ORIGINAL ARTICLE

Ehrlich tumor inhibition using doxorubicin containing liposomes

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Abstract Ehrlich tumors were grown in female balb mice by subcutaneous injection of Ehrlich ascites carcinoma cells. Mice bearing Ehrlich tumor were injected with saline, DOX in solution or DOX encapsulated within liposomes prepared from DMPC/CHOL/DPPG/PEG-PE (100:100:60:4) in molar ratio. Cytotoxicity assay showed that the IC50 of liposomes containing DOX was greater than that DOX only. Tumor growth inhibition curves in terms of mean tumor size (cm^3) were presented. All the DOX formulations were effective in preventing tumor growth compared to saline. Treatment with DOX loaded liposomes displayed a pronounced inhibition in tumor growth than treatment with DOX only. Histopathological examination of the entire tumor sections for the various groups revealed marked differences in cellular features accompanied by varying degrees in necrosis percentage ranging from 12% for saline treated mice to 70% for DOX loaded liposome treated mice. The proposed liposomal formulation can efficiently deliver the drug into the tumor cells by endocytosis (or passive diffusion) and lead to a high concentration of DOX in the tumor cells. The study showed that the formulation of liposomal doxorubicin improved the therapeutic index of DOX and had increased anti-tumor activity against Ehrlich tumor models.

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1. Introduction

Anthracycline-based antibiotics such as doxorubicin (DOX), pirarubicin and daunorubicin have found widespread applica-

tion in cancer chemotherapy. DOX is an anthracycline commonly used to treat several solid tumors, acute leukemia and malignant lymphoma (Kawano et al., 2003; Daemen et al., 1997; Mady et al., 2012). DOX can inhibit the replication process of cells by crossing the tumor cellular membrane and intercalating into the base pairs of DNA (Shi et al., 1993). However, severe clinical side effects, such as cardiotoxicity and myelosuppression, are of major concern. The prevailing liposomal drug delivery system can provide a solution to this problem.

Three basic requirements need to be fulfilled if liposomes are to be successful in delivering drugs specifically to cancerous tissue: (i) prolonged blood circulation, (ii) sufficient tumor accumulation, (iii) controlled drug release and uptake by tumor

cells with a release profile matching the pharmacodynamics of the drug. Passive targeting can result in increases in drug concentrations in solid tumors of several-fold relative to those obtained with free drugs (Rahman et al., 1986; Sapra and Allen, 2003). The mechanism of action of the liposomal drugs is thought to be due to sustained release of drug from the liposomes and diffusion of the released drug throughout the tumor interstitial fluid, with subsequent uptake of the released drug by tumor cells.

However, liposomes have been found to be plagued by rapid opsonization and by their being taken up by the reticuloendothelial system (RES) cells located mainly in the liver and spleen. In general, this rapid uptake of the liposomes leads to their having a short circulation time. This problem has been resolved by incorporating lipid-grafted polyethyleneglycol (PEG) into the liposome membrane. The incorporation of lipid-grafted PEG reportedly reduced the opsonization of the liposomes and consequently increased their circulation time (Allen et al., 1991; Papahadjopoulos et al., 1991; Mercadal et al., 1999; Lu et al., 2004).

PEG liposomal DOX has revealed an increased therapeutic efficacy and reduced cardiotoxicity compared to free DOX (Gabizon et al., 2003; Ogawara et al., 2008). However, there is little information on the therapeutic efficacy of PEG liposomal DOX in multidrug-resistant tumor-bearing animal model.

Generally, chemotherapeutic drugs are administered to cancer patients for a long term with low dosage to prevent severe side effects, which often causes the cancer cells to acquire the resistance against the chemotherapeutic drug and the effectiveness of the drug gradually decreases. Resistance acquisition of the cancer cells by long-term exposure of the chemotherapeutic drugs, called "multidrug resistance", has been considered as a major obstacle in the current clinical cancer chemotherapy (Chung et al., 1997).

Therefore, in order to improve the entrapment efficiency, we investigated a new liposomal composition of DMPC/CHOl/DPPG/PEG (100:100:60:4) in molar ratio. As the chemical composition, charge, structure and mode of preparation are all known variables that can modify the physicochemical, biological and pharmacological properties of the liposomes and therefore of the encapsulated drug, the present study was undertaken to evaluate the new liposomal doxorubicin formulation for its modulation of efficacy and pharmacokinetic behavior in Ehrlich tumor bearing mice.

However, except for the limited number of successful approaches that have launched into the clinical trial (Barraud et al., 2005), the outcomes from most of the approaches especially in the in vivo studies have been found to be still unsatisfactory to overcome the multidrug resistance of cancer cells.

The present study was undertaken to evaluate a new liposomal doxorubicin formulation, composed of DMPC/CHOL/DPPG/PEG and summarized that DOX loaded liposomes were able to enhance the intracellular uptake of the entrapped DOX by HCT-15 cells and improve the therapeutic efficacy of Ehrlich carcinoma treatment.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphoglycerol, (DPPG), L-α-dimyristoyl phosphatidyl-choline (DMPC) lipid and cholesterol (CHOL) type 99% pure were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Distearoyl phosphatidylethanolamine derivatized at the amino position with 2000 molecular weight segment of poly (-ethylene glycol), PEG-PE, was obtained from Calbiochem (La Jolla, CA). Adriblastina that consists of DOX hydrochloride was obtained from Pharmacia Italia, SPA, Italy. Dimethylsulfoxide (DMSO), RPMI-1640 medium, Sodium bicarbonate, Trypan blue, Fetal Bovine Serum, Penicillin/Streptomycin, Trypsin, Acetic acid, Sulforhodamine-B (SRB) and Trichloroacetic acid (TCA) were obtained from Sigma Chemical Co., St. Louis, Mo, U.S.A. Chloroform ($CHCl_3$) was purchased from Sigma Aldrich (St. Louis, MO, USA). All other reagent and solvents were of analytical grade and were used without further purification.

2.2. Liposomal preparation

We prepared multilamellar vesicles (MLV) of DMPC/CHOL/DPPG/PEG-PE (100:100:60:4) in molar ratio, according to the reverse phase evaporation method (Szoka and Papahadjopoulos, 1978). The lipids were first dissolved and mixed in chloroform to ensure a homogeneous mixture of lipids. The organic solvent was removed by rotary evaporation to yield a thin lipid film on the sides of a round bottom flask. Hydration of the dry lipid film was accomplished by simply adding deionized water to the container of dry lipid and agitating at a temperature greater than the phase transition temperature of the lipid. The final lipid concentration was 5 mg/ml. The resulting lipid suspension was extruded through 200 nm polycarbonate membranes (Nucleopore GmbH, Germany), using a commercially available extruder LiposoFast, Avestin Inc., Canada). Size measurement was done by dynamic laser light scattering (Zetasizer 3000 HS, Malvern Instruments, Germany) and the size was in the range of 200 ± 30 nm. For the DOX liposomal sample, hydration of the dry lipid thin film was achieved with 2 ml of deionized water containing DOX (5 mg/ml). The flask was mechanically shaken for 15 min at 45 °C. The suspension was then centrifuged at 13,000 rpm three times for 15 min each to remove non-encapsulated drug.

2.3. Cell culture and inoculation of mice with tumor cells

Ehrlich ascites tumor was chosen as a rapidly growing experimental tumor model where various experimental designs for anticancer agents can be applied (Elbialy et al., 2010). Ehrlich ascites carcinoma cells (1×10^6 cells) obtained from the National Cancer Institute "NCI"-Cairo University, were intraperitoneally injected into female balb mice. Ascites fluid was collected on the 7th day after injection. The Ehrlich cells were washed twice and then resuspended in 5 ml saline. Female balb mice of 22–25 g body weight and 6–8 weeks old (obtained from the animal house of NCI) were then injected subcutaneously in their right flanks where the tumors were developed in a single and solid form. Tumor growth was monitored post-inoculation until the desired volume was about 0.3–0.6 cm^3. All animal procedures and care were performed using guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee at Cairo University.

2.4. Sulforhodamine-B (SRB) assay of cytotoxic activity

The sensitivity of the human tumor cell lines to thymoquinone was determined by the SRB assay. SRB is a bright pink aminoxanthrene dye with two sulfonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content. HCT-15 (colon cancer cell line) cells were seeded in 96-well microtiter plates at a concentration of 5×10^4–10^5 cell/well in a fresh medium and left to attach to the plates for 24 h. After 24 h, cells were incubated with DOX and DOX loaded liposome samples, completed to total of 200 μl volume/well using fresh medium and incubation was continued for 48. Following 48 h treatment, the cells were fixed with 50 μl cold 50% trichloroacetic acid for 1 h at 4 °C. Wells were washed 5 times with distilled water and stained for 30 min at room temperature with 50 μl 0.4% SRB dissolved in 1% acetic acid. The wells were then washed 4 times with 1% acetic acid. The plates were air-dried and the dye was solubilized with 100 μl/well of 10 mM tris base (ph 10.5) for 5 min on a shaker (Orbital shaker OS 20, Boeco, Germany) at 1600 rpm. The optical density (O.D.) of each well was measured spectrophotometrically at 564 nm with an ELIZA microplate reader (Meter tech. Σ 960, U.S.A.). The mean background absorbance was automatically subtracted. The percentage of cell survival was calculated as follows:

$$\text{Survival fraction} = \text{O.D.(treated cells)}/\text{O.D.(control cells)}.$$

IC50 values are the concentrations of thymoquinone required to produce 50% inhibition of cell growth. The experiment was repeated 3 times for each cell line.

2.5. In vivo anti-tumor activity

As tumors reached the desired volume (0.3–0.6 cm^3) treatment was started. Sixty mice were initially used and randomly divided into four groups A, B, C and D. The treatment groups (A, B and C) were intravenously given a single dose injection of DOX loaded liposomes (2.5 mg/kg, 1.36 mg/kg) and DOX (2.5 mg/kg) respectively. Mice of group D were injected with saline solution.

2.6. Tumor size measurements

Due to the high growth rate in Ehrlich tumor model, change in tumor volume (ΔV) was monitored over 21-day period for the four groups A, B, C and D. Ellipsoidal tumor volume (V) was assessed every three days and calculated using the formula:

$$V = (\pi/6)(\text{d})^2(\text{D})$$

(Montgomery et al., 2000; Ogawara et al., 2009; Elbialy et al., 2010) where D and d are the long and short axes, respectively measured with a digital caliper (accuracy 0.01 mm). Fisher's LSD (least significance difference) multiple-comparison test was conducted to check the significance between group pairs. SPSS version 17 was used for statistical analysis.

2.7. Histopathological examination

Three days post injection, 2 mice of the treated groups A, B and C were sacrificed in order to investigate the tumor cell necrotic percentage. Tumors were excised, fixed in 10% neutral formalin, embedded in paraffin blocks and sectioned. Tissue sections were stained with hematoxylin and eosin (H&E). The previous procedures were repeated for control group D. All tissue sections were examined using light microscope (CX31 Olympus microscope) connected with a digital camera (Canon).

3. Results and discussion

The efficacy of drug delivery system has been assessed in vitro using cell viability assay (SBR assay). The cytotoxicity of free DOX and DOX loaded liposomes on HCT-15 colon cancer cell line has been measured. Compared with free drug, the synthesized Dox liposomes exhibit remarkable cytotoxicity. Two days post drug application, the IC50 values of free drug and DOX loaded liposomes formulation were 4.5 μg/ml and 35.5 μg/ml, respectively, (Fig. 1). For free doxorubicin treated cells, the percentage of cell viability decreased as the drug concentration increased. This is due to the anticancer effect of doxorubicin. For DOX loaded liposome treated cells, the marked increase in the IC50 value may be attributed to the sustained release of doxorubicin from liposomes. So, as the concentration of the encapsulated drug increases, the amounts of released drug also increase and consequently the cell viability decreases when the encapsulated drug concentration increases.

An important site of cytotoxic action of the anticancer drug doxorubicin is the nucleus, where doxorubicin intercalates into DNA, forming DNA adducts and inhibiting topoisomerase II (Gewirtz, 1999). When free doxorubicin reaches the tumor site, doxorubicin that is released from liposomes within the tumor interstitial space is capable of diffusing widely within the tumor. Doxorubicin can diffuse into surrounding cell
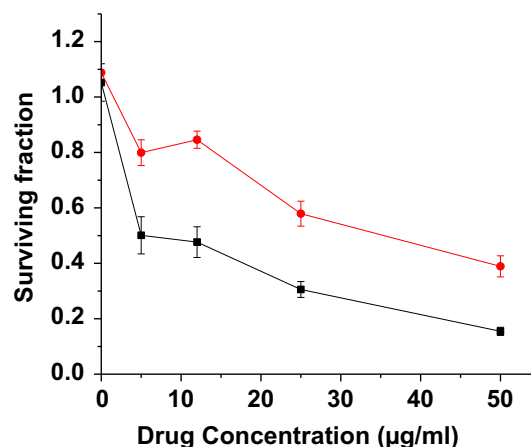

Figure 1 Dose dependent cytotoxicity of free DOX (●) and DOX loaded liposomes (■) in HCT-15 cell line. The growth inhibition was measured two days post treatment. The inhibition was calculated with respect to control. Data were expressed as mean ± S.E.

membrane or protein associated, or diffuses into subcellular compartments such as mitochondria and nuclei.

DPPG is a lipid with high phase transition temperature and can increase the rigidity of the bilayer. It has also negative charges that can bind electrostatically with the positive charges of doxorubicin. Cholesterol condenses the acyl chains of DMPC above the phase transition temperature due to an interaction between cholesterol and the acyl chains of the phospholipid molecules (Ghannam et al., 1999; Mady, 2007). The limited freedom of acyl chains causes the membrane to condense, with a reduction in area, closer packing and decreased fluidity (Ghannam et al., 1999; Mady, 2007).

The efficacy of the drug delivery system used in this study has been assessed in vivo by following up the change in Ehrlich tumor volume over 21-day period for the four groups (Fig. 2). Under our experimental conditions, a pronounced inhibition in tumor growth was demonstrated in the DOX loaded liposomes treated groups A and B. Such marked decrease in Ehrlich tumor volume upon treatment was attributed to antitumor activity of the encapsulated doxorubicin. The accumulation of DOX loaded liposomes within Ehrlich tumor cells was due to the enhanced permeability and retention (EPR) of tumor vasculature. Moreover, DOX loaded liposomes destabilized in slightly acidic environment of tumor, resulting in triggered DOX release. While for treated group C administrated with free DOX, a slight inhibition in tumor volume up to day 6 was observed. Then, the tumors began to grow slowly at day 9 when compared with that of control group D. This result would be attributed to the rapid elimination of free DOX solution from plasma by being excreted into bile and urine and as a consequence the amount of DOX delivered to tumor tissues was quite low (Fig. 2). Control group D showed a marked increase in tumor volume (growth) with time (Fig. 2).

Statistical analysis clearly demonstrated that the administrated two DOX loaded liposome formulations, in the treated groups A and B, have a high significant suppression effect on tumor growth rate up to 21 days with significant p-values of $p < 0.0001$ and $p < 0.0001$ respectively. Such difference was

not statistically observed in group C compared with group D during the same period.

DOX-loaded liposomes have enhanced efficacy in some solid tumors compared with free doxorubicin, because they passively target solid tumors through the enhanced permeability and retention effect (Maeda et al., 2000, 2001), resulting in increased drug payloads delivered to tumors. The enhanced permeability and retention effect are a result of defective vascular endothelial linings of growing tumors, resulting in gaps in the endothelium up to f800 nm in diameter, which are large enough to permit the extravasation of liposomes with diameters in the range of 100 nm (Ishida et al., 1999). In addition, growing tumors have defective lymphatic drainage, which contributes to the extended residence time of extravasated liposomes in the interstitial space of the tumor. Liposomes residing in the interstitial space gradually release their entrapped drug, exerting antitumor effects.

Histopathological examinations confirmed the observed inhibition of tumor growth rate for treated groups in addition to the high growth rate of the control group. Examination of the entire tumor sections for the various groups revealed marked differences in cellular features accompanied by varying degrees in necrosis percentage. Fig. 3 (a–d) shows a histopathological examination for the four experimental mice groups using a light microscope. The images show tumor tissue sections excised from mice of group D (Fig. 3a), group C (Fig. 3b), group B (Fig. 3c) and group A (Fig. 3d). The calculated necrotic percentages for the experimental groups A, B, C, and D were 12%, 45%, 65% and 70%, respectively. Control group (D) showed tissue architectural disarray, as well as marked degree of cellular anaplasia, pleomorphism and anisocytosis, with nuclear dyschromasia. Also giant form is encountered, at the upper right field, with multiple atypical nuclei. Additionally, minimum necrosis was revealed at the lower field with a necrosis percent of about 12 (mean field count) (Fig. 3a). For free doxorubicin treated group C, microscopic examination revealed an increase in necrosis percent up to 40–45% with nodules of viable round cell infiltrate surrounded by ghosts of degenerated cells (Fig. 3b). For treated group B, microscopic examination revealed sheets of round cell infiltrate admixed with red necrotic areas with ghosts of degenerated cells representing about 65% of tumor area (Fig. 3c). Treated group A that has the highest necrosis percent showed a complete loss of cellular details "ghosts" leaving only island of tumor viability with mean field count∼ 70% (Fig. 3d).

Based on the results obtained in the present study, the following mechanisms may be behind the anti-tumor effects of DOX loaded liposomes recognized in tumor bearing mice. First, the PEG liposome in the blood circulation gradually extravasated into the interstitial space of the tumor tissue due to EPR effect as reported previously (Maeda et al., 2000; Iyer et al., 2006; Northfelt et al., 1998; Schmidt et al., 1998). Therefore, DOX encapsulated in the liposome must be first released into the interstitial space of the tumor to be taken up by the tumor cells via passive diffusion. This speculation was supported by the facts that PEG liposomal DOX exhibited very large value of IC50 in control mice because these results reflect that cytotoxic effect would be dependent on DOX slowly released out of the PEG liposome. In the case of control tumor, DOX passively taken up by control would have exerted its anti-tumor activity against the tumor cells directly, leading to the apoptosis of tumor cells throughout
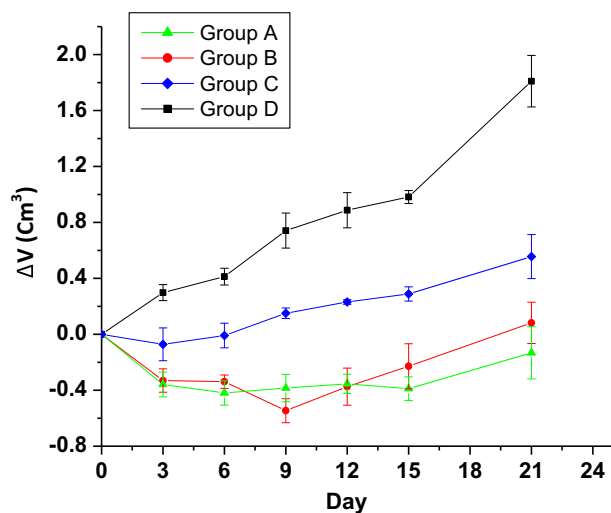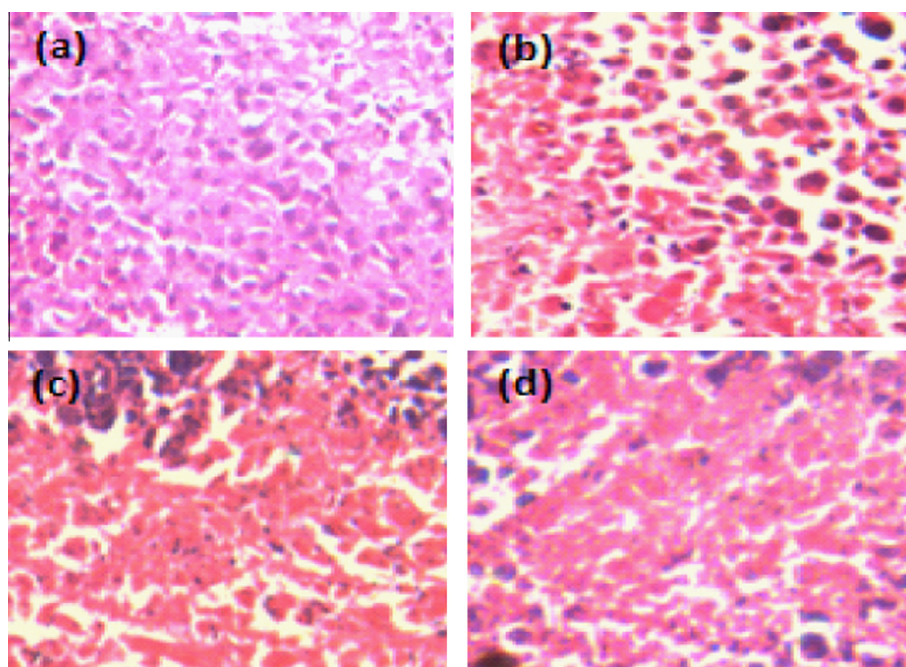

Figure 2 The average changes in Ehrlich tumor volume as a function of time for the three treated groups A, B, C and untreated group D throughout a period of 21 days.

Figure 3 Sections of Ehrlich tumor tissues excised, 21 days post treatment, from mice of (a) control group D, (b) treated group C (free doxorubicin), (c) treated group B (DOX loaded liposomes 2.5 mg/kg) and (d) treated group A (DOX loaded liposomes 1.36 mg/kg).

the tumor (Figs. 2 and 3). On the other hand, DOX passively taken up by Ehrlich tumor would have been subjected to the efflux out of the cells, which would have led to the much less apoptotic cell death in the tumor (Figs. 2 and 3). Subsequently, the resultant larger amount of DOX accumulated in the interstitial space of the tumor tissue would have penetrated into the neighboring vascular endothelial cells.

It is clear that the preparation of such nano-sized DOX loaded liposomal formulation facilitate their passive targeting to the tumor. In addition to their successful accumulation inside tumor tissues due to the enhanced permeability and retention effect (EPR) which slow down their lymphatic drainage and providing a prolonged time of circulation in blood over free DOX solution. All the above mentioned factors promote the use of such formulations as an efficient oncological modality over the other traditional chemotherapeutic agents.

4. Conclusion

The proposed liposomal formulation can efficiently deliver the drugs into the tumor cells by endocytosis (or passive diffusion) and lead to a high concentration of DOX in the tumor cells. Administration of these DOX loaded liposomes to tumor-bearing mice could be used to deliver DOX effectively to the targeted site, significantly increasing the DOX content in tumor. This would improve the therapeutic index of DOX, and is a proof of principle in support of administering liposomally co-encapsulated drug. Based on the results in this study, it is suggested that the proposed liposomal formulation could be employed to enhance the intracellular delivery of anticancer agents such as cytotoxic drugs, antisense nucleic acids and ribozymes or imaging agents.

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