Quantitation of Doxorubicin Uptake, Efflux, and Modulation of Multidrug Resistance (MDR) in MDR Human Cancer Cells

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ABSTRACT

P-glycoprotein (Pgp), a membrane transporter encoded by the MDR1 gene in human cells, mediates drug efflux from cells, and it plays a major role in causing multidrug resistance (MDR). Confocal microscopy was used to study in vitro and in vivo drug accumulation, net uptake and efflux, and MDR modulation by P-glycoprotein inhibitors in MDR1-transduced human MDA-MB-435mdr (MDR) cancer cells. The MDR cells were approximately 9-fold more resistant to the anticancer drug doxorubicin than their parental wild-type MDA-MB-435wt (WT) cells. Doxorubicin accumulation in the MDR cells was only 19% of that in the WT cells. The net uptake of doxorubicin in the nuclei of the MDR cells was 2-fold lower than that in the nuclei of the WT cells. Pgp inhibitors verapamil, cyclosporine A, or PSC833 increased doxorubicin accumulation in the MDR cells up to 79%, and it reversed drug resistance in these cells. In living animals, doxorubicin accumulation in MDA-MB-435mdr xenograft tumors was 68% of that in the wild-type tumors. Administration of verapamil, cyclosporine A, or PSC833 before doxorubicin treatment of the animals increased doxorubicin accumulation in the MDR tumors up to 94%. These studies have added direct in vitro and in vivo information on the capacity of the transporter protein Pgp to efflux doxorubicin and on the reversal of MDR by Pgp inhibitors in resistant cancer cells.

Drug resistance is a significant factor that limits the effectiveness of current chemotherapeutic drugs. Although resistance can develop through a variety of mechanisms, multidrug resistance due to the overexpression of drug transporters such as P-glycoprotein (Pgp) is an established cause of drug resistance (Broxterman et al., 1995). Pgp is a transmembrane protein encoded by the MDR1 gene in human cells. As a member of the ATP-binding cassette family of drug transporters, Pgp effluxes a variety of hydrophobic, neutral, and positively charged drugs from the cell. Pgp expression is a component of the normal cellular defense system against xenobiotics (Gottesman et al., 2002). However, in some human cancers, the overexpression of Pgp is correlated with decreased survival and poor prognosis (Diestra et al., 2003; Leonessa and Clarke, 2003).

Intracellular drug accumulation is a complex process including drug uptake into the cell, retention and distribution in the cell, and efflux from the cell. At any given time, the net uptake (accumulation) of a drug in cells is the difference between the amount of drug uptake and efflux from the cell. Pgp-mediating drug efflux decreases intracellular net drug uptake, and it causes cells to be drug-resistant. In an attempt to overcome the resistance that can develop due to Pgp overexpression, Pgp inhibitors have been developed (Teodori et al., 2006). The rationale to develop inhibitors is straightforward; it is hypothesized that if the action of Pgp (drug efflux) is blocked, it will result in an increased net uptake of drugs and greater clinical efficacy of chemotherapeutic agents in tumors overexpressing Pgp. First-generation inhibitors included drugs such as cyclosporine A, quinidine, tamoxifen, and verapamil (Ferry et al., 1996). Although these drugs were active inhibitors of Pgp, they were not sufficiently active in vivo, and the doses required for inhibition of Pgp resulted in unacceptable toxicity. Conse-
quenty, more specific and effective inhibitors were developed. However, second-generation inhibitors, including dexverapamil, PSC-833 (valspodar), and biricodar (VX-710), altered the pharmacokinetics of the chemotherapeutic agents, necessitating dose reductions (Rowinsky et al., 1998). A variety of third-generation Pgp inhibitors are currently in clinical development, including tarquidin, zosuquidar, elacridar, laniquidar, lonafarnib, and ONT-093 (Thomas and Coley, 2003; Jabbour et al., 2004). Several of these compounds have entered phase III clinical trials (Gerrard et al., 2004). However, some of these agents have had disappointing results (Doll et al., 2004; Pusztai et al., 2005), and the future clinical development of others is in jeopardy.

Over the past 15 years, there have been more than 150 clinical trials with approximately 30 different inhibitors, but a Pgp inhibitor has yet to be approved by the Food and Drug Administration (Oza, 2002). The outcome suggests that improved preclinical screening of potential inhibitors would be a valuable contribution to improving agent selection for clinical trials. This improvement would not only better use the resources available for clinical studies but also may prevent patients from participating in an unsuccessful clinical trial. Although there are a variety of assays for determining the intracellular accumulation and efflux of drugs mediated by Pgp and for evaluating the effectiveness of Pgp inhibitors, only a limited number of techniques allow for direct visualization and assessment of these processes. Of the techniques, confocal microscopy is a widely used and valuable tool for these studies.

Studies on drug accumulation in cultured resistant cancer cells have been published by other investigators; however, studies on visualizing in real time how Pgp processes anti-cancer drugs and how Pgp inhibitors modulate MDR in vitro and in vivo have not been reported. This article presents studies using confocal microscopy to visualize and assess doxorubicin net uptake and efflux in vitro and to evaluate the effectiveness of first- and second-generation Pgp inhibitors in modulating drug efflux in vitro and in vivo.

Materials and Methods

Reagents, Cell Lines, and Animals. Doxorubicin hydrochloride, verapamil, and cyclosporine A were purchased from Sigma-Aldrich (St. Louis, MO). PSC833 was a gift to W.T.B. from Dalia Cohen (Novartis Pharmaceuticals, Florham Park, NJ). Stock solutions were made by dissolving doxorubicin (Bouhadir et al., 2001) and verapamil in saline and by dissolving cyclosporine A and PSC833 in dimethyl sulfoxide, and they were stored at −20°C until use. The final concentration of dimethyl sulfoxide in the treated cells was less than 0.1%. Cyclosporine A and PSC833 were also dissolved in the vehicle of 7:3 sterile water to 95% ethanol for animal injections (Hermanussen et al., 2004).

Wild-type MDA-MB-435 human cancer cells (MDA-MB-435wt) and the MDA-MB-435 cells retrovirally transduced with the MDR1 gene to express Pgp (MDA-MB-435mdr; MDR cells) were obtained from Dr. George Sledge (Indiana University, Indianapolis, IN). MDA-MB-435 cells were widely accepted previously as human breast cancer cells. However, some investigators have recently suggested that this cell line may have originated from a melanoma (Ellison et al., 2002). MDA-MB-435 cells were grown in α-minimal essential medium with 10% bovine calf serum (HyClone Laboratories, Logan, UT), 1 mM glutamine, and 2 mM sodium pyruvate (Gemini, Calabasas, CA), 100 μM penicillin, and 100 μM streptomycin (Gibco BRL, Gaithersburg, MD).

Animal studies were carried out with protocols approved by the Animal Care and Use Committee of Indiana University. Female nude/nude mice (6–8 weeks of age) were injected subcutaneously with human MDA-MB-435 cancer cells. Each mouse was injected with 15 × 10^6 MDA-MB-435wt cells in one flank and with 15 × 10^6 MDA-MB-435mdr cells in the other flank. The injection produced a palpable tumor within 3 to 4 weeks. Confocal image studies were then conducted on the mice.

Western Blot Analysis of Pgp Expression. Cell lysates used for Western blot analysis were prepared from crude cell membranes following established procedures (Pineira et al., 2001). Briefly, cells were harvested, washed, and centrifuged. The pellet was resuspended in hypotonic lysis buffer and centrifuged at 4000g to remove cell debris. The supernatant from the second centrifugation was retained and centrifuged again to obtain crude cell membranes that were then resuspended in sucrose buffer and used as lysates for Western blot.

Protein concentration in the cell lysates was determined with the Bradford assay (Bradford, 1976), and equal amounts of proteins were loaded on gels. Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The blot was then probed with primary monoclonal antibody C219 (dilution 1:1000) (ID Labs Biotechnology, London, ON, Canada), followed by reaction with horseradish peroxidase-conjugated secondary antibody. The signal was detected using enhanced chemiluminescence and exposure of X-ray film.

Cytotoxicity of Doxorubicin and Reversal of MDR by Pgp Inhibitors. Colony formation assay was used to evaluate doxorubicin cytotoxicity and the reversal of resistance by Pgp inhibitors. In total, 3 × 10^5 cells were seeded in 5 ml of culture medium in each 25-cm² flask, and then they were incubated under standard conditions for 24 h. Each flask of cells was treated with a different dose of doxorubicin for 1 h. To assess the reversal of MDR by Pgp inhibitors, the cells were pretreated with verapamil, cyclosporine A, or PSC833 30 min before doxorubicin exposure. The cells were then washed with PBS, trypsinized, counted, and seeded into triplicate 100-mm cell culture dishes at densities of 500 and 750 cells/dish in culture medium. After incubation under standard conditions for 14 days, colonies were fixed with methanol, stained with 10% methylene blue in PBS, and visually counted.

Doxorubicin Intracellular Distribution, Accumulation, and Their Modulation by Pgp Inhibitors. MDA-MB-435 cells were seeded on coverslips in six-well plates, and they were allowed to grow overnight. On the following day, cells were washed with PBS, incubated with or without 5 μM verapamil, 2.5 μM cyclosporine A, or 3 mg/ml PSC833 for 30 min before treatment with 5 μM doxorubicin for 2 h, and then they were examined using confocal microscopy.

Dynamic Assessment of Doxorubicin Net Uptake, Efflux, and Effects of Pgp Inhibitors. MDA-MB-435 cells were seeded on coverslips overnight. The coverslips were mounted in microscope chambers. The chambers were placed on the microscope stage and perfused sequentially with doxorubicin-free medium for 6 min, with medium with 5 μM doxorubicin for 2 h (uptake perfusion), and then with doxorubicin-free medium again for 1 h (efflux perfusion). Serial images at 1-min intervals were collected and analyzed. To study the effect of Pgp inhibitors on the time course of doxorubicin accumulation and efflux, MDA-MB-435mdr cells grown on coverslips were pre-exposed to 5 μM verapamil, 2.5 μM cyclosporine A, or 3 mg/ml PSC833 for 30 min before being mounted in microscope chambers for perfusion as described above.

Doxorubicin Accumulation and Effects of Pgp Inhibitors in Tumor Xenografts in Living Nude Mice. Living nude mice bearing subcutaneous MDA-MB-435wt and MDA-MB-435mdr tumor xenografts on opposite flanks were injected with 4, 8, or 16 mg/kg doxorubicin intravenously. To study the effect of Pgp inhibitors, tumor-bearing mice were administered with or without 10 mg/kg verapamil, 50 mg/kg cyclosporine A, or 50 mg/kg PSC833 intraperitoneally 1 h before receiving intravenous injection of 8 mg/kg doxor-
rubricin. Confocal tumor images were taken 2 h after the mice had received doxorubicin. To obtain the images, the mice were anesthetized, and a small skin incision was made to expose the xenografts. The mice were placed on the microscope stage connected to a water circulator set to 37°C.

Confocal Microscopy and Image Analysis. Images were collected using an LSM510 NLO confocal microscope with a C-Apo 40× water immersion lens (Carl Zeiss, Jena, Germany). Doxorubicin fluorescence was excited with an argon laser at 488 nm, and the emission was collected through a 530-nm long-pass filter. The same confocal settings (excitation, laser power, detector gain, and pinhole size) were used to image the wild-type and the MDR cells in vitro and the wild-type and MDR xenograft tumors in vivo. Post-data acquisition image analysis was performed using MetaMorph software (Molecular Devices, Downingtown, PA). Cells images were analyzed as mean doxorubicin fluorescent intensity per pixel in a circular 314 pixel region of the nucleus or cytoplasm of the same cell (approximately doxorubicin fluorescent intensity per pixel in a circular 314 μm2). Multiple fields containing at least 15 cells/field were imaged in each experiment. The tumor images were analyzed as mean doxorubicin fluorescent intensity per pixel in a circular 785×785-pixel region of the tumor. Three regions of each tumor were randomly picked for image analysis. Results were obtained from analyzing images from three to five experiments.

Statistics. All data are expressed as mean ± S.E. from three or more experiments, and they were statistically evaluated by Student’s t test. Differences were considered significant when p < 0.05.

Results

Pgp Expression and Resistance to Doxorubicin. The expression of Pgp in MDA-MB-435 cells was demonstrated with Western blot analysis (Fig. 1). Monoclonal antibody C219 specifically detected a 180-kDa P-glycoprotein. The additional bands of lower molecular weight detected by the antibody represent degraded products of Pgp.

The IC₅₀ values of doxorubicin toxicity in MDA-MB-435wt cells and MDA-MB-435mdr cells were 0.60 ± 0.04 μM (mean ± S.E.) and 5.29 ± 0.85 μM, respectively (Table 1). These results indicate that MDA-MB-435mdr cells were approximately nine times more resistant to doxorubicin than MDA-MB-435wt cells, despite the modest expression of Pgp in these cells.

Reversal of the Resistance to Doxorubicin by Pgp Inhibitors. Pretreatment with verapamil, cyclosporine A, or PSC833 decreased the IC₅₀ of doxorubicin toxicity in MDA-MB-435mdr cells to 2.41 ± 0.43, 3.24 ± 0.26, or 3.71 ± 0.47 μM (Table 1), respectively, and reduced the IC₅₀ of doxorubicin in MDR cells. The pretreatment with Pgp inhibitors had no significant effect on the IC₅₀ of doxorubicin in MDA-MB-435wt type cells.

Doxorubicin Intracellular Distribution and Accumulation. Confocal cell images were used to determine intracellular doxorubicin localization and accumulation in MDA-MB-435wt and MDA-MB-435mdr cells. Most wild-type cells collected doxorubicin specifically in their nuclei. However, MDR cells accumulated doxorubicin in their nuclei and cytoplasm. In addition, the MDR cells showed much weaker doxorubicin-related fluorescent intensity than the wild-type cells (Fig. 2).

Effects of Pgp Inhibitors on Doxorubicin Intracellular Distribution and Accumulation. Pretreatment of MDA-MB-435mdr cells with Pgp inhibitors relocalized doxorubicin to the nuclei and significantly increased the drug accumulation in these cells. In nonpretreated MDR cells, doxorubicin fluorescent intensity was significantly low and amounted to only 19% of that in MDA-MB-435wt cells. Pretreatment with verapamil, cyclosporine A, or PSC833 increased doxorubicin accumulation in the MDR cells to 79, 73, and 66% of that in the wild-type cells, respectively (Fig. 3). The Pgp inhibitors had no significant effects on the intracellular distribution or accumulation of doxorubicin in the wild-type cells (data not shown).

Dynamic Assessment of Doxorubicin Net Uptake and Efflux in Cytoplasm and Nuclei of MDA-MB-435 Cells. Sequential cell images were used to assess and compare the real-time net uptake and efflux of doxorubicin in the nuclei and cytoplasm of MDA-MB-435wt and MDA-MB-435mdr cells. In both types of cells, the accumulation of doxorubicin differed markedly in the nuclei (Fig. 4A), but it was similar in the cytoplasm (Fig. 4B). Nuclear net uptake of doxorubicin in the MDR cells was slower than that in the wild-type cells, and at the end of 2-h doxorubicin uptake perfusion, drug accumulation in the nuclei of MDR cells was approximately 2-fold less than that in the nuclei of wild-type cells (Fig. 4C).

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TABLE 1

Toxicity of doxorubicin and modulation of drug resistance by verapamil, cyclosporine A, and PSC833 in human MDA-MB-435 cancer cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC₅₀ MDA-MB-435wt Cells</th>
<th>FS*</th>
<th>IC₅₀ MDA-MB-435mdr Cells</th>
<th>RF**</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td></td>
<td>μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.60 ± 0.04</td>
<td>1.0</td>
<td>5.29 ± 0.85</td>
<td>8.8*</td>
<td>1.0</td>
</tr>
<tr>
<td>+ 5 μM verapamil</td>
<td>0.52 ± 0.03</td>
<td>1.2</td>
<td>2.41 ± 0.43</td>
<td>4.6***</td>
<td>1.9</td>
</tr>
<tr>
<td>+ 2.5 μM CsA</td>
<td>0.53 ± 0.04</td>
<td>1.1</td>
<td>3.24 ± 0.26</td>
<td>6.1***</td>
<td>1.4</td>
</tr>
<tr>
<td>+ 3 mg/ml PSC833</td>
<td>0.57 ± 0.04</td>
<td>1.1</td>
<td>3.71 ± 0.47</td>
<td>6.5***</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Statistically significant increase (p < 0.05) in resistance between the wild type and the MDR cell lines.
** Statistically significant decrease (p < 0.05) in resistance between the MDR cell line and the MDR cell line pretreated with Pgp inhibitors.
† The fold sensitization was calculated as IC₅₀ of doxorubicin divided by IC₅₀ of doxorubicin in the same cells pretreated with Pgp inhibitors.
‡ Resistance factor was calculated as IC₅₀ of doxorubicin in MDA-MB-435 cells divided by IC₅₀ of the same drug in MDA-MB-435 cells pretreated with Pgp inhibitors.
Doxorubicin efflux occurred immediately in the MDR and wild type cells after removal of doxorubicin from the perfusates. However, the efflux was faster in MDR cells than in the wild-type cells. Doxorubicin efflux in the nuclei of MDR and wild-type cells occurred at about the same rate (Fig. 4A), and it led to a loss of 30 and 20% doxorubicin accumulation in the nuclei of MDR and wild-type cells, respectively (Fig. 4D). In contrast, in the cytoplasm of MDR cells, the efflux quickly reduced doxorubicin accumulation to near baseline levels (Fig. 4B), and it caused a loss of 47% of cytoplasmic doxorubicin accumulation at the end efflux perfusion. In the cytoplasm of wild-type cells, a much slower efflux was observed.
with a loss of only 17% cytoplasm doxorubicin accumulation (Fig. 4D). Because of the lower doxorubicin net uptake in their nuclei and stronger drug efflux in their cytoplasm, the MDR cells quickly and almost completely removed intracellular doxorubicin.

**Effects of Pgp Inhibitors on Doxorubicin Net Uptake and Efflux in Cytoplasm and Nuclei of MDA-MB-435mdr Cells.** Increased doxorubicin net uptake and decreased drug efflux were observed in MDA-MB-435mdr cells pretreated with Pgp inhibitors. At the end of the 2-h uptake perfusion, the fluorescent intensity of doxorubicin in the nuclei of the nonpretreated MDR cells was 1.8-fold that of the baseline level. Pretreatment with verapamil, cyclosporine A, and PSC833 increased the intensity 4.6-, 3.4- and 3.4-fold, respectively (Fig. 4C). Increased doxorubicin accumulation in the cytoplasm of pretreated MDR cells was also observed under the same conditions. Compared with the nonpretreated MDR cells, increased doxorubicin retention at the end of efflux perfusion was observed in the nuclei and cytoplasm of the MDR cells pretreated with verapamil and PSC833 (Fig. 4D). Surprisingly, cyclosporine A increased uptake of doxorubicin but reduced its retention in MDR cells.

**Doxorubicin Accumulation in Xenograft Tumors in Living Mice.** Confocal tumor images were used to evaluate in vivo doxorubicin accumulation and the effect of Pgp inhibitors on the accumulation in MDA-MB-435mdr tumors. Images of the tumors demonstrated similar distribution of doxorubicin in MDR and wild-type tumor cells. Doxorubicin in both types of tumors was located around the periphery of the nuclei (Fig. 5). Accumulation of doxorubicin in both wild-type and MDR tumors increased with the increase of dose of doxorubicin injected to animals (Fig. 6). However, the accumulation in the MDR tumors was only 71, 64, and 57% of that in the wild-type tumors, respectively, in the mice intravenously receiving 4, 8, and 16 mg/kg doxorubicin ($p < 0.05$).
Effects of Pgp Inhibitors on Doxorubicin Accumulation in Xenograft Tumors in Living Mice. Increased doxorubicin fluorescent intensity in MDA-MB-435mdr xenograft tumors was observed in mice preadministered verapamil, cyclosporine A, or PSC833. Verapamil, cyclosporine A, or PSC833 increased doxorubicin accumulation in the MDR tumors to 90, 84, or 94% of that in the wild-type tumors, respectively (Table 2). The preadministration of these Pgp inhibitors to mice eliminated the significant difference in doxorubicin fluorescent intensity between the MDR and wild-type tumors. In addition, verapamil and PSC833 significantly increased doxorubicin accumulation in MDR tumors compared with the nonpretreated MDR tumors.

**Discussion**

Failure of chemotherapy in cancer patients has been observed due to the presence and/or development of drug resistance. Potential resistance mechanisms in cancer patients are those involving the multidrug resistance phenotype, including expression of Pgp. Doxorubicin is a Pgp substrate frequently used in the clinic to treat cancer patients (e.g., breast cancer, ovarian cancer, and Kaposi’s sarcoma). In this study, we have taken advantage of the innate fluorescence of doxorubicin to evaluate how wild-type and genetically engineered resistant human cancer cells accumulate, distribute, and efflux doxorubicin and to study how Pgp inhibitors reverse multidrug resistance in vitro and in vivo. ATP-binding cassette transporters such as multidrug resistant protein (MRP) and breast cancer resistant protein are also known to be involved in causing MDR in cancer cells (Sharma et al., 2003). However, the current study only focused on evaluating the role of Pgp in mediating doxorubicin transport and multidrug resistance.

**MDR1-transduced human MDA-MB-435mdr cancer cell line** was used in the study to make it more relevant for investigating clinically resistant tumors. MDA-MB-435mdr cells only have a modest resistance to doxorubicin compared with drug-selected resistant cell lines, which seem to have much higher levels of resistance than actual clinical tumors.

**TABLE 2**

Doxorubicin accumulation in MDA-MB-435 xenograft tumors

<table>
<thead>
<tr>
<th>Preinjections</th>
<th>Doxorubicin Fluorescent Intensity in WT Tumor</th>
<th>Percentage of Nonpretreated WT Tumor</th>
<th>Doxorubicin Fluorescent Intensity in MDR Tumor</th>
<th>Percentage of WT Tumor</th>
<th>Percentage of Nonpretreated MDR Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>75 ± 4.7</td>
<td>100</td>
<td>51 ± 6.0</td>
<td>68*</td>
<td>100</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>82 ± 6.5</td>
<td>109</td>
<td>74 ± 7.3</td>
<td>90*</td>
<td>145**</td>
</tr>
<tr>
<td>PSC833</td>
<td>75 ± 5.1</td>
<td>100</td>
<td>65 ± 6.4</td>
<td>87</td>
<td>127</td>
</tr>
</tbody>
</table>

* Statistically significant difference (p < 0.05) in fluorescent intensity of doxorubicin in MDA-MB-435wt tumors and MDA-MB-435mdr tumors.

** Statistical significant difference (p < 0.05) in fluorescent intensity of doxorubicin in nonpretreated MDA-MB-435mdr tumors and pretreated MDA-MB-435mdr tumors.
In addition, a clinically achievable dose of doxorubicin, 5 \( \mu \text{M} \), was used in the in vitro cell imaging studies (Rahman et al., 1990).

The observation that some MDR cells displayed altered intracellular distribution and accumulation of doxorubicin compared with their parental wild-type cells is consistent with that reported by other investigators (Meschini et al., 1994), and it may indicate that Pgp reduces doxorubicin access to nuclear targets in the cells. A number of studies have shown that doxorubicin intercalates into DNA molecules. The binding of doxorubicin to DNA inhibits DNA polymerase and nucleic acid synthesis. In addition, doxorubicin stabilizes the cleavable complex between DNA and topoisomerase II enzyme subunits, resulting in the formation of protein-linked DNA double-strand breaks (De Beer et al., 2001; Cutts et al., 2003).

To our knowledge, this is the first report on directly visualizing and dynamically assessing doxorubicin net uptake, efflux, and modulation of MDR by Pgp inhibitors in cell nuclei and cytoplasm. Transmembrane protein Pgp is well known for mediating drug efflux and for causing a reduction of intracellular drug accumulation, which explains the decreased net doxorubicin uptake and increased drug efflux in cytoplasm and nuclei of MDR cells. Compared with the same cell compartment of wild-type cells, doxorubicin net uptake in the nuclei of MDR cells showed relatively more reduction than that in the cytoplasm. However, compared with the nuclei, the cytoplasm of the MDR cells lost a greater fraction of the accumulated doxorubicin during the efflux perfusion. In the wild type cells the extent of reduction of doxorubicin accumulation in nuclei and cytoplasm in the same period was about the same (Fig. 4B). The data suggest that the reduced total intracellular doxorubicin accumulation was mainly caused by decreased net drug uptake in the nuclei and increased drug efflux in the cytoplasm of MDR cells. The action of doxorubicin binding DNA in nuclei and the efflux of doxorubicin mediated by Pgp on cell membrane (Sharma et al., 2003) may explain, at least in part, the obvious decreases of drug net uptake in the nuclei and increase of drug efflux in the cytoplasm of MDR cells.

The modulation of Pgp activity to reverse MDR has been intensively studied. Inhibition of Pgp in MDR cells sensitizes the cells to chemotherapeutic drugs in vitro and in vivo (Tan et al., 2000). Combined therapy with MDR-related antineoplastic agents and inhibitors shrinks tumors, and it prolongs the life span in some animal models. Pgp inhibitors verapamil, cyclosporine A, and PSC833 reverse MDR and restore intracellular accumulation of drugs in MDR cells, possibly by competitively binding to Pgp with drugs and/or by causing conformational changes in the transport protein (Tsuruo et al., 1981; Rao and Scarborough, 1994). Our results indicate that the above-mentioned three Pgp inhibitors mainly modulate MDR by reversing drug net uptake in the nuclei of MDR cells. The modulating potential of Pgp inhibitors on MDR reported by other investigators (Keller et al., 1992) indicated that PSC833 was most effective, followed by cyclosporine A and verapamil. This rank order does not agree with our data. The discrepancy may be partially due to the dual inhibitory effects of verapamil and cyclosporine A on Pgp and MRP and to the specific inhibitory effects of PSC833 only on Pgp, which is supported by some investigators (Qadir et al., 2005). Indeed, modest MRP expression was found in MDA-MB-435mdr cells (data not shown). Cyclosporine A increased net drug uptake in both nuclei and cytoplasm like verapamil and PSC833; however, it surprisingly enhanced drug efflux (Fig. 4B). Morphological changes were observed in the MDR cells pretreated with cyclosporine A at the end of efflux perfusion experiment. Cyclosporine A may have induced toxicity to the cells and damaged the cell membrane and in turn caused doxorubicin to “leak” out of MDR cells.

To minimize differences in drug presentation and physical characteristics between animals in the in vivo study, experiments were conducted in mice that carried wild-type tumor on one flank and MDR tumor on the other flank. The approach to evaluate drug accumulation in tumors on living animals makes the animal study more relevant to the clinical situation. A difference in intracellular doxorubicin distribution between in vitro wild-type cell and in vivo wild-type tumor images was observed. It is possible that the doxorubicin dose administered to mice did not lead to a plasma doxorubicin level of 5 \( \mu \text{M} \) (the concentration used to treat cells in the in vitro study). Although cell nuclei have been considered the main target of doxorubicin, many studies have demonstrated that doxorubicin can also interact with other subcellular targets, such as cytoskeleton and membranes (Molinari et al., 1990), which may account for the distribution of doxorubicin observed in this study.

The results of tumor confocal images demonstrated that the increase of doxorubicin accumulation in MDA-MB-435 tumors was proportional with the dose of doxorubicin injected in the animal, which may suggest that in vivo confocal imaging approach can be used to facilitate identification of a MDR tumor phenotype and, more importantly, enables a screen for inhibitors of either MDR1 or other targets that increase the accumulation of doxorubicin in tumors.

This study showed that the increases of doxorubicin accumulation in MDR cells by Pgp modulators, verapamil, cyclosporine A, and PSC833 in vitro was more apparent than that in vivo. The results may be explained, at least in part, by doses of inhibitors injected intraperitoneally in mice not yielding a plasma concentration similar to that used to treat the cells in vitro. In future in vivo studies, monitoring the plasma concentration of these inhibitors in mice should be considered.

This article provides direct information on the capacity of modest levels of the transporter protein Pgp to decrease intracellular drug accumulation in vitro and in vivo. The study developed a new approach to examine in real time how MDR cancer cells accumulate and efflux substrate anticancer drugs and how they respond to transport inhibitors. This new approach will facilitate drug uptake and efflux studies in vitro and in vivo, and it may help the future development and evaluation of new inhibitors of MDR transporters.

References


De Beer EL, Bottone AE, and Voest EE (2001) Doxorubicin and mechanical perfor-


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