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Physical state changes of membrane lipids in human lung adenocarcinoma A_{549} cells and their resistance to cisplatin

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Abstract

The properties of membrane lipids in sensitive A_{549} and resistant A_{549}/DDP cells to *cis*-dichlorodiammine platinum[II] (cisplatin) were examined by combining different approaches. The results showed that fluorescence intensity (ΔF) of Merocyanine 540 (MC540) was 93.5 ± 21.8 for the sensitive A_{549} cells and 49.5 ± 11.2 for the resistive A_{549}/DDP cells, monitored by flow cytometry, which may indicate that membrane lipid packing of the sensitive A_{549} cells were looser than that of the resistant A_{549}/DDP cells. Diffusion rate of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-hexadecanoyl-Sn-glycero-3-phosphatidyl-ethanolamine (NBD-PE) was slower in A_{549}/DDP cells than in A_{549} cells as detected by fluorescence recovery after photobleaching (FRAP) technique. Fatty acid analysis of the membrane lipids showed 21.6, 27.0 and 31.8% increase in the amount of $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ fatty acid, respectively, in A_{549} cells as compared to A_{549}/DDP cells. The total amount of unsaturated fatty acids in the plasma membrane lipid is 69.13% ± 2.2% for A_{549}, and 55.08% ± 1.8% for A_{549}/DDP cells, respectively. The resistance to cisplatin in A_{549}/DDP cells was confirmed by the measurements of the transmembrane influx of Rhodamine-123, cisplatin or Bodipy-cisplatin by fluorescence assay and inductively coupled plasma mass spectrometry (ICP-MS). From the results described previously, it is concluded that changes in the membrane lipids "composition" cause a change in the physical state of the plasma membrane lipids and that this may be associated with the resistance of A_{549}/DDP cells to cisplatin.

Keywords: Human lung adenocarcinoma A_{549} cells; Cisplatin resistance; Membrane lipids

1. Introduction

The therapy of various human malignancies is often hampered by inherent or acquired resistance to chemotherapeutic agents. Considerable evidences now suggest that P-glycoprotein (Pgp) and/or the multidrug resistance protein (MRP), known as drug transporters overexpressed in a number of multidrug resistant tumor cell lines, are the major causes of multiple-drug resistance (MDR) [1,2]. Those proteins belong to the ABC superfamily of membrane proteins [3].

Abbreviations: MDR, multidrug resistance; Pgp, P-glycoprotein; MC540, merocyanine 540; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-hexadecanoyl-Sn-glycero-3-phosphatidyl-ethanolamine; cisplatin, *cis*-dichlorodiammine platinum[II]; ICP-MS, inductively coupled plasma mass spectrometer; DPH, 1,6-diphenyl-1,3,5-hexatrience; *P*, fluorescence polarizations; FRAP, fluorescence recovery after photobleaching

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Chemotherapeutic drugs perturb cells in many ways, therefore, it is very difficult to consider a "pure" (solely MDR protein-mediated) phenotype, responsible for the MDR [4]. The interaction of hydrophobic compounds with biological membranes induces a variety of physico-chemical effects that can influence binding and diffusion of drugs in cells. Cisplatin, the DNA-targeting drug, has membrane effects [5] and that may be due to ionic interactions of drugs with the head groups of phospholipids which are asymmetrically distributed in either leaflet of the bilayer [6]. Such perturbation could have a significant influence on the transmembrane partitioning of the drug by a non-channel diffusion-mediated process [7]. For MDR cells, resistance to chemotherapeutic agents may also be related to altered plasma membrane compositions and physico-chemical status as previously suggested [8].

Cisplatin resistance of some tumors is an important factor in deciding the efficiency of the treatment in the cancer clinic and in the elucidation of the resistance mechanism of this drug. Resistance to cisplatin is characterized by a lower intracellular concentration of the drug. The plasma membrane plays a key role in the control of intracellular concentration, and its lipid compositions or physical state may alter drug transmembrane influx [9]. For this reason, we measured and compared the difference of biophysical properties of plasma membranes between cisplatin-sensitive A_{549} and resistant A_{549}/DDP cells, and further analyzed the effects of membrane lipid physical state on the drug transmembrane diffusion at the cellular and liposomal level.

2. Materials and methods

2.1. Materials

MC540 was from Molecular Probes (Eugene, OR). 1,6-Diphenyl-1,3,5-hexatrience (DPH) was from Aldrich (Milwaukee). Penicillin, streptomycin, trypsin and cisplatin were from Sigma (St. Louis, MO). *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-hexadecanoyl-Sn-glycero-3-phosphatidyl-ethanolamine (NBD-PE) was purchased from Avanti Polar Lipids Co. Bodipy-cisplatin was synthesized in our laboratory. RPMI-1640 medium and fetal bovine serum were from Gibco Co. Other reagents were local products of analytical grade.

2.2. Methods

2.2.1. Cell lines and cell culture

The human lung adenocarcinoma cell line A_{549} and the MDR cell line A_{549}/DDP, which is selected from A_{549} cells by cisplatin (CDDP or DDP), were obtained from Beijing Tumor Institute, the two cell lines were grown as adherent monolayers on 75 cm^2 flasks in RPMI-1640 (pH 7.35) medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin in a humidified incubator (Life Science Co.) with 5% CO_2 at 37 °C. Cell viability was assessed by the ability to exclude trypan blue (0.5% (w/v); Sigma). Exponentially growing cells were used in all experiments.

2.2.2. Membrane lipid packing measurement

According to the method described by Schlegel et al. [10]. A_{549} and A_{549}/DDP cells were harvested from flasks with 0.25% trypsin and 0.02% EGTA, and suspended to 1×10^6 cells/ml in PBS containing 0.2% bovine serum albumin (fatty acid-free, Sigma), then 10 μl of the MC540 stock solution (stored in the dark at 4 °C, 1 mg/ml in 50% ethanol) was added. After 10 min at room temperature, the cell suspensions were pelleted and washed twice with PBS containing 0.2% bovine serum albumin and diluted to 1 ml for flow cytometric analysis.

Flow cytometric analysis of the stained tumor cells was performed using FACS-420 flow cytometer with confocal optics and a logarithmic amplifier (Becton Dickinson). Excitation was provided with an argon ion laser operating at 488 nm. MC540 fluorescence was monitored through a 575 nm bandpass filter. Histograms were collected for 10,000 viable cells. Data were analyzed with a HP-300 Consort 30 computer program.

2.2.3. Lateral diffusion of NBD-PE in cell membranes by fluorescence recovery after photobleaching (FRAP)

An amount of 100 μl of NBD-PE (1 mg/ml in chloroform) was added to 800 μl of the purified asolectin

(125 mg/ml in chloroform:methanol in the ratio of 4:1, respectively) and then evaporated to dryness under nitrogen. The liposomes containing NBD-PE were prepared by sonication in the buffer consisting of 130 mM NaCl, 40 mM Tris/HCl. NBD-PE was transferred from liposomes mostly into the outer leaflet of the plasma membrane of the cells by simple incubation of the cells and the NBD-PE containing liposomes for 30 min at 37 °C. The FRAP was measured by using the laser confocal scanning microscopy (LSCM Leica, TCS-NT type, Germany). Scanning of the chosen cells were taken immediately after the photobleach until the recovery is completed as judged by lack of further change in fluorescence. The effects of photobleach and the recovery can be monitored within the membrane of the intact cells. Four cells of each cell line were subjected to FRAP analysis.

2.2.4. Analysis of fatty acid compositions

Determination of the fatty acid compositions of the plasma membrane was based on the methods of Epand and co-workers [11]. Preparation of A_{549} and A_{549}/DDP crude membrane was performed as described by Shen et al. [12]. Briefly, crude membranes were dissolved in 2 ml of 1:2:0.8 (v/v/v) of chloroform:methanol:H_2O mixture for overnight, then chloroform:H_2O (1:1, v/v) was added to the final concentration of chloroform:methanol:H_2O as 2:2:1.8. The lower phase was collected and dried under vacuum. An amount of 200 μl of 1:1 (v/v) phenol:petroleum ether and 200 μl of 0.5 M KOH (dissolved in methanol) were then added for methyl esterification. After incubating in water at 50 °C for 50 min, 2 ml H_2O was added, the upper phase was collected and dried in vacuum. The residue was dissolved in 50 μl ethyl acetate. Analysis was carried out on a Shimadzu GC-9A gas chromatograph with a Grob injector for splitless injection. Detection was carried out with a frequency pulsed electron capture detector (Shimadzu, Japan).

2.2.5. Bodipy-cisplatin and cisplatin transmembrane influx

The active influx of Bodipy-cisplatin, a fluorescence dye labeled cisplatin, was measured using a Hitachi F-4010 spectrofluorometer. The exponentially growing A_{549} or A_{549}/DDP cells were harvested from flasks with 0.25% trypsin and 1 mM EDTA. The single cells suspension in RPMI-1640 (3.5×10^6 cells/ml) was incubated in 50 μM Bodipy-cisplatin at 37 °C. At different times of the incubation, the collected cells were washed three times with PBS buffer. The labeled tumor cells were analyzed immediately using Hitachi F-4010 spectrofluorometer (Ex/Em = 488 nm/515 nm, slide width = 3 nm, response = 2 s, temperature = 25 °C). In parallel experiments, intracellular cisplatin and Bodipy-cisplatin concentrations in the two cell lines were measured by inductively coupled plasma mass spectrometer (ICP-MS; PQII turbo type, Fisons Instrument Co., Middlewich, UK) according to the Bungo method with some modification [13]. Briefly, 3.5×10^6 cells treated for different time with 50 μM cisplatin or 50 μM Bodipy-cisplatin were washed twice with PBS, centrifuged and solubilized in 40 μl fuming nitric acid for 6 h at 80 °C. The final volume was then adjusted to 3 ml with distilled water. The platinum content of the samples was determined by comparison with an internal standard which was prepared by dilution of a cisplatin solution of defined concentration. The detection limit was 40 ppt in each sample. Experiments were repeated three times with each cell line in duplicate. The obtained data were auto-analyzed and -integrated by the internal Fisons PQ visions version 4.1.1 software.

2.2.6. Measurements of accumulation of the fluorescence dyes into asolectin liposomes and fluidity of the liposomes with DPH

The asolectin liposomes were prepared by sonication in the presence of 1 μM or 1 mM Ca^{2+} as described [14]. Then the liposomes with defined Ca^{2+} concentration were re-suspended in the buffer containing 1 mM Ca^{2+} and the liposomes were incubated with Rhodamine-123 (15 μM) or Bodipy-cisplatin (50 μM) for 10 min and DPH (3 μM) for 30 min at room temperature. The pellets obtained after centrifugation (100,000 × *g*, 20 min) were washed three times with PBS containing 1 mM Ca^{2+} and the fluorescence changes of the dyes were detected with fluorometer (Hitachi F-4010) at 25 °C. Ex/Em = 504 nm/525 nm for Rhodamine-123, 488 nm/515 nm for Bodipy-cisplatin and 360 nm/430 nm for DPH.

3. Results

3.1. Packing degree of phospholipids in resistant A_{549}/DDP cell membrane is higher

Merocyanine 540 (MC540) is a lipophilic fluorescence dye binding to the surface of the plasma membrane. MC540 can be used quantitatively to study phospholipid packing and membrane phases with lipid vesicles and to sense subtle differences in the arrangement of phospholipids in biological membranes [15].

The flow cytometry technique was utilized to examine the "packing" of lipids in resistant A_{549}/DDP and sensitive A_{549} cells. The total fluorescence intensity (ΔF) of MC540 stained in A_{549} cells was 93.5 ± 21.8 and 49.5 ± 11.2 of the A_{549}/DDP cells. The "packing degree" was markedly lower in A_{549} sensitive cells in contrast to A_{549}/DDP resistant cells. Increased binding of the MC540 dye is a consequence of the probe's property of binding more strongly to loosely packed lipid bilayer than to those more tightly packed [10]. It can be concluded that the packing of phospholipid molecules in A_{549}/DDP cells is tighter than that in A_{549} cells.

3.2. Diffusion rate of NBD-PE in A_{549}/DDP cell membrane was relatively slow

Physical state of plasma membranes is associated with the dynamic character of lipid molecules in membranes which can be monitored by the FRAP technique using a fluorescence dye like NBD labeled PE. Results in Fig. 1 indicate that fluorescence recovery rate of the probe in the A_{549} cell membranes was obviously faster within 10 min compared with that in the A_{549}/DDP cell membranes. It would mean that diffusion rate of NBD-PE in A_{549}/DDP cell membranes was relatively slow. The percentage recovery rate was also lower for these cells.

3.3. Unsaturation degree of fatty acids in plasma membrane of A_{549}/DDP resistant cells decreased

The fatty acid compositions of A_{549} and A_{549}/DDP cells grown under normal conditions were analyzed and is as shown in Fig. 2. As a chromatography marker, the 17:0 fatty acid:water was chosen, since this fatty acid is not present in plasma membranes of most cells grown at physiological conditions [16]. Fig. 2 shows that there was a significant difference in the proportion of $C_{16:0}$ and $C_{18:0}$ fatty acid species in A_{549} and A_{549}/DDP cell plasma membrane. In addition, there was an increase of 21.6, 27.0 and 31.8% in the amount of $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ fatty acid, respectively, for

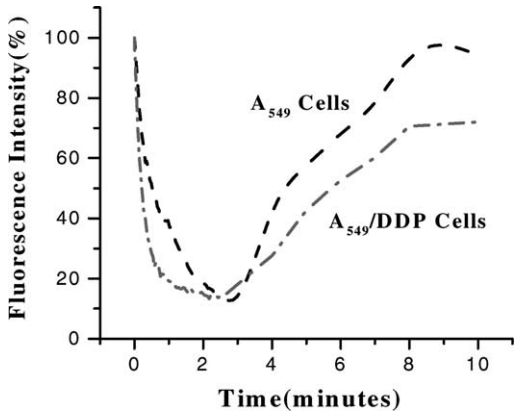

Fig. 1. Measurement of FRAP of NBD-PE in sensitive A_{549} and resistant A_{549}/DDP cells. The effects of photobleach and the recovery can be monitored with a part of the membrane on the intact cell using the LSCM Leica. Scanning of the chosen cells were taken immediately after the photobleach until the recovery was completed. Data are shown as the result of a representative single experiment ($n = 3$).

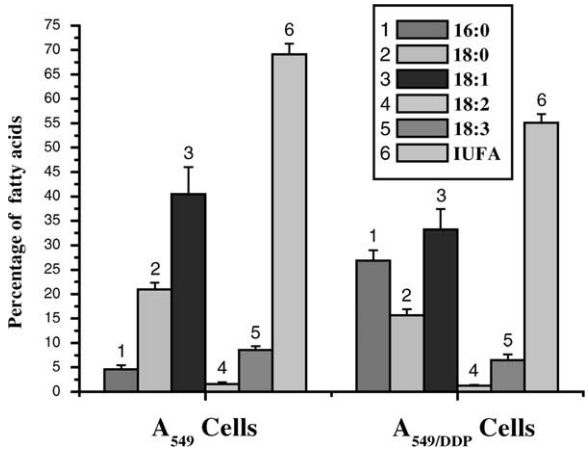

Fig. 2. Fatty acid compositions in plasma membranes of A_{549} and A_{549}/DDP cells. Quantity of each composition was expressed as a percentage of total fatty acids. The data represent mean $\pm$ S.D. of three independent experiments ($P < 0.05$).

Table 1
Transmembrane influx of Bodipy-cisplatin into sensitive A_{549} and resistant A_{549}/DDP cells examined by spectrofluorometer

	Time		
	10 min	40 min	60 min
A_{549} cells	41.0 ± 2.5	60.9 ± 4.2	73.5 ± 3.6
A_{549}/DDP cells	34.8 ± 2.6	49.9 ± 0.4	55.2 ± 2.8

The data were average ± S.D. of three experiments ($n = 3$; $P < 0.01$).

A_{549} cells compared with A_{549}/DDP cells. According to the method for calculating the "index of unsaturated fatty acid" [17], the unsaturated degree of plasma membrane fatty acids in A_{549} cells is 69.1 ± 2.2, and 55.1 ± 1.8 in A_{549}/DDP cells. The results indicate that the fluidity of membrane lipids decreased in resistant A_{549}/DDP cells compared to sensitive A_{549} cells.

3.4. Transmembrane influx of Bodipy-cisplatin in A_{549}/DDP cells is slower than that in A_{549} cells

We measured the transmembrane influx of the Bodipy-cisplatin in the two cell lines monitored by the ΔF change of the dye in the cells. Table 1 shows that the difference of transmembrane influx of Bodipy-cisplatin can be seen after the incubation for 10 min between the two cell lines. The transmembrane influx into A_{549} cells increased about 33% as compared with the A_{549}/DDP cells after 60 min incubation. This result was further confirmed by the measurement of intracellular concentrations of cisplatin or Bodipy-cisplatin using inductively coupled plasma mass spectrometry (ICP-MS, PQII turbo type, Fisons instrument Co.). As shown in Table 2, the concentration of cisplatin or Bodipy-cisplatin in the A_{549} or A_{549}/DDP cells was increased with incubation time. But, it was also obvious that accumulation of either cisplatin or Bodipy-cisplatin in the sensitive A_{549} cells was markedly faster than in the resistant A_{549}/DDP cells.

Table 3
Accumulation of Rhodamine-123 and Bodipy-cisplation related to fluidity of liposomes

Fluorescence probes	$[Ca^{2+}]_i$ (1 μM)	$[Ca^{2+}]_i$ (1 mM)
DPH (P)	0.287 ± 0.014	0.235 ± 0.021
Rhodamine-123 (ΔF)	43.75 ± 2.18	53.06 ± 1.31
Bodipy-cisplatin (ΔF)	20.41 ± 1.5	23.72 ± 0.33

The asolectin liposomes were prepared as described in Section 2.2. The experimental procedure was described in Section 2.2: P; ΔF; the data were mean ± S.D. of six experiments ($n = 6$; $P < 0.01$).

3.5. Accumulation of the dyes into asolectin liposomes depends on fluidity of the lipid bilayer

For further understanding relationship of cisplatin resistance with physical property of membrane lipids, accumulation of Rhodamine-123 and Bodipy-cisplatin into liposomes and fluidity of the liposomes were measured. It can be seen in Table 3 that more accumulation of the dyes into liposomes containing higher Ca^{2+} concentration which showed more fluidity of the membrane lipids as the polarization (P) change detected by DPH. This would mean that physical state change of membrane lipids may influence influx of drugs.

Table 2
Concentration of Bodipy-cisplatin or cisplatin in the sensitive A_{549} cells and resistive A_{549}/DDP cells monitored by ICP-MS

	Time		
	10 min	40 min	60 min
A_{549} cells			
Cisplatin	329.24 ± 9.89	404.91 ± 3.36	501.24 ± 26.85
Bodipy-cisplatin	877.32 ± 6.39	1281.92 ± 32.43	1377.39 ± 47.0
A_{549}/DDP cells			
Cisplatin	139.68 ± 9.75	258.75 ± 10.62	269.49 ± 0.99
Bodipy-cisplatin	609.24 ± 20.02	1098.27 ± 11.57	1174.60 ± 26.74

Concentration of the Bodipy-cisplatin or cisplatin in the cell lines were measured by ICP-MS and expressed as cisplatin atoms calculated with an internal standard. The data were average ± S.D. of the three experiments ($n = 3$; $P < 0.01$).

4. Discussion

At present, chemotherapy is still an important approach to cancer treatment, complementing surgery and radiotherapy. A major impediment to successful chemotherapy is occurrence of resistance of most of the cancer cells to a wide range of chemotherapeutic drugs [18].

The demonstration that the drugs do not move freely across lipid membrane may raise a possibility that the physical state of cell plasma membrane can modulate accumulation of drugs into cells resulting in drug-resistance [19]. For example, Epand and co-workers indicated that accumulation of drugs in Pgp expressing cells was related to alteration of membrane biophysical properties [20]. Romsicki and Sharom [21] and Luker et al. [22] reported that the function of Pgp is correlated with components, properties of phospholipids and their interactions, respectively. Drori et al. also showed that perturbation of plasma membrane biophysical properties was able to modify the activity of Pgp [23]. Contrary to these, Rintoul and Center reported that alteration in biophysical properties of membrane lipids was not an essential component of the adriamycin resistance in Chinese hamster lung cells [24]. It should be noted that these results reported from different laboratories are not consistent due to using different cells or the same cell line but treated with different drug. The previous studies examined the relation of Pgp activity and the physico-chemical status of cell membranes. No such studies were made for cisplatin compounds, in which compound's accumulation may not be influenced by Pgp. Therefore, we studied the relation of the physico-chemical status of plasma membrane and cisplatin up-take in sensitive A_{549} and resistant A_{549}/DDP cells.

We have shown previously that fluidity of lipid bilayers of resistant A_{549}/DDP cells is less than that of the sensitive A_{549} cells, and the surface and middle layer of the plasma membrane of the later were more fluid than that of the former detected by using fluorescence amphipathic molecular probes N-AS series probes [25]. This difference was further confirmed by the results here indicating faster lateral diffusion of NBD-PE in the sensitive A_{549} cell membranes compared to resistant A_{549}/DDP cell membranes (Fig. 1) and the lower packing of the membrane lipids of the sensitive A_{549} cells (as shown in Section 3). To determine the molecular reasons of the previous difference, we studied fatty acid compositions of these cells. Fatty acids composition analyzed by gas chromatography indicates that percentage of $C_{18:1}$ and $C_{18:3}$ fatty acid and the unsaturation degree (IUFA) of fatty acids of the plasma membrane of A_{549} cells are higher than that of A_{549}/DDP cells. However, no difference in cholesterol concentration was found in the membranes between the two cell lines (data not shown). This difference was mainly related to the difference of the compositions and the ratio of different types of the phospholipids. It also would be deduced that lipid phase of the membrane of the two cell lines might be different as indicated by the compositions and property of the fatty acids and the lateral diffusion of membrane lipids, although the phase change of the membranes of the two cell lines was not directly studied. The previous results provided evidence for the molecular basis for the found differences of the physical states of the membrane between the two cell lines.

Resistance of tumor cells to chemotherapy may be due to increase efflux or decreased up-take of drugs. Therefore, we studied the difference of up-take of cisplatin and Bodipy-cisplatin into the two cell lines. Results indicate that both substrates are taken up to a lower degree into resistive A_{549}/DDP cells (Tables 1 and 2). Our results are in good agreement with those of other investigations [12,13]. It seems that there is a correlation between the rate of transport of cisplatin and its fluorescent derivative into the studied cells and the physical status of the membrane of these cells. Cells with more "fluid" membranes take-up more cisplatin than the resistant cells which have membranes of lower fluidity.

In order to further explore the relationship between cisplatin resistance of A_{549}/DDP cells and physical property of the membrane lipids, following these findings previously, accumulation of fluorescent probes Rhodamine-123 and Bodipy-cisplatin into asolectin liposomes containing different concentration of Ca^{2+} were carried out. It was reported that Ca^{2+} can affect lipid fluidity of liposomes [26]. Ca^{2+} gradient may play an important role in modulating conformation and activity of membrane proteins reconstituted into liposomes by mediating the physical state of the membrane [27]. Therefore, Ca^{2+} concentration

difference in cells may be mimicked by that in liposomes and may result in changes in lipid fluidity, up-take of Rhodamine-123 and Bodipy-cisplatin into liposomes, which reflects resistance to drugs. As can be seen in Table 3 that both of the substrates were taken up to a higher concentration into the liposomes with higher concentration of Ca^{2+} which showed more fluidity as indicated by the measurement of polarization of DPH probe. It is proposed from the previous results that effect of Ca^{2+} on cisplatin resistance of the A_{549}/DDP cells might be related to the role of Ca^{2+} in mediating the fluidity change of the membrane phospholipids which in turn results in up-take difference of the drug. As discussed previously, more fluid plasma membrane is characteristic of the cisplatin-sensitive A_{549} cells. We find it interesting that differences in fatty acid compositions, the physical status of the membrane and Ca^{2+} concentration all influence cisplatin up-take and are related to similar DPH polarization differences between low and high drug up-take into the cells or liposomes. Possibly, physical state change of the membrane lipids mediated by the change of Ca^{2+} concentration resulted in functional change of MDR protein, which is also a transmembrane protein [14,27]. We postulate that decrease of Ca^{2+} concentration in A_{549} /DDP cells [28] induced the decrease of fluidity of the cell membranes which would then affect drug, like cisplatin, up-take. Gottsman et al. reported that the substrate for Pgp is not mainly cisplatin [29]. It should also be mentioned that very low ATP-hydrolyzing activity of Pgp and weak reaction of Western blotting with monoclonal antibody ($McAbF_4$, Sigma) against Pgp of the membranes of the two cells and no difference between them was found (data not shown). These results may further indicate that cisplatin resistance of A_{549}/DDP cells may not be associated with Pgp activity. Experiments to find out whether Ca^{2+} affects directly the cisplatin resistance of purified MRP reconstituted into liposomes are in progress.

In summary, the results obtained here by a combination of various approaches indicate that there is a significant difference in the physical state of the membrane lipids between the sensitive A_{549} and the resistive A_{549}/DDP cells. The differences in the physical states of the membranes of the two cell lines may be related to the resistance of the A_{549}/DDP cells to cisplatin.

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