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ORIGINAL ARTICLE

The Change of Intracellular pH is Involved in the Cisplatin-Resistance of Human Lung Adenocarcinoma A549/DDP Cells

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We had reported that the intracellular pH (pH_i) of human lung adenocarcinoma A549 cells, which is sensitive to cisplatin, was more acidic than that of cisplatin-resistant A549/DDP cells. The correlation between the change of the pH_i and cisplatin-resistance of A549 /DDP cells was further studied by altering pH_i in consequence of the change of CO_2 concentration of the incubator. The pH_i alterations of the cells were monitored by using the fluorescence probe of BCECF-AM. The results indicated that the pH_i was more alkaline at lower CO_2 concentration (2% CO_2 in the incubator) and more acidic at higher CO_2 concentration (8% CO_2 in the incubator) for both A549 and A549/DDP cells compared with those of both A549 and A549/DDP cells cultured at 5% CO_2 as the normal condition. Accumulation of bodipy-cisplatin, a fluorescence probe used for drug resistance assays, in A549 cells incubated at 2%, 5%, and 8% CO_2 was increased 8.4%, 17.4%, and 23.5% compared to A549/DDP cells, respectively. Intracellular sequestration and distribution of bodipy-cisplatin imaged by laser scanning confocal microscopy indicated that bodipy-cisplatin was more encapsulated in acidic compartments of A549/DDP cells as shown with acridine orange, a dye that specifically labels acidic organelles in the cells. These results can be further confirmed in liposome systems with different pH gradients. It is proposed from the above results that the change of pH_i in especially more acidic compartments in A549/DDP cells involves their cisplatin resistance.

Keywords A549/DDP Cells; pH_i; Cisplatin; MDR

INTRODUCTION

At present, chemotherapy is still one of the main treatments for malignant tumors. Normally, cancer cells are more sensitive to chemotherapeutic drugs than normal cells. However, during chemotherapy, tumor cells often lose this sensitivity and become no more vulnerable to not only the original drug but also to a broad class of other drugs than normal cells. This phenotype was named after multidrug resistance (MDR), which remains a major cause of treatment failure in cancer patients. Although it initially was believed that MDR was due to a single molecular mechanism such as overexpression of P-glycoprotein, it is now clear that in any kind of drug-selected cell lines, MDR is always multifactorial, with at least two resistance mechanisms present in the same tumor cell.[1] The MDR mechanisms are always related to the cell lines and the drugs used to select.

Cisplatin, which is an alkaline anticancer drug, has established its role in the treatment of testicular, ovarian, bladder, small cell lung cancers as well as head and neck cancers.[2] However, intrinsic or acquired resistance to cisplatin reduces its efficacy.[3] Several biochemical alterations have been reported to be involved in cisplatin resistance, including reduced accumulation of the drug,[4,5] increased levels of glutathione (GSH) or enzymes involved in GSH metabolism,[6] enhanced DNA repair,[7,8] and so on. It is surprisingly that cisplatin is not a substrate for P-glycoprotein and MRP1, which are frequently overexpressed in multidrug-resistant cell and function as a drug efflux pump.[9–11] Recently, results have shown, however, that MRP2, another member in MRP family, can confer resistance to cisplatin.[12]

Altered intracellular distribution of anticancer to which was recently paid more attention provides another important MDR mechanism for tumor cells to escape the anticancer drugs. The research results indicated that intracellular sequestration, distribution and exclusion of the anticancer drugs are related not only to drugs properties also to intracellular physical and chemical environments.[13] Some chemotherapeutics (especially for alkaline drugs) would be expected to accumulate in the more acidic cytoplasm of tumor cells.[14] The intracellular pH of tumor cells is considerably more acidic than that of normal or multidrug-resistant tumor cells.[15] Therefore, it is

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an intriguing idea whether the changes of intracellular pH or transmembrane pH gradients plays a role in anticancer drug sequestration and distribution, consequently, to modulate the response of MDR cells to anticancer drugs. We had previously reported that pH_i in human lung adenocarcinoma A549 cells is lower than that in cisplatin-resistant A549/DDP cells.[16] To confirm if the alteration of intracellular in A549/DDP cells take part in the regulation of their cisplatin resistance, we further studied the correlation between the cisplatin resistance of A549/DDP cells and the change of their pH_i.

MATERIALS AND METHODS

Materials

Cisplatin, acridine orange, asolectin, nigericin, penicillin, streptomycin, and trypsin were from Sigma (St. Louis, MO). Bodipy-cisplatin was synthesized in our laboratory. 2,7′-Bis (carboxyethyl)-5-carboxy fluorescein (BCECF-AM) was from Molecular Probes (Eugene, OR). The other reagents were local products of analytical grade.

Cell Lines and Cell Culture

The human lung adenocarcinoma cell line A549 and the MDR cell line A549/DDP, which was resistant to cisplatin (CDDP or DDP), were obtained from Beijing Tumor Institute. The two cell lines were grown as an adherent monolayer on 75-cm flasks in RPMI-1640 (pH 7.35) medium supplemented with 2 mM/L L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 U/mL streptomycin in a humidified incubator (Forma Scientific, Inc.) with 5% CO_2 (unless mentioned) at 37°C.[17] The resistance of A549/DDP cells to cisplatin was 8 times more than that of the sensitive A549 cells. Exponentially growing cells were used in all experiments.

Intracellular pH Measurements

Intracellular pH (pH_i) measurements in the two cell lines were performed fluorimetrically as described.[18,19] In brief, the exponentially growing A549 and A549/DDP cells were harvested from the flasks with 0.25% trypsin and 0.02% EDTA, and aliquoted into three sterile eppendorf tubes with RPMI-1640 (5×10^5 cells/mL), and then the cells in tubes were cultured for 4 hours at three different CO_2 concentrations (2%, 5%, and 8%), respectively. After that, the cells were loaded with 2 μM BCECF-AM for 40 minutes at 37°C with gentle shaking in Hepes bicarbonate buffer (15 mM Hepes, pH 7.4, 110 mM NaCl, 20 mM $NaHCO_3$, 5 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5.5 mM glucose). BCECF-AM-loaded cells were pelleted by centrifugation at $500 \times g$ for 5 minutes, resuspended in the same buffer at 5×10^5 cells/mL, and introduced in a cuvette at 37°C with constant stirring for the continuous recording of fluorescence change on a F-4010 fluorescence spectrophotometer. Intracellular pH was estimated from the ratio of the 540-nm fluorescence signals obtained at 485 nm and 437 nm as excitation wavelengths.[20] The signal was calibrated in high potassium Hepes buffer (25 mM Hepes, 145 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose) containing the H^+/K^+ ionophore nigericin (10 μg/mL) at defined pH (6.64, 7.28, and 7.50).[19]

Measurement of the Bodipy-Cisplatin Entrapped in the Two Cell Lines

Exponentially growing A549 and A549/DDP cells were harvested and aliquoted into three sterile eppendorf tubes (5×10^5 cells/mL) with RPMI-1640 without fetal calf serum, and then cells in the tubes were cultured for another 4 hours at three different CO_2 concentrations (2%, 5%, and 8%), respectively. After that, the cells were loaded with 10 μM bodipy-cisplatin at respective CO_2 concentrations for 30 minutes at 37°C. Cells were washed three times with PBS. The fluorescence of bodipy-cisplatin entrapped in the cells was monitored immediately on a F-4010 fluorescence spectrophotometer (Ex = 488 nm; Em = 525 nm; split = 5 nm; Tem = 37°C). The cells treated as above were also measured on a FACS-420 flow cytometer (Becton Dichinson) (Ex=488 nm; Em=525 nm). One- or 2-parameter correlated histograms (dual-dimensional histogram and frequency distribution diagram) were collected with 1.5% of CV intensity for 1×10^4 viable cells. Data were analyzed with HP-3-consort 30 computer program.

Laser Scanning Confocal Microsocopy (LSCM) Imaging of Intracellular Distribution of Bodipy-Cisplatin and Acridine Orange

Five hundred microliters (1×10^6 cells/mL) A549 or A549/DDP cells were placed on a sterile Petri dish and cultured for 4 hours in the incubator. The adherent cells were loaded with 10 μM bodipy-cisplatin or 2 μg/mL acridine orange (from 4 mg/mL stock in water) in RPMI-1640 medium without serum for 30 minutes at 37°C and then washed three times with RPMI-1640 medium without serum. The stained tumor cells were observed immediately by using a LSCM (LSCM Leica, TCS-NT type, Germany). The excitation was at 488 nm, and emission was at 525 nm for bodipy-cisplatin. For acridine orange,[21] excitation was at 488 nm, and dual emission confocal images were sequentially recorded by using both a 530-nm band-pass barrier filter (green fluorescence) and a 605-nm long-pass barrier filter (red fluorescence).

Bodipy-Cisplatin Encapsulated in Liposomes Under Different Transmembrane pH Gradients

Large unilamellar vesicles (LUVs) were prepared as described,[22,23] and the asolectin lipids concentrations were measured as described.[24] To study the effects of the transmembrane pH gradients on the encapsulation of bodipy-cisplatin in the liposomes, we made four kinds of LUVs with different tansmembrane pH gradients. LUV A (pH 4.6 on both

sides); LUV B (pH 7.5 on both sides); LUV C (pH4.6 inside, pH 7.5 outside); LUV D (pH 7.5 inside, pH 4.6 outside). The transmembrane pH gradients formed in LUV C and D remained unchanged throughout the experiments 10 μM bodipy-cisplatin was added into these LUVs suspended in their outside buffer, respectively, and incubated for 30 minutes at 37°C. Then the LUVs were pelleted by centrifugation (33,000 × g for 30 minutes) and resuspended with the corresponding outside pH buffer. The fluorescence of bodipy-cisplatin entrapped in these LUVs was monitored by an F-4010 fluorescence spectrophotometer (Ex = 488 nm; Em = 525 nm; split = 5 nm; Tem = 37°C) after adding 0.1% Triton.

RESULTS

Change of CO_2 Concentration in Incubator Leads to Intracellular pH Shift

Changing CO_2 concentration in the incubator can mimic the intracellular pH shift.[14] Regarding of the cell viability measured by trypan blue (figures were omitted), both A549 and A549/DDP cells can survive at least 24 hours after the CO_2 concentration shift from 5% to 2% or 8%. CO_2 can quickly equilibrate across cellular membranes, and the high activity of cytosolic carbonic anhydrase and the other cellular mechanisms to regulate bicarbonate exchange ensure that change in CO_2 concentration rapidly affects intracellular pH.[14] Figure 1 shows that pH_i of cisplatin-sensitive A549 cells was 7.1, and that of the cisplatin-resistant A549/DDP cells was 7.4 when the cells of both are cultured at normal CO_2 concentration (5%). It can be clearly seen that when CO_2 concentration in the culture lowered to 2% or rose to 8%, pH_i of the sensitive A549 cells was shifted to 7.3 and 6.8, respectively, whereas pH_i of A549/DDP cells was shifted to 7.6 and 7.1, respectively. Returning the cells to 5% CO_2 caused the intracellular pH to revert rapidly to the starting level. This means that intracellular pH values of either A549 or A549/DDP can be changed through altering the CO_2 concentrations in the incubator. Therefore, this would provide an available method to study the effect of pH_i changes on cisplatin resistance of A549/DDP cells through adjusting CO_2 concentration in incubators.

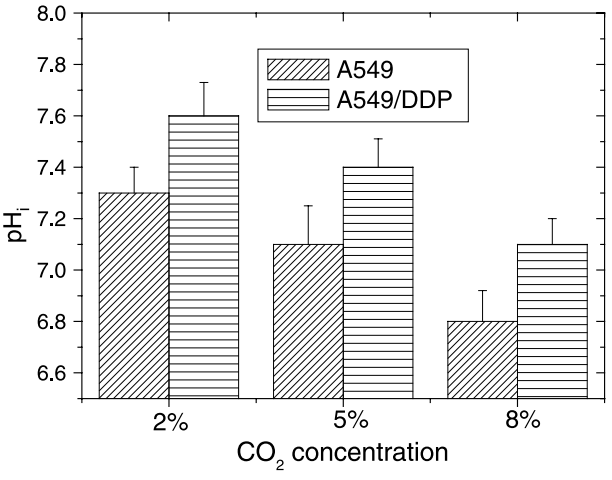

FIG. 1. Effect of CO_2 concentration on pH_i change of A549 and A549/DDP cells. The intracellular pH was measured as mentioned in Materials and Methods, and the pH_i is plotted for the A549 cells (▨) and A549/DDP cells (▤) at 2%, 5%, and 8% CO_2. Data points represent mean values (±SE) of three independent determinations.

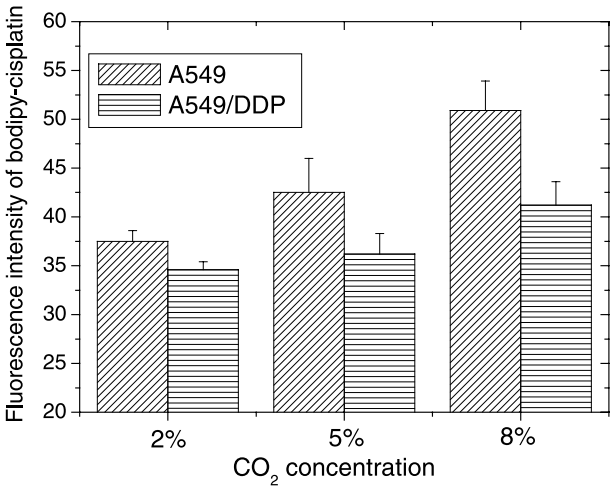

FIG. 2. Effect of CO_2 concentrations on bodipy-cisplatin entrapped in A549 and A549/DDP cells. The cells cultured at different CO_2 concentrations (2%, 5%, and 8%) were loaded with 10 μM bodipy-cisplatin as mention in Materials and Methods. After being washed for three times with PBS, the fluorescence of bodipy-cisplatin entrapped in the cells was monitored on an F-4010 fluorescence spectrophotometer (Ex = 488 nm; Em = 525 nm). The fluorescence of bodipy-cisplatin is plotted for the A549 cells (▨) and the A549/DDP cells (▤) at 2%, 5%, and 8% CO_2. Data points represent mean values (±SE) of triplicate determinations.

Change of CO_2 Concentration in the Cell Culture Correlates with the Accumulation of Intracellular Bodipy-Cisplatin

Bodipy-cisplatin (a fluorescence probe) can be accumulated in tumor cells.[17] The fluorescence intensity of bodipy-cisplatin in A549 and A549/DDP cells at different CO_2 concentrations were measured by using F-4010 fluorescence spectrophotometer and flow cytometer. Figure 2 shows the fluorescence intensity (42.5±3.5) of bodipy-cisplatin trapped in A549 cells was 17% higher than that (36.2±2.1) of A549/DDP cells when both of them were incubated at 5% CO_2. When the concentration of CO_2 in the incubator was shifted from 5% to 2%, fluorescence of the bodipy-cisplatin entrapped in both of the cells decreased slightly. When the CO_2 concentration in the incubator was increased to 8%, the fluorescence intensity (50.9±3.0) of bodipy-cisplatin in A549 cells was increased more significantly than that in the cells cultured at 2% or 5% CO_2 concentration, and the accumulation of bodipy-cisplatin in the cisplatin-sensitive

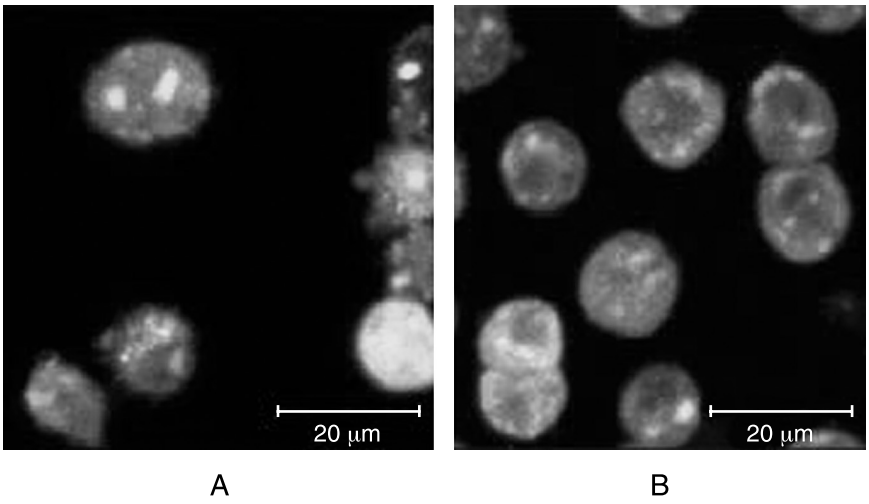

FIG. 3. Laser scanning confocal microscopy (LSCM) imaging cells labeled with acridine orange. The A549 and A549/DDP cells cultured at 5% CO_2 concentration were loaded with 2 μg/mL acridine orange for 30 minutes at 37°C. After being washed for three times with RPMI-1640 medium without serum, the cells were observed on an LSCM as mentioned in Materials and Methods. A: A549 cells. B: A549/DDP cells.

A549 cells was much higher than that in the cisplatin-resistant A549/DDP cells. These changes were further confirmed by the results measured with flow cytometer (data not shown). The results indicated that changing the CO_2 concentration (i.e., changing the pH_i) could cause accumulation alteration of bodipy-cisplatin in both cell lines.

The Intracellular Distribution of Acridine Orange Imaged by Laser Scanning Confocal Microscopy (LSCM)

Acridine orange demonstrates a concentration-dependent long-wavelength shift in the fluorescence emission; it shows a red fluorescence after it was accumulated within acidic cellular compartments to a high concentration and a green fluorescence when bound at a lower concentration to membrane and/or nucleic acids.[21] Figure 3A shows a homogeneous and weaker fluorescence (more green fluorescence) of the acridine orange and a distribution of the dye in more scattered vesicles of the cisplatin-sensitive A549 cells, whereas a stronger red fluorescence intensity and more vesicles labeled with higher red fluorescence of the dye in cisplatin-resistant A549/DDP cells can be seen (Figure 3B). These images from LSCM might indicate that the numbers of acidic vesicles in cisplatin-resistant A549/DDP cells are more than those in cisplatin-sensitive A549 cells.

The Intracellular Distribution of Bodipy-Cisplatin Imaged by LSCM

The distribution of the bodipy-cisplatin in A549 and A549/DDP cells at 5% CO_2 concentration was further measured and imaged by LSCM after the fluorescence intensity of bodipy-cisplatin entrapped in both A549 and A549/DDP cells was

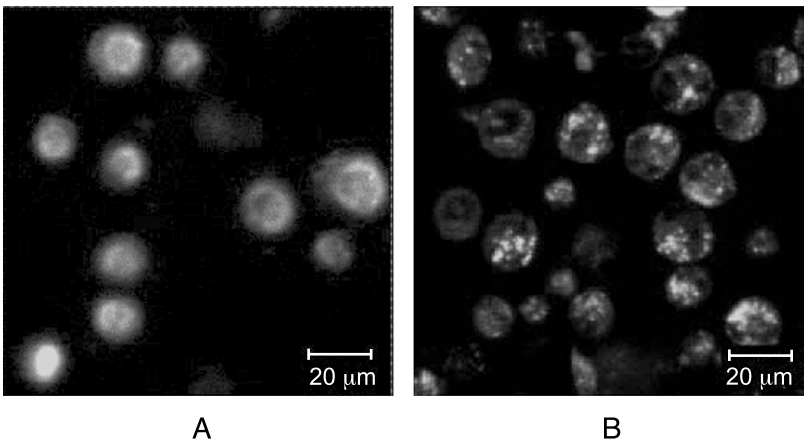

FIG. 4. Laser scanning confocal microscopy (LSCM) imaging cells labeled with bodipy-cisplatin. The protocol is the same as Figure 3. A. A549 cells. B. A549/DDP cells.

TABLE 1
Encapsulation of bodipy-cisplatin into liposomes with different pH gradient at 37°C

Liposomes	A	B	C	D
[pH]in	4.6	7.5	4.6	7.5
[pH]out	4.6	7.5	7.5	4.6
Fluorescence intensity	368.7±7.5	552.6±17.5	609.9±20.9	256.9±45.3

LUVs A (pH 4.6 inside and outside), LUVs B (pH 7.5 inside and outside), LUVs C (pH 4.6 inside and 7.5 outside), LUVs D (pH 7.5 inside and 4.6 outside). Data represent mean values (±SE) of three independent determinations.

measured by using fluorescence spectrophotometer and flow cytometer. Figure 4A shows that the fluorescence intensity of bodipy-cisplatin in A549 cells was higher than that in A549/DDP (Figure 4B), and the distribution of the dye was more homogeneous in the former in contrast to the latter when both cells were cultured at 5% CO_2. However, it is also clear that the more dotted and dense fluorescence could be seen in the cisplatin-resistant A549/DDP cells compared with the cisplatin-sensitive A549 cells. It would indicate that the distribution of bodipy-cisplatin in the two cell lines was significantly different. Combining the observations of more acidic vesicles in the A549/DDP described as above, may suggest that bodipy-cispaltin were more trapped in the acidic compartments of cisplatin-resistant A549/DDP cells.

To determine whether the dotted pattern of bodipy-cisplatin shown in Figure 4 corresponds to its accumulation in intracellular acidic compartments, a double staining (using bodipy-cisplatin and acridine orange) of the acidic compartments in both A549 and A549/DDP cells was carried out. Unfortunately, the green dots of bodipy-cisplatin shown in both A549 and A549/DDP cells could not be differentiated from the green fluorescence of acridine orange because both probes are excited at the same wavelength (488 nm). To further confirm whether bodipy-cisplatin is more trapped easier in acidic compartments, experiments about the effects of pH gradients on the accumulation of bodipy-cisplatin into liposomes with different pH gradients were performed.

Bodipy-Cisplatin Was More Entrapped into Liposomes with Acidic pH Inside

It is clear that there were differences between liposomes and biological membranes. But it is well known and accepted that there is much similarity between them, especially in biophysical properties, so liposomes are usually to be used as a biomembrane mold to study structure and function of biomembranes. Table 1 shows the encapsulatoin of bodipy-cisplatin in four kinds of liposomes with different pH gradients. It demonstrates clearly that the fluorescence intensity of bodipy-cisplatin encapsulated in LUVs C (pH 4.6 inside; pH 7.5 outside) was the highest among four kinds of the LUVs. It indicates that the encapsulated capacity of the LUVs was in the order: LUVs C>LUVs B>LUVs A>LUVs D.

DISCUSSION

Most anticancer agents target DNA or nuclear and cytoplasmic enzymes, and sequestration of drug in cytoplasmic organelles will lead to decrease of relative effective concentration of drugs and thereby decrease cytotoxity. Therefore, cytotoxity of the drugs depends strongly on their intracellular distribution. Changes of intracellular drug concentration and distribution are usually associated with overexpression of drug efflux pumps, such as the P-glycoprotein[25] and the multidrug resistance-associated protein.[26] Another modification in MDR cells is alkalization of the intracellular pH.[14] Because the equilibrium between the uncharged and charged forms of the drug is pH dependent, drug accumulation in acidic vesicles is favored by a large pH gradient between the cytoplasm and the acidic compartment, whereas acidification of the cytoplasm and alkalization of the acidic vesicles would decrease drug accumulation in these organelles.

Cisplatin is a widely used anticancer agent that is highly effective in the treatment of testicular, ovarian, bladder, germ cell, head and neck, and small cell lung cancers.[27] Cisplatin can be passively transported into cells and plays role in aquated form.[28] Much attention had been paid to interaction of cisplatin with DNA or −SH groups of proteins[27] and peptides as well as even membrane.[28] However, the correlation between cisplatin-resistance and the changes of intracellular pH has not yet been considered. We had reported that the intracellular pH of the cisplatin-sensitive A549 (pH 7.1) and the cisplatin-resistant A549/DDP cells (pH 7.4) was significantly different.[16] On this basis, further evidences for the change of intracellular pH (pH_i) associated with cisplatin resistance of the A549/DDP cells in this article are presented as follows: 1) pH_i change of the cisplatin-sensitive A549 or the cisplatin-resistant A549/DDP cells can be induced by altering CO_2 concentration in the culture. The results indicated that the pH_i of both cell lines reduced when CO_2 concentration in the incubator increased and rose when CO_2 concentration decreased (Figure 1). This findings provides an available strategy to study the effect of pH_i change on cisplatin resistance of the A549/DDP cells. 2) Bodipy-cisplatin that accumulated in both A549 and A549/DDP cells was enhanced when pH_i decreased (at higher CO_2 concentration) and reduced when pH_i increased (at lower CO_2 concentration). The results indicated that acidification of the

cells is in favor of the intracellular accumulation of bodipy-cisplatin into the cells (Figure 2). 3) The confocal images of the A549 and the A549/DDP cells labeled with acridine orange (orange-red fluorescence represents acidic compartments) indicated that the acidic compartments in the A549 cells were much less than those in the A549/DDP. In addition, the confocal images of the two cell lines labeled with bodipy-cisplatin showed that the bodipy-cisplatin (green fluorescence) that accumulated in the A549 cells is more than that in the A549/DDP cells, but it is of interestingly that the green fluorescence of bodipy-cisplatin in the A549/DDP is much more concentrated, in contrast to the few scattering green fluorescence in the A549 cells. On the basis of the above observations, it could be deduced that bodipy-cispaltin tends to be entrapped in acidic compartments/vesicles much more in the A549/DDP cells than those in the A549 cells. Many anticancer drugs are weak lipophilic bases. A substantial fraction of the drug molecules are uncharged at normal intracellular pH, allowing them to freely penetrate the membranes of cytoplasmic organelles. When the drug encounters an acidic environment, such as the interior of acidic vesicles, it is converted into a charged form that is unable to cross internal membranes. This results in the sequestering and accumulation of such anticancer agents in cytoplasmic acidic organelles, which include the lysosomes, recycling endosomes, the trans-Golgi network, and secretory vesicles, followed by transportation to the cell surface and extrusion into the extracellular environment. We deduce that the mechanism might be suitable for cisplatin. 4) To further confirm the above deduction, we test the capacity of liposomes with different transmembrane pH to encapsulate bodipy-cisplatin. Our results clearly indicated that the fluorescence intensity of bodipy-cisplatin entrapped in LUVs C (pH 4.6 inside; pH 7.5 outside) is the highest and LUVs D (pH 7.5 inside; pH 4.6 outside) is the lowest among the four kinds of LUVs mentioned as above, suggesting that liposomes with acidic inside and alkaline outside should be optimal to encapsulate more bodipy-cisplatin compared with those liposomes with alkaline inside and acidic outside. The results indicated that bodipy-cisplatin would become a positively charged, weak acid, which is not easy to transmenbrane, when it is located in acidic environment.

In summary, the above results provide stronger evidences for the intracellular pH change that especially in more acidic compartments in human lung adenocarcinoma A549/DDP cells may play an important role in their cisplatin resistance. It may also provide a new clue to further explore how cisplatin entrapped in the acidic organelles is exported out from the cells by a secretory pathway.

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