OVEREXPRESSION OF CAVEOLIN-1 INDUCES ALTERATION OF MULTIDRUG RESISTANCE IN Hs578T BREAST ADENOCARCINOMA CELLS

Chuanxi CAI and Jianwen CHEN*
National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China

Caveolin-1 is a major caveolae-coat protein involved in a variety of cell signaling processes. Some studies have suggested that the level of caveolin-1 expression positively correlates with multi-drug resistance in cancer cells. We demonstrated for the first time that Hs578T doxorubicin resistant cells (Hs578T/Doxo), which contain low levels of endogenous caveolin-1 and high levels of P-glycoprotein, are rendered drug-sensitive by overexpression of exogenous caveolin-1. MTT assays showed that after overexpressing caveolin-1, the drug resistance of Hs578T/Doxo cells to doxorubicin and cisplatin was reduced from 25.4 ± 1.5 and 65.3 ± 2.5 μg/ml to 0.8 ± 0.15 and 23.2 ± 2.1 μg/ml, respectively (i.e. reduced by 97% and 64%, respectively). Furthermore, using rhodamine-123 efflux assays, we observed a significant decrease in P-glycoprotein activity in caveolin-1 overexpressing cells, similar to that observed with 5 μM cyclosporine A or 10 μM verapamil, 2 inhibitors of P-glycoprotein activity. Using confocal microscopy, subcellular fractionation and co-immunoprecipitation assays, a possible physical interaction between caveolin-1 and P-glycoprotein in the caveolae membrane was observed in Hs578T/Doxo cells overexpressing caveolin-1. These results suggest that overexpression of caveolin-1 changes the state of the cells from drug-resistant to drug-sensitive by inhibiting P-glycoprotein transport activity.

© 2004 Wiley-Liss, Inc.

Key words: caveolin-1; Hs578T breast adenocarcinoma cells; MDR; P-glycoprotein

A major pitfall with chemotherapy in cancer patients is the development of tumor cells that are resistant to a broad range of therapeutic drugs commonly used in clinical treatment. Although the precise mechanism of multi-drug resistance (MDR) remains largely unclear, there is abundant evidence that many cases of MDR are due to overexpression of a plasma membrane ATPase called P-glycoprotein (P-gp). As an energy-dependent drug efflux transporter, P-gp decreases the effective concentration of active drugs in the cytosol and thereby reduces their cytotoxic efficacy. This is a very important mechanism of MDR in cancer cells.

Caveolae are invaginations of the plasma membrane that are enriched in cholesterol and sphingolipids, which impart unique physical properties to these membrane sub domains. Caveolae have been shown to play an important role in many signal transduction processes, by acting as a compartmentalization center for signaling molecules. Caveolin-1 is a major caveolar coat protein that has the ability to engage in complex interactions with other protein components in the caveolae and to affect their functions. Some studies have shown that the expression level of caveolin-1 closely correlates with the development of MDR in cancer cells. High expression levels of caveolin-1 and high surface density of caveolae have been identified in a number of MDR cancer cell lines, including adriamycin-resistant MCF-7 breast adenocarcinoma cells, colchicine-resistant HT-29 colon carcinoma cells, vinblastine-resistant Lovo colon carcinoma cells and Taxol-resistant A549-T24 lung carcinoma cells. Thus, it has been proposed that the acquisition of resistance to different drugs by various cell types might be associated with high expression of caveolin-1. A recent clinical study, however, showed that caveolin-1 expression in ovarian carcinoma showed no correlation with MDR. Although several studies have suggested colocalization of caveolin and P-gp in caveolae, the functional effect of caveolin-1 interaction with P-gp and its overall impact on the development and progression of MDR in cancer cells is largely unknown. To identify the possible effects of caveolin-1 on multidrug resistance and P-gp transport activity, we overexpressed caveolin-1 in Hs578T breast adenocarcinoma cells, and examined the changes of drug resistance and P-gp transport activity in these cells. In addition, we examined colocalization, co-fractionation and co-immunoprecipitation of caveolin-1 and P-gp. Our results suggest that overexpression of caveolin-1 in Hs578T breast cancer cells renders the cells drug-sensitive by inhibiting P-gp drug transport activity.

MATERIAL AND METHODS

Materials
Monoclonal antibody (mAb) and polyclonal antibody (pAb) against caveolin-1 were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-human P-gp mAb (C219) was obtained from Signet Laboratories (Dedham, MA). FITC-labeled goat anti-mouse IgG and Texas Red-labeled goat anti-rabbit IgG were purchased from Amersham Pharmacia Biotech. BCRP/NBT reagents were purchased from Promega. The pCI-neo and pCI-neo-cav-1 plasmids were generous gifts from Dr. Eric J. Smart (Kentucky Medical School, KY). Protein-G Sepharose, verapamil, cyclosporine A and Rhodamine-123 were purchased from Sigma (St. Louis, MO). All other reagents were obtained from regular commercial sources with highest purity.

Cell lines, plasmids and transfections
The parental human breast cancer cell line Hs578T/S and its doxorubicin-resistant subclone Hs578T/Doxo were the generous gifts of Dr. Kjell Grankvist (Umea University, Sweden). The cells were all cultured at 37°C, 5% CO2 in RPMI 1640 medium

Abbreviations: Cav-1, caveolin-1; CsA, cyclosporine A; DDP or cis, cisplatin; DMSO, dimethyl sulfoxide; Doxo, doxorubicin; mAb, monoclonal antibody; MDR, multidrug resistance; pAb, polyclonal antibody; P-gp, P-glycoprotein; Rh-123, rhodamine-123; S, sensitive; Vm, verapamil.

Grant sponsor: National Laboratory of Biomacromolecules; Grant number: 30070186, 30230120.
*Correspondence to: National Laboratory of Macromolecules, Institute of Biophysics, Chinese Academy of Sciences, Datun Road 15, Chaoyang District, Beijing 100101, China. Fax: +86-10-6487-2026. E-mail: chenmaci@sun5.ibp.ac.cn

Received 20 August 2003; Revised 17 December 2003, 12 February 2004; Accepted 26 February 2004

DOI 10.1002/ijc.20300
Published online 4 May 2004 in Wiley InterScience (www.interscience.wiley.com).
supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin. The entire cDNA sequence of caveolin-1 was cloned into the EcoRI site of pcI-neo, to generate pCI-neo-cav-1. pCI-neo-cav-1 and its empty vector were transfected into Hs578T cell lines as described.14 Briefly, 5 μg of pCI-neo-cav-1 plasmid was transfected into ~106 cells in a 100-mm dish, using Lipofectamine reagent according to the manufacturer’s instruction. Twenty-four hours after transfection, the culture medium was changed to a selection medium containing 1.5 mg/ml genetin for two days. Stable clones of cells overexpressing caveolin-1 were subsequently maintained in a culture medium containing 500 μg/ml genetin. As controls, the cells were transfected with the mock pCI-neo vector, and subjected with same selection treatment with genetin.

**Isolation of caveolae-enriched membrane fraction**

Detergent-free caveolae fractions were prepared using the modified method of Smart et al.15 Briefly, cells were homogenized with a tight Dounce homogenizer. The post-nuclear supernatant fraction, obtained after centrifugation at 1,000g for 10 min, was layered on top of a 30% Percoll gradient for caveolin-1. After centrifugation for 90 min in a SW41 Beckman swinging bucket rotor. The plasma membrane (PM) fraction was collected from the top of the gradient, and then sonicated with a Vibra Cell sonicator (Sonic & Materials, Danbury, CT). The sonicate was mixed with 50% Opti Prep to make a 23% Opti Prep solution. This was placed on the bottom of a Beckman SW41 centrifuge tube, and a linear 20–10% Opti-Prep gradient was layered on the top. The sample was then centrifuged at 52,000g for 90 min in a SW41 Beckman swinging bucket rotor. A sample of the bottom fraction (fractions 12 and 13) was collected and designated non-caveolae membrane (NCM). The top 5 ml of the gradient (fractions 1–6) was collected and mixed with 50% Opti-Prep. This was overlaid with 2 ml of 5% Opti-Prep and centrifuged at 52,000g for 90 min. An opaque band located just above the 5% interface was collected and designated caveolae fraction (CM).

**Western blot and immunoprecipitation**

Proteins were separated by SDS-PAGE with a standard reducing condition protocol. Samples were resuspended in sample buffer and loaded on 7.5% acrylamide-bisacrylamide gels for P-gp or 12.5% acrylamide-bisacrylamide gels for caveolin-1. After electrophoresis, proteins were electroblotted on to a nitrocellulose membrane. Blots were blocked by 5% nonfat dry milk, 0.05% Tween 20 in Tris-buffered saline (10 mM Tris, pH 8.0, 135 mM NaCl). Immunoblotting was carried out with designated antibodies and loaded on 7.5% acrylamide-bisacrylamide gels for caveolin-1. After electrophoresis, proteins were electroblotted on to a nitrocellulose membrane. Blots were blocked by 5% nonfat dry milk, 0.05% Tween 20 in Tris-buffered saline (10 mM Tris, pH 8.0, 135 mM NaCl). Immunoblotting was carried out with designated antibodies and loaded on 7.5% acrylamide-bisacrylamide gels for P-gp or pCI-Cav-1. pCI-Cav-1 cells were harvested by trypsin/EDTA solution and 12.5% acrylamide-bisacrylamide gels for caveolin-1. After electrophoresis, proteins were electroblotted onto PVDF membranes for the detection of caveolin-1 or P-gp.15

**Cell survival MTT assay**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) colorimetric assay was used to assess the sensitivity of the cells to agents in vitro as described.19 Cells were trypsinized and harvested during exponential growth, and equal numbers of cells (5,000 for Hs578T/HS, Hs578T/S + pCI-neo and Hs578T/S + pCI-Cav-1 or 10,000 for Hs578T/Doxo, Hs578T/Doxo + pCI-neo and Hs578T/Doxo + pCI-Cav-1) were inoculated into each well with 150 μl of culture medium. After overnight incubation, 100 μl of culture medium containing various concentrations of drugs were added, and the cells were incubated for 48 hr. Thereafter, 20 μl of MTT (2 mg/ml in PBS) were added to each well and incubated for a further 4 hr. The resulting formazan product was dissolved in 100 μl of dimethyl sulfoxide after aspiration of the culture medium. Plates were placed on a plate shaker for 5 min and read immediately at 550 nm using a microplate reader. The survival percent was calculated by the following equation: cell survival percent = (mean OD of one grade) / (mean OD of the blank control) × 100%. The IC50 for doxorubicin or cisplatin was defined as the doxorubicin or cisplatin concentration at which the survival rate was 50%.

**Efflux of Rhodamine-123**

To examine the transport activity of P-glycoprotein, efflux of Rhodamine-123 (Rh-123) was measured as described.20 Briefly, the cells were harvested and adjusted to 106/ml cells, then incubated with 2 μM of Rh-123 for 30 min at 37°C. After 3 times of washing to remove the extracellular free dye, the cells were incubated in dye-free media or media containing 10 μM verapamil or 5 μM cyclosporine A (dissolved in DMSO) at 37°C. The efflux of Rh-123 was analyzed at successive time points by flow cytometry using a Becton-Dickinson FACS-420 instrument.

**Transmission electron microscopy**

Samples were fixed with glutaraldehyde, postfixed with osmium tetroxide, and stained with uranyl acetate and lead citrate, as described.12 Samples were examined under a Philips TECAI 20 TEM.

**Confocal microscopy imaging**

Cells were cultured in 35-mm glass-bottom dishes or on a cover glass and the following protocol was used:2 fixation was in PBS containing 4% paraformaldehyde at room temperature for 20 min. Fixed cells were rinsed with PBS and treated with 25 mM NH4Cl in PBS for 10 min to quench free aldehyde groups. The cells were permeabilized by incubation for 15 min in freshly prepared 0.1% Triton X-100/ PBS. The cells were precultured for 1 hr in PBS containing 5% normal goat serum, and then incubated for 2 hr in diluted P-gp (1:25) or caveolin-1 (1:100) antibody in PBS containing 2.5% goat serum. Immunostaining was carried out by incubation for 1 hr in 1:200 diluted FITC-labeled goat anti-mouse IgG or Taxus Red-labeled goat anti-rabbit IgG. After extensive PBS washes, immunostained cells were examined with a Bio-Rad Radiant-2100 confocal microscope. More than 100 cells were inspected per experiment, and the results displayed are typical.

**Data analysis**

All data points represented the mean value of at least 3 independent experiments, with 5 duplicates for each. Values are represented as the mean ± SE. Statistical significance was determined by Student’s t-test with p < 0.05.

**RESULTS**

**Resistance to cisplatin and doxorubicin in Hs578T cells**

Through subclonal selections, we have obtained the following stable clones of cancer cells: parental human breast cancer cell line Hs578T (Hs578T/S) and its doxorubicin-resistant derivative cell line Hs578T/Doxo. The drug sensitivity or resistance of these cells was assayed using the MTT method. As shown in Table I, the
Hs578T/Doxo cells were around 2-fold and 24-fold more resistant to cisplatin and doxorubicin than the parental Hs578T/S cells, respectively, suggesting that doxorubicin is a more specific substrate of P-gp than cisplatin.

**Overexpression of caveolin-1 and increase of caveolae organelles in Hs578T/S and Hs578T/Doxo cells**

Hs578T cells were transfected with the pCI-neo-cav-1 plasmid, or with the pCI-neo vector as a control. After culture in geneticin selection medium for two weeks, the expression of caveolin-1 and P-gp were detected by Western-blot assay. As shown in Figure 1a (lane 1, 3), the expression levels of endogenous caveolin-1 were similar in Hs578T/S and Hs578/Doxo cell lines transfected with pCI-neo vectors, but the expression level of P-gp was significantly higher in the drug-resistant cells (Hs578/Doxo + vector) than in the drug-sensitive cells (Hs578T/S + vector).

The different expression patterns of P-gp and caveolin-1 in these cells raise an intriguing possibility that the mutual interaction between caveolin-1 and P-gp may impact the development of MDR in these cells. To test this possibility, we introduced exogenous caveolin-1 into these cells and therefore altered the ratio of caveolin-1 to P-gp. The subsequent response to different drugs was then studied. The densitometry results confirmed that the expression level of caveolin-1 in Hs578T/S + pCI-Cav-1 or Hs578T/Doxo + pCI-Cav-1 cells was 2.5–4-fold higher than in Hs578T/S + pCI-neo or Hs578T/Doxo + pCI-neo cells. Under the same conditions, the expression level of P-gp in Hs578T/Doxo + pCI-neo and Hs578T/Doxo + pCI-Cav-1 cells was about 20-fold higher than in Hs578T/S + pCI-neo and Hs578T/S + pCI-Cav-1 cells, as detected by densitometry, irrespective of whether these cells were transfected with pCI-Cav-1 or empty vector (Fig. 1a, lane 1 vs. lane 2, lane 3 vs. lane 4).

To further test the impact of overexpressing caveolin-1 on the formation of caveolae in Hs578T cells, we studied the changes of caveolae organelles using electron microscopy. As shown in Figure 1b, the number of caveolae increased greatly in Hs578T cells overexpressing caveolin-1. These caveolae appeared as attached omega-shaped flasks and bunches like clusters of grapes. Interestingly, a significant amount of caveolae-like intracellular vesicles could be detected in the cells overexpressing caveolin-1. Studies from Lavie et al. have shown that the caveolae-like vesicular compartments contain both caveolin-1 and P-gp.12,15 The changes in membrane ultrastructure could in principle alter the MDR property of the Hs578T cells.

**TABLE 1 – RESISTANCE TO CISPLATIN AND DOXORUBICIN IN Hs578T CELLS**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>IC50(μg/ml) Hs578T/S</th>
<th>IC50(μg/ml) Hs578T/Doxo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cisplatin</td>
<td>32.7 ± 1.52</td>
<td>63.2 ± 1.7 (1.9×)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>1.03 ± 0.07</td>
<td>24.8 ± 1.4 (24×)</td>
</tr>
</tbody>
</table>

1Cell survival was determined by MTT assay. – 2Data are displayed as the mean ± SD of 3 determinations, each obtained from 5 duplicate cultures. – 3The values in parentheses represent the relative resistance, determined by dividing the IC50 value of the resistant cells by that of the sensitive cells.

**FIGURE 1 – Stable overexpression of caveolin-1 in Hs578T cells and the changes in caveolae organelles.** (a) Drug sensitive (Hs578T/S) and drug resistant (Hs578T/Doxo) cells were stably transfected with caveolin-1, or vector as control. The expression levels of caveolin-1 in Hs578T/S and Hs578T/Doxo were about 2.5- or 4-fold higher than their control cell lines, respectively. The expression level of P-gp in Hs578T/Doxo is about 20-fold higher than in Hs578T/S cells, regardless of the caveolin-1 expression level. Each lane contained 30 μg of total protein from whole cell lysates. The results shown are representative of three separate experiments. (b) Cells were fixed with osmium tetroxide, and stained with uranyl acetate and lead citrate. Representative electron micrographs display views of the plasma membrane, showing a large increase in the number of caveolae organelles in cells overexpressing caveolin-1.
Reduced multi-drug resistance in Hs578T cells overexpressing caveolin-1

We examined the effect of overexpressed caveolin-1 on the drug resistance in Hs578T cells. We selected a specific substrate of P-gp, doxorubicin, and a non-specific substrate, cisplatin, to study the changes of multidrug resistance in these cells. As shown in Figure 2a, the IC_{50} values for cisplatin in Hs578T/S + pCl-neo and Hs578T/S + pCI-Cav-1 cells were about 40.3 ± 2.0 and 30.5 ± 1.5 μg/ml, respectively (p < 0.05) (left panel), and the IC_{50} values for doxorubicin in Hs578T/S + pCl-neo and Hs578T/S + pCl-Cav-1 cells were about 1.15 ± 0.10 and 0.86 ± 0.09 μg/ml, respectively (p < 0.05) (right panel). This indicated that although the Hs578T/S cells overexpressing caveolin-1 seem to be more sensitive to these drugs than the Hs578T/S cells transfected with the pCl-neo vector, the difference is not very large. In contrast, the difference in drug sensitivity could be observed more clearly in Hs578T/Doxo cells. The IC_{50} values for cisplatin and doxorubicin in Hs578T/Doxo + pCl-neo were 65.3 ± 2.5 μg/ml and 25.4 ± 1.5 μg/ml, respectively, whereas those in Hs578T/Doxo + pCl-Cav-1 cells were reduced to 23.2 ± 2.1 and 0.8 ± 0.15 μg/ml, respectively (p < 0.01) (Fig. 2b). This equates to a decrease in drug resistance in terms of the IC_{50} values of 64% for cisplatin and 97% for doxorubicin (Fig. 2c). These data demonstrate that overexpression of caveolin-1 markedly reduces the drug resistance in Hs578T/Doxo cells, particularly for doxorubicin, a specific substrate of P-gp. This suggests that overexpressed caveolin-1 may inhibit P-gp transport activity in Hs578T/Doxo cells. This would also be consistent with the high expression of P-gp in these cells compared to the parent strain, after selection in the presence of doxorubicin.

Reduced multi-drug resistance in Hs578T cells overexpressing caveolin-1

Reduced P-gp transport activity in Hs578T cells overexpressing caveolin-1

Because the overexpression of caveolin-1 did not change the protein levels of P-gp (Fig. 1a, lane 1 vs. lane 2, lane 3 vs. lane 4), the reduced drug resistance in cells overexpressing caveolin-1 is likely to be due to the inhibition of P-gp transport activity. To verify this hypothesis, we examined the activity of P-gp by mea-
Figure 3.
suring the efflux of rhodamine-123,21–23 a P-gp specific substrate, in Hs578T cells. Cells were loaded with Rhodamine-123, and then washed with dye-free medium to remove the extracellular dye. The fluorescence of remaining Rh-123 in the cells was monitored by flow cytometry. The fluorescence intensity change of Rh-123 reflects the change of P-gp transport activity in the membrane. The higher the fluorescence intensity of remaining Rh-123 in the cells, the lower the P-gp transport activity. As shown in Figure 3a, the Rh-123 fluorescence intensities at time 0 min were different in the 4 cell lines: F<sub>Hs578T/</sub><sup>Doxo</sup> > F<sub>Hs578T/S</sub> > F<sub>pCI-Cav-1</sub> > F<sub>pCI-neo</sub>. This indicates that P-gp transport activity is lower in Hs578T/S cells than in Hs578T/Doxo cells, consistent with the relative expression levels of P-gp. It also indicates, however, that P-gp transport activity is lower when the expression level of caveolin-1 is increased. The Rh-123 fluorescence intensity at time = 0 is related to the equilibrium of dye uptake and efflux, and the passive dye uptake rate is expected to be identical in the 4 cell lines. Therefore, the reduced intensity of Rh-123 fluorescence in drug-resistant cells and in those overproducing caveolin-1 implies a reduction in efflux rate, which may be mediated by the function of P-gp. This was investigated further by measuring the rate of decrease of Rh-123 fluorescence intensity in the 4 cell lines (Fig. 3b,c). As shown in Figure 3b (right vs. left), Hs578T/S + pCI-Cav-1 cells and Hs578T/S + pCI-neo cells had similar rates of decrease of Rh-123 fluorescence. This suggests that overexpression of caveolin-1 has little effect on the efflux of Rhodamine-123 when P-gp expression is low. Compared to drug-resistant cells transfected with only the pCI-neo vector, however, Hs578T/Doxo + pCI-Cav-1 cells had a much lower rate of decrease of Rh-123 fluorescence (Fig. 3c, right vs. left). The rate of fluorescence decrease in Hs578T/Doxo + pCI-neo cells was significantly faster than in Hs578T/S + pCI-neo cells, indicating that the rate of efflux correlates with the level of P-gp expression. To quantify these changes, we took the mean fluorescence intensity of each sample at time = 0 as 100% and plotted the relative fluorescence intensity at various time points as a percentage of this (Fig. 3d). The data demonstrate that Hs578T/Doxo + Cav-1 cells have a similar fluorescence decline rate to Hs578T/S cells. This suggests that over-expression of caveolin-1 reduces the efflux rate of Rhodamine-123 in Hs578T/Doxo cells to the level observed in drug-sensitive cells.

To compare the inhibitory effect of caveolin-1 on the Rh-123 efflux from Hs578T cells with that of P-gp inhibitors, we used the drugs, verapamil and cyclopodine A, 2 known inhibitors of P-gp transport activity.21 The results are shown in Figure 3e. We found that 5 µM cyclopodine A or 10 µM verapamil were able to reduce the efflux rate of Rh-123 from Hs578T/Doxo cells to the level of Hs578T/S cells. This not only suggests that the drug resistance of Hs578T/Doxo cells can be attributed to augmented P-gp levels, but also confirms that overexpressing caveolin-1 in Hs578T/Doxo cells has a similar effect on the activity of P-gp as known inhibitors of P-gp transport activity (Fig. 3d,e).

Localization of P-gp in caveolae membrane and co-immunoprecipitation of P-gp and caveolin-1

Our data show that overexpression of caveolin-1 can functionally inhibit the transport activity of P-gp in Hs578T cells. This inhibition effect could be due to direct interaction, which would require physical contact between caveolin-1 and P-gp, presumably in the caveolar membrane region. To test this possibility, we used true co-localization confocal microscopy experiments of intact cells to detect P-gp and caveolin-1 in the caveolar plasma membrane. As shown in Figure 4a, we found that P-gp colocalizes with caveolin-1.

We also found that P-gp and caveolin-1 co-fractionate in the caveolar fraction of the plasma membrane. As shown in Figure 4b, we employed a detergent-free method to isolate the caveolar fraction from Hs578T/Doxo + pCI-Cav-1 cells. The data show that caveolin-1 and P-gp could be detected in the whole plasma membrane fraction (PM). The strong bands of caveolin-1 and P-gp immunoreactive proteins in the caveolar membrane fraction (CM) indicates, however, that the majority of caveolin-1 and P-gp is localized in the caveolar membrane subdomain. Caveolin-1 and P-gp proteins were undetectable in the non-caveolar membrane fraction (NCF). We also carried out co-immunoprecipitation in Hs578T/Doxo + pCI-Cav-1 whole cell lysates using protein G-Sepharose beads and antibodies against caveolin-1 and P-gp. As shown in Figure 4c, caveolin-1 protein could be pulled down by anti-P-gp antibody and P-gp could also be co-immunoprecipitated with caveolin-1 by anti-caveolin-1 antibody. Taken together, these results suggest that caveolin-1 and P-gp may be able to interact directly within the caveolar membrane compartment.

**DISCUSSION**

Controlling the overexpression of particular proteins in the cell is increasingly employed as a tool to investigate their function and physiological significance.24,25 We used this method in Hs578T carcinoma cells and their MDR derivatives to investigate the role of caveolin-1 in the reduction of the transport activity of P-gp, and hence the ability of caveolin-1 overexpression to cause multidrug resistant Hs578T/Doxo cells to become drug sensitive. We also observed that P-gp and caveolin-1 co-localize and co-fractionate in the caveolar membrane fraction, meaning that a direct physical interaction between these proteins is possible. It is generally believed that development of MDR in cells is due to the increased expression of P-gp. Our data, however, suggest that changes in the expression of caveolin-1 also play an important role in regulating the resistant nature of cells in response to multi-drug treatment.

Conflicting results have been reported in the literature on the role of caveolins in the development of MDR. On the one hand, the caveolin-1 expression level is upregulated in MDR phenotypes of a number of human cell lines, including colon, ovarian, breast and lung carcinoma.12–14 On the other hand, expression of caveolin-1 and caveolin-2 was not detected in several MDR cell lines that express high levels of P-glycoprotein, such as J7.V1-1 and J7/T3-16 derived from murine macrophage, and Caco-V100 derived from human colon carcinoma cells.13 Unlike epothilone B-resistant A549 cells, epothilone B-resistant MCF-7 cells do not express any caveolins. It has also been shown that caveolin-1 expression has no association with that of P-gp protein or MDR1 mRNA in human ovarian carcinoma.14 In our study, we observed comparable amounts of caveolin-1 in Hs578T cells, irrespective of whether they were drug-sensitive or drug-resistant (Fig. 1a, lane 2). Thus, it is possible that the expression level of caveolin-1 in these cells...
varies in a tissue-dependent, cell-dependent or drug-dependent manner. Most importantly, our data show that overexpression of caveolin-1 reduces P-gp transport activity and renders multi-drug resistant cancer cells sensitive to anticancer drugs. The inhibitory effect of overexpressing caveolin-1 on P-gp transport activity was found to be similar to the effect of verapamil or cyclosporine A, which are known inhibitors of P-gp activity (Fig. 3e). These functional studies not only provide some clues as to the role of caveolin-1 in multi-drug resistance of cancer cells, but also indicate that caveolin-1 may function as an important regulator of P-gp transport activity.

Co-fractionation and co-immunoprecipitation assays in Hs578T/Doxo-Cav-1 cells (Fig. 4) indicate that P-gp and caveolin-1 are predominantly found in the caveolae membrane fraction. Confocal microscopy confirms that caveolin-1 and P-gp colocalize in intact cells, meaning that direct association of these proteins in the caveolae membrane may occur. This is consistent with previous findings that caveolin-1 and P-gp colocalize in the low density detergent-insoluble membrane fraction and that there is a physical interaction between these proteins in resistant CHRC5 cells and brain capillaries. This indicates that co-localization of caveolin-1 and P-gp as well as their physical association is not depend on the cells selected for their resistance. In our study, the functional inhibitory effect of caveolin-1 on P-gp transport activity was further investigated. Caveolin-1, as an important structural protein of caveolae, plays a key role in signal transduction and lipid homeostasis. There is a growing body of evidence that a number of proteins, such as Ras, Src, protein kinase C, eNOS, and the epidermal growth factor receptor, can directly interact with a specific scaffolding domain of caveolin-1, which consequently results in a strong inhibition of their biological activities. The proteins interacting with caveolin-1 have a consensus caveolin-binding motif, termed ‘scaffold’ site, which contains the sequences XXXXXA or XXA, where Φ is an aromatic residue, and X is any amino acid. By BLAST searching, we found that this caveolin-binding motif is also present in human P-gp between amino acid residues 36 and 44 (FSMFYSNW). This is similar to the equivalent residues in hamster MDR1 (FTMFYAGW), which has already been identified as a putative caveolin-binding motif. Taken together, this implies that the mechanism of this functional inhibition of the enzymatic activity may be caused by direct interaction between caveolin-1 and P-gp.

Nevertheless, other possible mechanisms of the inhibitory effects of caveolin-1 on P-gp transport activity and drug-resistance in cancer cells must be considered. One possible mechanism is that caveolin-1 regulation of cellular cholesterol may be important in the regulation of P-gp drug transport activity. Caveolin-1 has been reported to functionally bind to cholesterol. In addition, recombinant expression of caveolin-1 has been shown to facilitate the transport of newly synthesized cholesterol from the ER to the plasma membrane, where cholesterol rapidly diffuses out of caveolae. Thus, it is possible that the overexpression of caveolin-1 might indirectly affect P-gp transport activity through modulation of the availability of cholesterol at the level of the plasma membrane.
Although the precise mechanism by which overexpression of caveolin-1 alters multidrug resistance is worthy of further investigation, our present data suggest that enhancing the expression or function of caveolin-1 could downregulate the multidrug resistance of cancer cells and thus might serve as an alternative mechanism for improving the efficacy of chemotherapy in cancer patients.

REFERENCES

ACKNOWLEDGEMENTS
We thank Dr. E.J. Smart (Kentucky Medical School, Kentucky) for providing the pCI-neo and pCI-neo-Cav-1 expression vectors and Dr. K. Grankvist (Umea University, Sweden) for providing Hs578T cell line. We are grateful to Dr. Zui Pan (Piscataway, NJ) and Dr. Sarah Perrett (Institute of Biophysics, Beijing, P.R. China) for editing the manuscript.