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Liposome-based intracellular kinetics of doxorubicin in K562/DOX cells

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Liposomes can improve the intracellular concentration of cytotoxic drugs, and are regarded as a possible pharmacological approach to overcome drug resistance. The kinetic analysis of subcellular drug uptake and efflux helps to elucidate the resistance mechanism which is associated with the ATP-dependent membrane transporter P-glycoprotein (P-gp). However, there are only few reports about the intracellular kinetics of liposomes. In this work, the kinetics of drug uptake and active efflux of doxorubicin (DOX) encapsulated in liposomes in both intact cells and nuclei were studied using P-gp expressing K562/DOX cells. The results show that liposomes enhanced drug accumulation in intact cells and nuclei, and improved DOX retention in nuclei after withdrawal. Furthermore, the nuclei levels of liposomal drug rose slowly and reached a plateau after 2 h incubation, whereas the free drug reached the plateau in 15 min, suggesting that it takes time for the liposomes to get from the cytoplasm to the nuclei. Our results demonstrated that liposomes not only increase DOX levels allocated to nuclei but also extended retention in the nuclei of resistant cells.

1. Introduction

Doxorubicin (adriamycin, DOX) is an anthracycline antibiotic widely used in the treatment of solid tumours (Bender et al. 1978). However, the use of the drug is limited by tumor cell resistance. One mechanism widely studied is the classical form of multidrug resistance (MDR), which is usually mediated by the overexpression of P-glycoprotein (P-gp). P-gp is a membrane drug efflux transporter that reduces intracellular levels of a number of structurally unrelated compounds (Leonessa and Clarke 2003), including DOX, daunorubicin, paclitaxel, and others. A number of agents such as verapamil and cyclosporine A have been shown to depress the activity of P-glycoprotein and, thus, to diminish drug resistance, which has been shown to reverse the MDR phenotype (Nobili et al. 2006). However, the use of these compounds is significantly compromised because of their toxic side effects at optimal clinical doses (Praet et al. 1996).

Recently, there have been several reports on the administration of doxorubicin encapsulated in liposomes in experimental and clinical cases, which could overcome drug resistance and improve the effectiveness and safety of cancer chemotherapy (Thierry et al. 1993; Beyer et al. 2001).

Some researchers demonstrated that the intracellular accumulation and the subcellular distribution of anti-cancer agents might play a significant role in their cytotoxic potency and contributes to their pharmacological features (Croce et al. 2004; Zhao et al. 2006). For example, DOX works by interfering with the growth of rapidly growing cancer cells where it binds and intercalates into the DNA strand, thus, inhibiting further DNA and RNA biosynthesis, eventually causing cell death (Bender et al. 1978). Studies of *in vitro* cytotoxicity and intracellular drug uptake indicated that increasing intracellular anticancer drug level is likely to lead to an reduced activity of P-gp (Lundberg et al. 2003; Shabbits and Mayer 2003; van Ark-Otte et al. 1998; Gabr et al. 1997; Pratesi et al. 2004). Therefore, the kinetic analysis of subcellular drug uptake and efflux, particularly the nuclear concentration, will help to understand multidrug resistance associated with the ATP-dependent membrane transporter P-gp. However, there are only few studies about the subcelluar distribution of drug and the time course of the nuclei concentration of different pharmaceutical formulations (Pakunlu et al. 2006; Goren et al. 2000; Beyer et al. 2001).

In the present work, we prepared DOX-encapsulating liposomes and determined the time course of subcellular (cytoplasm and nuclei) drug distribution of liposomal DOX compared with that of free drug in K562/DOX (DOX-resistant) cells.

2. Investigations and results

2.1. Quantitative uptake studies of the DOX or liposomal DOX

In this experiment, we followed free and liposomal DOX access to K562/DOX cells by high-performance liquid chromatography with a fluorescence detector. As shown in Fig. 1, the amount of drug uptaken by K562/DOX cells exposed to DOX liposomes was significantly higher than that of DOX solution ($p < 0.05$) at the same drug concentration. The data of DOX solution demonstrated a rapid

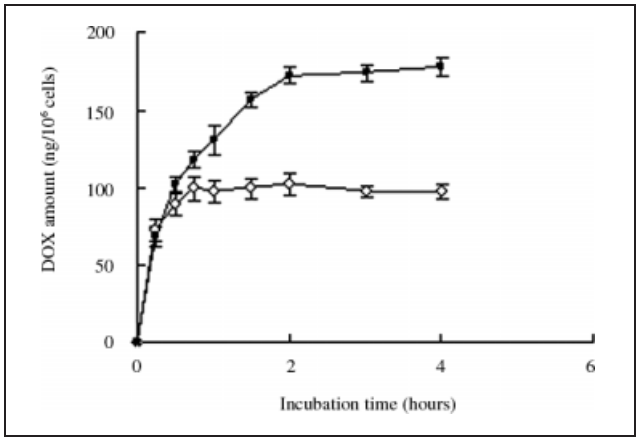

Fig. 1: Time course of cellular uptake of the free DOX or liposomal DOX in K562/DOX cells with 5 μg/ml DOX or liposomal DOX, the intracellular concentration was measured by HPLC. Results are represented as means ± S.D. of three different experiments
—◇— Free drug
—■— Liposome/DOX

accumulation and reached a plateau within 0.5 h, whereas in cells treated with liposomal DOX, the cellular DOX levels continued to increase up to 2 h. After 4 h of treatment, nearly the 1.7-fold amount of DOX delivered by liposomes was transported into cells compared to that produced by DOX solution (172.3 ± 5.2 μg/ml versus 102.3 ± 6.7 μg/ml). Significant differences were found except for 0.25 and 0.5 h.

To investigate the delivery of liposomal DOX to the nucleus quantitatively, cell fractionation experiments were carried out. As shown in Fig. 2, a majority of drug was found in the nuclear fraction with both free DOX and liposomal DOX already after 0.5 h of incubation. The nuclear drug concentration mediated by liposomes obviously overwhelmed that by free DOX, especially after 4 h of incubation. In fact, the drug concentration in cells treated with free DOX did not increase at all when incubation was prolonged from 0.5 h to 4 h. In contrast, there was a 2.2-fold increase during the same period in the case of cells treated with liposomal DOX. The data in nuclei was in accordance with that in intact cells.

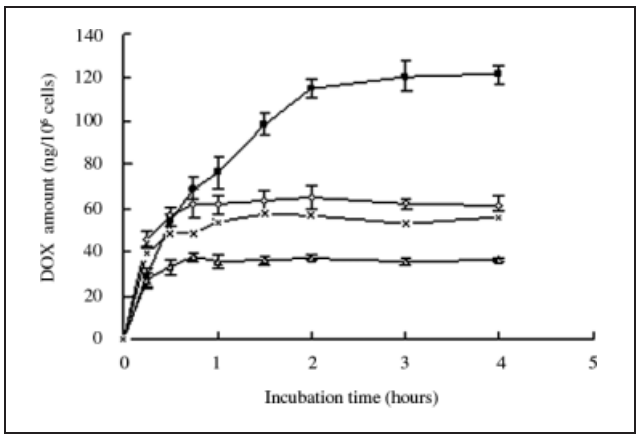

Fig. 2: Time course of nuclei and cytoplasm drug levels exposure to the DOX as free or loaded in liposome in K562/DOX cells. The concentration of nuclei and cytoplasm was measured by HPLC. Results are represented as means ± S.D. of three different experiments
—◇— Nuclei of cells exposed to free DOX
—△— Cytoplasm of cells exposed to free DOX
—■— Nuclei of cells exposed to liposomal DOX
—×— Cytoplasm of cells exposed to liposomal DOX

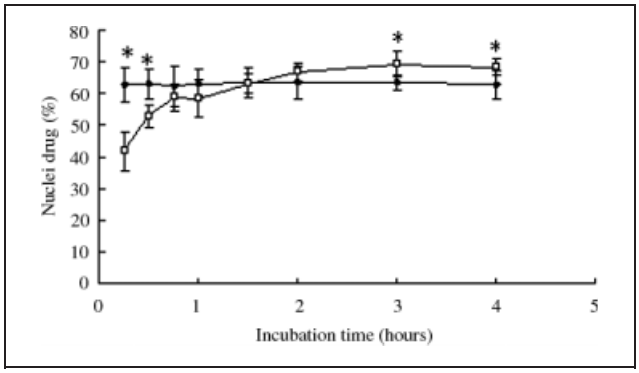

Fig. 3: Time course of the percent of nuclear drug concentration in the total DOX uptaken by K562/DOX cells. Results are represented as means ± S.D. of three different experiments. * $P \leq 0.05$
—◆— Free drug
—□— Liposome/DOX

Fig. 3 shows the percent of nuclear drug concentration in the total DOX uptaken by K562/DOX cells. The nuclei levels of liposome rose slowly, and reached a plateau after 2 h, whereas after DOX solution they increased rapidly and reached a plateau after 15 min. The percent of nuclear drug concentration in the total DOX of liposome was 41.9%, 53.0% and 66.9%, respectively, versus 65.5%, 65.3% and 65.4% produced by free drug, when accumulation time is 0.25, 0.5 and 2 h, respectively. The results indicated that it may take a longer time for the liposomal DOX to accumulate into nuclei.

To determine whether this effect was simply due to ingredients of the liposomes, we also treated K562/DOX cell lines with blank liposome/DOX solution combination. No significant increase in DOX uptake was observed with the liposome/DOX mixture compared with that with DOX solution (data not shown).

2.2 Quantitative efflux studies of the DOX or liposomal DOX

To examine drug efflux, K562/DOX cells were exposed to liposome-DOX or free DOX for 2 h and then further incubated in drug-free medium for 9 h. As expected, liposomes had stronger retention ability than free drug. A quick drop of cellular drug level, decreased approximate

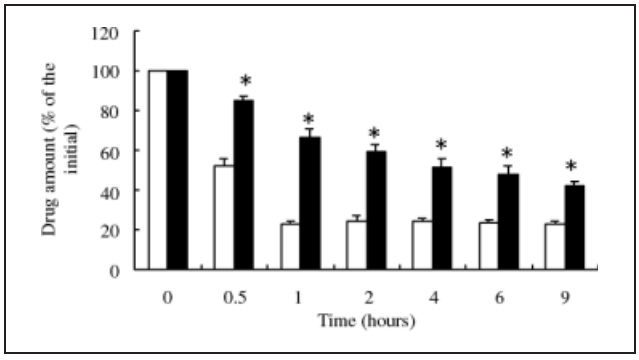

Fig. 4: Time course of DOX of the DOX solution or liposomal in K562/DOX cells after 2 h incubation. Cells are incubated with 5 μg/ml DOX or liposomal DOX for 2 h at 37 °C. The medium was replaced by drug-free-medium consequently for times. The DIX concentration at initial time was considered as 100%. The amount of cellular concentration was determined by HPLC method. Results are represented as means ± S.D. of three different experiments. * $P \leq 0.05$
—□— Free drug
—■— Liposome/DOX

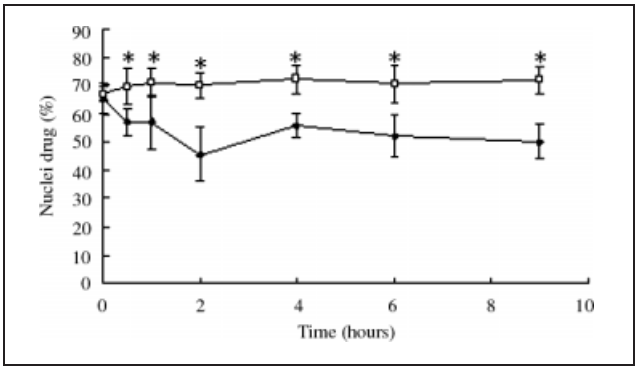

Fig. 5: Time course of the percent of nuclear drug concentration in the total DOX uptaken by K562/DOX cells after 2 h incubation. Results are represented as means ± S.D. of three different experiments. * $P \leq 0.05$
—◆— Free drug
—□— Liposome/DOX

50% of DOX in 30 min, was observed. In contrast, the efflux of liposomal DOX was slowly (Fig. 4). Significant differences were observed at every time point. 42.2% of DOX still remained in the cells exposed to liposomes after incubation with drug-free medium for 9 h.

The nuclei efflux kinetics of free drug and liposomes were studied as shown in Fig. 5. The percent of nuclear drug concentration obtained with liposomal DOX was obviously higher than that with free DOX, especially after 2 h of efflux. To be more exact, the percent of nuclei concentration of liposomal DOX did not drop at all when efflux was prolonged to 9 h. However, about 15% decrease was found in the case of cells treated with free DOX during the same period.

3. Discussion

P-gp is known to reduce cellular drug accumulation by acting as an ATP-dependent efflux pump of a great many structurally distinct hydrophobic compounds (Goren et al. 2000). The manner in which Pgp recognizes these different substrates is unknown. Some researchers demonstrated that liposomal preparations could overcome the bypass P gp-mediated efflux of DOX from the cells, thus circumventing MDR of tumor cells (Kobayashi et al. 2007).

The results described above showed that a liposomal preparation leads to higher intracellular DOX concentrations than free drug.

The crucial finding of this study was that liposomes can retain DOX in nuclei. About 42% of the intracellular DOX from the liposomal preparation still remained in the cells after 9 h incubation with drug-free medium, whereas the residual of DOX solution was only 23%. Part of the liposomal drug might escape from the recognition of P-gp as well as the degradation by lysosomes, resulting in accumulation in cells and a continuous release from liposomes which could maintain a dynamic balance between nuclei and cytoplasm. It provided a micro-environment with relatively high DOX concentration in cytoplasm surrounding nuclei, thus more DOX could bind tightly with single-strand or double-strands which was hard to dissociate from nuclei. However, during exocytosis, free DOX was rapidly pumped out before accessing the target which caused the low concentration and short half time in nuclei.

It is also noteworthy that the intracellular distribution of free and liposomal doxorubicin was similar, about 70% free drug and liposomal DOX penetrated the cell membrane and accumulated into the nucleus after 2 h incubation. But during drug uptake, free drug reached a plateau concentration quickly compared to the liposomal DOX. It might be supposed that liposomal DOX needs time to be internalized and then let drug released and enter the nucleus. The small size of the pores of the nuclear membrane makes this compartment inaccessible to liposomes. Therefore, this observation suggests that during the process of vesicular docking on cell surface and/or subsequent internalization path, destabilization of the liposomal carrier occurs, resulting in release of entrapped DOX and accumulation of the drug in the nucleus. It is unclear what triggers drug release from endocytosed liposomes.

In conclusion, this work demonstrated that liposomes delay DOX allocation into nuclei, but the levels of liposomal DOX in nuclei are higher than those of free drug after 2 h incubation. Furthermore, liposomes prolong the retention of drug in nuclei when exposed to resistance cells. Our results also support the proposition that liposomes offers an attractive means of delivering DOX into tumor cells, which are sensitive to P-gp-mediated drug efflux. Liposome-mediated drug delivery has the potential to circumvent multidrug resistance.

4. Experimental

4.1. Materials

DOX was a gift from Zhejiang Hisun Pharmaceutical Co. Ltd. (Zhejiang, China). Fetal calf serum and heat-inactivation were purchased from Hangzhou Sijiqing Biotech Co. Ltd. (Hangzhou, China). Other chemicals were obtained from commercial sources. Methanol were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Cholesterol and Sephadex G50 were purchased from Pharmacia Biotech (Piscataway, NJ, USA), and Soya phosphatidylcholine from Lipoid GmbH (Ludwigshafen, Germany). All other reagents were of analytical grade. Ultrapure water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA).

4.2. Cell lines and culture conditions

The multidrug resistant human chronic myelogenous leukemia cell lines K562/DOX (DOX-resistant) were kind gifts from Prof. Hu Xun (The Cancer Institute, The Second Affiliated Hospital, Zhejiang University School of Medicine). K562/DOX cells were maintained in the presence of 1 μg/ml of DOX and grown in RPMI 1640 containing 10% fetal calf serum, 75 μg L-glutamine/ml, 100 U penicillin/ml, 100 μg streptomycin/ml, and 0.5 mg sodium bicarbonate/ml (pH 7.2). Cells were cultured at 37 °C in a humidified CO_2 incubator.

4.3. Nuclei and cytoplasm isolation and determination

Nuclei were isolated by modifying a method that employed isotonic conditions and detergent lysis (Lahiri and Ge 2000; Spector et al. 1998). K562/DOX cells were collected by centrifugation at 2000 rpm for 5 min in a centrifuge (HermleZ323K Germany). The resulting cell pellet was washed three times with 3 ml ice-cold PBS. The pellet was resuspended in 300 μl TM-2 buffer (0.01 M Tris-HCl, pH 7.4, 2 μM $MgCl_2$, 0.0005 M phenylmethyl sulfonyl fluoride) and incubated at room temperature for 1 min. The tube containing cell in TM-2 buffer was incubated in ice water for 5 min. Triton X-100 was added to the suspension to a final concentration of 0.5% (v/v) and the suspension was incubated in ice water for an additional 5 min. The nuclei were isolated from the cytosol by centrifugation at 800 rpm at 4 °C for 10 min. The supernatant was collected for determining the drug amount in cytoplasm. The pellet of the isolated nuclei was resuspended in 1 ml of TM-2 buffer for determining the level of drug in the nuclei.

The HPLC system consisted of a pump (LC-10ATvp pump), and a Jasco FP-2020 fluorescence detector (Hachioji City, Japan). The stationary phase was composed of 5-μm particle size Diamond ODS column material, packed in a 250 × 4.6 mm stainless steel column (Dikma, Beijing, China). The mobile phase consisted of methanol-water-acetic acid (50 : 50 : 0.25, v/v/v). The flow rate of the mobile phase was set at 1.0 ml/min, and the eluent was monitored fluorimetrically at an excitation wavelength of 497 nm and an emission wavelength of 555 nm. Detection and integration of chromatographic peaks was performed by the Beckman system Gold data analysis system. Calibration curves were plotted using the ratio of the area of doxo-

rubicin and daunorubicin (IS) versus the nominal concentration (x), by using weighted ($1/x^2$) least-squares linear-regression analysis.

4.4. Liposome preparation

Liposomes were prepared by thin lipid film hydration followed by sonication and extrusion as described previously (Amselem et al. 1990; Xiong et al. 2005). Briefly, Soya phosphatidylcholine and cholesterol were dissolved in chloroform, dried into a thin film in a round bottom flask on a rotary evaporator under reduced pressure at 50 °C. The dried lipid film was rehydrated in 150 mM ammonium sulfate (pH 5.2), sonicated with a bath type sonicator, and then sequentially extruded five times through a 0.2-Ampore size polycarbonate filter (Nulcepore, USA). The resulting liposomes were passed through a Sephadex G50 gel-filtration column pre-equilibrated in PBS to exchange the external phase. DOX was remote-loaded via an ammonium sulfate gradient method and the free DOX was removed by gel-filtration.

4.5. Intracellular distribution and influx and efflux kinetics of DOX

Cells were grown in the absence of drugs for at least 7 days before the experiment. For determining the intracellular drug distribution, K562/DOX cells from monolayer growing cultures were harvested and seeded in 6-well culture plates (Costar Corp., Combridge, MA) in RPMI 1640 with 10% fetal bovine serum at concentration of 1×10^6 cells/well.

For the time course study, K562/DOX cells were incubated with 5 μg/ml DOX or liposomal DOX for 0.25, 0.5, 0.75, 1, 1.5, 2, 3 and 4 h, respectively. To examine the efflux processes, the culture medium containing 5 μg/ml DOX or liposomal DOX was replaced by an drug-free medium 2 h after incubation. Cells were harvested at 0, 0.5, 1, 2, 4, 6 and 9 h later, respectively. The fluorescence of DOX in cells was measured by HPLC. The determination process was described as above. The DOX concentration was given by the standard curve.

4.6. Statistical analysis

The statistical analysis of experimental data was carried out using the Student's t-test at $p < 0.05$. Data are presented as mean ± S.D.

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