Comparative Study of the Sensitivities of Cancer Cells to Doxorubicin, and Relationships between the Effect of the Drug-Efflux Pump P-gp

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Multi-drug resistance (MDR) of cancers to chemotherapy including doxorubicin (DOX) is mediated by several factors. To design an effective therapy for the treatment of chemotherapy-resistant cancers, it is essential to explore the elements responsible for mediating MDR. However, exploring these factors in detail in a wide range of tumor types is challenging as several critical analytical steps are involved. Here, we demonstrated the way of exploring the factors mediating MDR in the tumor types without performing the analysis at the molecular level of cells. The sensitivities of 15 different types of cancer cells to DOX were evaluated, and the role of P-glycoprotein (P-gp), one of the major efflux-pumps, was explored. A correlation curve was developed between the intracellular amounts of DOX and the sensitivities of cells, and, based on this correlation, the cells were classified in response to the involvement of P-gp that mediates MDR. P-gp plays an active role in mediating MDR of cancer cells where a correlation between the sensitivities of cells and the accumulated DOX exists. In contrast, in cells that show a resistance to DOX but whose sensitivities are independent of the amount of accumulated drug, it was reasonably presumed that mechanisms other than P-gp are likely to be involved in mediating MDR. Based on the correlation between the availability of a drug and cell sensitivity, it would be reasonable to explore the factors governing cancer MDR, which is essential in designing an effective therapeutic approach for treating chemotherapy-resistant cancers using chemotherapeutic drugs.

Key words multi-drug resistance; doxorubicin; cytotoxicity; drug availability–cell sensitivity correlation; P-glycoprotein (P-gp) dependence

Chemotherapeutic drugs having different chemical structures and functions are widely used in the treatment of various types of cancers. However, cancer cells become resistant either acquired or even intrinsically to structurally and functionally unrelated drug molecules, which is referred to as multidrug resistance (MDR) that leads to the failure of the treatment clinically and patient mortality. In mediating MDR in cancer cells, several mechanisms, including membrane transporters (ATP-binding cassette (ABC) transporters), metallothionein (MT), glutathione-S-transferases (GST), thymidylate synthase (TS), DNA topoisomerase II (Topo II) etc. are responsible, which function by reducing the intracellular drug concentration as well as by deactivating the drug molecules. The ABC transporters P-glycoprotein (P-gp)/MDR1; Multidrug resistance-associated proteins (MRPs); Breast cancer resistance protein (BCRP) present on the cell membrane play a pivotal role in the circulation and excretion of drug molecules in the cellular compartments.

Doxorubicin (DOX) an important chemotherapeutic drug that is used in clinics for the treatment of various types of cancer, functions against Topo II, mediates DNA damage and finally leads to cell apoptosis. Unfortunately, the efficacy of DOX is eventually hampered by the that cancer cells become resistant to the drug. The drug is actively expelled from the cells that overexpress the ABC transporters. It is evident that, in cancer cells, P-gp plays a role in the development of simultaneous resistance to multiple cytotoxic drugs, including DOX. To circumvent the MDR of cancer cells, verapamil (a calcium channel blocker) is used which modulates the acquired as well as intrinsic resistance of cancer cells by inhibiting the expression and function of P-gp, thereby enhancing cell cytotoxicity by decreasing drug efflux. This suggests that to develop the effective chemo-therapeutics, the identification of the precise mechanism of the MDR should be required. However, a great effort should be needed to identify the mechanisms of the MDR in large panel of tumors, since exploring the expression of abnormal genes, pathways of analyses, proteins using several molecular biological analyses are severe, time consuming and complicated.

In the present study, we presented a way of predicting the involvement of the mechanism of MDR by performing a simple correlation using the sensitivity of cells to drug as well as the amount of drug internalization without considering the molecular biological analysis. This regard, 15 different types of cancer cells were used to observe and compare the dose–response, drug retention efficiency upon exposure to DOX. In addition to these, as a major drug-efflux pump, the role of P-gp on the viability of cells was also evaluated. In an effort to understand these phenomena, we focused on the classification of the cells based on the involvement of possible factors responsible for causing the cells to become resistant to DOX.

MATERIALS AND METHODS

Materials Doxorubicin hydrochloride and Fetal bovine serum (FBS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Hyclone Laboratories (Logan, UT, U.S.A.), respectively. Verapamil hydrochloride and RPMI 1640 were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, U.S.A.). Dulbecco’s Modified Eagle’s

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Medium (DMEM), EGM-2 and EGM-2 MV medium were purchased from Lonza (Walkersville, MD, U.S.A.). Hoechst 33342 were purchased from Dojindo (Kumamoto, Japan). The protein content of cell monolayers and lysates was assessed using a BCA kit from Thermo Scientific (IL, U.S.A.). The anti-human P-gp antibody and Alexa Fluor 488 labeled goat anti-mouse Immunoglobulin G (IgG) were purchased from Calbiochem (cat. no. 517312) and Invitrogen (Carlsbad, CA, U.S.A.), respectively.

**Cell Lines and Culture**

Human cancer cells; small cell lung cancer (H69AR) (American Type Culture Collection (ATCC), VA, U.S.A.); breast cancer (MDA-MB-231; ATCC), ovarian cancer (SKOV-3; ATCC), and renal cell carcinoma (OSRC-2; Riken cell bank, Tsukuba, Japan) were cultured in RPMI 1640. Human prostate cancer (PC-3; ATCC), human non-small cell lung cancer (A549; ATCC) cells were cultured in F12K medium. Human liver cancer (Hep-3B, Huh-7, Hep-G2; Riken cell bank), colon cancer (HCT-116; ATCC), pancreas cancer (PANC-1; ATCC), cervical cancer (Hela; Riken cell bank), murine osteosarcoma (LM-8; Riken cell bank) and mouse melanoma (B16F10, Riken cell bank) cells were cultured in DMEM. Human malignant melanoma (A375-SM cells, were a gift from Dr. Isaiah J. Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.A.) cells were cultured in Minimum Essential Medium (MEM) (GIBCO, Grand Island, NY, U.S.A.). All of the medium mentioned above were supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/mL), streptomycin (100 μg/mL). The cells were cultured under an atmosphere of 5% CO₂ at 37°C.

**In Vitro Cytotoxicity Assay of Cells Using Free DOX**

The sensitivity of cells to DOX was evaluated by following the in vitro WST-8 assay protocol, as described previously. In a typical experiment, 5000 cells were seeded in 96-well plates and incubated overnight. The next day, the cells were exposed to different concentrations of DOX at 37°C for 8 h, followed by re-incubation for 16 h in the presence of fresh media. The cells were washed with PBS and re-incubated with a cell counting kit-8 (CCK-8) solution (Dojindo) for 2 h and the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific Inc.).

**Evaluation of the Growth Rate of the Cancer Cells**

To evaluate the effect of verapamil on the viability of cells, 5000 cells were plated in 96-well plates and incubated overnight. The next day, the cells were exposed to verapamil (0.1 mM) for 1 h at 37°C. After 1 h, cells were reincubation in the presence of verapamil (0.1 mM) and different concentrations of free DOX for 8 h at 37°C, followed by re-incubation for 16 h in the presence of fresh media. The cells were washed with PBS and re-incubated with a cell counting kit-8 (CCK-8) solution (Dojindo) for 2 h and the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific Inc.).

**Intracellular Accumulation of DOX**

To observe the effect of verapamil on the viability of cells treated with DOX, 40000 cells were seeded in 24-well plates over night. The next day, the cells were exposed to verapamil (0.1 mM) for 1 h at 37°C, followed by re-incubation with free DOX (10 μg/mL) in presence of verapamil (0.1 mM) for 8 h at 37°C. Finally, the cells were washed twice with PBS. The cell supernatant was collected by centrifugation at 12000 rpm, 4°C for 5 min and fluorescence intensity of accumulated DOX was measured (450 nm and Em: 590 nm).

**Detection of the Expression of P-gp**

For the detection of P-gp on the cell surface, 100000 cells were seeded on a 35-mm glass-bottom dish (Iwaki, Chiba, Japan) in 2 mL of culture medium for 24 h. The next day, the cells were washed with PBS and incubated with verapamil (0.1 mM) for 1 h at 37°C, followed by concomitant incubation with free DOX (10 μg/mL) and verapamil (0.1 mM) for 8 h at 37°C. After 7.5 h of incubation, 5 μL of Hoechst 33342 (1 mg/mL) (Dojindo Laboratories, Kumamoto, Japan) was added to stain the nuclei and the cells were re-incubated for an additional 30 min. The medium was then removed followed by washing with PBS. Finally, 1 mL of Krebs buffer was added and the cells were observed by confocal laser scanning microscopy, CLSM (A1 Confocal Laser Microscope System, Nikon Instruments Inc., Tokyo, Japan).

**Statistical Analysis**

Results are mainly expressed as mean ± Standard Deviation (S.D.). Data were analyzed by unpaired Student’s t-test. Differences among means were consid-
RESULTS

In Vitro Cytotoxicity Assay  To determine the cytotoxic activities of DOX, 15 types of cancer cells were incubated with different concentrations of DOX. DOX concentrations leading to 50% cell-death (EC\textsubscript{50}) were determined from concentration-dependent cell survival curves (Fig. 1, Table 1). A wide range in the sensitivities (EC\textsubscript{50}) of the cells to DOX was observed. Among the cells tested, Hep-G2 cells showed the lowest EC\textsubscript{50} value (0.0002 µg/mL) and OSRC-2 cells exhibited the highest EC\textsubscript{50} (71.15 µg/mL) to DOX. Based on the EC\textsubscript{50} values, cells were categorized into 3 major groups, as shown in Table 1 and in Figs. 1 and 2A. Cells having EC\textsubscript{50} values within the range of 0.0001–0.007 µg/mL and 0.02–0.50 µg/mL are considered to be sensitive and moderately sensitive to DOX, respectively. In contrast, cells that showed substantially higher EC\textsubscript{50} values (>5 µg/mL) were classified as being resistant to DOX.

In Vitro Cellular Accumulation of DOX  To determine the intracellular concentration of the drug, cells were incubated with DOX (10 µg/mL) for 8h (Fig. 2B), followed by measurement of the fluorescence of DOX accumulated by simple diffusion. The results indicated that, depending on the cell type, the amount of DOX in the cell cytosol varied from cell to cell. A very minor amount of DOX (0.0016 mg DOX/mg of cellular protein) was detected in the cytosol of H69AR cells. On the other hand, Huh-7 and Hep-3B cells contained a much higher amount of DOX (0.069 mg and 0.049 mg DOX/mg of cellular protein, respectively) in their cytosol (Fig. 2B). Among the treated cells, a lower amount of DOX was quantified in Hep-G2, PANC-1, SKOV-3, A375SM cells. A comparatively higher amount of DOX was detected in PC-3, LM-8, Hela, B16F10 and HCT-116 cells.

Effect of the Growth Rate of the Cells on the Sensitivity to DOX  To evaluate the effect of the cellular growth rate on the cytotoxic effect of DOX, we calculated the growth rate of the cancer cells following the protocol as we reported before (20). Based on the results presented in Fig. 3, it was observed that the cells which show resistance to DOX exhibited relatively slower rate of growth as compared to those which are sensitive or moderately sensitive to DOX. Among the cells,  

Cells were incubated with different concentrations of DOX for 8h followed by reincubation for 16h in presence of fresh media. The viability of cells was determined by following the WST-8 assay protocol (n=3). The dose–response curve was drawn using the Sigma-plot 12.0 software. A wide variation in the responses of cells to DOX was observed.

Table 1. The Values of the Concentration of DOX Required to Kill 50% (EC\textsubscript{50}) of the Cancer Cells

<table>
<thead>
<tr>
<th>Types</th>
<th>Cancer cells</th>
<th>EC\textsubscript{50} (µg/mL)</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal cell carcinoma</td>
<td>OSRC-2</td>
<td>71.15±52.84</td>
<td>Resistant</td>
</tr>
<tr>
<td>Small cell lung carcinoma</td>
<td>H69AR</td>
<td>41.42±27.75</td>
<td></td>
</tr>
<tr>
<td>Ovarian carcinoma</td>
<td>SKOV-3</td>
<td>35.94±35.66</td>
<td></td>
</tr>
<tr>
<td>Breast adenocarcinoma</td>
<td>MDA-MB-231</td>
<td>25.72±20.27</td>
<td></td>
</tr>
<tr>
<td>Pancreas carcinoma</td>
<td>PANC-1</td>
<td>5.58±6.68</td>
<td></td>
</tr>
<tr>
<td>Melanoma (human)</td>
<td>A375-SM</td>
<td>0.37±0.061</td>
<td>Moderately sensitive</td>
</tr>
<tr>
<td>Melanoma (mouse)</td>
<td>B16F10</td>
<td>0.10±0.034</td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Hep-3B</td>
<td>0.050±0.028</td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Huh-7</td>
<td>0.049±0.034</td>
<td></td>
</tr>
<tr>
<td>Colon carcinoma</td>
<td>HCT-116</td>
<td>0.027±0.045</td>
<td></td>
</tr>
<tr>
<td>Cervix adenocarcinoma</td>
<td>Hela</td>
<td>0.0066±0.0028</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>LM-8</td>
<td>0.0029±0.0017</td>
<td></td>
</tr>
<tr>
<td>Non-small cell lung carcinoma</td>
<td>A549</td>
<td>0.0008±0.0005</td>
<td></td>
</tr>
<tr>
<td>Prostate adenocarcinoma</td>
<td>PC-3</td>
<td>0.0008±0.0003</td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>Hep-G2</td>
<td>0.0002±0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Cells were incubated with different concentrations of DOX for 8h followed by reincubation in the presence of fresh media for the next 16h. The viability of cells was counted by following the WST-8 assay protocol. EC\textsubscript{50} values, calculated by using the Sigma-plot 12.0 software, indicated the existence of differences in the sensitivities of cells to the drug. Based on the responses to DOX (EC\textsubscript{50} values), cells were classified into three major groups.
H69AR cell exhibited the least rate of growth which are resistant to DOX, and Hela cell exhibited the fastest growth rate which are sensitive to DOX. Likely, A549, Hela, A375SM and B16F10 cells demonstrated a faster growth rate; however, their sensitivities to DOX are different (Fig. 3). Despite the variations in sensitivities to DOX (Fig. 1), PC-3, PANC-1, MDA-MB-231 and H69AR cells exhibited a similar growth rate. Likely, a similar growth rate was also observed in Hep-G2, LM-8, SKOV-3, OSRC-2, Huh-7, Hep-3B and HCT-116 cells regardless their sensitivities to DOX (Fig. 3). Despite similar growth rate, A549 cell exhibited about 460 folds more sensitivity to DOX as compared to that of A375SM cell (Table

Fig. 2. Comparisons of the EC_{50} Values as Well as the Amounts of DOX Taken up by the Cancer Cells
A. Cells were incubated with different concentrations of DOX for 8h followed by reincubation in the presence of fresh media for 16h. Cell viability was determined by the WST-8 assay protocol. The EC_{50} values were calculated using the Sigma-plot 12.0 software. Based on the sensitivities to DOX, cells were classified into three groups.
B. The intracellular accumulation of DOX. The cells were incubated with free DOX (10µg/mL) for 8h, and the amount of drug present in the cell cytosol was calculated by measuring the fluorescence intensity of the internalized DOX.

Fig. 3. Evaluation of the Growth Rate of Different Types of Cancer Cells
Five thousand cells were seeded in 96-well plate and incubated for 24h at 37°C. The cells were washed with PBS followed by reincubation with cell counting kit-8 (CCK-8) in respective culture media for 2h and the absorbance of the generated color was measured at 450nm using a microplate reader. The intensity of the yellow-color formazan dye, generated by the tetrazolium salt present in the CCK-8, is directly proportional to the number of living cells present in each well.
Likely, Huh-7 cell showed about 735 and 835 folds more sensitivity to DOX as compared to SKOV-3 and H69AR cell, respectively. On the contrary, SKOV-3 cell exhibited 3 folds higher growth rate as compared to that of H69AR cell, however, they demonstrate a similar level of sensitivity (EC50) to DOX. These results indicated that the growth rate of the tumor cells plays very negligible effect on the sensitivities to DOX, rather the sensitivities

![Fig. 4. Correlation between the EC_{50} Values and the Amounts of Intracellular Accumulation of DOX](image)

A. The EC_{50} values of cancer cells were plotted against the amounts of DOX present in the respective cells. Depending on the correlation, three distinct groups (G-I, II, III) of cells were found. B. Statistical analysis indicating a linear correlation ($R^2=0.84$) between the EC_{50} and the amount of internalized DOX in the cells of G-II.

![Fig. 5. Effect of Verapamil (Ver) (Inhibitor of P-gp) on the Intracellular Accumulation of DOX](image)

Cells were pre-incubated with Ver followed by co-incubation with Ver and DOX (10µg/mL) for 8h. The accumulation of DOX (white) in the cell cytosol was determined by observing the cells under a microscope (A) as well as quantitatively by measuring the fluorescence intensity of the internalized DOX (B). Verapamil induces a remarkable amount of DOX accumulation in SKOV-3 cell, as compared to others. Statistical analysis was performed by the Unpaired Student’s $t$-test. N.S.: not significant, **$p<0.01$. Scale bars 20µm.

![Fig. 5. Effect of Verapamil (Ver) (Inhibitor of P-gp) on the Intracellular Accumulation of DOX](image)
Correlation between EC\textsubscript{50} Values and DOX Uptake

The cytotoxic activity of DOX against the cells literally depends on the amount of the drug that is taken up by the cells. Cells that are able to internalize a higher amount of DOX should show a lower EC\textsubscript{50} or sensitivity to the drug. Therefore, it appears that the sensitivities of cancer cells are correlated to the amounts of DOX present in the cellular compartments. However, based on the results presented in Fig. 2, at first glance it seems that the EC\textsubscript{50} values are independent of the cellular uptake of DOX. Therefore, a graph was drawn by plotting the EC\textsubscript{50} values against the accumulated amounts of DOX (Fig. 4A), where three distinct clusters of cells were observed, and are categorized into 3 groups (G-I, -II, -III). In the case of G-I cells, the EC\textsubscript{50} values were higher, even though they could internalize higher amounts of DOX, indicating their resistance to DOX. On the other hand, the G-III cells internalize higher amounts of DOX which functions efficiently thus making them sensitive. Therefore, a correlation curve using the G-I and -III cells could not be drawn precisely, as was done for the G-II cells (Fig. 4B), where a good correlation ($R^2 > 0.8$) between the amount of accumulated DOX and the EC\textsubscript{50} value of a respective cell was observed. This result suggests that the degree of sensitivity of cells categorized in G-II depends on the uptake amount of DOX. It is well known that the uptake amount of DOX in the cell cytosol is primarily governed by P-gp. Taken together, it can be hypothesized that the degree of sensitivity to DOX of the cells in G-II depends mainly on the expression and function of P-gp. If this is true, the higher amount of DOX uptake induced by the inhibition of P-gp would increase the sensitivity of cells present in G-II, consequently, the blockage of the expression and function of P-gp would be an effective strategy to overcome DOX resistance of cells in G-II (H69AR, SKOV-3, PANC-1 etc.). On the other hand, the EC\textsubscript{50} is not correlated with the uptake amount of DOX in the case of cells categorized into G-I and G-III (Fig. 4A). This suggests that resistance or sensitivity of these cells to DOX is not governed by P-gp.

Relationship of P-gp to DOX Uptake

To evaluate the role of P-gp on the availability of accumulated DOX; OSRC-2, SKOV-3 and A549 cells were selected from the G-I, -II and -III groups, respectively. The cells were pre-incubated with verapamil to block the function of P-gp, followed by incubation with DOX (Fig. 5). Based on qualitative and quantitative analyses, the verapamil treatment significantly enhanced (by 6-fold) the accumulation of DOX in SKOV-3 cell (Figs. 5A, B). On the contrary, no significant difference in accumulation of DOX was observed in the cytosol of OSRC-2 and A549 cells. Based on these results, we used these cell lines for further investigation.

Fig. 6. Effect of Verapamil (Ver) on the Sensitivities of Cancer Cells to DOX

Cells were pre-incubated with verapamil followed by co-incubation with Ver and different concentrations of DOX. The dose–response curves were drawn using the Sigma-plot 12.0 software (A). Inhibition of P-gp by verapamil increases the cytotoxic effect of DOX to SKOV-3 cell, as compared to others (B).
Role of P-gp on Cell Viability  We further investigated the effect of uptake amount of DOX induced by the inhibition of P-gp. To provide an insight into the involvement of P-gp in cell viability, cells were pre-treated with verapamil followed by incubation with different concentrations of free DOX (Fig. 6). Based on the results of WST-8 assays, it was found that the inhibition of P-gp by verapamil increases the cytotoxic effect of DOX to SKOV-3 cells by 15 folds (EC50 changes from 33.84 µg/mL to 2.31 µg/mL) (Figs. 6A, B). Therefore, it can be said that the increased uptake of DOX enhanced the cytotoxicity of DOX resistant SKOV3 cells categorized in G-II (Fig. 5A). The varied EC50 and uptake amounts of DOX in SKOV-3 with verapamil is well correlated with the G-II correlation curve (Fig. 5B). On the other hand, verapamil had only a very minor effect on the sensitivity of OSRC-2 (by 2.5 fold) and A549 (by 1.5 folds) cells to DOX. These results suggest that the effect of verapamil on the cytotoxicity of cells in G-I and G-III is minor as compared to that of cells in G-II.

Evaluation of the Expression of P-gp  To confirm the relevancy of drug resistance to DOX with P-gp expression, we finally evaluated the expression of P-gp by CLSM (Fig. 7). Cells were treated with anti-P-gp and Alexa Flour 488 labeled antibodies followed by flow cytometric analyses (Fig. 7). A remarkable level of expression of P-gp was detected in SKOV-3 cells. On the contrary, the expression level of P-gp in OSRC-2 and A549 cells was quite low or almost undetectable.

DISCUSSION

The activity of chemotherapeutic drugs, including DOX, against cancer cells primarily depends on its availability in the cellular compartments as well as its distribution to the site of action. Therefore, cancer cells that are able to uptake higher amounts of DOX should exhibit greater sensitivity to the drug. However, cancer cells express various types of proteins to protect them from cytotoxic agents such as DOX.2-5 One such class of proteins is the transmembrane efflux pumps in which P-gp is a viable candidate.6,13 The efflux pumps expel drug molecules from the interior of the cell to the exterior and due to that fact that the concentration of the accumulated drug is decreased, a sufficient amount of drug can not reach the site of action, finally the cells become resistant to therapy. Hence, in the pathway for producing drug resistance, efflux pumps, including P-gp, play the major role. However, the elucidation of precise mechanisms of drug resistance in the wide range of tumor types is not easy.

In this study, 15 different types of cancer cells were incubated in presence of free DOX and their sensitivities were evaluated and compared in response to the amounts of drug that was accumulated. Based on the EC50 values, the cells showed a wide variation in sensitivity (Figs. 1, 2A) to DOX and it was possible to classify the cells into 3 distinct groups. Among the cells, PANc-1, SKOV-3, MDA-MB-231, H69AR and OSRC-2 cells showed (EC50>5 µg/mL) resistance to DOX (Table 1). In contrast, Hep-G2, PC-3, A549, LM-8 and Hela cells were found to be very sensitive (EC50<0.007 µg/mL) to the drug. The other cells were found to be moderately sensitive to DOX. To provide a therapeutic effect, the drug molecules should be located in the cellular compartment. Therefore, it would be expected that the availability of DOX in the cytosol of the resistant cells would be lower than those of cells that show a sensitivity to DOX. To explore the possibility of intracellular drug concentration-dependent cells sensitivities, we next determined the amount DOX that is taken up by each type of cells (Fig. 2B). The cells that showed resistance to DOX exhibited a lower accumulation of DOX in their cytosol as compared to the others, indicating that a reduced uptake of the drug by resistant cells might be an important
determinant for resistance to DOX. Among the resistant cells, PANC-1, SKOV-3 and H69AR cells contained a much lower amount DOX in their cytosol as compared to MDA-MB-231 and OSRC-2 cells (Fig. 2B). On the other hand, the cells that were moderately sensitive to DOX exhibited a higher amount of drug internalization than those of the sensitive cells. Moreover, a big difference in sensitivities to DOX was observed in Hep-G2, PANC-1 and SKOV-3 cells, despite their similar level of accumulated DOX (Figs. 2A, B). A similar phenomenon was also observed in the cases of A549, LM-8, MDA-MB-231 and OSRC-2, as well as in the PC-3, Hela, B16F10 and HCT-116 cells. These results indicate that the sensitivities of cancer cells are not always proportionally related to the amount of DOX taken up by the cells. There are certain types of cancer cells where such a relation exists. To expose such a relation, we prepared a curve using the EC_{50} values and the amounts of DOX taken up by the cells (Fig. 4A), where three distinct positions of cells observed (G-I–III). The cells classified as G-I (MDA-MB-231, OSRC-2) show resistance to DOX despite their abilities to internalize higher amounts of drug. On the other hand, the amounts of DOX taken up by G-III cells were found to be effective in inducing a higher degree of drug sensitivity. A good correlation between the sensitivities to internalized DOX was observed only in the G-II cells (Fig. 4B). DOX is one of the major substrates of P-gp,^{6,11–13} therefore, it is possible that P-gp might be actively involved in internalizing DOX specifically by G-II cells. To verify this, OSRC-2, SKOV-3 and A549 cells were selected from the G-I, -II and -III classes, respectively, and the involvement of P-gp on the availability of the drug in the cytosol (Fig. 5) as well as on the cell viabilities was explored (Fig. 6). To inhibit the expression and function of P-gp, the cells were treated with Verapamil.^{16–18}

In the case of OSRC-2 cells, a class of RCC, which are intrinsically resistant to chemotherapeutic,^{20–24} the EC_{50} to DOX was calculated to be 71.15 μg/mL. Even though, the inhibition of P-gp by verapamil induces a 2-fold increment in the amount of internalized DOX (Figs. 5A, B), however no remarkable change in the viability of OSRC-2 cells was observed (Figs. 6A, B). These results support the view that the resistance of this type of cell to DOX might not be mediated by P-gp, since the expression level of P-gp was quite low (Fig. 7). The resistance of OSRC-2 cells to DOX might be mediated by the factors including the GST, Topo II, overexpression of anti-apoptotic gene Bcl-2, and of cell survival gene clusterin and Signal Transducer and Activator of Transcription 1 (STAT1) as well as of hypoxia-inducible factor 2α (HIF-2α).^{25–29} It was previously reported that MDA-MB-231 cells do not express P-gp under normal conditions, and where the MDR1 genes (mdr1 mRNA)^{30–33} as well as MRP-1 and BCRP^{30} were barely detected; rather the cell expresses P-gp only after a long period of incubation with the drug^{31} or when transduced with the MDR1 gene,^{32} or under exposure to hyposic conditions.^{33} In MDA-MB-231 cells, the activity of GST, Bcl-2 protein which protects the cell from programmed death and detoxification enzyme cytochrome P450 that rapidly metabolize and inactivate the internalized drugs^{36–38} would be the possible factors mediating the resistance to DOX. Therefore, it is entirely possible that the resistance of G-I cells to DOX is independent of the expression of efflux pumps, and that other MDR proteins present in the cell cytosol or in the nuclei (GST, TS, Topo II etc.) are responsible.^{5}

On the other hand, the inhibition of P-gp makes SKOV-3 cells about 15-fold more sensitive to DOX (Fig. 6A), thereby reversing the resistance of this cell to DOX (Fig. 6B) which is presumably due to the significant enhancement in intracellular drug accumulation (Figs. 5A, B). Therefore, a large shift in the EC_{50} value, corresponding to the internalized DOX, after verapamil treatment was observed in case of SKOV-3 cells, as compared to OSRC-2 (Fig. 6B), indicating that the sensitivity of SKOV-3 cells to DOX is dependent on the intracellular concentration of the drug. Moreover, a huge amount of P-gp was detected in these cells (Fig. 7), which is in general agreement with findings reported by several other groups.^{39–41} Therefore it is possible that the sensitivity of SKOV-3 cells to DOX is mediated by the expression and function of P-gp. It was previously reported that PANC-1 cells express P-gp^{42,43} and MRP-1.^{43,44} In addition, HCT-116 cells express several proteins, including P-gp, MRP, LRP.^{45–47} Similarly, the expression of ABC transporters including P-gp was detected in B16F10 cells.^{48–50} Therefore, based on the above information, it can be concluded that in the case of G-II cells, efflux pumps play an active role in mediating their sensitivities to DOX in a drug-concentration dependent manner.

Compared to either OSRC-2 or SKOV-3 cells, in A549 cells, it was observed that the amount DOX present in the cytosol likely is a factor in inducing cell sensitivity (EC_{50}=0.0008 μg/mL) (Fig. 5A, Table 1). Therefore, after the verapamil treatment, no remarkable change in drug accumulation or in cell viability was observed. Moreover, no detectable level of P-gp was found on these cells (Fig. 7), consistent with findings reported in a previous study.^{51} Considering these, it can be said that the sensitivity of A549 cells to DOX is independent on the level of expression of P-gp. The sensitivity of this cell to DOX can also be explained by the fact that this cell does not produce a detectable level of the Bcl-2 protein^{52,53} which can protect it from programmed death. Similarly, the sensitivity of PC-3 cell to DOX can also be explained by at least one factor that this cell generally does not have the Bcl-2 protein.^{54} In other cells, such as Hep-G2 and Hela cells, no expression of ABC transporters or LRP was detected.^{55–57} Therefore, considering the above information, it can be predicted that in the cells of G-III cells, MDR proteins might not have any role in their viability to DOX.

CONCLUSION

Several proteins or enzymes are involved in mediating the resistance of cancer cells to chemotherapeutic drugs such as DOX. Drug-efflux pumps, more importantly P-gp, exhibit an active role in inducing MDR of cancer cells (SKOV-3, H69AR, PANC-1, HCT-116 etc.) where the sensitivities of the cells are dependent on the availability of the drug in the cellular compartment. On the contrary, mechanisms other than the drug-efflux pumps are presumably involved in certain other cells (OSRC-2, MDA-MB-231 etc.) whose sensitivities to the drugs are independent of the amount of drug that is internalized. Based on the correlation of cell sensitivity with the internalized drug, it is reasonable to explore the factors responsible for mediating MDR in cancer cells. Depending on the identification of the factors in a cell type, it would be reasonable to develop an effective drug delivery system for the treatment of
chemotherapy resistant cancer using chemotherapeutic drugs.

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