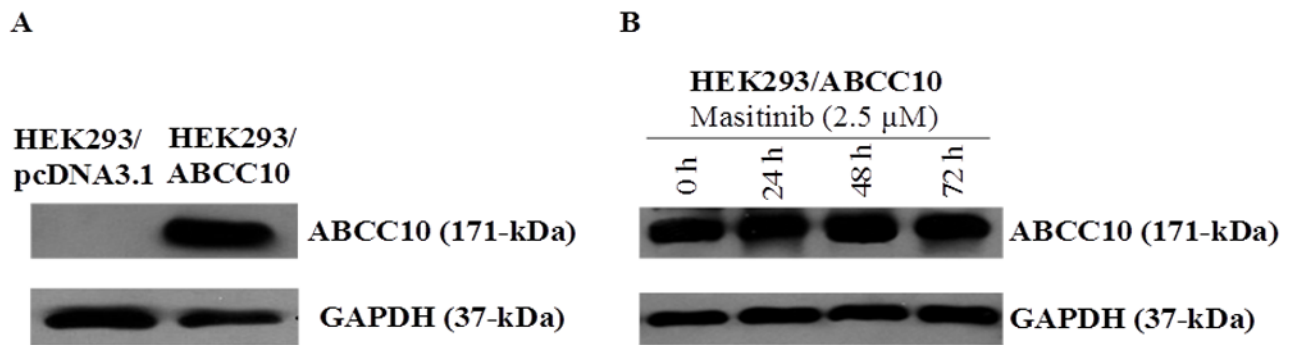


Figure 4.

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 μ M) for the indicated period of time, determined by immunoblot. GAPDH was used as a loading control. Data presented are representative of 3 independent experiments.

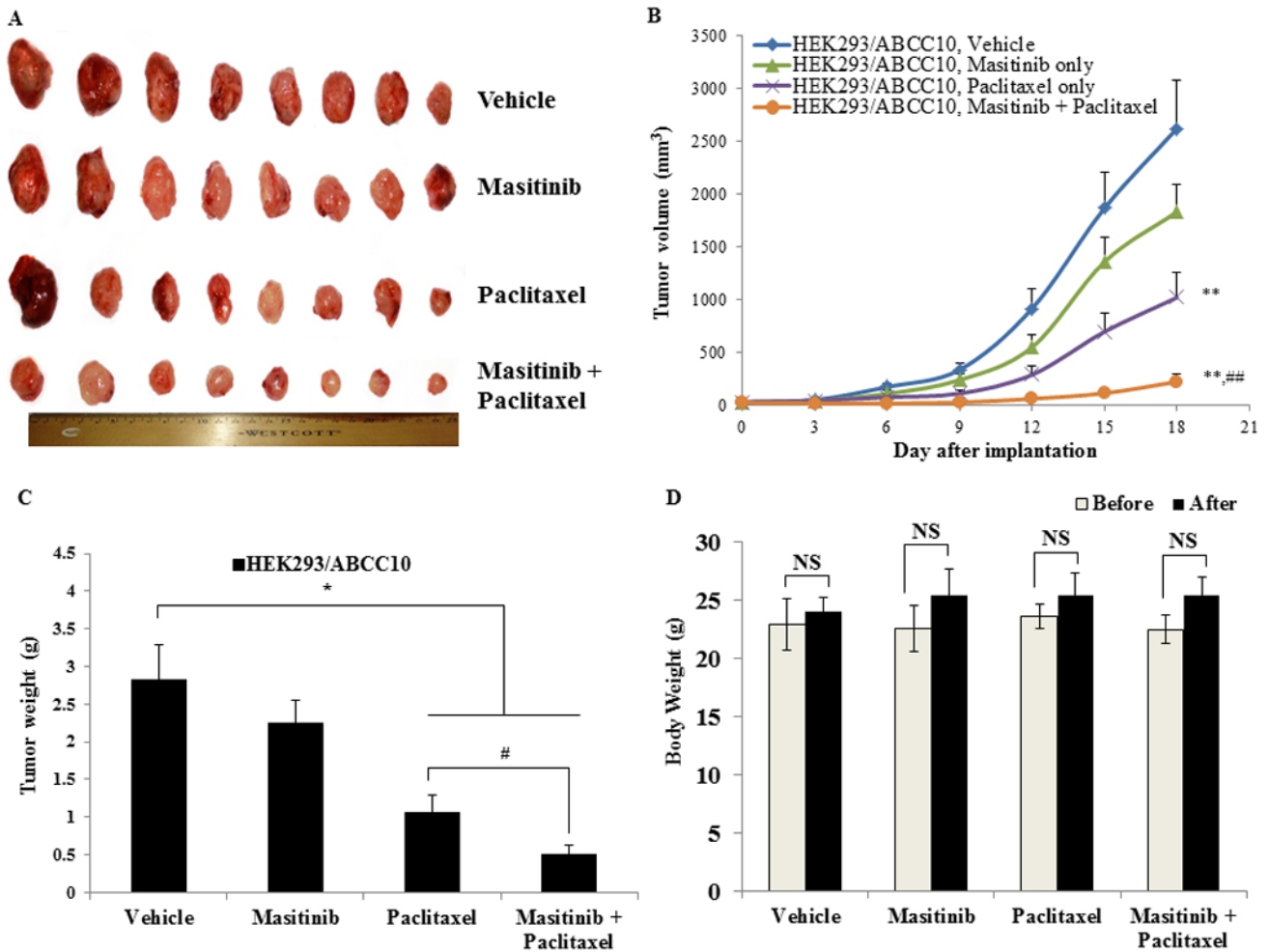


Figure 5.

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 combination of masitinib plus paclitaxel, at the end of the 18-day treatment period. Results are representative of 3 independent experiments performed. B, the changes in tumor volume over time following implantation. Data points represent mean tumor volume for each treatment group ($n = 8$). Error bars represent SEM. **: $p < 0.01$ versus the vehicle group; ##: $p < 0.01$ versus paclitaxel alone group. C, mean weight ($n = 8$) of the excised HEK293/ABCC10 tumors from the mice treated with with vehicle, paclitaxel, masitinib or combination of masitinib plus paclitaxel, at the end of the 18-day treatment period. Error bars represent 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 treatment. NS: not statistically significant ($p > 0.05$).

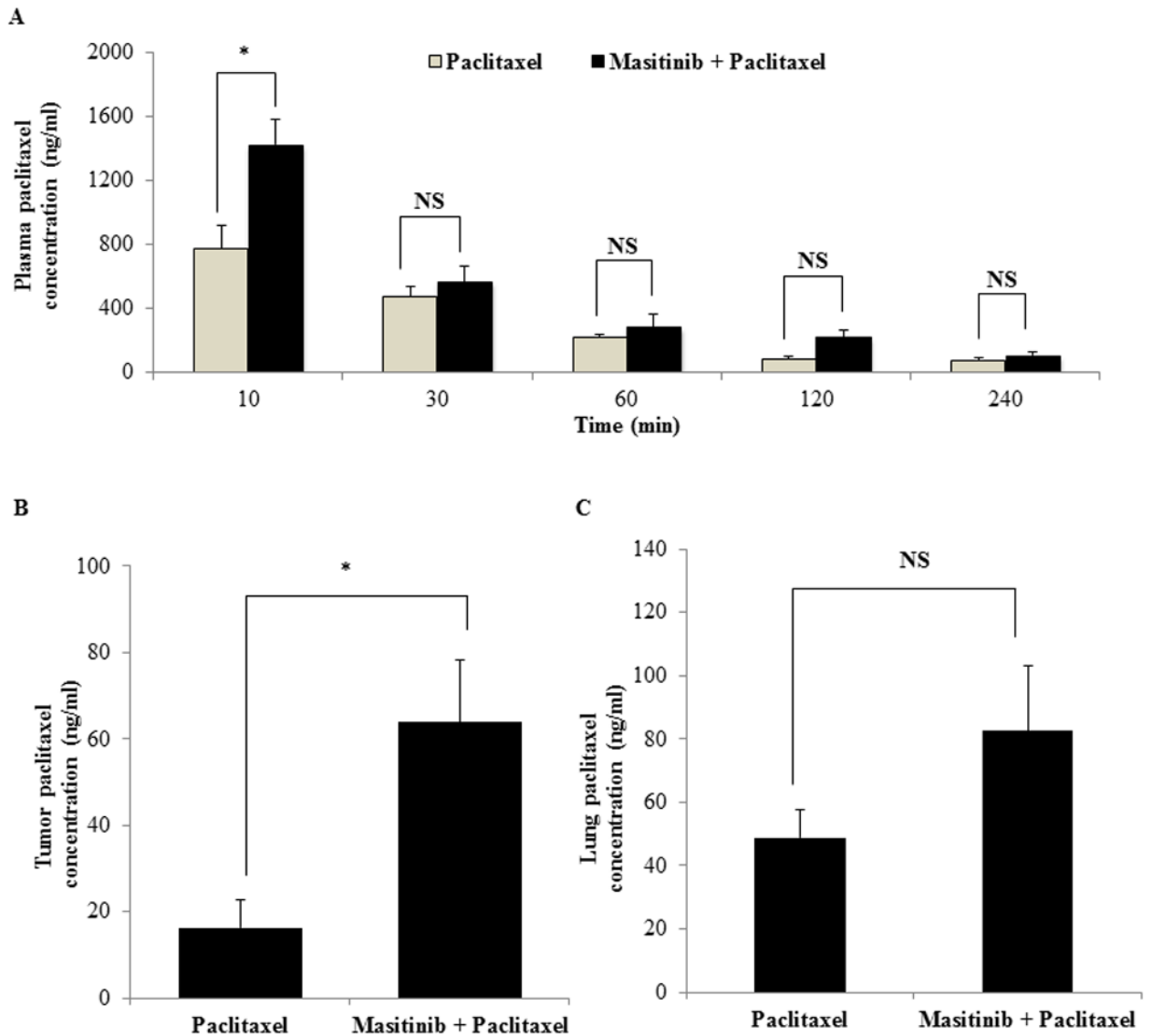


Figure 6.

The effect of masitinib and paclitaxel co-administration on plasma, intratumoral, and lung paclitaxel concentrations in mice. A, plasma at 10, 30, 60, 120, 240 min following administration ($n = 7$), B, paclitaxel concentrations in HEK293/ABCC10 tumors ($n = 7$) after 240 min following administration, and C, lungs ($n = 7$) after 240 min following administration. Columns and error bars represent mean \pm SEM. *: $p < 0.05$ versus paclitaxel only group. NS: not statistically significant ($p > 0.05$).

Table 1

Masitinib significantly sensitizes HEK293/ABCC10 cells to paclitaxel and docetaxel but cisplatin with no or minimal effect on HEK293/pcDNA3.1 cells.

Compounds	HEK293/pcDNA3.1		HEK293/ABCC10	
	IC ₅₀ ± SD ^a (nM)	FR ^b	IC ₅₀ ± SD (nM)	FR
Paclitaxel	8.86 ± 0.9	[1.0]	87.06 ± 3.18	[9.9]
+Masitinib 0.625 μM	8.53 ± 1.1	[1.0]	39.43 ± 2.7**	[4.4]
+Masitinib 1.25 μM	7.67 ± 1.5	[0.9]	20.20 ± 2.3**	[2.3]
+Masitinib 2.5 μM	6.69 ± 0.7	[0.7]	10.06 ± 1.4**	[1.1]
+Cepharanthine 2.5 μM	5.65 ± 0.4*	[0.6]	8.92 ± 0.8**	[1.0]
Docetaxel	8.69 ± 1.2	[1.0]	87.54 ± 3.4	[10.1]
+Masitinib 0.625 μM	7.44 ± 1.6	[0.8]	43.22 ± 1.4**	[5.0]
+Masitinib 1.25 μM	6.81 ± 0.8	[0.8]	12.35 ± 0.7**	[1.4]
+Masitinib 2.5 μM	5.62 ± 0.9*	[0.6]	9.62 ± 1.4**	[1.1]
+Cepharanthine 2.5 μM	7.22 ± 0.5	[0.8]	9.53 ± 0.7**	[1.1]
Cisplatin	5244.6 ± 426.3	[1.0]	5743.3 ± 528.1	[1.0]
+Masitinib 2.5 μM	5523.0 ± 159.3	[1.0]	6245.7 ± 728.5	[1.2]
+Cepharanthine 2.5 μM	4532.1 ± 181.8	[0.9]	5878.9 ± 407.0	[1.3]

^aIC₅₀: The drug concentration that inhibited cell survival by 50% (means ± SD).

^bFR: fold-resistance 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 3 independent experiments, each performed in triplicate.

* and ** indicate significant statistical difference from the IC₅₀ values of HEK293/ABCC10 without reversal drug (* *p* < 0.05; ** *p* < 0.01).