

Alteration of membrane lipid biophysical properties and resistance of human lung adenocarcinoma A₅₄₉ cells to cisplatin

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Abstract Alterations of membrane lipid biophysical properties of sensitive A₅₄₉ and resistant A₅₄₉/DDP cells to the Cis-dichlorodiammine platinum (Cisplatin) were performed by measurements of fluorescence and flow cytometry approaches using fluorescence dyes of DPH, N-AS and Mero-cyanine 540 (MC 540) respectively. Fatty acids of membrane lipid of the two cell lines were analyzed by gas chromatography. The results indicated clearly that fluorescence polarization (P) of the DPH probe is 0.169 for the sensitive A₅₄₉ cell and 0.194 for the resistant A₅₄₉/DDP cells. Statistical analysis showed significant difference between the two cell lines. The polarizations of 2-AS and 7-AS which reflect the fluidity of surface and middle of lipid bilayer are 0.134 and 0.144 for the sensitive A₅₄₉ cells as well as 0.171 and 0.178 for the resistant A₅₄₉/DDP cells respectively, but there is no significant difference of the polarization of 12-AS between the two cell lines. This shows that alterations of the membrane fluidity of both cells were mainly located on the surface and middle of the lipid bilayer. In addition, the packing density of phospholipid molecules in the membrane of the two cell lines detected by MC540 probe indicated that lipid packing of A₅₄₉ cell membranes was looser than that of the A₅₄₉/DDP cells. And unsaturation degree of plasma membrane fatty acids of the A₅₄₉/DDP cells was also lower than that of A₅₄₉ cells. Taken together, it was proposed that the alteration of membrane lipid biophysical state may be involved in the resistance of A₅₄₉/DDP cells to cisplatin.

Keywords: multidrug resistance, DPH, MC540, N-AS probes, lipid ordering, fatty acids unsaturation.

Malignant tumor is characterized by resistance to most anticancer drugs with different structure, function and mechanism if it is treated by chemotherapy for a long period. This phenomenon is known as multidrug resistance (MDR)^[1]. The multidrug resistance of tumor cells to drugs has become a key factor in leading to failure of chemotherapy of malignant tumor. The rates of illness and death of cancer still show a higher general trend in the world due to multidrug resistance.

Non-small cell lung cancer (NSCLC) is almost 75% of lung cancers; at present, cisplatin and taxol, etc. are more efficient drugs for patients with NSCLC. However, it is a serious problem needed to be understood whether the multidrug resistance to chemotherapeutic drugs is related to the influence of drugs on cellular membrane. It is well-known that the major reason for tumor MDR is usually the result of glycoprotein (Pgp) overexpression in the membrane of tumor cells^[2]. In view of the multitude of factors involved in drug resistance of tumor cells, it is very important to study the changes in structure and function of cell membranes between the resistant and sensitive (non-

resistant) cells, which are treated repeatedly and long by drugs, for fully elucidating the molecular mechanism of tumor MDR.

Dudeja et al. reported that the change of membrane lipid fluidity caused by polyoxyethylene surfactants is one of the reasons for reversal of MDR mechanisms^[3]. However, studies on the relationship between MDR and change of membrane lipid biophysical state have been paid less attention to and the experimental results are also contradictory. In this work, we measured the changes of membrane lipid ordering, packing density as well as fatty acid components and its saturation degree of membrane lipids between the sensitive A₅₄₉ and resistant A₅₄₉/DDP cell membranes, and we also tried to elucidate the correlation between MDR and change of the membrane lipid biophysical state.

1 Materials and methods

1.1 Chemicals

DPH (1,6-diphenyl-1,3,5-hexatriene) was purchased from Aldrich Chemical Co. (Milwaukee, WZ). 2-AS(DL-2-(9-anthroyl)-stearic acid), 7-AS(DL-7-(9-anthroyl)-stearic acid), 12-AS(DL-12-(9-anthroyl)-stearic acid and MC540 (Merocyanine 540) were from Molecular Probes (Eugene, OR). Penicillin, streptomycin, Trypsin, Rhodamine-123 were from Sigma (St. Louis, MO).

1.2 Cell lines and cell culture

The human lung adenocarcinoma cell line A₅₄₉ and the MDR cell lines A₅₄₉/DDP, which were resistant to cisplatin (CDDP or DDP), were obtained from Beijing Tumor Institute. The two cell lines were grown as adherent monolayers on 75-cm flasks in RPMI-1640 (pH 7.35) medium supplemented with 2 mmol/L 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% CO₂ at 37°C. A₅₄₉/DDP was cultured for 20 generations and does not alter its resistive type. The resistance of A₅₄₉/DDP cells to cisplatin was 8 times more than that of A₅₄₉ cells. Exponentially growing cells were used in all experiments.

1.3 Measurement of fluorescence polarization of DPH and N-AS

In brief, stock solution of the probes was stored at 4°C in the dark: 2 mmol/L DPH in THF (tetrahydrofuran), 2 mmol/L N-AS in DMSO (Dimethylformamide). For labeling, the 2 μmol/L final concentration of DPH and N-AS were added to A₅₄₉ and A₅₄₉/DDP cell suspension (1×10⁶ cell/mL) in the phosphate buffer (PBS). After incubation for 30 min at 37°C, the suspension was centrifuged at 1000 g for 10 min. It was resuspended in PBS after washing twice with PBS.

The fluorescence anisotropy was immediately measured in a Hitachi F-4010 spectrofluorometer at 30°C. The excitation wavelengths were 360 nm for DPH and N-AS, the emission wavelength was 430 nm for DPH and 470 nm for N-AS, and the slide width was 5 nm. The polarization (P) can be calculated from the results according to refs. [4, 5].

1.4 Measurement of packing density of membrane lipids of A₅₄₉ and A₅₄₉/DDP cells labeled with MC540 by flow cytometry^[6]

The cells of A₅₄₉ and A₅₄₉/DDP were harvested from flasks with 0.25% trypsin and 0.02% EGTA, and suspended to 1×10^6 cell/mL in PBS containing 0.2% bovine serum albumin (fatty acid-free, Sigma), then 10 μ L 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 immediate flow cytometric analysis.

Flow cytometric analysis of stained tumor cells was performed using a FACS-420 flow cytometer (Becton Dickinson). Excitation was provided with an argon ion laser operating at 488 nm. MC540 fluorescence was monitored through a 575 nm bandpass filter. 1- or 2-parameter correlated histograms (dual-dimensional histogram and frequency distribution diagram) were collected with 1.5% of CV intensity for 10 000 viable cells. Data were analyzed with a HP-300 Consort 30 computer.

1.5 Analysis of fatty acid components and unsaturation degree in A₅₄₉ and A₅₄₉/DDP cell membrane

Preparation of A₅₄₉ and A₅₄₉/DDP crude membrane was performed by sucrose density gradient centrifuge. Briefly, crude membranes (0.2 mg) were dissolved in 2 mL of 1 : 2 : 0.8 (v/v) chloroform : methanol : H₂O overnight, then chloroform : H₂O (1 : 1) was added to the final concentration of chloroform : methanol : H₂O at 2 : 2 : 1.8. The lower phase was collected and dried at vacuum for 4 h 200 μ L of 1 : 1 (v/v) phene : petroleum ether and 200 μ L 0.5 mol/L KOH (dissolved in methanol) were then added for methyl esterification. After incubating in water at 50°C for 50 min, 2 mL H₂O was added, the upper phase was collected and dried in vacuum. The residue was dissolved in 50 μ L 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.

1.6 Measurement of efflux rate of Rhodamine-123 by flow cytometry

It was basically based on the method of Dudeja et al.^[3]. In brief, the single cell suspension in RPMI-1640 (1×10^6 cell/mL) was incubated with 0.5 μ g/mL Rhodamine-123 for 60 min at 37°C and centrifuged at 1000 g, then washed three times with RPMI-1640 medium. The stained tumor cells were analyzed immediately using a Coulter Epics flow cytometer.

2 Results and discussion

2.1 Fluorescence assay of membrane fluidity of A₅₄₉ and A₅₄₉/DDP cells labeled by DPH or N-AS

Fluidity of biomembrane reflects the orientational order of whole or partial acyl chain of the phospholipid molecules and the rotational and lateral diffusional motion of the acyl chain. The

relative change in fluorescence anisotropy or polarization can be easily assessed by the binding of fluorescence probes with membrane phospholipid molecules. DPH is a hydrophobic molecule and shows a double hard-cone wobbling movement to incorporate hydrocarbon inside the membrane bilayer. Orientation of the probe in the membrane is related to the change of physical state of lipid bilayer. It reflects average changes of fluidity, and ordering of phospholipid molecules in the lipid bilayer, namely fluidity change of membranes.

N-AS (2-AS, 7-AS, 12-AS) is a series of fluorescence probes with anthrance at different positions of carbon on the alkane chain. After the membrane is labeled by the probes, the polarization change shows the fluidity change at different depths of lipid bilayer because 2-AS, 7-AS and 12-AS are located at 2, 7 and 12 of different carbon positions respectively.

Table 1 shows that the polarization of DPH is 0.162 for A_{549} and 0.194 for A_{549}/DDP , which means that the fluidity of membrane lipid in the A_{549}/DDP cell resistant to cisplatin decreases compared with that of A_{549} cells. In order to confirm the difference, amphiphatic molecular probes N-AS (2-AS, 7-AS, and 12-AS) were used to monitor the anisotropic changes of the probes in different depth of lipid bilayer. The polarization of 2-AS and 7-AS is 0.134 and 0.144 for the sensitive A_{549} cells, and is 0.171 and 0.178 for the resistant A_{549}/DDP cells respectively, but there is no significant difference with the 12-AS probe between both the cells. This indicated that the changes of membrane fluidity in both cells were mainly located on the surface and middle layer of plasma membranes.

Table 1 Fluorescence measurement of membrane fluidity in A_{549} and A_{549}/DDP cells

| Fluorescence probes | Fluorescence polarization (P) | |
|---------------------|-------------------------------|---------------|
| | A_{549} | A_{549}/DDP |
| DPH | 0.162±0.008 | 0.194±0.009 |
| 2-AS | 0.134±0.017 | 0.171±0.006 |
| 7-AS | 0.144±0.018 | 0.178±0.005 |

Fluorescence polarization (P) was measured by using a Hitachi F-4010 spectrofluorometer after the cells were labeled for 30 min at 37°C in the dark. The excitation and emission wavelength were 360 and 430 nm for DPH, 360 and 470 nm for N-AS probes respectively. The data represent an average ± SD of three experiments. $P < 0.05$.

The research on the relationship between tumor MDR and physical state of plasma membrane is limited, and the results are also controversial. Sentjurc et al. reported that Vinblastine treatment did not significantly affect the membrane fluidity of Hela CA cell resistant to the drug by using electron paramagnetic resonance method^[7]. However, Dudeja et al. indicated that MDR reversal agent can decrease the membrane lipid fluidity of resistant KB 8-5-11 cells by steady state fluorescence technique^[3]. Regev et al. showed that membrane fluidizer can modulate the drug efflux from Pgp overexpressing cells by membrane fluidization^[8]. It should be mentioned that the correlation between MDR and membrane physical state in lung adenocarcinoma cancer cells has not yet been reported up to now. It might be proposed from the above results that it is very significant to further explore the molecular mechanism on MDR of different or the same cells to different anticancer drug.

2.2 Packing of membrane lipids for A₅₄₉/DDP cell increased significantly

MC 540 as a hydrophobic fluorescence probe is used to label the phospholipid molecules in the membrane and binds to the headgroup of phospholipid molecules. The fluorescence intensity of MC540 is very low in water phase but increases when it binds to phospholipid molecules. Fluorescence intensity change of the probe can reflect the packing change of phospholipid molecules in the membrane^[9]. Lagerberg et al. demonstrated that increase of fluorescence intensity of MC540 means looser packing density of outer phospholipid molecules of bilayer after MC540 bound with membrane^[10]. The looser the lipid molecules pack density, the stronger the fluorescence intensity of MC540. Therefore, fluorescence intensity of MC540 reflects the packing density of lipid molecule headgroup^[11].

The fluorescence change of MC540 bound with phospholipid is usually measured by steady-state fluorescence spectrum method. In this work, it was measured by a new method named flow cytometry which can measure the forward angle light scatter, dual-dimensional histogram and frequency distribution diagram of the cells labeled by a fluorescence dye. Forward angle light scatter is linearly proportional to surface area or size of cells. The change of cell number with the same fluorescence intensity is shown by the frequency distribution of membrane lipid. Therefore, we can accurately measure the packing density or fluidity of membrane lipid by dual-dimensional histograms and frequency distribution diagram.

Figs. 1 and 2 show the measurements of dual-dimensional histograms of A₅₄₉ and A₅₄₉/DDP cells respectively. The results indicated that A₅₄₉ cells are divided into two subpopulations: one is located in the region with low fluorescence intensity, the other is in the region with high fluorescence intensity. The result in fig. 2 indicates that A₅₄₉/DDP cell population labeled by MC540 is

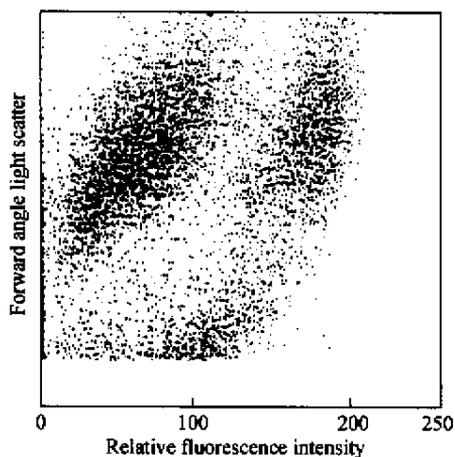


Fig. 1. Dual-dimensional histogram of A₅₄₉ cellular membrane labeled by MC540.

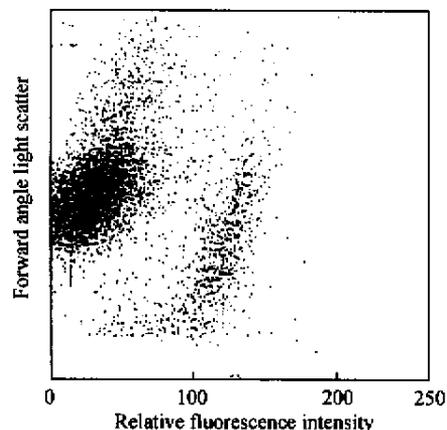


Fig. 2. Dual-dimensional histogram of A₅₄₉/DDP cellular membrane labeled by MC540. Ordinate indicates the intensity of forward angle light scatter, abscissa indicates the relative fluorescence intensity. Data were obtained from 10 000 viable cells for dual-dimensional histogram. Data are shown as the result in a single experiment that was repeated 3 times.

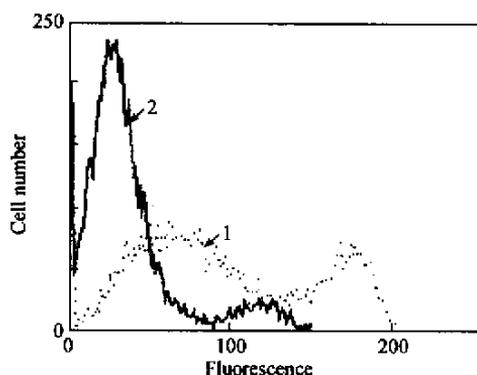


Fig. 3. Frequency distribution diagrams of A_{549} (1) and A_{549}/DDP (2) cellular membrane labeled by MC540. Ordinate indicates the cell numbers with the same relative fluorescence intensity, abscissa indicates the relative fluorescence intensity. Data were obtained from 10 000 viable cells for frequency distribution diagram. Data are shown as the result in a single experiment repeated 3 times.

mainly located in the region with low fluorescence intensity. This difference can be quantitatively analyzed further by HP-300 Consort 30 computer and C-30 analysis data software shown as in fig. 3.

The frequency distribution result of fig. 3 shows that A_{549} cells (fig. 3-1) labeled by MC540 are located in low (50—80) and high (180) fluorescence intensity regions, but A_{549}/DDP cells (fig.3-2) are located mainly in the region with 20 — 50 fluorescence intensity. Therefore, fluorescence intensity can be measured from frequency distribution diagram by the treatment of C-30 multimode program. The total fluorescence intensity of A_{549} cells ($93.49 \pm$

21.80) is significantly higher than that of A_{549}/DDP cells (49.48 ± 11.18), which indicates that membrane fluidities of both cells are significantly different. These results are consistent with the measurements of DPH and N-AS as above.

2.3 Unsaturation of fatty acids of resistant A_{549}/DDP cells decreased

Unsaturation degree of membrane fatty acids is one of the important factors to maintain stability of the plasma membrane. It is associated with the phase transition and fluidity of membrane lipid^[12]. So, the components and unsaturation degree of membrane fatty acids of both cells were further analyzed. The results in table 2 showed that fatty acids of A_{549} and A_{549}/DDP were composed of 16 : 0 (palmitic acid), 18 : 0 (stearic acid), 18 : 1 (oleic/vaccenic acid), 18 : 2 (linoleic acid), 18 : 3 (α/γ -linolenic acid). But the percentages of 18 : 1 (oleic/vaccenic acid) and 18 : 3 (α/γ -linolenic acid) for A_{549} cell are 40.44% and 8.53%, as well as 33.25% and 6.47% for A_{549}/DDP cell respectively. It should be noticed that the unsaturation degree of fatty acids calculated from IUFA equation showed that the fatty acid unsaturation degree of A_{549} (69.10) clearly increased compared with that of A_{549}/DDP (55.08). The difference of membrane fluidity between two cell lines measured as above was further confirmed by the measurement of membrane fatty

Table 2 Fatty acid analysis of plasma membrane in A_{549} and A_{549}/DDP cells by gas chromatograph

| Cell lines | Fatty acid composition | | | | | |
|---------------|---------------------------|--------------------------|---------------------------------|---------------------------|----------------------------|----------------------------------|
| | palmitic acid (16 : 0) | stearic acid (18 : 0) | oleic/vaccenic acid (18 : 1) | linoleic acid (18 : 2) | linolenic acid (18 : 3) | fatty acid unsaturated degree |
| A_{549} | 4.58 ± 0.9 | 20.93 ± 1.4 | 40.44 ± 5.6 | 1.55 ± 0.4 | 8.53 ± 0.8 | 69.10 ± 2.2 |
| A_{549}/DDP | 26.87 ± 2.1 | 15.72 ± 1.2 | 33.25 ± 4.2 | 1.22 ± 0.2 | 6.47 ± 1.2 | 55.08 ± 1.8 |

17:0 fatty acid was chosen as a marker for analysis. Components are expressed as a percentage of total fatty acids. The determined intensity represent mean \pm SD of 3 independent experiments. $P < 0.05$.

acid unsaturation degree, which also provided a molecular basis of the difference of lipid biophysical properties of the two cell lines.

2.4 The efflux rate of Rhodamine-123 from A₅₄₉/DDP cells increased significantly

Rhodamine-123 is a cationic fluorescence dye with similar structure of most anticancer drugs. It has no significant death effect on tumor cells and can be effectively effluxed by proteins associated with multidrug resistance^[13]. Therefore, anti-drug properties of MDR phenotype of tumor cells are always monitored by measuring efflux rate of Rhodamine-123. Flow cytometry, as an important tool for studying MDR mechanism of tumors can be used to measure change of the fluorescence intensity of intracellular probes^[14]. The fluorescence change of intracellular Rhodamine-123 measured by Becton-Dickinson FACS-420 type flow cytometry showed that the efflux rate of Rhodamine-123 in 20 min in A₅₄₉/DDP cells increased 3 times compared with that in A₅₄₉ cells. It would be suggested that the difference of efflux rate of Rhodamine-123 between two cell lines might be associated with fluidity property of the cell membranes.

Le Moyec et al. demonstrated that the increase of fatty acid unsaturation in sensitive K562 cells was associated with the increase of membrane fluidity compared with the resistant K562/adr cells and the change of membrane lipid components benefited the absorb of drugs or the modulation of Pgp on drug transport to improve the cellular sensitivity to drugs^[15, 16]. Sinicrope^[17] and Romsicki^[18] et al. also indicated that the change of plasma membrane physical state may affect the MDR of tumor cells. It is well known that classical MDR is attributed to the overexpression of plasma membrane P-glycoprotein (Pgp or P170) in resistant cells^[19, 20]. Although the existence of Pgp protein on the membrane of lung adenocarcinoma cancer cell has not yet been completely identified till now, there is some evidence for the existence of an MRP protein similar to Pgp on the membrane^[21]. Cisplatin, the DNA-targeting drug can affect the physical state of plasma membrane due to the electrostatic interaction with the plasma membrane or weak hydrophobic action with the phospholipid by negative charges^[22]. Correlation between the change of membrane lipid physical state and the multidrug resistance of lung adenocarcinoma cancer cells has not yet been reported up to now.

In the present paper, by using flow cytometry, spectrofluorometer and gas chromatograph, all of the results indicate consistently that the membrane fluidity of sensitive A₅₄₉ cell is significantly different from that of resistant A₅₄₉/DDP cells, and the efflux rate of Rhodamine-123 is also different between them. It is suggested that the changes of membrane lipid physical properties might be one of the reasons for the resistant lung adenocarcinoma cancer cell under repeated and long treatment of the cells with cisplatin. Our preliminary experimental results also demonstrated that the transport ability of different drugs into liposomes is related to the phospholipid compositions made of liposomes having different fluidities. Related studies are still in progress.

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