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In vitro cytotoxicity of liposome-encapsulated doxorubicin: dependence on liposome composition and drug release

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We have investigated the in vitro cytotoxicity of free doxorubicin (DOX) and liposome-entrapped DOX (L-DOX) against a human ovarian carcinoma cell line (OV-1063) using a colorimetric assay. DOX was encapsulated in the inner water phase of liposomes by an ammonium sulfate-generated proton gradient. Liposomes varied in phospholipid composition but were of a similar size. It was found that the cytotoxic activity of L-DOX is substantially decreased when liposomes containing phospholipids of high phase-transition temperature (T_m) are used. The type of negatively charged headgroup did not have any significant influence on the cytotoxicity observed. Experiments using resin beads that bind free and protein-bound DOX, but do not interact with L-DOX, indicated that the cytotoxic effect is mediated by the release of drug from the liposomes into the extracellular medium; no evidence was found for direct cellular uptake of liposome-encapsulated drug. The use of the ionophore nigericin to induce the release of DOX from high-T_m liposomes increased cytotoxicity to a level comparable to free DOX, suggesting that 'remote release' techniques may substantially improve the efficiency of liposome-mediated drug delivery and allow for the full exploitation of the favorable pharmacokinetic properties of specific high-T_m formulations.

Introduction

Numerous studies have examined the in vivo pharmacology of doxorubicin (DOX) encapsulated in liposomes (L-DOX) (reviewed in Ref. 1). In recent years, evidence gathered from several reports indicates significant localization of long-circulating, small (< 100 nm) liposomes in animal and human tumors [2,3]. In addition, there are data suggesting increased tumor localization and enhanced therapeutic efficacy of anthracyclines encapsulated in these long-circulating liposomes [4,5]. It is, therefore, important to understand how liposome-encapsulated anthracyclines interact with tumor cells and to quantify their cytotoxic effect relative to free drug. For that purpose, in vitro experiments examining the mechanism of interaction of L-DOX with tumor cells and the effect of liposome composition on cytotoxicity are an essential step. Some of these issues have been addressed previously by Storm et al. [6] using liposome formulations with DOX incorporated by passive encapsulation.

In this report we examine the in vitro cytotoxic activity and cellular drug uptake resulting from exposure of a human ovarian cancer cell line to various formulations of liposome-encapsulated anthracyclines. Special emphasis was placed on those liposome formulations with favorable pharmacokinetic properties, i.e., long circulation time and good tumor localization, which have been referred to as Stealth^R liposomes[1] [7]. To restrict the number of variables affecting the drug delivery process, we concentrated in this study on liposome preparations in which drug was encapsulated in the aqueous phase through an ammonium sulfate-generated proton gradient [8], as opposed to preparations in which the drug is associated to the liposome bilayer [9].

Materials and Methods

Cell line and growth conditions

A human ovarian carcinoma cell line, OV-1063, was used [10]. Cells were grown in RPMI-1640 medium supplemented with penicillin 50 U/ml, streptomycin

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[1] Stealth liposomes is a registered trademark name of Liposome Technology Inc., Menlo Park, CA.

50 μg/ml, L-glutamine 2 mM and 10% bovine fetal serum. Cells were routinely passed by treatment with trypsin (0.05%)-EDTA (0.02%) solution in calcium- and magnesium-free phosphate-buffered saline. Culture media components were purchased from Biological Industries (Beyt Ha'emek, Israel) and Gibco (Grand Island, NY). The cultures were routinely passed at a split ratio of 1:10, although confluent cultures could be achieved at a split ratio as low as 1:50.

Chemosensitivity assays

The cytotoxic effect of free DOX or L-DOX was assayed colorimetrically by a Methylene blue (MB) staining method described previously [11], with slight modifications and by the XTT microculture tetrazolium assay [12]. In some experiments, both assays were combined. Upon completion of the XTT assay, the cells were fixed and evaluated using the MB staining assay. There was a very good correlation between the results obtained with these two assays with regard to OV-1063 cells and other adherent cell lines (data not shown).

In our assays, 5000 OV-1063 cells (from exponentially growing cultures) in 200 μl aliquots were plated onto 96-well flat-bottom microtiter plates. Following 20 h in culture, during which cells attached and resumed growth, 20 μl of the tested drug was added to each well. For each 10-fold increase in drug concentration, four drug concentration points were tested. Each test was performed in triplicate wells and in two parallel plates. The cells were treated continuously for 72 h (or as stated). The cultures were fixed by the addition of 50 μl 2.5% glutaraldehyde to each well for 10 min. The plates were washed three times with deionized water, once with 0.1 M borate buffer (pH 8.5) and then stained for 60 min with 100 μl Methylene blue (1% in 0.1 M buffer borate, pH 8.5) at room temperature. The plates were rinsed in five baths of deionized water to remove non-cell bound dye and were then dried. The dye was extracted with 200 μl 0.1 M HCl for 60 min at 37°C and the optical density was determined using a microplate spectrophotometer.

In preliminary experiments, it was found that the cell number determined by counting cells with a hemocytometer correlated well with the spectrophotometric absorbance. The initial cell plating density was chosen to ensure a linear relationship between cell number and absorbance at the end of the experiment. In each experiment, 10 wells were fixed before drug was added to determine the initial average absorbance. This value was used to calculate doubling times (DT) of control and drug-treated cells using the following equation:

$$DT = \ln 2/\ln[(OD_t/OD_c)/h]$$

where: DT = doubling time in h; OD_t = optical density of test well at the end of the experiment; OD_c = optical density of control well at the start of the experiment; h = duration of incubation in h.

The growth rate was calculated by dividing the doubling times of drug-treated cells with those of the control cells. The drug concentration which caused a 50% inhibition of the control growth rate (IC_{50}) was calculated by interpolation of the two closest values of the growth inhibition curve.

In some experiments, cells were co-cultivated in the presence of Dowex resin beads prepared as previously described [9], which avidly bind free DOX [13] and thus prevent drug entry into cells. In these experiments, cells were plated in 35-mm plates and the results were obtained by counting the cells with a hemocytometer. The MB staining method could not be used because the Dowex beads are themselves stained by the dye.

DOX accumulation in cells

Cellular accumulation of DOX was measured as described by Chambers et al. [14] with slight modifications. Monolayers of OV-1063 cells (exponentially growing cultures of about 10^6 cells in 35-mm plates) were incubated with free DOX or L-DOX, 1–10 μg/ml, for 1 h at 37°C. The cells were rinsed three times with ice-cold PBS and the drug was extracted from the cells with 1 ml acidified isopropanol (0.075 M HCl in 90% isopropanol), for 20 h at 4°C. DOX concentration was determined spectrofluorometrically using an excitation wavelength of 470 nm and an emission wavelength of 590 nm. The fluorescence intensity emitted was translated into DOX-equivalents based on a DOX standard curve, after readings of untreated background cells were subtracted.

To determine non-specific adsorption of DOX, control plates with and without cells were cooled to 5°C and then treated with DOX or L-DOX for 1 h at 5°C. Cell-associated DOX was expressed as ng DOX/10^6 cells.

Liposome preparations

Sources of liposome components were as follows: egg phosphatidylcholine (EPC), egg-derived phosphatidylglycerol (EPG), hydrogenated soybean phosphatidylcholine (HPC) and phosphatidylinositol (HPI), distearoylphosphatidylglycerol (DSPG), and dipalmitoylphosphatidylcholine (DPPC) from Avanti Polar Lipids (Birmingham, AL); cholesterol (Ch), monosialoganglioside (GM1) and α-tocopherol from Sigma (St. Louis, MO). PEG/DSPE (poly(ethylene glycol) derivative of distearoylphosphatidylethanolamine) was provided by Liposome Technology (Menlo Park, CA). The phospholipid/cholesterol molar ratio was between 10:7 to 10:8. The molar fraction of negatively-charged phospholipid (HPI, PEG/DSPE, GM1, EPG, DSPG)

was 7.5–10% of total phospholipid. Liposomes were prepared by thin lipid film hydration and downsized by extrusion as previously described [3]. DOX was actively encapsulated in the water phase of preformed liposomes as a result of a proton gradient generated by liposome-entrapped ammonium sulfate [15]. Mean liposome size was 70–100 nm. Details regarding the preparation and characteristics of the liposomes are as reported previously [5].

Results

Effect of liposome composition on the in vitro cytotoxicity of L-DOX

Fig. 1 depicts the growth inhibition curves for free DOX and DOX encapsulated in three types of liposomes: PEG/DSPE-EPC-Ch, PEG/DSPE-DPPC-Ch and PEG/DSPE-HPC-Ch. The cytotoxicity decreased (EPC > DPPC > HPC) as the phase-transition temperature (T_m) of the main lipid component increased (EPC < DPPC < HPC, approximate T_m, 5°C, 41°C and 52°C, respectively). Table I shows the IC_{50} values for DOX encapsulated in a variety of liposome formulations. It may be seen that the IC_{50} values for liposomes containing HPC were 4–10-fold greater than that for free DOX; in contrast, the IC_{50} values for EPC-containing liposomes were of the same order of magnitude as for free DOX. It thus appears that the cytotoxicity of DOX encapsulated in liposomes whose main component is a low phase-transition temperature (T_m < 37°C) phospholipid, such as EPC, is similar to that of free DOX. However, when the main liposome component is a high phase-transition temperature (T_m > 37°C) phospholipid, such as HPC, the cytotoxicity is significantly reduced. This is true regardless of the type of negatively-charged phospholipid used (EPG, DSPG, HPI, GM1, PEG/DSPE), as seen in Table I. These results also indicate that the negatively-charged head group does not appear to play a significant role in the degree of cytotoxicity observed. Liposome preparations with or without α-tocopherol yielded similar results. To continue our investigations on the mechanism of cytotoxicity of L-DOX, we chose the HPI-HPC-Ch and PEG/DSPE-HPC-Ch formulations.

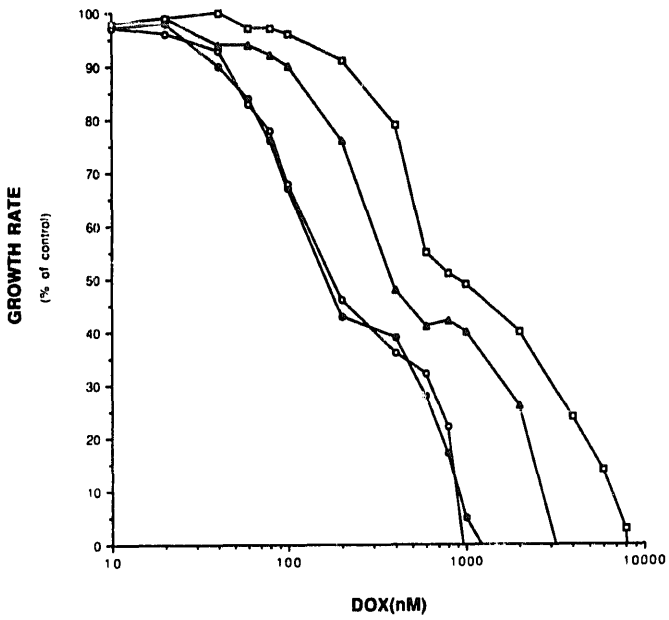

Fig. 1. Growth inhibition curves for OV-1063 cells (5000 cells/well) treated for three continuous days with free DOX (●) or DOX encapsulated in PEG/DSPE-EPC-Ch (○), PEG/DSPE-DPPC-Ch (▲) or PEG/DSPE-HPC-Ch (□).

TABLE I

Effect of liposome composition on the in vitro cytotoxicity of L-DOX on OV-1063 human tumor cells

	$IC_{50} \pm$ S.D.[a] (nM)
Free DOX	190 ± 70
L-DOX-liposome composition[b]	
EPG-EPC-Ch	300 ± 20
DSPG-HPC-Ch	1100
PEG/DSPE-EPC-Ch	180
PEG/DSPE-DPPC-Ch	400 ± 40
PEG/DSPE-HPC-Ch	1210 ± 400
HPI-HPC-Ch	1410 ± 320
GM1-HPC-Ch	840
HPC-Ch	2200 ± 100

[a] The IC_{50} values were calculated from the MB assay. S.D. values are shown for formulations tested in 4 or more plates and two or more experiments.

[b] All liposomes contain 1% α-tocopherol. DOX is encapsulated in the aqueous phase by remote loading through a proton gradient (see Materials and Methods).

Drug concentration in tumor cells incubated with L-DOX

To investigate the reason for the decreased toxicity of the high phase-transition temperature (solid phase) L-DOX formulations, we examined the cell-associated drug concentration after incubation with free DOX and DOX encapsulated in HPI-HPC-Ch liposomes. As seen in Fig. 2, the amount of drug associated with tumor cells was between 5- and 10-fold less when the drug was presented in liposomes. In both cases, there was a linear correlation between the amount of cell-associated drug and the drug concentration in the medium within the tested range. Therefore, the reduced cytotoxicity observed with DOX encapsulated in solid phase liposomes is probably accounted for by decreased cell exposure to the drug.

Dependence of cytotoxicity on the release of DOX from liposomes

Next, we examined whether the cytotoxic activity of L-DOX was due entirely or partially to drug released

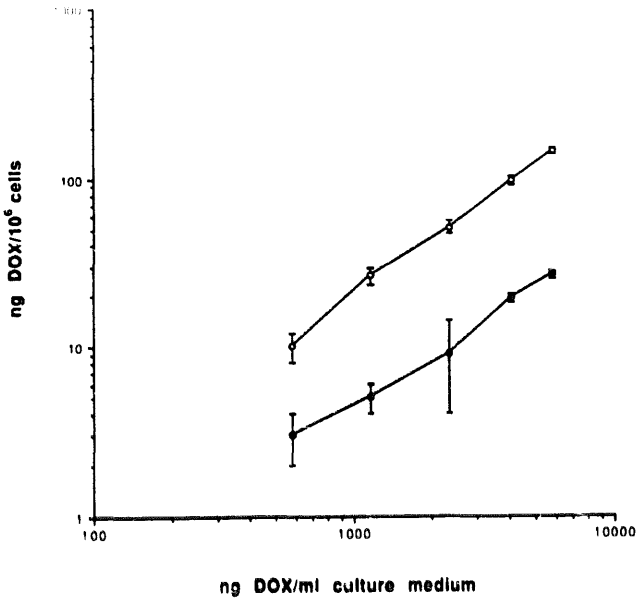

Fig. 2. DOX accumulation in OV-1063 cells. Monolayers of OV-1063 cells were incubated for 1 h at 37°C with free DOX (●), or DOX encapsulated in HPI-HPC-Ch liposomes (○), 1–10 μg/ml. DOX was extracted directly from washed monolayers with acidified isopropanol and measured spectrofluorometrically.

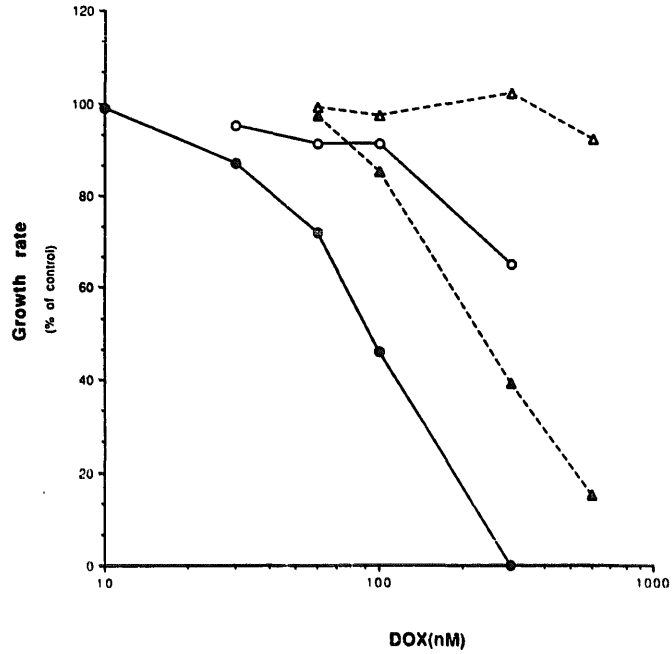

Fig. 3. Cytotoxicity of free DOX (○, ●) and L-DOX (HPI-HPC-Ch) (△, ▲), in the presence (○, △) or absence (●, ▲) of Dowex (0.4%, v/v) beads. $2 \cdot 10^5$ OV-1063 cells were seeded onto 35-mm dishes. 20 h after seeding, the number of cells was counted in three plates to establish the cell count for time 0 when drugs and Dowex were added. After 72 h, the cell number was counted in triplicate plates for each point.

by liposomes into the extracellular medium, without any direct cellular uptake of DOX in liposome-associated form. For this purpose, we incubated the cells with Dowex beads, which bind irreversibly free and protein-bound DOX but do not interact with L-DOX. The concentration of Dowex beads was carefully titered in order to avoid any significant inhibition of the beads on cell growth. Results shown in Fig. 3 indicate that in the presence of the Dowex beads the cytotoxicity of both free DOX and L-DOX was almost completely inhibited (80–100% inhibition factor). Therefore, it seems that most of the cytotoxic effect of L-DOX depends on extracellular release of drug and that the liposome does not carry the drug into the cell. Drug release is limited due to the improved stability of high-T_m liposomes and consequently bioavailability and cytotoxicity are both reduced.

Effect of incubation time on the cytotoxicity of L-DOX and free DOX

If the reduced cytotoxicity of L-DOX is due to slow drug efflux from liposomes, prolonging the incubation time of cells in the presence of the test agent should narrow the differences between the cytotoxicity of free and L-DOX. Fig. 4 shows the results of such experiments, expressed as the ratio of the IC_{50} of L-DOX (PEG/DSPE-HPC-Ch) to the IC_{50} of free DOX along four incubation time points (1, 2, 3 and 4 days). Prolonging the incubation time of the cells with L-DOX from one to 3 days reduced the difference between free DOX and L-DOX from an IC_{50} ratio of 15.4 ± 2.2

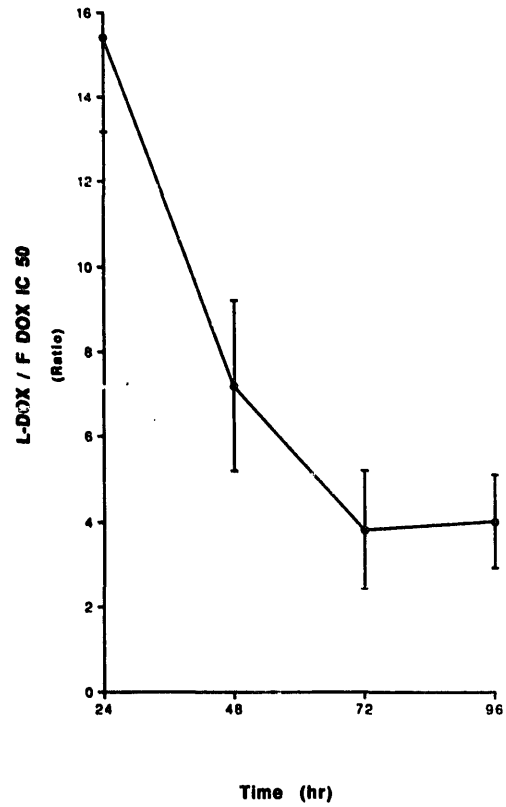

Fig. 4. Ratios of IC_{50} values for L-DOX versus free DOX treated cells after incubation periods of 1–4 days. IC_{50} values were obtained from growth inhibition curves as described in Fig. 1. The liposome formulation used is PEG/DSPE-HPC-Ch.

to 3.8 ± 1.4. These data suggest that the time required for drug release from high T_m liposomes is a significant factor with respect to the cytotoxic effect.

Enhancement of drug release from liposomes using the ionophore nigericin

To investigate the effect of enhancing drug release from liposomes on cellular drug uptake and cytotoxicity, we performed experiments in which nigericin, an ionophore that accelerates K^+/H^+ exchange, was added to the medium containing liposomes and cells. As reported previously [15], high-T_m liposomes normally release DOX very slowly due to their proton gradient, which keeps DOX in its ionized form. Nigericin causes the proton gradient of liposomes to collapse and this in turn leads to DOX deprotonation and leakage. Exposure to nigericin (10 μM, in the presence of 5 mM K^+) results in > 90% leakage of DOX from liposomes within 30 min. The addition of nigericin (10 μM) to the culture medium greatly enhanced (5-fold) the uptake of DOX by OV-1063 cells exposed to L-DOX; while, the uptake of free DOX was not affected significantly by the ionophore. Furthermore, nigericin was able to augment the cytotoxic effect of L-DOX on OV-1063 cells (Fig. 5), when PEG/DSPE-HPC-Ch (high-T_m) liposomes were used. Fig. 5 also shows that the cytotoxicity of free DOX was not significantly increased by the ionophore nigericin. Similar results were obtained with HPI-HPC-Ch DOX liposomes (data not shown). As seen in Fig. 6, the cytotoxicity of PEG/DSPE L-DOX in the presence of nigericin matched that of free DOX. Thus, these experiments demonstrate that controlling drug release from liposomes is critical in determining drug bioavailability to cells and the magnitude of cytotoxicity.

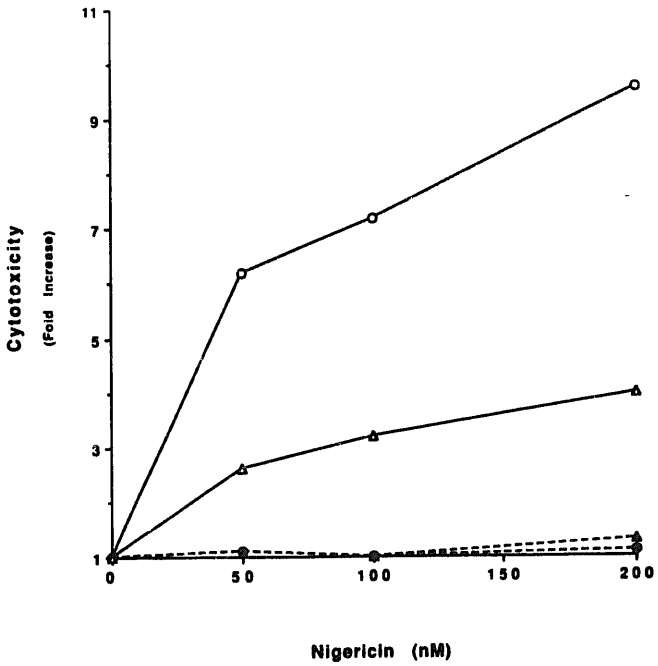

Fig. 5. Ratios of IC_{50} values for nigericin-untreated versus nigericin-treated cells exposed to free DOX (●, ▲) or L-DOX (○, △), for 48 h (●, ○) or 72 h (▲, △) incubation. The liposome formulation used is PEG/DSPE-HPC-Ch.

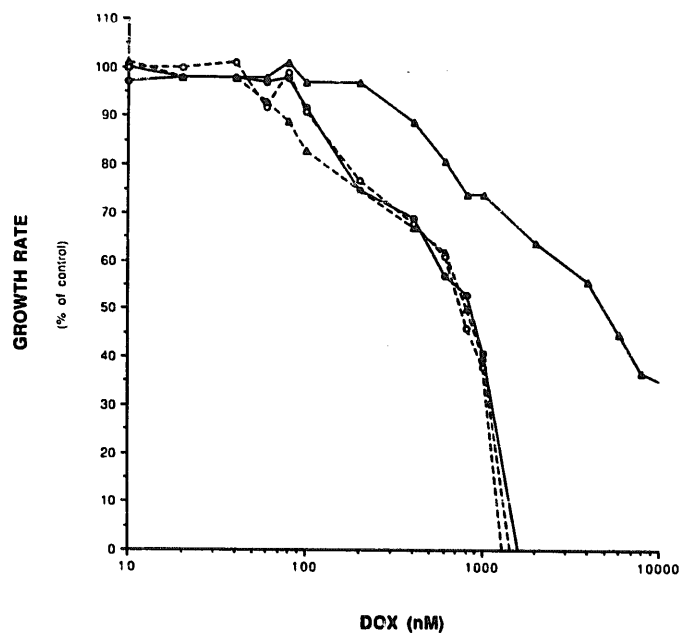

Fig. 6. Cytotoxicity of free DOX (●, ○) and L-DOX (▲, △) in the presence (broken lines, ○, △) or absence (solid lines, ●, ▲) of nigericin (50 nM). Nigericin was added immediately after L-DOX. OV-1063 cells were exposed to the drugs for 72 h. The liposome formulation used is PEG/DSPE-HPC-Ch.

Discussion

In previous studies, we found that liposome formulations in which DOX is intercalated in the lipid bilayer and PC is the main lipid component show an in vitro cytotoxic activity equal to free DOX on mouse J6456 lymphoma [16], P388 leukemia [17] and OV-1063 carcinoma cells (data not shown). In the present study we investigated the cytotoxicity of DOX entrapped in the water phase of liposomes containing high or low phase-transition temperature phospholipids using the OV-1063 cell line. We found that all DOX formulations containing high phase-transition temperature phospholipids showed a decreased cytotoxic activity as compared to free drug. In contrast, when low phase-transition temperature phospholipids were used there was no substantial loss of cytotoxic activity. These results are in agreement with previous findings by Storm et al. [6] comparing a 'fluid' with a 'solid' DOX liposome preparation.

Allen et al. [18] have shown that the cytotoxic effect of various non-anthracycline liposome-encapsulated drugs is mediated in most instances by the release of drug into the extracellular medium. The results of the

present study indicate that the same mechanism operates for DOX entrapped in the liposome aqueous phase. In line with this, we have not observed any significant changes in the sensitivity of DOX-resistant cells to L-DOX (Horowitz and Gabizon, unpublished data). In contrast, Thierry et al. [19] have reported reversal of drug resistance using a DOX-cardiolipin liposome preparation. The reason for this discrepancy may rest on the fact that DOX and cardiolipin form a high-affinity complex [20], likely to be stable in culture medium.

The experiments presented here point to slow drug release as the main reason underlying the reduced cytotoxicity of DOX encapsulated in high-T_m liposomes. A slower efflux rate of DOX from HPI-HPC-Ch liposomes as compared to EPG-EPC-Ch liposomes has been reported previously [15]. If these results are extrapolated to other HPC- and EPC-containing liposomes, one may account for the differences in cytotoxic activity between solid-phase and fluid-phase liposomes. A slow rate of drug efflux may explain the reduced accumulation of DOX in tumor cells exposed to an HPC-containing formulation for 1 h (Fig. 2).

Small-sized liposomes made with high phase-transition temperature phospholipids and bearing specific headgroups, such as GM1, HPI and PEG/DSPE, show stable retention of contents, prolonged circulation time [4,5,21–24] and increased accumulation in tumors [4,5]. The results of these studies raise an important point regarding the therapeutic value of DOX encapsulated in these long-circulating liposomes. From this and previous studies [5], it appears that extracellular release of drug from liposomes is the key step to allow drug access to cells, whether in the culture medium in vitro or in the tumor interstitial fluid in vivo. If we extrapolate the in vitro observations of reduced cytotoxicity of L-DOX to the in vivo situation, one may infer that the in vivo therapeutic potential of L-DOX is not fully exploitable due to reduced bioavailability. However, this simplistic view disregards the complexity of the in vivo scenario. It is likely that differences exist between the in vivo and in vitro kinetics of drug efflux. In vivo, liposomes are exposed to much higher concentrations of plasma and interstitial fluid proteins and to tumor-associated macrophages, factors which may accelerate the drug efflux rate of liposomes and therefore enhance the antitumor effect. In addition, the kinetics of tumor cell growth may also play a role. Fast-growing tumors, as in the case of many mouse leukemias, may significantly increase their cell burden before most of the drug becomes bioavailable, thus diminishing the net anti-tumor effect. This will not be the case with slower growing tumors, which are more appropriate models of human malignant diseases.

Besides the absolute or cumulative drug bioavailability during the total incubation period, another important issue relevant to the cytotoxic effect is the peak bioavailable concentration achieved by free and liposomal drug at any given time. Net drug accumulation in the tumor cells depends on drug influx and efflux processes. In the case of DOX, influx is mainly governed by a passive, concentration-dependent diffusion gradient while efflux appears to depend on carrier-mediated active transport [25]. It is likely that the peak bioavailable drug concentration is lower when cells are exposed to L-DOX, instead of free DOX, given the slow release of drug from high-T_m liposomes [15] and the drug subcellular compartmentalization, metabolic and degradative processes taking place in the meantime. This may confer a survival advantage to tumor cells exposed to L-DOX as opposed to free DOX. The fact that free DOX retained part of its cytotoxic advantage over L-DOX when the incubation period was prolonged from 1 to 4 days (Fig. 4) may be due to a difference in the peak bioavailable concentration.

The potential for increased cytotoxicity of L-DOX is demonstrated by the nigericin experiments (Figs. 5 and 6). The nigericin-induced 'remote release' of DOX from liposomes resulted in a subsequent 8-fold increase in cytotoxicity. Although the systemic use of nigericin in vivo is probably unfeasible given its toxicity, these experiments indicate that a significant improvement of the therapeutic effect of L-DOX may be obtained if effective ways of manipulating the rate of drug release in vivo (hyperthermia [26], pH-sensitive liposomes [27]) become applicable. Developing a 'remote release' technology may allow full therapeutic exploitation of the increased localization of L-DOX in tumors.

Acknowledgements

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