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Tumorigenesis and Neoplastic Progression

Increased Expression of P-Glycoprotein Is Associated with Doxorubicin Chemoresistance in the Metastatic 4T1 Breast Cancer Model

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Development of drug resistance is one of the major causes of breast cancer treatment failure. The goal of this study was to understand the chemoresistance mechanism using the highly metastatic 4T1 breast cancer model, which emulates stage IV breast cancer in humans. The metastatic 4T1 breast cancer cell line treated with either doxorubicin or 5-FU showed a concentration-dependent reduced cell proliferation, with induced G2-phase growth arrest (doxorubicin) or G1-phase growth arrest (5-FU). Doxorubicin treatment partially suppressed the multiorgan metastasis of 4T1 breast cancer cells in the lung, heart, liver, and bone, compared with either 5-FU or cyclophosphamide. We isolated and characterized 4T1 breast cancer cells from doxorubicin-resistant metastatic tumors (cell line 4T1-R). Multiorgan metastasis of drug-resistant 4T1 breast tumors was totally resistant to doxorubicin treatment. Our results indicate that doxorubicin is localized exclusively in the cytoplasm of resistant 4T1 breast cancer cells and that it cannot reach the nucleus because of increased nuclear expression of P-glycoprotein. Pretreatment of doxorubicin-resistant 4T1-R breast cancer cells with verapamil, a general inhibitor of P-glycoprotein, increased nuclear translocation of doxorubicin and cellular cytotoxicity. Thus, impaired nuclear translocation of doxorubicin due to increased expression of P-glycoprotein is associated with doxorubicin resistance of highly metastatic 4T1 breast cancer. ***(Am J Pathol 2011, 178:838–852; DOI: 10.1016/j.ajpath.2010.10.029)***

Breast cancer is the second leading cause of cancer-related mortality among women throughout the world.[1,2] The majority of breast cancer patients present with early-stage disease that can be cured by surgery. Although surgery is the first line of treatment for primary breast cancer, it can also increase the dissemination of tumor cells into the bloodstream, resulting in the seeding of tumor cells in distant organs.[3,4] Adjuvant chemotherapy for breast cancer after surgery has been the standard of care, and can effectively prevent the occurrence of tumor cell dissemination and metastasis.[5] Nonetheless, a considerable number of patients experience recurrence of cancer metastasis, despite adjuvant chemotherapy.[6] The development of resistance to anticancer drugs is a major problem in the use of chemotherapy for metastatic breast cancer.[7,8] Furthermore, the mechanisms of chemoresistance for breast cancer are not completely understood. Chemoresistant malignancy is now the leading cause of death after otherwise successful breast cancer surgery.[1,2] Identification of a molecular marker that will predict at an early stage the chemotherapy response of breast cancer should allow selection of alternative treatment strategies to overcome chemoresistance.

Among the various chemotherapy agents used for the treatment of breast cancer, doxorubicin achieves a response rate of 40% to 50% as a single agent and 60% to 80% in combination with other chemotherapeutic agents.[9,10] The combination of doxorubicin with 5-fluorouracil (5-FU) and cyclophosphamide leads to a response rate of 50% to 80%.[11] Understanding the mechanisms of doxorubicin action and resistance in a relevant animal model system could lead to identifying a molecular marker to predict the response to chemotherapy. The major obstacle to date has been the lack of a suitable animal model system that faithfully recapitulates the multiorgan metastasis of human breast cancer. Over time,

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various animal models of breast cancer have been extensively used to study the mechanisms of chemoresistance. The majority of studies have used the xenograft mouse models, in which human breast cancer cells are implanted in immunodeficient mice.[12] However, human tumor cells metastasize poorly in these mouse models,[13,14] and thus these models do not recapitulate the multiorgan metastasis of human breast cancer. Furthermore, the immune system plays an important role in the growth of cancer in humans, and the mechanisms of resistance to therapy may be related to interactions with the immune system. A model that includes a functional immune system may be more suitable for the study of the action of chemotherapy agents.

In the present study, we used a highly metastatic 4T1 murine mammary carcinoma cell line that develops metastases in lung, liver, and bone when injected systemically in BALB/c mice.[15,16] The highly metastatic 4T1 cell line was isolated from a BALB/cfC3H mouse at the Karmanos Cancer Institute, University of Michigan.[16] Cancer metastasis in this mouse model closely resembles that of advanced human breast cancer and therefore serves as an animal model for stage IV breast cancer. Recently, Tao et al[15] showed that the 4T1-luciferase breast tumor in BALB/c mice metastasizes to multiple organs (including lung, liver, and bone), which can be measured in a real-time manner with whole-body imaging.

We decided to use this syngeneic 4T1 mouse model to address the overall success of a chemotherapy response for metastatic breast cancer. We first evaluated the effect of doxorubicin and 5-FU, singly and in combination, on the growth of 4T1 cells in culture. The mechanism of action of each chemotherapy drug in inducing 4T1 cell growth arrest was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and by cell cycle analysis. The effect of single or combination chemotherapy of doxorubicin, 5-FU, and cyclophosphamide on proliferation of 4T1 cells in several visceral organs of BALB/c mice was determined by parameters that measure the survival of mice, quantitation of chemoresistant tumor cells and tumor nodules in the metastatic organs by colony assay, staining by India ink, and histological evaluation of tumor metastasis.

Using this model, we determined that doxorubicin chemotherapy is highly effective in reducing the multiorgan metastasis of 4T1 tumor cells, compared with a combination treatment with 5-FU, doxorubicin, and cyclophosphamide. Of these three drugs, doxorubicin has the highest anticancer properties, followed by 5-FU. Cyclophosphamide has very little effect in inhibiting the growth of 4T1 metastatic cancer in the BALB/c mice. With this 4T1 experimental metastasis model in BALB/c mice, we found evidence suggesting that a fraction of metastatic tumors develop resistance to doxorubicin, whether singly or in combination chemotherapy. We therefore isolated and characterized a 4T1 doxorubicin-resistant cell line (4T1-R) from the metastatic tumors; this line is completely resistant to doxorubicin-induced cell killing, and the cells do not undergo G2/M growth arrest. We showed that impaired nuclear translocation of doxorubicin in the 4T1-R cells is due to increased expression of P-glycoprotein. We propose that the genetic analysis of highly metastatic 4T1-R cells should provide a deeper understanding of the chemoresistance mechanisms of human breast cancer.

Materials and Methods

Cell Culture

The 4T1 mouse breast cancer cell line[16,17] was obtained from Dr. Fred Miller at the Karmanos Cancer Institute. Cells were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere with 5% CO_2. Tumor cell suspension with >90% viability was prepared from subconfluent cultures by the treatment of trypsin-EDTA solution (Invitrogen). The 4T1 cells were stably transfected with green fluorescence protein (GFP)-containing plasmid (pEGFP-N1; Clontech Laboratories, Palo Alto, CA) and were selected in medium containing 250 μg/ml of G-418 antibiotic.

Drug Treatment

We chose the three drugs 5-FU, doxorubicin (trade name, Adriamycin), and cyclophosphamide, because their use corresponds to the human FAC chemotherapy regimen.[18] Doxorubicin (catalog no. 44583) and fluorouracil (5-FU) (catalog no. F6627) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared in distilled water: doxorubicin at 1 mg/ml (1.7 μmol/L) and 5-FU at 7 mg/ml (54 μmol/L). Cyclophosphamide (catalog no. 150749) was purchased from MP Biomedicals (Solon, OH). Cyclophosphamide is activated to 4-hydroxy cyclophosphamide by cytochrome P450 isozymes in the liver, and therefore this drug was used only *in vivo*.[19,20] A stock solution of cyclophosphamide at 0.8 mg/ml (2.8 μmol/L) was prepared in distilled water. Stock solution for each drug was sterilized with a 0.2-μm syringe filter, aliquoted, and stored at −20°C until use. Four sets of drug combinations were freshly prepared by serial dilution with medium to reach final concentrations ranging from 0.086 μmol/L to 0.690 μmol/L for doxorubicin and 0.228 μmol/L to 1.820 μmol/L for 5-FU.

MTT Proliferation Assay

Cell viability was measured by the MTT assay. The tetrazolium compound in this assay is reduced intracellularly to formazan by mitochondrial dehydrogenase. The conversion of blue tetrazolium into purple formazan by metabolically active cells indicates the extent of cell viability. Viability of tumor cells is measured by the quantification of formazan dye by the colorimetric method. We used this MTT assay and colorimetric method to measure the effect of the chemotherapy drugs on cell proliferation.

The 4T1 cells were seeded on 24-well plates at a density of 2×10^4 cells/well in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and were allowed to adhere by incubation at 37°C overnight. After 24 hours, culture me-

dium was replaced and cells were then treated with four different concentrations of the drugs (doxorubicin and 5-FU), either singly or in combination. After 48-hour incubation, cultures were washed twice with PBS and 100 μL of MTT solution (MTT solution concentration is 5mg/ml dissolved in PBS; thiazolyl blue tetrazolium bromide, catalog no. M5655; Sigma-Aldrich), along with 900 μL of growth medium added in each well. Cells were incubated at 37°C for 3 hours. The tumor cells were then washed in PBS, and plates were directly photographed to visualize the number of cell colonies that survived the drug treatment. The cells were solubilized with 1 ml of MTT solubilization buffer (anhydrous isopropanol containing 10% Triton X-100, 0.1 N HCl) for 5 minutes. Absorbance of converted dye was measured in a spectrophotometer (DU-530 UV/VIS Life Science spectrophotometer; Beckman Coulter, Brea, CA) at a wavelength of 570 nm. Experiments were repeated three times for each drug alone and in combination. The percentage of cell viability was determined by comparison with untreated controls. The median inhibitory concentration IC_{50} (ie, 50% reduction in cell viability) was determined for each chemotherapy drug.

Analysis of the Combinatorial Response

To determine the combinatory effect of doxorubicin and 5-FU, 4T1 cells were treated with 0, 0.043 μmol/L, 0.086 μmol/L, 0.172 μmol/L, 0.344 μmol/L, 0.616 μmol/L, or 1.230 μmol/L of doxorubicin and 0, 0.038 μmol/L, 0.076 μmol/L, 0.228 μmol/L, 0.546 μmol/L, 0.912 μmol/L, or 1.824 μmol/L of 5-FU in a 7×7 factorial design. Out of these 49 cultures, the MTT assay results of 3 escalating doses of doxorubicin, 3 escalating doses of 5-FU, and 9 combination treatments of doxorubicin and 5-FU were analyzed, using CalcuSyn software version 4.2 (Biosoft, Cambridge, UK). The software uses the median-effect principle to delineate the interaction between two drugs. For each combination, the program generates a combination index (CI) based on the equation of Chou and Talalay,[21,22] where (D)1 and (D)2 represent the doses of drug 1 and drug 2 that inhibit cell growth by *x*% based on empirical observations if used in combination and (Dx)1 and (Dx)2 represent the theoretical doses of drug 1 and drug 2 that will achieve *x*% inhibition if used alone. (Dx)1 and (Dx)2 are calculated from the median-effect formula,[21] based on the dose curve of each drug. The combination index indicates synergism ($CI < 1$), additive effect ($CI = 1$), or antagonism ($CI > 1$).

Flow Cytometry

4T1 cells (1×10^5) were seeded into 100-mm plates in DMEM with 5% FBS at 37°C overnight. On the next day, the medium was aspirated and replaced with 10 ml of fresh media containing the appropriate drug or drugs (doxorubicin and 5-FU). After 24 hours of treatment, cells were washed with PBS and trypsinized using trypsin-EDTA (Invitrogen). The cells were fixed in 70% ethanol in PBS and were incubated for 2 hours on ice. The fixed cells were subsequently washed twice with PBS and centrifuged at 2000 rpm for 5 minutes. The cells were resuspended in a commercially available propidium iodide staining solution (BD Pharmingen catalog no. 550825; BD Biosciences, San Diego, CA) and were incubated in the dark at room temperature for 30 minutes. The cell cycle analysis was performed using 2×10^4 cells in a flow cytometer (BD LSR II; BD Biosciences). The percentages of 4T1 tumor cells present in the G1, S, and G2 phases of the cell cycle after drug treatment were analyzed using Modfit LT 3.0 software (Verity Software House, Topsham, ME).

Western Blot Analysis

Briefly, 4T1 breast cancer cells were cultured in a 6-well plate, lysed with 200 μL of nuclear and cytoplasmic extraction reagents (NE-PER; Pierce Biotechnology, Rockford, IL). Total protein in the lysate was quantified using a protein assay kit (Bio-Rad, Hercules, CA). Approximately 10–30 μg of protein from each sample was mixed in 4× SDS-loading buffer. Proteins were separated by NuPAGE 4-12% Bis-Tris Gel (Invitrogen) and then transferred onto a nitrocellulose membrane (GE Health Care, Buckinghamshire, UK). The membrane was blocked with 5% fat-free milk powder in 50 mmol/L Tris-buffered saline, pH 7.6, with 0.1% Tween 20 at room temperature for 1 hour. The membrane was washed three times and incubated overnight at 4°C with either mouse monoclonal antibody to P-gp (C219; Abcam, Cambridge, MA) at 1:500 dilution or a mouse monoclonal anti-β-tubulin clone (TUB 2.1; Sigma-Aldrich) at 1:1000 dilution in Tris-buffered saline-Tween 20 containing 5% fat-free milk powder. After this step, the membrane was washed three times with Tris-buffered saline-Tween 20 and reacted for 1 hour with the secondary antibody (anti-mouse, supplied in the enzymatic chemiluminescence kit) that was conjugated with horseradish peroxidase at a dilution of 1:2000. The bound antibodies were detected using the ECL Plus Western blotting detection system (GE Health Care), and the chemiluminiscent signals were detected using high-performance chemiluminescence film (GE Health Care).

Immunocytochemistry

The 4T1 cells were cultured on chamber slides overnight. The next day, slides were fixed with chilled acetone and were treated with 1 ml of blocking reagent (Background Sniper BS966; Biocare Medical, Concord, CA) for 10 minutes at room temperature. The slides were then incubated with a primary mouse monoclonal antibody (C219; Abcam) at 1:50 dilution for 1 hour at room temperature, followed by a secondary reagent mouse probe (MACH 4 mouse probe, UP534; Biocare Medical) for 10 minutes, then tertiary reagent horseradish peroxidase polymer (Mach 4 HRP polymer, MRH534; Biocare Medical) for 20 minutes. At the final step, the slides were counterstained with hematoxylin and bluing. All images were taken at 20× magnification.

TUNEL Assay

For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, 4T1 cells were cultured in the chamber slide for overnight. On the next day, the cells were treated with doxorubicin (100 ng/ml). After 24, 48, and 72

hours, culture slides were removed and washed with PBS, then fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) for 1 hour at 15–25°C. After fixation, cells were rinsed with PBS and incubated in 0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared. Staining was performed using the protocol supplied with a Roche *in situ* cell death detection kit (catalog no. 11684809910; Roche Applied Science, Indianapolis, IN). The color was developed using Vulcan Fast Red chromogen (Biocare Medical).

Animal Model and Drug Treatment

The testing for a chemotherapy drug using BALB/c mice was conducted after receiving approval from the Institutional Animal Care and Use Committee (IACUC), Tulane University Health Sciences Center. Female BALB/c mice, 4 to 6 weeks old, were obtained from Charles River Laboratories International (Wilmington, MA) and were maintained in a pathogen-free environment at the Department of Comparative Medicine, Tulane University Health Sciences Center. The mice were handled and euthanized according to ethical guidelines. The mice were housed five to a cage, fed *ad libitum*, and observed daily. The cages were kept in a climate-controlled, warm animal suite and were cleaned on a weekly basis.

Tumor metastasis was induced in BALB/c mice by inoculation of 4T1 breast cancer cells by two different routes. In the first model, 4T1 cells (5×10^5) were inoculated into the orthotopic mammary fat pad of BALB/c for tumor development; tumors were surgically removed when they had reached 10 mm in size, after 3 to 4 weeks. At 1 week after surgery, chemotherapy was administered every other day for 21 days. The effect of chemotherapy on the growth of established solid metastasis in different visceral organs was examined. In the second model, 4T1 cells (5×10^5) tumor cells in 100 μL of PBS were injected directly, via the tail vein. At 1 day after 4T1 cell injection, the mice were injected with 100 μL of the chemotherapy drug by intraperitoneal injection, as described previously.[23]

For each model, chemotherapy treatment of BALB/c mice was randomized into five groups of five mice each. The control group was injected with 0.9% saline. In the three single-drug treatment groups, one group was injected with doxorubicin (5 mg/kg body weight),[24] one group was injected with 5-FU solution (35 mg/kg body weight,[25] and one group with cyclophosphamide (40 mg/kg body weight).[26] The fifth group was injected with a combination of the three drugs (5 mg/kg + 35 mg/kg + 40 mg/kg). The mice were treated with chemotherapy drugs every other day for 21 days. The mice were closely monitored. They were weighed thrice weekly and were assessed according to the following criteria: lethargy or body weight loss of >20% of initial body weight. The mice were sacrificed and the organs (lung, heart, liver, kidney, spleen, bone, and brain) were collected for colony assay and histological evaluation. Antitumor activity after chemotherapy was evaluated by using an assay that quantitatively measures the viable tumor cells in each metastatic organ.

Measuring Lung Metastasis by India Ink Staining

To observe tumor metastasis in lung, mice were sacrificed and their lungs were injected with 1 ml of 15% India ink (Yasutomo, San Francisco, CA) to visualize individual tumor nodules using a standard protocol described previously.[27,28] Briefly, 1 ml of India ink solution was injected through the trachea using a 20G x 1 Surflash IV catheter (Terumo Medical Products, Somerset, NJ) and was allowed to fill the lungs. The lungs were removed and placed in Fekete's solution [100 ml 70% alcohol, 10 ml 10% buffered formalin (Stephens Scientific, Wayne, NJ), and 5 ml glacial acetic acid] for 5 minutes for destaining. Tumor nodules do not absorb 15% India ink, and therefore normal lung tissue stains black and tumor nodules remain white.

Quantitation of Tumor Metastasis by Colony Assay

To recover 4T1 tumor cells from each organ, we used a previously described protocol that involves a combination of mechanical and enzymatic digestion.[29] In brief, tissue was placed in a Petri dish containing 10 ml of medium containing heparin (5 units/ml) to maintain the viability of cells and to prevent coagulation, and the tumor cell isolation was performed as quickly as possible to maximize the viability of the tumor cells. The tissues were disaggregated mechanically using forceps and then were subjected to proteolytic enzyme digestion. Tissues were cut into small pieces (1 to 3 mm^3) and were digested with collagenase type 1 (Invitrogen) for 30 minutes at 37°C. The cell suspension was then filtered through a 22-μm nylon mesh. Tumor cells were harvested at low-speed centrifugation (500 rpm) using a Beckman tabletop centrifuge. The cell pellet was suspended and cultured in 100-mm Petri dishes in a growth medium containing 10% FBS and 1% penicillin-streptomycin before incubation at 37°C. The next day, the culture was washed, and the growth medium was replaced with fresh medium. The cells were cultured for 2 weeks, until visible cell colonies were obtained. The colonies were stained using Giemsa dye (Sigma) and were counted using an SC6 colony counter (Barloworld Scientific, South Africa).

Immunohistochemical Characterization of 4T1 Breast Cancer

At the end of chemotherapy treatment, the mice were sacrificed and the individual organs (lung, heart, liver, spleen, kidney, bone, and brain) were harvested, and fixed in 10% buffered formalin and embedded in paraffin. Paraffin blocks were prepared and 5-μm tissue sections were prepared and stained with hematoxylin and eosin. Tissue sections of treated and untreated mice were examined under a light microscope by two pathologists (K.M. and R.J.) for histopathological evaluation of tumor metastasis and then were photographed using a Nikon digital camera. Immunohistochemical staining for Ki-67, E-cadherin, estrogen receptor (ER), progesterone receptor (PR), p63, cytokeratin 5/6, HER2, and SMA was per-

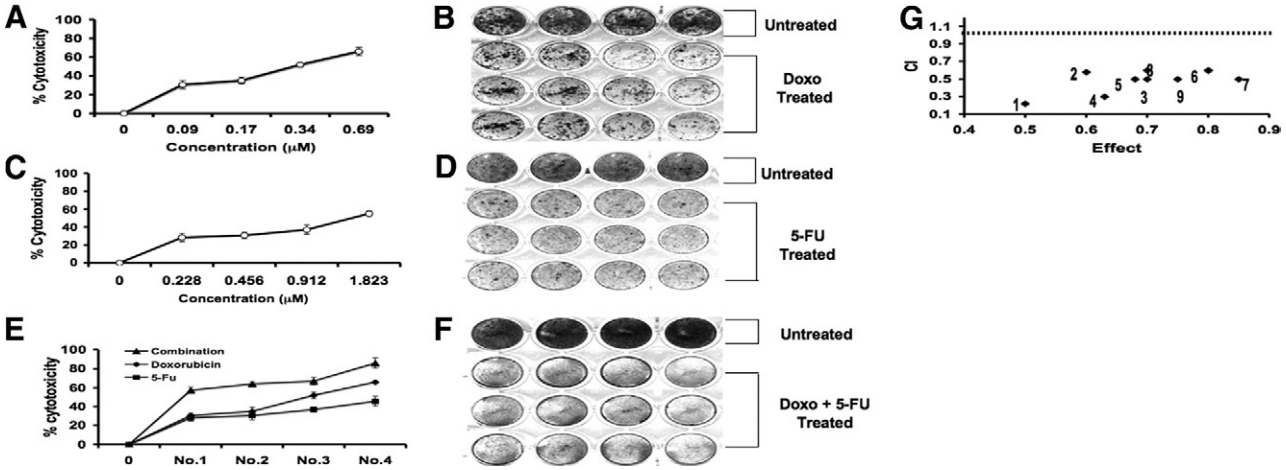

Figure 1. Doxorubicin and 5-FU together induce dose-dependent cell viability and toxicity of 4T1 cells in culture. **A**, **C:** 4T1 cells were plated in triplicate in 24-well dishes; on the next day, cells were cultured with fresh media containing various concentrations of doxorubicin or 5-FU alone. **E:** Cell toxicity of 4T1 culture when both doxorubicin and 5-FU were added. Cell viability and toxicity was assessed by MTT assay after 48 hours of drug treatment. Data are expressed as a percentage of optical density value obtained from the untreated control. **B**, **D**, **F:** We measured the presence of the viable 4T1 tumor cells in the culture after 48 hours of treatment with a single drug or in combination, in the concentrations shown in panels **A** and **C**. **G:** The combination index plot of doxorubicin and 5-FU. Combination index (CI) is <1, indicating that these two drugs work synergistically (see section on *Analysis of the Combinatorial Response*).

formed on paraffin-embedded tissue slides prepared from lung metastasis. The slides were deparaffinized for 15 minutes at 50–60°C, followed by two xylene treatments for 5 minutes. The slides were dehydrated by treatment with 100%, 95%, and 80% ethanol. Peroxidase quenching was performed with 3% H_2O_2 + 100% methanol for 5 minutes each treatment. The slides were put into a plastic Coplin jar with Reveal Decloaker RTU reagent (Biocare Medical) for 25 minutes at 95°C in the steamer for heated antigen retrieval. Slides were then allowed to stand at room temperature for 20 minutes. The slides were rinsed in deionized H_2O, and we used a PAP pen to mark around the tissue. The slides were incubated with blocking sniper (Biocare Medical) for 10 minutes and were incubated with a primary antibody for 1 hour at room temperature. After a washing with Tris-buffered saline, the slides were incubated with the secondary reagent mouse probe for 10 minutes and then the tertiary reagent horseradish peroxidase polymer for 20 minutes. Slides were counterstained with hematoxylin and bluing.

The following primary antibodies were used: P63 rabbit polyclonal antibody (ab5039; Abcam) at 1:500 dilution; anti-cytokeratin 5, 6 clone monoclonal antibody (catalog no. MAB1620; Millipore, Billerica, MA) at 1:200 dilution; HER2/ErbB2 (29D8) rabbit monoclonal antibody (catalog no. 2165; Cell Signaling Technology, Danvers, MA) at 1:200 dilution; estrogen receptor (SP1) rabbit monoclonal antibody (RM-9101; Thermo Scientific, Fremont, CA) at 1:200 dilution; progesterone receptor rabbit monoclonal (SP2) antibody (ab16661; Abcam) at 1:200 dilution; and Ki-67 rabbit monoclonal antibody (RM-9106; Thermo Scientific) at 1:500 dilution. In addition, E-cadherin rabbit monoclonal antibody (RM-2100) and SMA antibody (RB-9010-P0) were purchased from Thermo Scientific and were used at 1:200 dilution. The final images were taken at 20× magnification.

Statistical Analyses

Kaplan-Meier analysis was used to assess survival and the log-rank (Mantel-Cox) test was used to compare the survival distributions of the two groups to determine significance.

Results

Doxorubicin and 5-FU Each Inhibits Proliferation of 4T1 Cells in Culture

The cytotoxic effect of doxorubicin and 5-FU on the 4T1 tumor cell line was investigated by MTT assay after 48 hours (Figure 1). The dose-response data of doxorubicin-induced toxicity in 4T1 tumor cells from three separate experiments indicated that doxorubicin cytotoxicity reached a maximum of 70% at the highest concentration tested (0.69 μmol/L) (Figure 1A). The increase in doxorubicin concentration in the culture also correlated with a decrease in the number of viable 4T1 tumor cell colonies in the plate (Figure 1B). Similarly, for 5-FU the MTT assay indicated dose-dependent toxicity of the drug in 4T1 tumor cells, with maximum cytotoxicity of 60% at the highest concentration tested (1.82 μmol/L) (Figure 1C).We also found a decrease in the density of viable 4T1 tumor cells in the culture with an increase in the concentration of the 5-FU (Figure 1D). The concentration showing 50% growth inhibition of 4T1 cells in culture (IC_{50}) was determined to be 0.17 μmol/L for doxorubicin and 1.0 μmol/L for 5-FU. Thus, doxorubicin and 5-FU induce 4T1 tumor cell toxicity differently in culture, and doxorubicin induces a stronger toxic effect on 4T1 tumor cells at a significantly lower concentration, compared with 5-FU.

We then determined the cell toxicity of 4T1 cells in the culture by adding these two chemotherapy drugs in combination. The different concentrations of doxorubicin and

5-FU (Figure 1, A and C) were combined, and toxicity to 4T1 tumor cells with the four separate doses of combined drug was measured. With the addition of doxorubicin and 5-FU in combination, the MTT assay indicated increased cytotoxicity in 4T1 tumor cells in culture, reaching a maximum toxicity of 85% at the highest combined dose (Figure 1E). These results correlated very well with the colony assay, showing that concentration dependence decreased in viable cell density when they were combined (Figure 1F). The dose-dependent data to the median-effect principle for two-drug combinations were extended to generate CI values using CalcuSyn software version 4.2 (Biosoft). Combined treatment was defined as antagonistic if CI $>$ 1, additive if CI = 1, and synergistic if CI $<$ 1. Our analysis yielded a CI value of $<$1, indicating that these two drugs interact synergistically to arrest the growth of 4T1 cells in culture (Figure 1G).

Cell Cycle Analysis of 4T1 Cells Shows Dominant Effect of Doxorubicin-Induced G2/M Arrest

Cell cycle analysis by flow cytometry was performed 24 hours after treatment of 4T1 tumor cells with doxorubicin alone, and then in combination with 5-FU. The concentration of each drug that inhibited 50% proliferation of 4T1 cell in cell culture was selected for cell cycle analysis (Figure 2A). Of 4T1 tumor cells treated with doxorubicin, 99% showed increased accumulation in the G2/M phase of the cell cycle and subsequently showed a progressive decline in the G1- and S-phase populations, compared with the untreated control. Only 66% of 4T1 tumor cells treated with 5-FU showed increased accumulation in the G1/S phase of the cell cycle, compared with the untreated control cells. Thus, the potential growth inhibitory effect of the two drugs on the 4T1 cells appears to be due to a block at the G2/M phase of the cell cycle (doxorubicin) and the G1/S phase (5-FU). The change in the cell cycle distribution was distinct in the doxorubicin-treated 4T1 cells, compared with the 5-FU treatment. These results are in agreement with a previous study using the human breast cancer cell line MDA-MB-231.[30] Interestingly, the combination treatment with both doxorubicin and 5-FU lessened the pronounced G2/M growth arrest, from 99% to 65%. The cell cycle profile after combination treatment shifted more toward a block in the G2/M phase of the cell cycle, as originally observed for doxorubicin (Figure 2A). These findings thus suggest that the anticancer effect of doxorubicin is dominant in the combination treatment.

The TUNEL assay was performed to confirm the DNA strand break in 4T1 cell lines after doxorubicin treatment at different time intervals. The doxorubicin treatment induced a time-dependent increase in the number of 4T1 cells exhibiting DNA fragmentation in culture (Figure 2B). The most significant differences were observed at 48 and 72 hours for doxorubicin treatment. There was a positive correlation between the DNA content and the occurrence of TUNEL-positive cells showing the DNA break.

Doxorubicin Treatment Improves the Survival of BALB/c Mice with 4T1 Breast Cancer Metastasis

The success of combination chemotherapy using three drugs (doxorubicin, 5-FU, and cyclophosphamide) to prevent growth of 4T1 cells in multiple organ systems was examined in a BALB/c mouse model. First, the overall survival of mice in the experimental metastasis model

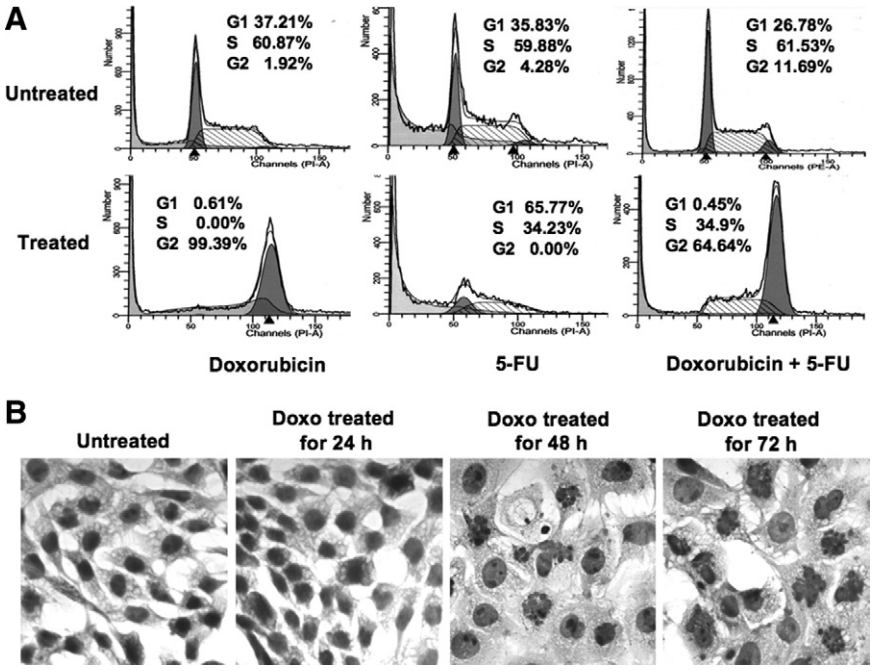

Figure 2. A: Flow cytometric analysis shows the effect of doxorubicin and 5-FU alone or combination on the cell cycle profile of 4T1 tumor cells in culture. 4T1 cells were treated with 0.17 μmol/L doxorubicin alone or 0.46 μmol/L 5-FU or a combination of doxorubicin and 5-FU for 48 hours. Cells were then stained with propidium iodide and cells in different phases of the cell cycle were analyzed by flow cytometry. Doxorubicin treatment resulted in G2/M phase growth arrest in 99% of 4T1 cells in culture. 5-FU treatment resulted in G1/S phase growth arrest in 66% of 4T1 cells in culture. Treatment with a combination of doxorubicin and 5-FU resulted in a round 65% G2/M and 35% G1/S phase growth arrest of 4T1 cells in culture. **B:** TUNEL staining shows DNA fragmentation and apoptosis of 4T1 cell culture after doxorubicin treatment.

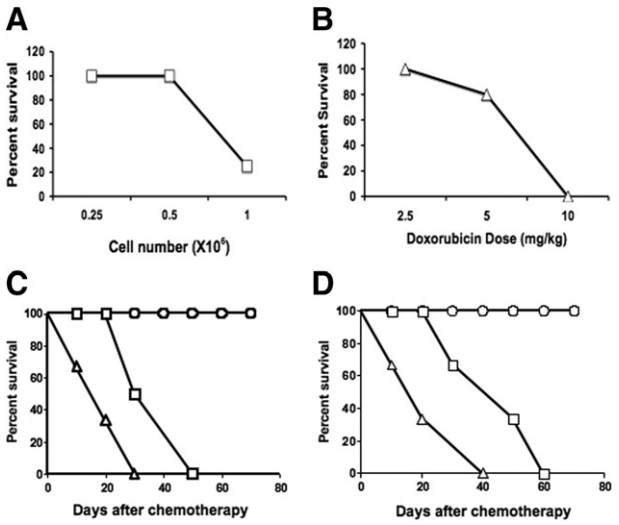

Figure 3. Effect of chemotherapy on overall survival of BALB/c mice with or without 4T1 breast cancer metastasis. **A:** Survival of mice after tumor cell injection was determined using 20 normal BALB/c mice (4 groups, 5 mice each). One group received no tumor cells; the remaining three groups were injected with different numbers of 4T1 breast cancer cells (0.25×10^6, 0.5×10^6, and 1.0×10^6) via the tail vein and overall survival at 2 weeks was determined. **B:** Doxorubicin toxicity studies in 20 normal BALB/c mice (4 groups, 5 mice each). One group received no treatment and three groups were treated with different doses of doxorubicin (2.5, 5.0, and 10 mg/kg) via intraperitoneal injection every other day and overall survival at 21 days was determined. **C:** Kaplan-Meier survival curves for combination chemotherapy using the 4T1 experimental metastasis model in BALB/c mice. Five mice were injected with 4T1 tumor cells via the tail vein. After 24 hours, mice were treated every other day with a combination of three drugs (doxorubicin, 5-FU, and cyclophosphamide) by intraperitoneal injection (open squares). Another group of five mice were injected with 4T1 tumor cells by the tail vein without treatment (open triangles). To assess overall toxicity of the chemotherapy, a third group of five mice, without tumor cell injection, received a similar treatment (open circles). The overall survival of mice treated with a combination of the three drugs was found to be significantly different from those without drug treatment ($P = 0.0025$; log-rank test). **D:** Kaplan-Meier survival curves for combination chemotherapy in the spontaneous tumor metastasis model in BALB/c mice (fat pad injection). Five mice were each injected with 4T1 tumor cells into the mammary fat pad. After the tumor grew to 10 mm in size, the primary tumor was removed by surgery. Mice were treated every other day with a three-drug combination (doxorubicin, 5-FU, and cyclophosphamide) by intraperitoneal injection (open squares). Another group of five mice similarly injected with 4T1 tumor cells into the mammary fat pad received no treatment (open triangles). To assess the overall toxicity of chemotherapy, a third group of five mice without tumor cell injection received similar treatment (open circles). Overall survival was significantly different after combination chemotherapy ($P = 0.0015$, log-rank test).

inoculated with a different number of 4T1 tumor cells was determined using a group of five BALB/c mice over 2 weeks. Results indicated that the overall survival of mice inversely correlated with the number of tumor cells used for inoculation in the experimental metastasis model (Figure 3A). We then performed drug toxicity studies using a group of five normal mice, to determine the optimum drug concentrations that are not toxic to the animals. Among the three chemotherapy drugs, the concentration of cyclophosphamide and 5-FU used in our study did not show any toxicity in the mice, because all animals survived the treatment. For doxorubicin, however, we found a dose-dependent toxicity in BALB/c mice (Figure 3B). Doxorubicin solution injected at a proportion of 10 mg/kg body weight was very toxic. The maximum tolerable dose of doxorubicin in the BALB/c mouse was found to be 5 mg/kg body weight. These preliminary experiments allowed us to select the use of a tumor cell number and drug concentration for subsequent studies in the 4T1 breast cancer metastasis model.

The success of chemotherapy was validated using the 4T1 breast cancer metastasis induced in mice by tail vein injection (experimental metastasis model) or fat pad injection (spontaneous metastasis model). The chemotherapy-mediated suppression of the 4T1 tumor metastasis in a different organ system was determined with a number of independent assays. The effect of chemotherapy on the survival of mice due to tumor metastasis was determined using a total of 30 mice, split into five groups. The survival results are summarized in Figure 3C. The overall toxicity of the combination chemotherapy treatment was assessed in a group of five normal mice. The overall survival rate was 100% in the normal tumor-free mice receiving combination chemotherapy (doxorubicin, 5-FU, and cyclophosphamide). The tumor-bearing mice treated with a combination of doxorubicin, 5-FU, and cyclophosphamide survived for 50 days. For single-drug treatment, survival was 30 days, identical across all three treatment groups. The group of mice that received no chemotherapy after tumor cell injection survived up to 27 days. The overall difference in the percentage of survival between the group of mice with or without combination chemotherapy was analyzed using Kaplan-Meier survival curves with the log-rank test and was found to be statistically significant (P value = 0.0025).

The effect of chemotherapy on tumor metastasis was examined using the mammary fat pad injection model, in which a solid metastasis of breast tumor occurred due to a natural spread of 4T1 tumor cells into multiple visceral organs after surgical removal of the primary tumor. The overall survival of mice due to tumor metastasis induced by fat pad injection was approximately 40 days without chemotherapy. The survival of mice after combination chemotherapy was improved, to >60 days (Figure 3D). The overall survival of mice with or without chemotherapy was analyzed by Kaplan-Meier curves with log-rank test and found to be significant ($P = 0.0015$).

Doxorubicin Treatment Inhibits Multiorgan Metastasis of 4T1 Breast Cancer

Because we did not observe 100% survival of mice after the chemotherapy treatment, the presence of residual tumor metastasis in multiple visceral organs was examined and quantified. For this purpose, residual 4T1-GFP tumor cells in each metastatic organ were isolated and cultured in a growth medium supplemented with G-418 antibiotic (250 μg/ml). The tumor cell colonies formed in the 100-mm plate were counted after staining with Giemsa dye. The percentage of viable tumor cell colonies reduced under chemotherapy treatment was determined. The results of the analysis were expressed as viable 4T1 cell clones after Giemsa staining (Figure 4A) and the relative number of viable tumor cells remaining after chemotherapy, compared with the untreated mice (Figure 4B).

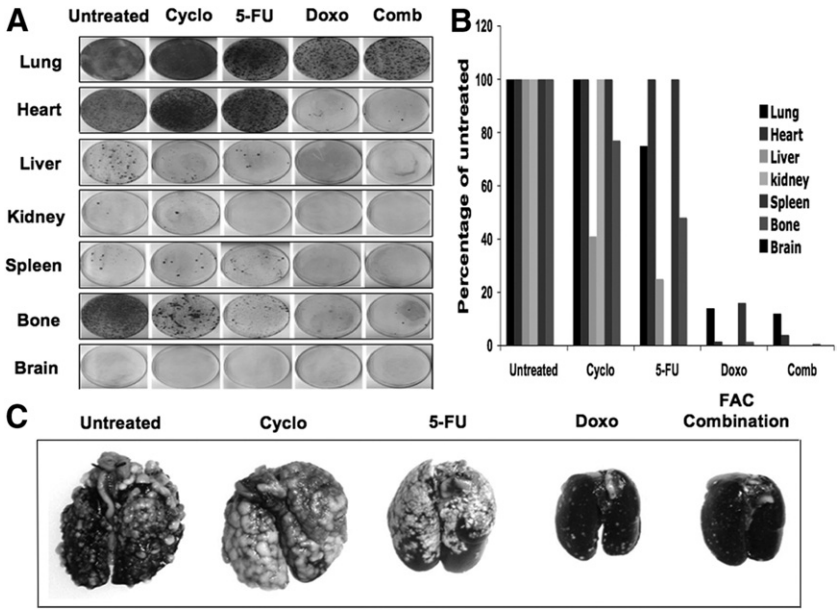

Figure 4. Quantitative analysis of residual 4T1 tumor cells in each metastatic organ in BALB/c mice after an intravenous injection of doxorubicin, 5-FU, cyclophosphamide, singly or in a combination of the three drugs. One group of mice were injected with 4T1 tumor cells via the tail vein. After 24 hours, they were injected with doxorubicin, 5-FU, cyclophosphamide, and a combination of the three drugs. After chemotherapy, 4T1-GFP tumor cells were isolated from each metastatic organ in BALB/c mice and cultured in a growth medium containing G-418 (250 μg/ml). Tumor cell colonies were stained with Giemsa dye. **A:** Cell colony density in treated and untreated mice; darker color indicates greater cell numbers. **B:** Relative number of tumor cell colonies in each metastatic organ. Tumor cell colonies were counted by using a colony counter; results were compared with the untreated group and are expressed as a percentage of the control. **C:** Lung metastasis of 4T1 tumor cells in mice with and without chemotherapy. Mice were injected with 4T1 tumor cells via the tail vein. After 24 hours, they were treated with doxorubicin, 5-FU, cyclophosphamide alone or in combination for 21 days. Lungs were then stained with India ink and were photographed using a digital camera. The mice treated with doxorubicin and combination with 5-FU and cyclophosphamide effectively suppressed 4T1 metastasis in the lungs, compared with other chemotherapy drugs.

Quantification of tumor cells by colony assay indicated that the highest number of viable 4T1 tumor cells were isolated from lung, followed by heart, bone, and liver (Figure 4A). Using this assay, we showed that the numbers of viable 4T1 cells isolated from mice with or without chemotherapy were significantly different, an indication that this assay can quantify residual tumor cells in each metastatic organ in mice. Mice treated with cyclophosphamide alone did not show a strong antitumor effect in lung and heart, because the number of colonies isolated from these two organs was not significantly different, compared with untreated mice, but cyclophosphamide treatment did reduce the number of viable 4T1 tumor cells in liver and bone. Mice treated with 5-FU alone showed a reduced growth of tumor cells in liver, kidney, spleen, and bone, but the antitumor effect was not strong enough to inhibit proliferation of 4T1 tumor cells in lung and heart. Treatment with doxorubicin alone significantly suppressed the growth of 4T1 tumor cells in heart, liver, kidney, spleen, and bone. Treatment with doxorubicin alone or in combination with 5-FU and cyclophosphamide significantly reduced the number of viable 4T1 tumor cells in heart, liver, kidney, spleen, and bone. The antitumor effect of doxorubicin alone was comparable to that of the combination drug treatment.

A significant number of residual viable 4T1 tumor cells were present in the lungs of mice that had received doxorubicin alone or in a combination treatment with 5-FU and cyclophosphamide, indicating that chemotherapy did not kill all of the 4T1 tumor cells in the lungs. From 10% to 15% of 4T1-GFP cells in the lungs remained resistant to the cytotoxic effect of doxorubicin or combination treatment with 5-FU and cyclophosphamide (Figure 4B). The reduction of tumor metastasis in the lung was due to the treatment with doxorubicin alone or combination with 5-FU and cyclophosphamide was examined by staining with India ink. Mice treated with either doxorubicin alone or in combination chemotherapy had a significant reduction in the tumor growth in the lungs, compared with 5-FU or cyclophosphamide treatment (Figure 4C). The results of the lung staining are in agreement with the results of cell colony assay and clearly show that significant numbers of 4T1-GFP cells are not killed by doxorubicin alone or in combination with 5-FU and cyclophosphamide.

The effects of chemotherapy on growth of 4T1-GFP cells in the lungs were confirmed by histological evaluation (Figure 5). Lung morphology after injection of chemotherapy drugs alone or in the three-drug combination appeared normal in control mice (Figure 5A), but mice injected with 4T1 cells contained multiple tumor nodules at 21 days (Figure 5B). Similarly, the 4T1-injected mice treated with cyclophosphamide and 5-FU had numerous tumor nodules (Figures 5C and 5D, respectively). Mice treated with doxorubicin alone (Figure 5E)or with combination chemotherapy (Figure 5F) showed a significant reduction of metastatic tumor nodules compared with 5-FU or cyclophosphamide treatments.

We established the immunohistochemical profile of the 4T1 tumor metastasis by determining the expression of ER, PR, Her-2, Ki-67, p63, smooth muscle actin (SMA), E-cadherin, and cytokeratin 5/6 (Figure 6). The chemoresistant 4T1 tumor was of ductal differentiation (positive E-cadherin) (Figure 6E). The tumor was negative for ER and PR and did not overexpress Her-2 (triple-negative) (Figure 6, D, F, and H). In addition, positive immunostaining with antibodies to p63, cytokeratin 5/6, and SMA placed it in the basal-like molecular category of tumors (Figure 6, A, C, and G). Those tumors are generally high grade, with a high proliferation rate (Figure 6B), and they are associated with an aggressive course, frequent metastasis, and overall poor prognosis. Many carcinomas in this group are associated, in humans, with *BRCA1* mutations. The results of these *in vivo* experiments led us to conclude that doxorubicin alone and in combination with 5-FU and cyclophosphamide significantly inhibits

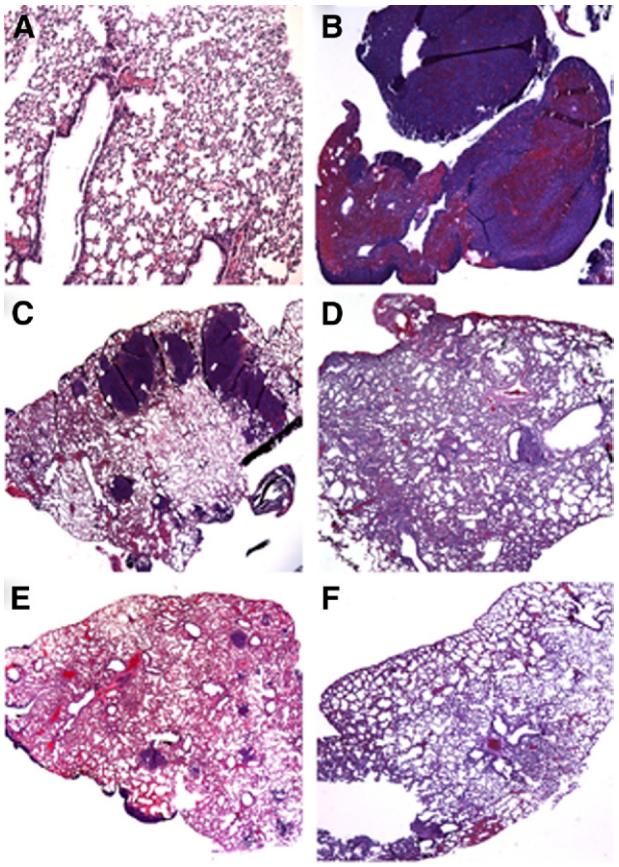

Figure 5. Histological evaluation of lung metastasis in BALB/c mice with or without chemotherapy. **A:** Lung of control mice without tumor cell inoculation but treated with doxorubicin, 5-FU, and cyclophosphamide combination chemotherapy. **B:** Lung metastasis of 4T1 tumor cells in mice without chemotherapy treatment. **C:** Lung metastasis of 4T1 tumor cells in mice treated with cyclophosphamide alone. **D:** Lung metastasis of 4T1 cells in mice treated with 5-FU alone. **E:** Lung metastasis of 4T1 cells in mice treated with doxorubicin alone. **F:** Lung metastasis of 4T1 cells in mice after treatment with doxorubicin, 5-FU, and cyclophosphamide combination chemotherapy.

4T1 tumor metastasis in the mice. The antitumor effect of doxorubicin was comparable with that of combination chemotherapy, suggesting that most of the anticancer properties in combination therapy are due to the action of doxorubicin. The 5-FU treated group also showed a moderate level of anticancer properties against 4T1 tumor cells in the mouse model. Cyclophosphamide had minimal effect on controlling proliferation of 4T1-GFP cells in these mice.

Isolation and Characterization of a Doxorubicin-Resistant 4T1 Breast Cancer Cell Line

A lack of complete inhibition of 4T1 tumor metastasis after chemotherapy led us to investigate the possible presence of chemoresistant 4T1 tumor cells. After doxorubicin treatment, 4T1 tumor cells were isolated from metastatic tumors of mouse lung and were cultured in the presence of doxorubicin (50 ng/ml). These cells had been cultured for >6 months in the growth medium supplemented with doxorubicin, to verify that they had become truly resistant to the drug treatment. Thus, we successfully isolated the 4T1 cell clones that are resistant to doxorubicin (cell line 4T1-R). Multiple 4T1 cell lines showing increased sensitivity to doxorubicin-mediated cell killing were also developed by using a limiting dilution method. Their sensitivity to doxorubicin was assessed by MTT assay. Cell clones showing the lowest optical density reading in the MTT assay were selected as doxorubicin-sensitive 4T1 cells (cell line 4T1-S). The 4T1-R and 4T1-S cell lines were further characterized, to better understand the mechanisms of doxorubicin resistance. First, the cytotoxicity effect of doxorubicin against 4T1-R and 4T1-S cell lines was measured by MTT assay. Doxorubicin-induced cytotoxicity in the 4T1-S cell line in a concentration-dependent manner (Figure 7A), whereas 4T1-R cells continued to grow in the culture in the presence of doxorubicin. No toxicity of doxorubicin was observed using the 4T1-R cell line when the MTT assay was performed at 24, 48, and 72 hours using 0.17 μmol/L doxorubicin (Figure 7B).

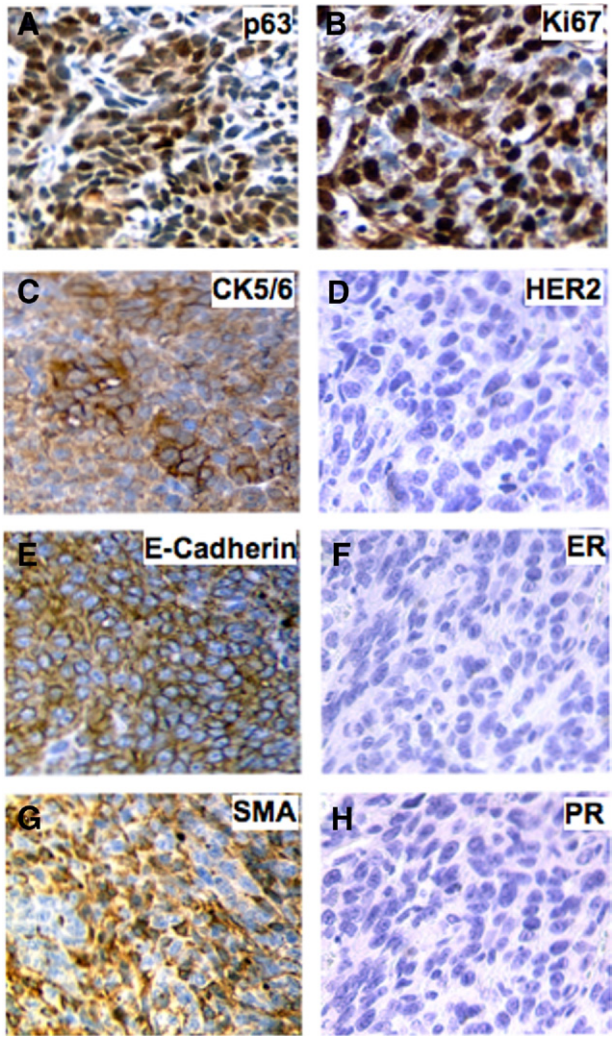

Figure 6. Immunohistochemical characterization of 4T1 metastatic breast cancer in the BALB/c mouse primary tumor. **A:** Nuclear expression of p63. **B:** Nuclear expression of Ki-67. **C:** Cytoplasmic and membrane staining for cytokeratin 5/6. **D:** Her-2-negative staining. **E:** Ductal carcinoma is indicated by E-cadherin-positive staining. **F:** ER-negative staining. **G:** Cytoplasmic staining of α-smooth muscle actin. **H:** PR-negative staining.

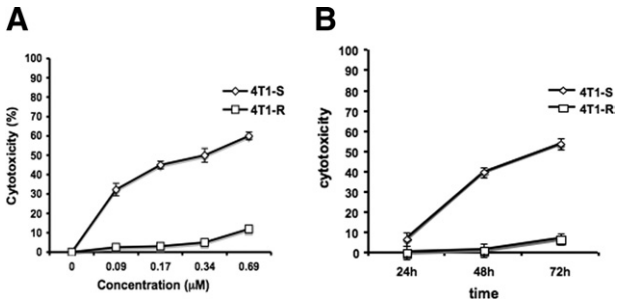

Figure 7. MTT assay of doxorubicin-mediated cellular toxicity in the 4T1-S (doxorubicin sensitive) and 4T1-R (doxorubicin resistant) cell lines. Both cell lines were cultured in a 24-well plate. On the next day, they were treated with doxorubicin. **A:** Cellular cytotoxicity of 4T1-R and 4T1-S cells in culture treated with different concentrations of doxorubicin. **B:** Cytotoxicity of the two cell lines at 24, 48, and 72 hours after doxorubicin treatment at the 0.17 μmol/L concentration.

We then performed cell cycle analysis, with propidium iodide staining, to see whether the doxorubicin-mediated cell cycle arrest was altered in 4T1-R versus 4T1-S cells, and performed TUNEL assay of the 4T1-R versus 4T1-S cell line at 24 hours after doxorubicin treatment (Figure 8, A and B). The 4T1-R cells did not show a block in the G2/M cell cycle after treatment with doxorubicin, and there were no noticeable differences in the cell cycle of the 4T1-R line before and after doxorubicin treatment (Figure 8A), whereas >77% of 4T1-S tumor cells showed a clear G2/M growth arrest after doxorubicin treatment. The TUNEL assay indicated that doxorubicin treatment induced a DNA break at 24, 48, and 72 hours only in the 4T1-S tumor cell line; no positive cells were detected in the 4T1-R tumor cell line (Figure 8B).

The Doxorubicin-Resistant 4T1-R Cell Line Develops Multiorgan Metastases That Are Resistant to Doxorubicin Chemotherapy

We examined the ability of doxorubicin to inhibit the growth of the 4T1-R cell line in mice after tail vein injection. The mice were treated with doxorubicin at 1 day after tail vein injection of tumor cells. Like the parent 4T1 cell line, the 4T1-R cells formed multiorgan metastasis; we isolated viable 4T1-R cells from lung, heart, liver, kidney, spleen, and bone. Doxorubicin efficiently suppressed the growth of the doxorubicin-sensitive 4T1-S tumor line in the heart, liver, kidney, spleen, and bone (Figure 9A). However, the number of viable 4T1-R cells isolated from the metastatic organs of mice with or without doxorubicin treatment did not differ, suggesting that the 4T1-R tumors could not be inhibited by doxorubicin (Figure 9).

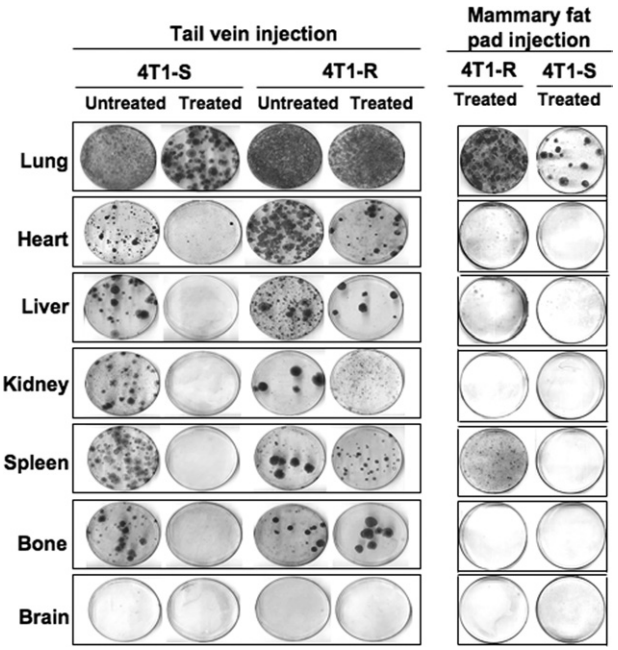

Figure 9. Effect of doxorubicin treatment on multiorgan metastasis of 4T1-R and 4T1-S cells in BALB/c mice. Mice were inoculated with either resistant or sensitive cells by tail vein injection. After 24 hours, the cells were treated with doxorubicin solution three times a week. After 21 days, mice were sacrificed and organs were harvested. Viable 4T1 tumor cells were harvested from different metastatic organs and cultured in growth medium. After 3 weeks, the viable tumor cell colonies were stained with Giemsa dye. Both 4T1-R and 4T1-S cell lines developed multiorgan metastasis in the mice, and there were no differences in their ability to form metastasis. Doxorubicin inhibited metastasis of 4T1-S cells in the lungs, heart, liver, kidney, spleen, and bone, but did not inhibit metastasis of 4T1-R cells. The experiments were repeated using fat pad injection of 4T1-R and 4T1-S breast cancer cells, and the doxorubicin effect on multiorgan metastasis was determined by cell colony assay. The results were essentially identical.

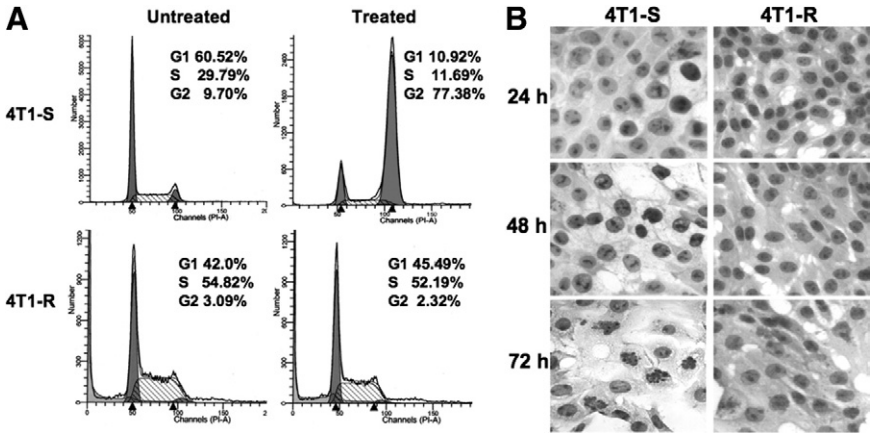

Figure 8. Characterization of the 4T1-R breast cancer cell line. **A:** Cell cycle analysis of 4T1-S and 4T1-R breast cancer cell lines with and without doxorubicin treatment. Cells were cultured in 100-mm tissue culture dishes. The doxorubicin treatment was at 200 μg/ml for 24-hours. Cells were then isolated, treated with propidium iodide, and analyzed by flow cytometric assay. In the doxorubicin-sensitive cell line (4T1-S), 77% of treated cells show G2/M growth arrest, with a corresponding decrease in the G1 and S phase cell cycle distribution relative to untreated cells. In the doxorubicin-resistant cells (4T1-R), the cell cycle profile did not change after doxorubicin treatment. **B:** TUNEL staining shows that doxorubicin induces DNA damage and apoptosis in 4T1-S cells but not in the 4T1-R cells.

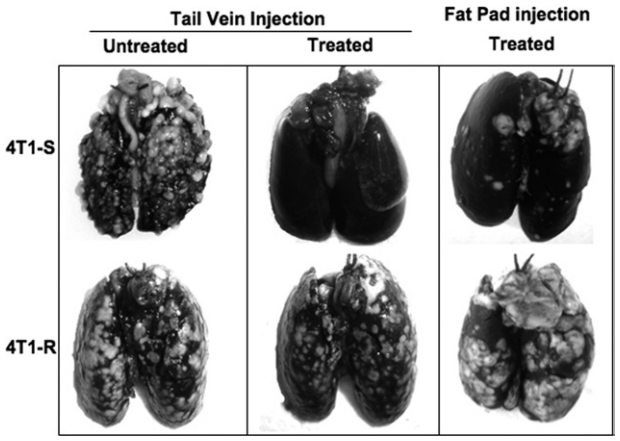

Figure 10. India ink staining of mouse lungs at 21 days after tumor cell inoculation via the tail vein or mammary fat pad injection. Doxorubicin treatment inhibited lung metastasis of 4T1-S cells but not in 4T1-R cells.

The doxorubicin antitumor effect on the 4T1-R cells was also examined in mice by inoculating tumor cells in the mammary fat pad (spontaneous breast cancer metastasis model). In this model, the 4T1 tumors were allowed to grow to 10 mm and to develop spontaneous micrometastasis, thus mimicking breast cancer metastasis in humans. Doxorubicin treatment was started at 1 day after surgery. The doxorubicin treatment did not alter the proliferation of 4T1-R cells in the various organ systems. These results of the colony assay were also confirmed by examining lung metastasis by staining with India ink (Figure 10). The 4T1-R tumor cells efficiently formed lung metastasis in the mice, and these tumors were totally resistant to doxorubicin treatment. There was also no difference in the ability to form lung metastasis in the presence and absence of doxorubicin treatment. In contrast, doxorubicin effectively inhibited lung metastasis of 4T1-S tumor cells in the mice. The fat pad injection model was also used to measure the effect of doxorubicin treatment on the tumor growth of 4T1-R and 4T1-S cells by lung staining and colony assay (Figures 9 and 10). The results were identical, except that the tumor burden was relatively high in the experimental metastasis model. Under both the experimental metastasis model and the fat pad injection spontaneous metastasis model, doxorubicin treatment did not inhibit lung metastasis of 4T1-R cells but did effectively inhibit metastasis of 4T1-S cells.

Increased P-Glycoprotein Expression Impairs Doxorubicin Uptake in 4T1-R Cells

To better understand the mechanisms of resistance, we first compared the cellular uptake of doxorubicin between the 4T1-S and 4T1-R cell lines in cell culture. Both the sensitive and the resistant 4T1 cell lines were treated with doxorubicin (400 μg/ml). After 24 hours, cells were washed, and intracellular doxorubicin fluorescence was measured by flow cytometry. There was a shift in the fluorescence peak, due to intracellular doxorubicin accumulation, in both the 4T1-S and 4T1-R cells (Figure 11A). This was confirmed by looking at the intracellular distribution of doxorubicin under a fluorescence microscope. In 4T1-S cells, doxorubicin efficiently localized in the nucleus; in 4T1-R cells, however, doxorubicin remained exclusively in the cytoplasm (Figure 11B). The differences in the intracellular drug trafficking and drug distribution between the 4T1-S and 4T1-R cell line were then examined in a kinetic study (Figure 12). In these experiments, we learned that doxorubicin enters the nucleus in both the 4T1-S and 4T1-R cells within 8 hours of treatment. Thereafter, in 4T1-R cells the doxorubicin was excluded from the nucleus and stayed mostly in the cytoplasm. In 4T1-S cells, the doxorubicin molecule stays in the nucleus at all times, leading to cellular toxicity.

One of the major problems in chemotherapy treatment of cancer is the eventual appearance of multidrug resistant tumor cells.[31] The phenomenon of acquired drug resistance has been attributed to overexpression of ATP-binding transporters such as MDR1/P-glycoprotein (P-gp, encoded by the *ABCB1* gene), multidrug resistance-associated protein ABC transporters MRP1 and MRP2, encoded by the ABCC1 and ABCC2. We therefore examined the expression level of the multidrug resistant proteins P-gp, MRP, and BCRP in the 4T1-S and 4T1-R cell lines by immunocytochemical staining, as well as by Western blot analysis. We observed very high-level expression of P-gp in both cytoplasm and nucleus of 4T1-R breast cancer cells, but negative expression in the 4T1-S cells (Figure 13A). This finding was confirmed by Western

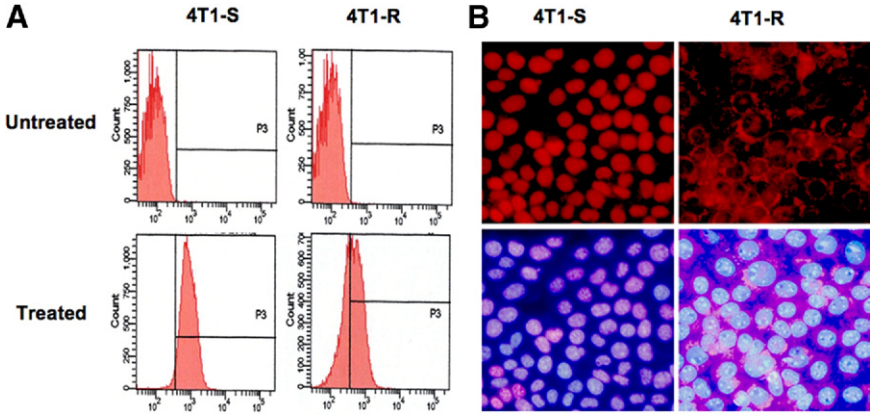

Figure 11. Intracytoplasmic accumulation of doxorubicin in the 4T1-R cells. Both 4T1-S and 4T1-R cells were treated with doxorubicin (400 μg/ml) for 24 hours and then cells were washed and analyzed for cellular uptake by flow cytometric analysis and under a fluorescence microscope. **A:** Intracytoplasmic accumulation of doxorubicin differs between 4T1-S and 4T1-R cells by flow cytometric analysis. **B:** Cellular uptake of doxorubicin differs between the 4T1-S and 4T1-R cells lines. Doxorubicin localized in the nucleus of 4T1-S cells, but intracytoplasmic accumulation of doxorubicin was seen in the cytoplasm of the 4T1-R cells. Red color (**top row**) is red fluorescence of doxorubicin. Purple color (**lower row**) is nuclear staining with Hoechst dye.

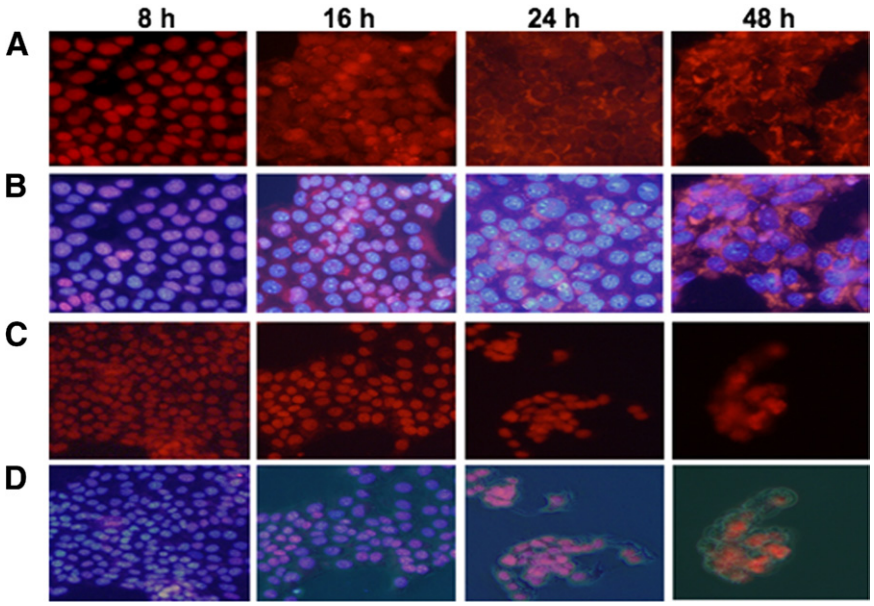

Figure 12. Comparison of intracellular trafficking and distribution of doxorubicin between 4T1-R (**A**, **B**) and 4T1-S (**C**, **D**) cells. **A:** Doxorubicin uptake of 4T1-R cells at 8, 16, 24, and 48 hours after drug exposure. **B:** The same treatment, with Hoechst nuclear staining. **C:** Doxorubicin uptake of 4T1-S cells at 8, 16, 24, and 48 hours after drug exposure. **D:** The same treatment, with DAPI nuclear staining.

blot analysis of P-gp, using both cytoplasmic and nuclear extracts of 4T1-R and 4T1-S cells (Figure 13B). Both the 4T1-R and 4T1-S cells showed negative expression of MRP and BCRP by immunocytochemistry and Western blot analysis (data not shown).

The final phase of our investigation was to examine whether inhibiting the expression of P-glycoprotein in the 4T1-R cells could increase doxorubicin uptake, thus increasing cellular cytotoxicity and reversing chemoresistance. For this experiment, we used verapamil, a general inhibitor of P-gp.[32] The 4T1-R cells in culture were pretreated with verapamil at a concentration of 20 μmol/L for 2 hours and then treated with doxorubicin at increasing concentrations. Nuclear translocation of doxorubicin in 4T1-R cells was significantly improved at 24 hours by pretreatment with verapamil (Figure 14A). Doxorubicin-induced cell toxicity was measured after 24 hours with the MTT assay. Verapamil indeed improved the nuclear translocation and toxicity of doxorubicin in the 4T1-R cell line (Figure 14B).

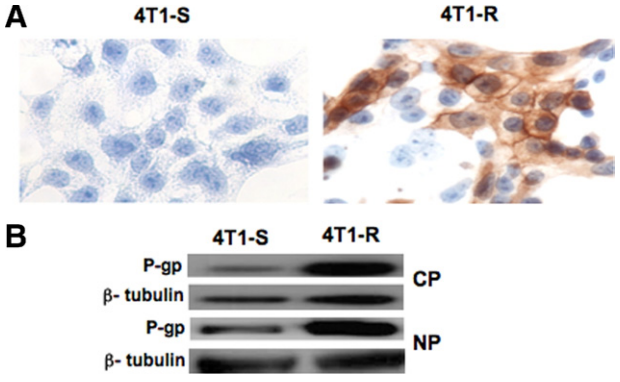

Figure 13. Expression of P-glycoprotein between the 4T1-S and 4T1-R cells, determined by immunocytochemical staining (**A**) and Western blot analysis (**B**). **A:** Expression of P-gp between 4T1-S and 4T1-R cells was examined by immunocytochemical staining using a monoclonal antibody. **B:** Western blot analysis of nuclear (NP) and cytoplasmic (CP) expression of P-gp between the 4T1-S and 4T1-R cells.

In summary, we have isolated and characterized a highly metastatic doxorubicin-resistant 4T1 cell line, 4T1-R, that forms multiorgan metastasis, in contrast to the doxorubicin-sensitive 4T1-S cells.

Discussion

Chemotherapy is the standard treatment option for metastatic breast cancer. Prolonged treatment with chemotherapeutic drugs leads to development of chemoresistance, however, and resistance to chemotherapeutic agents is a significant problem in the treatment of patients with breast cancer.[31,33] The mechanisms of chemoresistance against metastatic breast cancer treatments are not well understood, in part for lack of an experimental metastasis model system that faithfully recapitulates the multiorgan metastasis seen in humans. We have selected a 4T1 mouse model of cancer metastasis because this cell line forms multiorgan metastasis, similar to human breast cancer. The model is highly reproducible, and tumor metastasis can be accurately quantified to determine the overall success of chemotherapy drugs. This model is suitable for testing the success of different chemotherapy regimens, as well as for furthering understanding of the molecular, cellular, and pathological basis of breast cancer metastasis. The present studies conducted using this model should contribute to understanding the success of current treatment approaches and to developing innovative approaches to improved treatment.

Recently, Derksen et al[34] developed a mouse model for lobular breast carcinoma by conditional inactivation of E-cadherin and p53 in mammary epithelial cells. They showed by *in vivo* imaging that mice orthotopically transplanted with luciferase-labeled tumor cells develop distant metastasis to the lungs, peritoneal and thoracic cavity, and bone. However, the incidence of lobular carcinoma is only 10% to 15%, in contrast to ductal carcinoma, which is highly prevalent (>50% of all breast cancers). The 4T1 breast

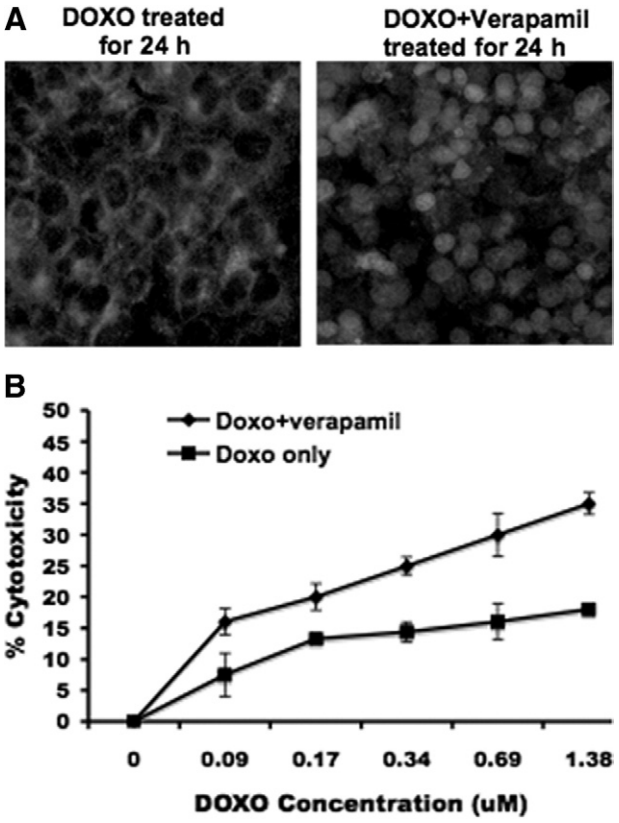

Figure 14. Verapamil pretreatment improves the nuclear translocation of doxorubicin and induces cellular cytotoxicity in the 4T1-R cell line. **A:** 4T1-R cells in culture were treated with verapamil at a concentration of 20 μmol/L for 2 hours and then treated with doxorubicin (100 μg/ml). After 24 hours, nuclear translocation was examined under a fluorescence microscope. **Left:** Without verapamil pretreatment, distribution doxorubicin in 4T1-R cells was cytoplasmic. **Right:** Pretreatment with verapamil induced nuclear translocation of doxorubicin in 4T1-R cells. **B:** MTT assay results showed that pretreatment with 20 μmol/L verapamil induced dose-dependent cellular cytotoxicity of doxorubicin in 4T1-R cells at 24 hours, compared with doxorubicin in 4T1-R cells without verapamil pretreatment.

cancer model used here is a basal type, triple-negative ductal carcinoma strongly positive for E-cadherin and cytokeratin 5/6 and negative for ER/PR and Her-2. The 4T1 tumor model represents a distinct subtype of invasive carcinoma that has poor prognosis and does not respond to chemotherapy.

Using this 4T1 breast cancer model, we determined the relative efficacy of doxorubicin in combination with cyclophosphamide and 5-FU (the FAC regimen used in human breast cancer) to inhibit multiorgan metastasis of 4T1 breast cancer cells in a BALB/c mouse model. Doxorubicin effectively induced cytotoxicity in the 4T1 cell line and inhibited proliferation of 4T1 cell growth, compared with 5-FU. The combination treatment of 5-FU and doxorubicin synergistically reduced the growth of 4T1 cells by nearly 90%. To understand the cellular target of each chemotherapy drug in the 4T1 cell, cell cycle analysis was performed after treatment with an individual chemotherapy drug. It was determined that doxorubicin treatment leads to the inhibition of cell proliferation by blocking the G2/M phase of the cell cycle, consistent with the fact that doxorubicin inhibits topoisomerase-II and this inhibition leads to G2/M growth arrest and apoptotic cell death. The present results are in agreement with previous reports.[30,35]

5-Fluorouracil is widely used in the treatment of many cancers, including colorectal cancer and breast cancer. This drug inhibits tumor growth by two different mechanisms.[36,37] First, 5-FU is converted to 5-fluoro-dUMP and 5-fluoro-dUTP in cells. The 5-fluoro-dUMP binds to the nucleotide-binding site of thymidylate synthase (an enzyme that catalyzes the reaction from dUMP to dTMP) and so inhibits its enzyme activity. Treatment with 5-FU leads to a decrease in the intracellular deoxynucleotide pool and impairs DNA synthesis and repair, resulting in DNA damage. Second, 5-FU is also converted to 5-fluorouridine triphosphate, which incorporates into ribosomal RNA and so interferes with the cellular RNA biosynthesis. Thus, 5-FU treatment leads to inhibition of rRNA processing, followed by cell cycle arrest and apoptosis. We show here that 5-FU treatment inhibits 4T1 cell proliferation by blocking at the G1/S phase of the cell cycle. These results are consistent with studies with human breast cancer cell lines.[36,37]

From these cell culture experiments, it appears that the anticancer properties of doxorubicin and 5-FU drugs in murine cells are equivalent to their properties and mechanisms in human breast cancer cells. The additive effects of doxorubicin and 5-FU in cell culture may be due to the fact that they have two different intracellular targets. Notably, when these two drugs were given in combination the doxorubicin mechanisms of action were dominant over the 5-FU mechanisms, because a majority of cultured cells treated with combination drugs showed a G2/M cell cycle arrest.

We performed experiments to examine the effect of FAC chemotherapy on the proliferation of mammary carcinoma cells in different organ systems of mice, using the 4T1 cell line. The chemotherapy treatment did not have significant toxicity, because 100% of the mice survived the treatment. The success of chemotherapy in this mouse model was evaluated by a quantitative assessment of the number of residual tumor cells in each metastatic organ. Tumor metastasis was the highest in lung, followed by heart, liver, and bone. Combination chemotherapy effectively inhibited growth of metastatic tumors in most of the visceral organs, excepting lung. The results of chemotherapy using a single agent suggest that doxorubicin has the maximum anticancer properties, compared with 5-FU and cyclophosphamide. Nonetheless, a considerable quantity of tumors in the lung remained resistant to the doxorubicin treatment.

To understand why such a considerable number of 4T1 cells in the mice survived doxorubicin treatment, we isolated a doxorubicin-resistant 4T1 cell line (4T1-R) from a tumor that survived treatment. The 4T1-R cells were cultured further in the presence of doxorubicin (50 ng/ml), to ensure drug resistance. The concentration of doxorubicin used in the culture medium was comparable to the human dose. For example, doxorubicin is usually given to patients with metastatic breast cancer by intravenous infusion at a dose of 60 mg/m^2, and the plasma concentration of doxorubicin was found to be 52.5 ng/ml after 8 hours of chemotherapy and 39.8 ng/ml after 24

hours of chemotherapy.[38] MTT assay and cell cycle analysis indicated that doxorubicin-mediated G2/M growth arrest failed in the 4T1-R cells. These cells cannot be killed by doxorubicin in cell culture. The chemoresistant phenotypes of these tumor cells were confirmed by *in vivo* experiments in mice: doxorubicin inhibited multiorgan metastasis of 4T1-S cells (our doxorubicin-sensitive cell line), but not the 4T1-R cells (our doxorubicin-resistant cell line). The 4T1-R cells do not respond to doxorubicin because in these cells the drug stays in the cytoplasm, in contrast to the efficient nuclear localization of doxorubicin observed in 4T1-S cells. We determined that the 4T1-R cells express a high level of MDR1/P-glycoprotein in the nucleus, which leads to development of multidrug resistance. The mechanism of doxorubicin resistance was confirmed when pretreatment of 4T1-R cells with verapamil, a general inhibitor of P-glycoprotein, improved the nuclear uptake of doxorubicin and led to cytotoxicity.

The overexpression of ATP-binding cassette (ABC) transporters, including P-glycoprotein, multidrug resistance-associated protein 1 (MDR1), and BCRP, is one of the most common causes of multidrug resistance.[31,39] Other drug-resistant mechanisms have been proposed, including misregulation of drug-induced DNA repair pathway, drug-induced interruption of the apoptotic pathway, and poor drug penetration through the extracellular matrix have been proposed.[40–42] The role of P-gp expression in the development of chemoresistance in breast cancer is also supported by a number of clinical studies.[43–48]

Chintamani et al[43] examined the expression of P-gp in 50 cases of locally advanced human breast cancer; they reported that the positive expression of P-gp was associated with poor clinical response to neoadjuvant chemotherapy (FAC regimen) in the breast cancer patients. Schneider et al[44] examined the expression of P-gp, multidrug resistant protein (MDR1), and lung cancer associated resistant protein (LRP) in formalin-fixed, paraffin-embedded tumor samples from 52 patients treated for locally advanced breast cancer by induction chemotherapy; MDR1 and P-gp expression was associated with poor response and lymph node metastasis. In a study of P-gp expression in 92 primary and 12 metastatic breast cancers by immunohistochemistry, Linn et al[45] found that P-gp expression was associated with tamoxifen resistance and shorter overall survival. In a study from Japan of 27 cases with breast cancer tissue treated with paclitaxel, Fujita et al[46] reported that 33% of the patients showed increased P-gp expression, specifically due to the translocation of transcription factor Y-box binding protein to the nucleus. In a critical review and meta-analysis of studies examining P-gp expression in breast cancer, to determine prevalence and clinical relevance, Trock et al[47] found that patients with tumors expressing MDR1/P-gp are three times more likely to fail to respond to chemotherapy than those whose tumors are negative for this protein. In a study of 104 cases of primary invasive breast cancer examined for the expression of MDR1/P-gp and Cox-2, Surowiak et al[48] documented that higher expression MDR1/P-gp is associated with high-grade tumor, lymph node metastasis, and shorter survival. Taken together, results from these various clinical studies suggest that high-level expression of MDR1/P-gp is associated with chemoresistance, high-grade tumor, and shorter survival.

In summary, we have established a chemoresistant 4T1-R cell line that forms multiorgan metastasis in mice and is resistant to doxorubicin. This model can be used to further understanding of the role of MDR1/P-gp in the mechanisms of chemoresistance in the mouse model. We also propose that the chemoresistant 4T1 breast cancer in BALB/c mouse model can be used to develop innovative approaches to overcome chemoresistance.

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