# Fast, Stable Induction of P-Glycoprotein-mediated Drug Resistance in BT-474 Breast Cancer Cells by Stable Transfection of *ABCB1* Gene

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**Abstract.** *Background: Patients with P-glycoprotein and HER2/neu (HER2) receptor-overexpressing breast cancer usually have poor clinical outcomes. However, there exist no commercially available breast cancer cell lines that are HER2/P-glycoprotein double-positive, which limits research in this field. Materials and Methods: We report on the development and characterization of a drug-resistant sub-line from an HER2-positive breast cancer cell line by stable transfection of the ATP-binding cassette (ABC) subfamily B member 1 (ABCB1) gene which encodes P-glycoprotein. Results: ABCB1 gene expression levels were higher after transfection, which led to a 40-fold increase in P-glycoprotein expression. Interestingly, the transfection of ABCB1 also led to a slight increase in HER2 gene and protein expression levels. The transfection of ABCB1 increased the P-glycoprotein expression levels significantly. Conclusion: The method used herein for developing this cell line is appropriate for fast, stable induction of P-glycoprotein-mediated drug resistance compared to traditional methods. The in vitro cytotoxicity test suggests this cell line has cross-resistance to a wide range of chemotherapeutic agents.*

Breast cancer is the most common cancer in women (1). Research has shown that ~25-30% of breast tumors overexpress HER2/neu (HER2) receptor (1-3). These breast tumors tend to be much more aggressive and fast-growing (4), therefore, patients with elevated HER2 levels usually have poor clinical outcomes compared to patients with HER2-negative disease (5).

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Chemotherapy is a major therapeutic approach for the treatment of localized and metastasized breast cancer. The major problem in the management of HER2-overexpressing breast cancer is its multidrug resistance (MDR), especially in patients with recurrent breast cancer (6). HER2 overexpression has been found to correlate with tumor growth and metastasis, as well as with the development of chemoresistance (7, 8). As evident from both laboratory and clinical studies, HER2 may render tumor cells resistant to a wide range of chemotherapeutic agents, such as paclitaxel, cyclophosphamide, methotrexate, 5-fluorouracil, and epirubicin (9-11).

ATP-binding cassette (ABC) subfamily B member 1 (ABCB1) gene-mediated overexpression of P-glycoprotein (P-gp) has been recognized as one of the major mechanisms in drug resistance in breast cancer (12). The 170-kDa *ABCB1* gene encodes transmembrane P-gp (14, 15). Although the exact mechanism is still not fully understood, *ABCB1* gene appears to play a key role in the development of drug resistance in breast cancer both *in vitro* and *in vivo* (13-18). P-gp resides in the cell membrane and can actively transport chemotherapeutic agents out of cancer cells using energy from ATP hydrolysis, leading to accelerated drug efflux and decreased net drug accumulation in tumor (19).

Although the role of HER2 in breast cancer has been extensively studied over the years, some of the underlying mechanisms of MDR development in the HER2-positive subtype are still far from being completely understood (20). Research in this area has been hampered, in part, by the fact that currently there exist no commercially available breast cancer cell lines that are both HER2- and P-gp-positive, which limits the scope of research in this field.

We report on the development and characterization of a drug-resistant subline from a HER2-positive breast cancer cell line by stable transfection of the *ABCB1* gene. This new drug-resistant cell line has the potential to serve as a model cell

line for studying targeted-drug delivery of chemotherapeutic agents using antibody-tagged immunoliposomes.

## Materials and Methods

*Cell culture and transfection.* HER2 overexpressing (2) human breast carcinoma cell line BT-474 (ATCC, Manassas, VA, USA) was cultured as monolayers in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/ streptomycin (Invitrogen) in a humidified atmosphere of 95% air and 5% $CO_2$ at 37˚C. Cells were subcultured twice weekly. For experiments, the cells were grown in plastic tissue culture flasks (FisherSci, Pittsburg, PA, USA) and used when in the exponential growth phase.

Unlike traditional transfection methods involving virus vector (21), BT-474 cells were transfected with ABCB1 gene packaged in a plasmid vector (EX-E2266-M02; Genecopoeia, Rockville, MD, USA) using Lipofectamine 2000 tranfection reagent (Invitrogen) according to the manufacturer's instructions. After transfection, cells were maintained in culture media containing 60 ng/ml of colchicine to select P-gp-positive cells.

*Western blot.* Cultured cells were washed three times with ice-cold phosphate buffered saline (PBS) and lysed in a lysis buffer cocktail containing protease inhibitor (50 mmol/L Tris-HCl (pH 7.5), 150 mmol/l NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/l EDTA, 1 mmol/l sodium fluoride, 1 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μl/ml phosphatase inhibitor, and 10 μl/ml protease inhibitor cocktail (all purchased from SigmaAldrich, St. Louis, MO, USA) for 30 min on ice. Proteins (15 μg for HER2; 50 μg for ABCB1) were separated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% nonfat dry milk plus 0.1% Tween 20 in Tris-buffered saline (TBS) and then incubated with primary antibodies (mouse anti-human ABCB1 monoclonal antibody, SC-55510; rabbit anti-human HER2/neu polyclonal antibody, SC-284; both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. Membranes were washed three times with TBS containing 0.1% Tween20 and then incubated for 1 h with secondary antibodies in blocking buffer. The signal was detected with a SuperSignal West Pico Chemiluminescent kit (Thermo Scientific, Rockford, IL, USA).

*Real-time reverse transcription polymerase chain reaction. ABCB1* and *HER2* gene expression level was quantified using quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) technique. Cells were first lysed using Trizol reagent (Life Technologies) to extract RNA according to the manufacturer's instructions. RNA concentrations were measured in a Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized from total RNA using the GoTaq® 2-Step RT-qPCR System (Promega, Madison, WI, USA). Relative mRNA levels were than measured with a realplex EP gradient S mastercycler (Eppendorf, Hauppauge, NY, USA) through the SYBR Green detection system. The relative amount of expression of each gene was calculated as the ratio of the studied gene to the control gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

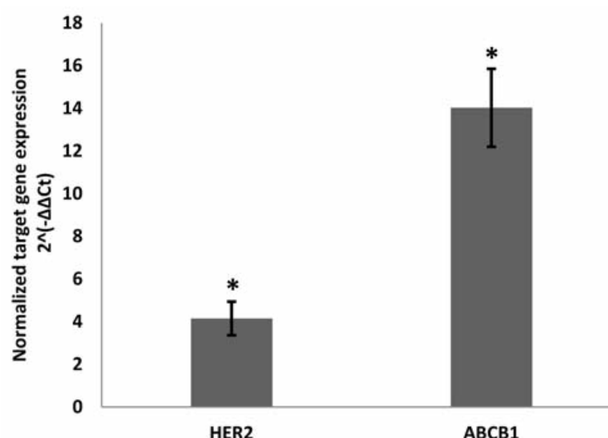


Figure 1. *Relative mRNA levels of HER2/neu (HER2) and ATP-binding cassette (ABC) subfamily B member 1 (ABCB1) transfected into BT-474 cells compared to non-transfected BT-474 cells using 2(-ΔΔCt) method, normalized to a control gene glyceraldehyde 3-phosphate dehydrogenase. *p<0.001 compared to non-transfected cells. Both HER2 and ABCB1 gene expression levels were higher after transfection.*

*Immunostaining.* The presence and distribution of P-gp was characterized by immunocytochemical techniques. Briefly, $5x10^4$ cells were seeded on a poly-D-lysine pre-coated glass coverslip (BD Biosciences, San Jose, CA, USA) in a 24-well tissue culture plate. Upon experiment, cells were washed with PBS three times and fixed in 4% paraformaldehyde for 15 min at room temperature. Following another three washes in PBS, cells were blocked with 1% Bovine serum albumin (SigmaAldrich) in PBS/Tween-20 (SigmaAldrich) for 30 min at room temperature to reduce nonspecific binding. Mouse anti-human ABCB1/HER2 monoclonal antibody (Santa Cruz Biotechnology) was then added to the fixed cells for staining overnight at 4˚C. The coverslips were then washed and mounted on glass slides with mounting medium for imaging purpose.

*Visualization of doxorubicin (DOX) subcellular localization.* In order to study the internalization of DOX, cells were seeded at a density of 50,000 cells/well on poly-D-lysine pre-coated glass coverslips which were placed in a 24-well tissue culture plate overnight to reach confluence. On the second day, the medium was replaced with 0.5 ml of DOX (Santa Cruz Biotechnology)-containing medium. The cells were then returned to culture for 2 h to allow for uptake. After incubation, cells were washed three times in PBS and fixed with 4% formaldehyde for 15 min, followed by three washes with PBS. The coverslips were then removed and mounted on glass slides with mounting medium for imaging purposes.

*Cytotoxicity asessment.* In order to test whether the transfection changed the resistance of BT-474 cells to chemotherapy, five commonly used chemotherapeutic agents DOX, docetaxol, carboplatin, lapatinib, and fluorouracil (all purchased from Santa Cruz Biotechnology) were used for the cytotoxicity assessment. Cytotoxicity was measured by means of sulforhodamine B (SRB) assay (Invitrogen), which colorimetrically quantifies total cellular protein.

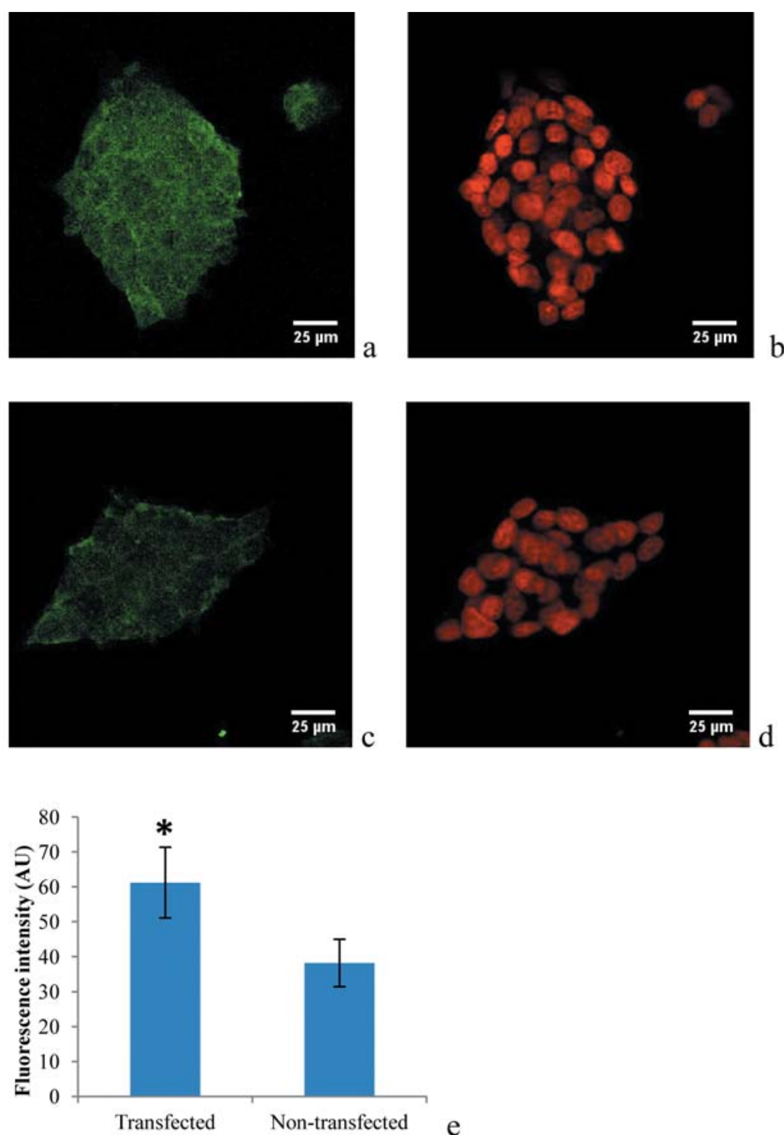


Figure 2. *Immunostaining for membranous HER2/neu (HER2) (green) in both transfected (a, b) and non-transfected (c, d) BT-474 cells. The transfection of ATP-binding cassette (ABC) subfamily B member 1 (ABCB1) gene changed HER2 expression level significantly. Images were taken using a confocal microscope. Membranous HER2 was stained with Dylight 488-conjugated antibody (green). The same cells were stained with DRAQ5 (red) to identify cell nuclei. a: Anti-HER2 staining on ABCB1 gene-transfected BT-474 cells; b: DRAQ5-stained nuclei of the same cells shown in (a); c: anti-HER2 staining of non-transfected BT-474 cells; (d) DRAQ5-stained nuclei of the same cells shown in (c); e: measured fluorescence intensity of HER2 staining indicates that protein levels of HER2 membranous receptor of transfected cells was much higher than that of non-transfected cells.*

*Imaging*. Antibodies were modified with a DyLight 488 antibody labeling kit (Thermo Scientific) according to the manufacturer's instructions. Images were taken either with a Nikon TE-200 inverted microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with Qimaging Retiga 1300 camera (Qimaging, Surrey, BC, USA) using Nikon Element software (Nikon Instruments Inc.) or with a confocal microscope. DRAQ5 (Thermo Scientific) far-red DNA staining dye was used for confocal imaging as counterstaining. Obtained images were further analyzed in ImageJ software to assess the expression levels of HER2 and P-gp by the levels of fluorescence intensity and the fluorescence distribution.

## Results

*HER2 and ABCB1 mRNA levels*. We transfected BT-474 cells with the ABCB1 gene to introduce P-glycoprotein overexpression, thereby expecting to cause MDR. To evaluate the gene expression capability of transfected *ABCB1* gene, RT-qPCR was used to measure the mRNA levels in the transfected cells and the results were compared against non-transfected cells (controls). At the same time,

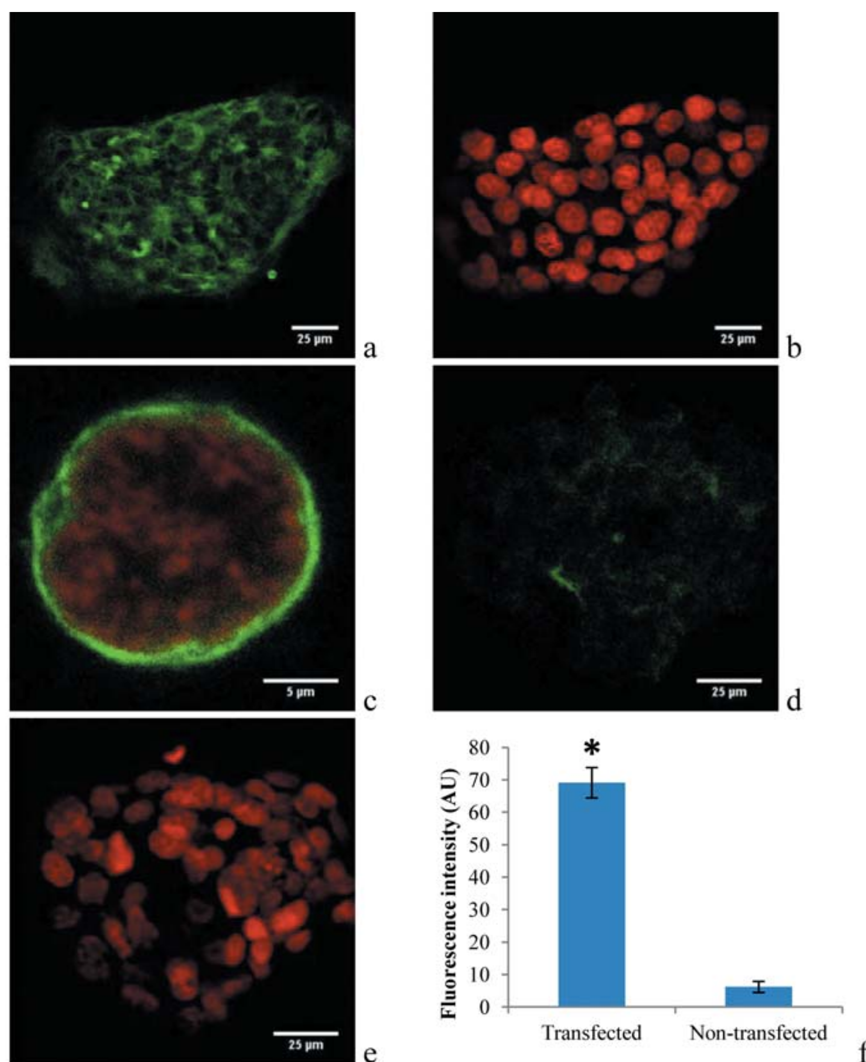


Figure 3. *Immunostaining for membrane P-glycoprotein (P-gp) in ATP-binding cassette (ABC) subfamily B member 1 (ABCB1)-transfected (a, b, c), and non-transfected (d, e) BT-474 cells, showing P-gp in the cell membrane (a, c, d). The transfection of ABCB1 increased P-gp expression significantly. Images were taken using a confocal microscope. Membranous P-gp was stained with Dylight 488-conjugated antibody (green). The same cells were stained with DRAQ5 (red) to identify cell nuclei (b, e). a: P-gp antibody staining of ABCB1-transfected BT-474 cells; b: DRAQ5-stained nuclei of the same cells shown in (a); c: a superimposed image of both green and red channels showing a single transfected BT-474 cell; d: P-gp staining of BT-474 cells; e: DRAQ5 staining of the same cells shown in (d); f: fluorescence intensity measured from P-gp-stained images.*

the mRNA level of *HER2* was also evaluated to ensure HER2 expression was not affected. As shown in Figure 1, the level of *HER2* and *ABCB1* mRNA in the transfected BT-474 cells were quantified and normalized to that of non-transfected cells. After transfection, *ABCB1* gene expression increased by ~14-fold. This result indicates that the plasmid transfection and the subsequent colchicine selection successfully induced expression of transfected *ABCB1* gene into BT-474 cells. Interestingly, the *HER2* mRNA level was also higher (4-fold) compared to that of the non-transfected cells.

*P-gp and HER2 protein expression levels*. In order to test whether the introduction of ABCB1 gene to BT-474 cells affects HER2 receptor expression, immunostaining experiments against HER2 protein were performed in both transfected (Figure 2a) and non-transfected BT-474 cells (Figure 2c). The HER2 receptor expression level (green fluorescence) of transfected cells (Figure 2a) was higher than that of non-transfected cells (Figure 2c). Semi-quantitative measurements (Figure 2e) of the fluorescence intensity of both groups confirmed that the protein level of HER2 membrane receptor of transfected cells was much higher than

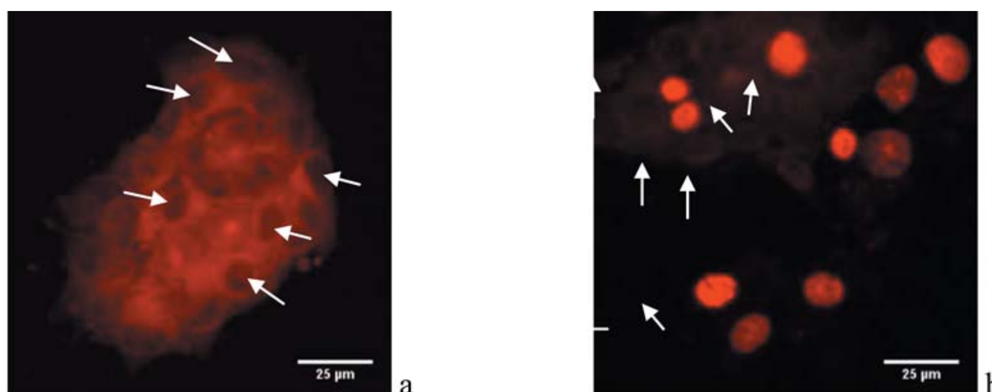


Figure 4. *Fluorescence images of doxorubicin (DOX) localization in P-glycoprotein (P-gp)-overexpressing BT-474 cells using fluorescence microscopy, showing cell membrane and cytoplasm localization of DOX after ATP-binding cassette (ABC) subfamily B member 1 (ABCB1) transfection. The location of cell nuclei are indicated by arrows. a: In transfected BT-474 cells, DOX signals were detected mainly on the cell membrane or cytoplasm; b: non-transfected BT-474 cells, DOX signals were mainly detected in cell nuclei.*

that of non-transfected cells, which was consistent with our data obtained from RT-qPCR experiments (Figure 1).

The existence of P-gp pump in *ABCB1* gene-transfected BT-474 cells was confirmed by immunostaining as shown in Figure 3. The green fluorescence in the non-transfected cells (Figure 3c) was very low, indicating that the non-transfected cells expressed a basal level of P-gp, whereas the green fluorescence was much higher in the transfected cells, indicating successful transfection and gene expression (Figure 3a). Superimposed images (Figure 3c) show that P-gp mainly resided in the cell membrane. The changes in P-gp and HER2 protein levels before and after transfection were also confirmed by western blot (data not shown).

*DOX uptake and subcellular localization.* To test the drug-resistance level after transfection, the cells were cultured with DOX and the DOX uptake and localization were studied. Since DOX has a unique fluorescence signature at around 580 nm, the uptake of DOX in both cell lines was evaluated in an *in vitro* setting using fluorescence microscopy. As shown in Figure 4, compared to that of the non-transfected cell line, doxorubicin-induced fluorescence intensity was much lower in transfected cell lines, indicating that the P-gp pumps can efficiently clear-out this drug. DOX was accumulated in the nucleus in non-transfected cells, whereas in transfected cells, DOX was observed mostly near the cell membrane and the cytoplasm, indicating that the overexpression of P-gp changed the subcellular distribution of DOX. These data strongly suggest enhanced P-gp pump function after transfection of *ABCB1*.

*Drug resistance after transfection.* To further confirm the acquired MDR of the transfected cells, both transfected and non-transfected cell lines were treated with five commonly used agents in breast cancer treatment. Figure 5 shows the cell survival rate of both transfected and non-transfected BT-474 cells treated by increasing concentrations of DOX, docetaxol, carboplatin, lapatinib and fluorouracil, respectively. With the presence of P-gp, transfected cells showed much lower growth inhibition across all concentrations tested (Figure 5). HER2-targeting drug lapatinib is the most effective chemotherapy drug against HER2-positive breast cancer cell lines. The increased resistance of transfected BT-474 cells to lapatinib suggests that acquired resistance through the P-gp pathway might lead to resistance to HER2-targeting drugs.

## Discussion

With the improvement of our knowledge and understating over the signaling pathways and molecular basis of metastatic breast cancer, the patient survival rate has been steadily improving in recent years (22). For patients with HER2-overexpressing tumors, the combination of chemotherapeutic agents together with receptor inhibitors, such as trastuzumab or pertuzumab, appears to be effective and offers some promise for the future (3, 23). However, drug resistance seems to be inevitable when tumor recurs (22). To date, despite intense efforts, there exist no clinically available agents that are capable of overcoming MDR. Since hormone-positive breast cancer progresses much faster than hormone receptor-negative cancer, it is extremely difficult to treat MDR/HER2 double-positive breast cancer. Thus, in order to develop a cell model for investigating the mechanism responsible for the development and progression of P-gp-mediated MDR, as well as for studying other biological properties of its gene product, we transferred *ABCB1* gene into BT-474 breast cancer cells and successfully maintained the MDR phenotype.

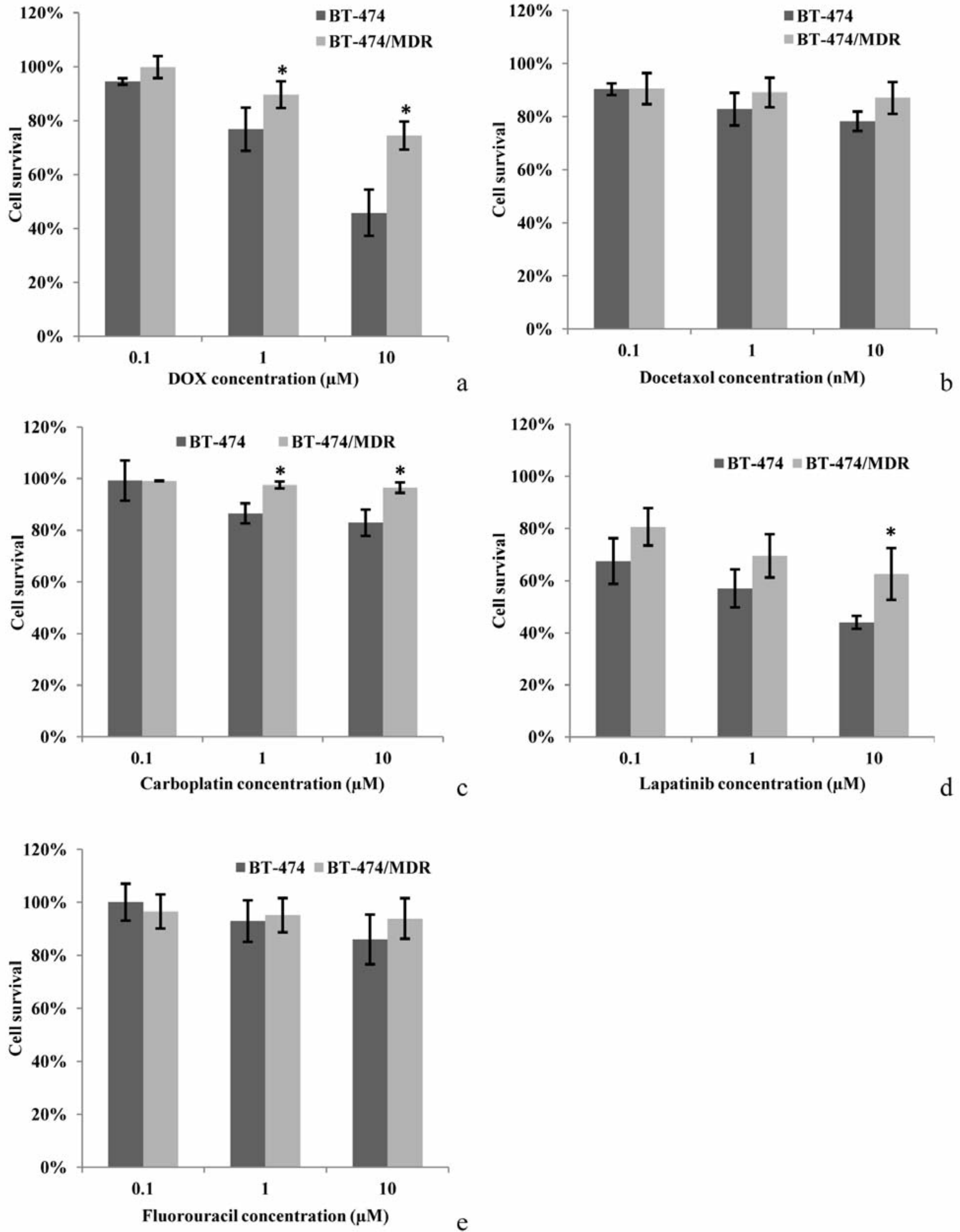


Figure 5. *Assessment of cytotoxicity of doxorubicin (DOX), docetaxol, carboplatin, lapatinib, and fluorouracil to both transfected and non-transfected BT-474 cells. Transfected cells showed much higher resistance to chemotherapy for all drugs tested. *p<0.05 compared to non-transfected cells.*

The method most commonly used for formation of drug resistance is prolonged cultivation of tumor cells in a medium with gradually increasing concentrations of anticancer agents (24). This method has several distinct disadvantages. Firstly, this process requires a very large amount of time, which also varies greatly from cell line to cell line. For example, the establishment of DOX resistance in MCF-7 human breast cancer cell line requires at least four months; development of cross-resistance in MCF-7 could take as long as two years (25). For A2780 human ovarian cancer cell, the duration is even longer (11-12 months for DOX and 6.5-8 months for cisplatin (24). Secondly, this method usually achieves resistance to only one drug, whereas the MDR phenotype in humans is often characterized by cross-resistance to a wide range of chemotheraputic agents. Last but not least, further cultivation of resistant cells in drug-containing medium is required, otherwise drug resistance will decline.

In this study, we report for the first time, a fast yet stable method for establishing MDR phenotype in HER2-positive breast cancer cells based on mRNA transfection. The transfection process takes two to three days, while the subsequent colchicine selection took about three weeks for BT-474 cell line in our experiment. The overall process takes less than a month to formulate a stable MDR cell line compared to at least four months with the traditional method. The RT-PCR results suggest stable *ABCB1* gene expression after transfection, and the western blot and immunostaining analyses indicated that the P-gp was consistently overexpressed in the cell membrane of the transfected cells, which maintained their HER2-positive phenotype.

We also conducted an *in vitro* cell culture experiment using five different chemotherapeutic agents, with each representing a unique tumor growth-inhibition pathway. Among them, DOX, docetaxol, and carboplatin all work by inducing DNA damage in tumor cells (26). Fluorouracil can inhibit cell mitosis, thus stopping cell replication (27). These four drugs are known P-group substrates and since they all target intracellular compartments, the increased cell survival after transfection (Figure 5) suggests a high level of P-gp activity on the cell membrane. Our *in vitro* cytotoxicity results on non-transfected cells matched those of existing reports (28-33).

Differently from the other four drugs, lapatinib is a tyrosine kinase inhibitor that works by interrupting HER2 receptor functionality (34). Interestingly, sensitivity to lapatinib also decreased after transfection with *ABCB1*. This may suggest that P-gp can recognize this HER2 inhibitor. Since P-gp can respond to a wide range of small water-soluble drug molecules, it is not surprising that lapatinib is one of its substrates. Although not explicitly confirmed, it has been observed by several groups that lapatinib can reverse ABCB1-and ABCG2-mediated MDR by directly inhibiting their transport function (23, 35), which demonstrates the possible association between lapatinib and P-gp.

## Conclusion

In the present study, we describe a method which is suitable for inducing MDR phenotype in breast cancer cells. The method is appropriate for fast, stable induction of P-gp-mediated drug resistance compared to the traditional methods. *In vitro* cytotoxicity testing suggests cross-resistance to a wide range of chemotherapeutic agents. Such a method has great potential for both *in vitro* and *in vivo* applications.

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