Reduced UV-Induced Degradation of Doxorubicin Encapsulated in Polyethyleneglycol-Coated Liposomes

Suzan Bandak,[1,3] Avner Ramu,[1] Yechezkel Barenholz,[2] and Alberto Gabizon[1]

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Purpose. The aim of this study was to investigate the stability of doxorubicin encapsulated in polyethyleneglycol-coated liposomes (Doxil™) under UV-A light.

Methods. High performance liquid chromatography and a fluorimetric method were used to quantify doxorubicin in bulk solution and doxorubicin in Doxil formulation.

Results. The photodegradation of Doxil was significantly lower in comparison to the photodegradation of the free drug and showed no concentration dependency at the measured concentration range of 5–50 μg/ml. During and after UV-A irradiation, there was no leakage of the drug from liposomes to the medium. After induced leakage of doxorubicin from the liposomes by the ionophore nigericin, the degradation kinetics of Doxil were identical to that of free doxorubicin.

Conclusions. High intraliposomal doxorubicin concentration and intraliposomal acidic pH are the two critical factors that protect DXR in Doxil from UV-A degradation.

KEY WORDS: doxorubicin; liposomes; photodegradation; UV-A light.

INTRODUCTION

Anthracyclines, consisting generally of a chromophoric anthraquinone moiety and a charged sugar, are light sensitive (1). There are several studies investigating the photodegradation of DXR when exposed to room light and intensive light (2,3). The mechanism of photodegradation of DXR is assumed to be related to radical formation (4). Wood *et al.* found that exposure to light, an increase in the pH and adsorption onto the container are significant factors which favor DXR loss (5). The high instability of DXR in biological solutions under the influence of artificial light or daylight was confirmed by Le Bot *et al.* (6). Ramu *et al.* studied the photoinactivation of DXR by ultraviolet light using different media and concluded that light-excited riboflavin and certain amino acids present in biological media enhance the UV-A effect on DXR by orders of magnitude over plain buffer (7).

One of the major obstacles in chemotherapy is the lack of selectivity and antitumor activity of the cytostatic agents. An increase in the therapeutic activity through selective extravasation in tumor sites with a slow drug release was successfully achieved by the encapsulation of cytotoxic drugs in long circulating Stealth® liposomes (8). Stealth® liposomes are sterically stabilized, polyethyleneglycol-coated liposomes which are very stable in blood and have minimal interaction with plasma components (9). One of the leading preparations in this field is Doxil™, prepared by encapsulating DXR at high concentrations in the water phase of polyethyleneglycol-coated liposomes using an ammonium sulfate gradient (10). In this study, we have investigated the *in vitro* photodegradation of different concentrations of free DXR in bulk solution and DXR in Doxil formulation after exposure to UV-A light under various conditions.

Studying the photodegradation of Doxil is of great relevance for the following reasons: (1) Pharmaceutical considerations regarding stability of the liposomal drug during infusion and storage if exposed to light. (2) Doxil circulates for prolonged periods of time and localizes in the skin at high concentrations. In fact, one of the major dose-limiting toxicities of Doxil is a form of skin toxicity, known as the "hand-foot syndrome" (11). These factors favor the possibility of increased exposure of Doxil to UV light. Thus, it is of interest to learn about the effects of UV irradiation on liposome-encapsulated DXR.

MATERIALS AND METHODS

Materials

DXR hydrochloride was obtained from Farmitalia-Carlo Erba (Milan, Italy) as a lyophilized powder for injection. Doxil™ and N-Carbamoyl-poly-(ethylene glycol methylether)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine triethyl ammonium salt (PEG-DSPE) were kindly provided as a gift from Sequus, Menlo Park (California, USA). Hydrogenated soybean phosphatidylcholine (HSPC) was obtained from Lipoid (Ludwigshafen, Germany). Cholesterol, Hepes buffer, Dowex 50-WX-4 and nigericin were all obtained from Sigma (St. Louis, MO, USA).

Methods

Experimental Conditions

The following concentrations of DXR and Doxil were studied: 5, 10, 25, and 50 μg/ml. Photodegradation kinetics were performed in phosphate-buffered saline (PBS) pH 7.0 and in 150 mM Hepes buffered saline (1:9), pH 7.0. Solutions of the various concentrations of DXR and Doxil were irradiated in scintillation vials containing 5 ml of each solution. Irradiation of the samples was carried out using a Vilber Lourmat 240 BLB lamp, with two 40 W 365 nm tubes, at a distance of 20 cm from the scintillation vial. The radiation was performed at 365 nm with an intensity of 1.3–1.4 mW/cm^2 as measured with a UVR 365 radiometer from Vilber Lourmat (Marne la Vallee, France). All irradiations were performed in iced water to avoid any possible effect of heat on the degradation. Solutions of DXR and Doxil, at 5 and 50 μg/ml, were left in roomlight for 5 hours. In roomlight studies scintillation vials were kept on a table in the laboratory at a two meter distance from twelve 45-watt ceiling fluorescent tubes. All experiments were done three times on three separate days.

[1] Sharet Institute of Oncology, Hadassah Hebrew University Medical Center, Jerusalem, Israel.

[2] Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

[3] To whom correspondence should be addressed. (e-mail: suzan@md2.huji.ac.il)

ABBREVIATIONS: doxorubicin, DXR.

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Duplicates of the concentrations 5 and 50 μg/ml DXR and Doxil were kept in absolute darkness for 5 hours and used as controls. Aliquots of DXR solution and Doxil were taken after the following irradiation times (or dark): 0, 1, 2, 3, 4, and 5 hours.

Quantification of DXR by HPLC

Aliquots of DXR solutions were diluted at a ratio of 1:10 with the mobile phase and directly chromatographed. DXR was extracted from aliquots of Doxil solutions at defined timepoints with acidic isopropanol (90% isopropanol, 10% 0.75 N HCl) at a volume ratio of 1:10. An aliquot of this solution was once again diluted with the mobile phase of the HPLC at a volume ratio of 1:10 and chromatographed as described by Goren *et al.* (12).

Quantification of DXR by Fluorimetry

In samples not subjected to HPLC, total fluorescence assay measurements were performed on the Kontron SFM 25 Spectrofluorimeter at an excitation wavelength of 470 nm and an emission wavelength of 590 nm. Samples for fluorescence assay were diluted with acidic isopropanol at a volume ratio of 1:10 and directly measured.

Quantification of DXR by Spectrophotometry

Samples for UV/VIS spectra were diluted with acidic isopropanol at a volume ratio of 1:10 and directly measured. UV/VIS absorption spectra were measured with a Hewlett Packard 8452A diode array spectrophotometer, at wavelengths between 200–600 nm.

Measurement of UV-A-Irradiation-Induced DXR Leakage

Dowex 50-WX-4 is a cation exchange resin which binds free DXR very efficiently (13,14). Dowex columns were preconditioned with 1 ml human plasma to avoid nonspecific binding of the liposomes. Aliquots of 1 ml of the Doxil solutions at 5 and 50 μg/ml ($\sim 10^{-5}$ and 10^{-4} M) were taken after 0, 3 and, 5 hours of irradiation and run through the Dowex columns. DXR was extracted from the liposomes in the eluate with acidic isopropanol at a volume ratio of 1:10 and DXR was determined by HPLC as described above.

Photodegradation of Doxil After Nigericin-Induced Leakage of DXR from Liposomes

Nigericin is an ionophore which accumulates in the lipid bilayer causing the proton gradient of the liposomes to collapse by K^+/H^+ exchange, leading to DXR deprotonation and leakage (15). To check nigericin-induced leakage, 5 ml of a solution of 50 μg/ml Doxil in Hepes buffered saline were incubated in the presence or absence of 200 μM nigericin and 5 mM KCl for 30 min at 50°C in a waterbath, under shaking at 80 strokes/min. To determine the release of DXR from the liposomes, 1 ml of each solution after heating was run on Dowex columns preconditioned with plasma. The vis absorption of the Dowex eluate at 490 nm was measured and compared to the absorption of the original solutions after heating. To rule out any interference of nigericin with the photodegradation of DXR, 10 and 50 μg/ml DXR in Hepes buffered saline were incubated in the presence or absence of 200 μM nigericin and 5 mM KCl for 30 min at 50°C under shaking, as described previously. Solutions were then irradiated. Fluorescence emission was measured as described previously.

For photodegradation studies, concentrations of 5 and 25 μg/ml Doxil in Hepes buffered saline were incubated in the presence of 200 μM nigericin and 5 mM KCl for 30 min at 50°C under shaking. As controls, same concentrations of DXR and Doxil were irradiated with UV-A light for 5 h. Samples for fluorometry were processed as indicated above.

Photodegradation of Liposomal Preparations with Different Drug:Lipid Ratios

Stealth liposomes with an ammonium-sulfate gradient were prepared as described elsewhere (16). Different concentrations of DXR were loaded in these stealth liposomes by remote loading above the phase transition temperature of the phospholipids (60°C). In preparation (I), a solution of 18.3 mM DXR was added to the liposome dispersion (71 mM phospholipids), in preparation (II), 1.83 mM DXR was added to 71 mM phospholipids, whereas in preparation (III), 0.183 mM DXR were added to 71 mM phospholipids. Free DXR was removed from liposome-associated DXR through binding of free DXR to the cation exchange resin, Dowex 50-WX-4 The phosphorus and liposomal DXR content were then determined as described by Barenholz and Amselem (17). The DXR:Phospholipid ratio was calculated from the phosphorus and liposomal DXR content of the preparations. With these preparations we were able to achieve a vast difference in the intraliposomal DXR concentrations, starting at a concentration at which most intraliposomal DXR exists in a gel-like state [~35 mg/ml, calculated by assuming an intraliposomal water volume of 2.1 μl/μmol phospholipids (18)] and gradually diluting it to a concentration (~0.5 mg/ml) at which DXR is present as a mixture of dimers and aggregates (19). With the remote loading method mentioned above, we were not able to achieve intraliposomal DXR concentrations at which DXR exists as a monomer. The photodegradation of different concentrations of 1, 2.5, and 5 μg/ml liposomal DXR, of these three preparations in Hepes buffered saline was studied.

Role of the Intraliposomal pH on the Degradation of Doxil

During the encapsulation process of DXR inside Stealth liposomes, an acidic pH of ~3.0–4.0 is achieved in the interior of the liposomes (10), which is maintained by the high concentration of sulfate (250 mM). To examine the influence of the acidic pH on the degradation of Doxil, in comparison to free DXR, the degradation-kinetics of 5 and 25 μg/ml DXR were studied both in Hepes buffered saline (pH 7.0) and in acetic buffered saline (pH 3.6).

Data Assessment

Data for the different concentrations of free DXR were adequately fitted to first-order kinetics by the exponential equation $[DXR]=[DXR]_0.e^{-kt}$. The rate constant for degradation (k) was calculated by nonlinear regression analysis for the first order plot. The data calculations for Doxil were best fitted to zero order kinetics by the equation $[DXR]=[DXR]_0 + k.t$ (k

is the reaction rate, t is the time of degradation and [DXR]o is [DXR] at t = 0). Statistical significance of the data was analyzed by the unpaired t-Test.

RESULTS

The drug concentrations chosen for this study are in the range of plasma concentrations of Doxil in the first week after injection. Besides, in this range we could follow with good confidence the drug degradation.

In roomlight free DXR was moderately stable: after 5 hours of irradiation 25% of the initial drug amount was degraded at a concentration of 5 μg/ml while in the same interval no photodegradation was observed at 50 μg/ml. At both concentrations 5 and 50 μg/ml, the DXR of Doxil over the same measured DXR concentrations was fully stable for 5 hours when exposed to roomlight. All DXR and Doxil solutions kept in the dark showed no photodegradation in the interval of 5 hours. When comparing the UV/VIS spectra of DXR and Doxil before and after UV-A irradiation, no UV/VIS absorbing metabolites can be seen. The degradation of DXR is revealed in the decline of the absorbance maximum of DXR at 490 nm.

As shown in Fig. 1, the rate of degradation of DXR when irradiated with UV-A light is inversely proportional to the concentration. Photodegradation kinetics of free DXR can be well described as nonlinear, with first order kinetics at lower concentrations ($<10^{-5}$M) and approaching zero order kinetics at higher concentrations ($>10^{-4}$M). While the degradation of DXR is concentration dependent in the range of 5–50 μg/ml, the degradation of Doxil shows no concentration dependency. As shown in Fig. 2, the rate constants for degradation of DXR in bulk solution and DXR in Doxil formulation calculated by linear regression are clearly different. The decay process of DXR in the Doxil formulation would be better described as linear with zero order kinetics.

Drug leakage from the liposomes was examined using the Dowex resin. Figure 3 shows that no significant leakage of the liposomes occurs within the interval of 5 hours UV-A irradiation. Similar results were obtained when leakage was examined at a concentration of 5 μg/ml Doxil, followed by a total fluorescence assay measurement (data not shown). These results indicate that the ammonium sulfate gradient which holds the encapsulated drug in the liposomes was not damaged by the UV irradiation, suggesting intactness of the liposome membrane.

Degradation studies of DXR performed in Hepes buffered saline showed a significantly stabilizing effect of Hepes buffered saline on the degradation in comparison to PBS (Fig. 4). The acceleration of the degradation of DXR in PBS may be due to chemical interactions between the phosphate group and

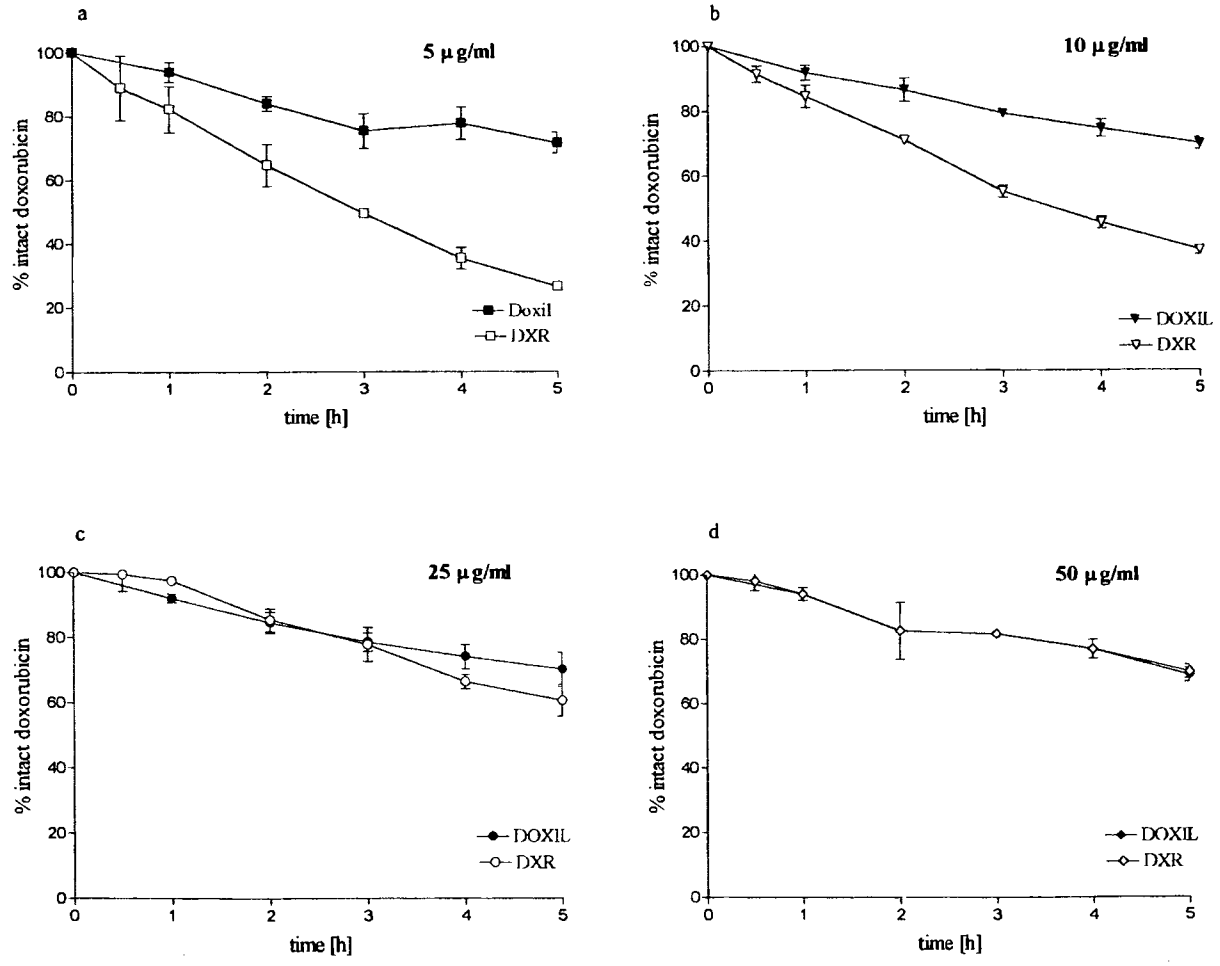

Fig. 1. Comparison of the photodegradation curves of different concentrations of doxorubicin in bulk solution (DXR) and doxorubicin in Doxil in phosphate buffered saline after UV-A irradiation, whereby 100% represents the initial drug concentration at timepoint 0.

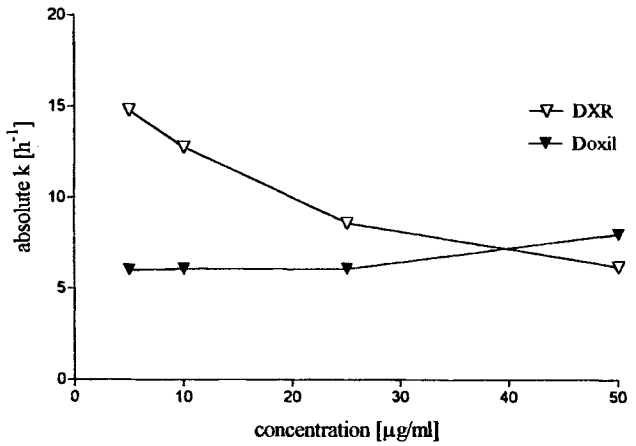

Fig. 2. Absolute values of the rate constants of degradation [k] of various concentrations of doxorubicin in bulk solution and doxorubicin in Doxil, calculated by fitting the data to the equation $[DXR] = [DXR]_0 + k.t.$

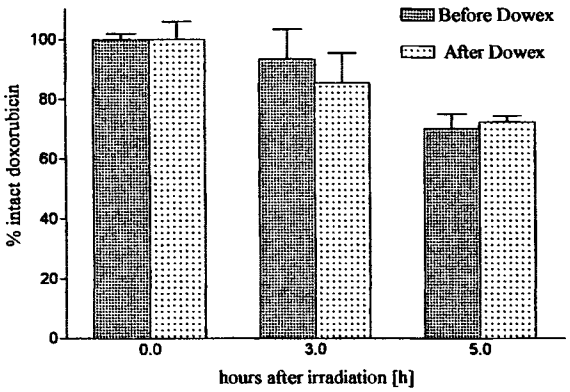

Fig. 3. Leakage of doxorubicin from liposomes at 50 μg/ml Doxil in phosphate buffered saline before and after UV-A irradiation.

DXR-molecules (19), with the phosphate ion acting as a base inducing alkaline hydrolysis of DXR. To avoid the confounding factor of the PBS effect, we decided to carry out the following experiments in Hepes buffered saline.

Exposure to 200 μM nigericin in the presence of 5 mM KCl under the influence of heat (50°C) resulted in the leakage of 80–90% of DXR from the liposomes. In separate experiments, it was found that nigericin does not interfere with the photodegradation of DXR.

After induced release of DXR from the liposomes with nigericin, the degradations of DXR released from liposomes and free DXR approach similar kinetics (Fig. 5), whereas no statistical significant difference between the means of the percents of intact DXR in both groups at the different time points could be observed ($p = 0.1–0.64$). Therefore, it can be concluded that the difference in the degradation kinetics between DXR in bulk solution and DXR in Doxil formulation is related to the liposomal encapsulation of DXR.

Finally, we investigated the role of intraliposomal factors on the degradation kinetics of DXR. Figure 6 represents the photodegradation curves of the three different liposomal preparations (I, II, and III) at a constant DXR-concentration in the liposomal suspension of 2.5 μg/ml. The degradation curves indicate that neither the physical state of DXR inside the liposomes (gel versus aggregates in solution), nor the change in the intraliposomal DXR-concentration in the range of ~35–0.5 mg/ml are critical in the protection of DXR from photodegradation. Intraliposomal DXR concentrations lower than 0.5 mg/ml were impossible to achieve using the current remote loading method.

Figure 7 shows that after 5 hours of irradiation, less than 20% of the initial drug concentrations in acetic buffered saline are degraded, while in Hepes buffered saline a more intensive degradation of DXR (30–50%) was observed. It can be concluded that, the acidic pH in the interior of the liposomes seems to play an important role in the photoprotection of DXR in Doxil. An additional photodegradation experiment done with DXR in the presence of ammonium sulfate salt at a similar concentration to the intraliposomal medium (100 mM) did not point at any protective effect (data not shown).

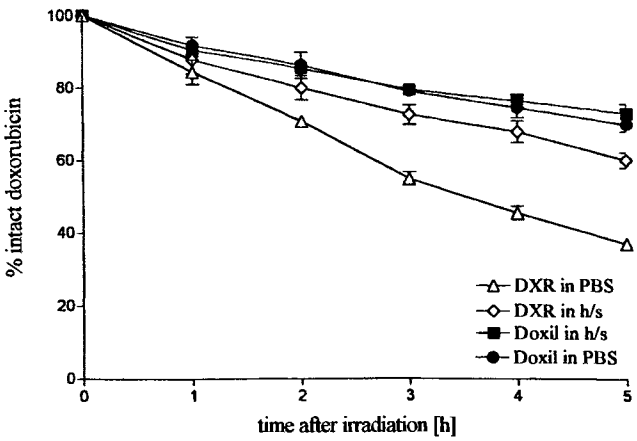

Fig. 4. Comparison of the degradation curves of 10 μg/ml doxorubicin (DXR) in bulk solution and 10 μg/ml doxorubicin in Doxil in Hepes buffered saline (h/s) and in phosphate buffered saline (PBS).

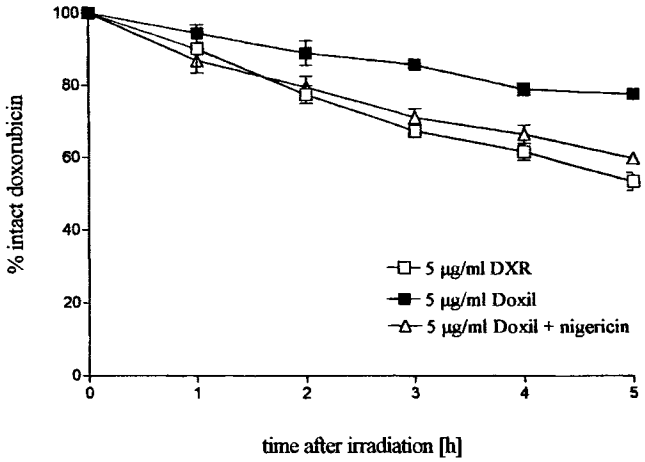

Fig. 5. Degradation of 5 μg/ml Doxil in Hepes buffered saline after induced leakage of doxorubicin from the liposomes with nigericin, in comparison to the degradation of the same concentrations of Doxil and doxorubicin (DXR).

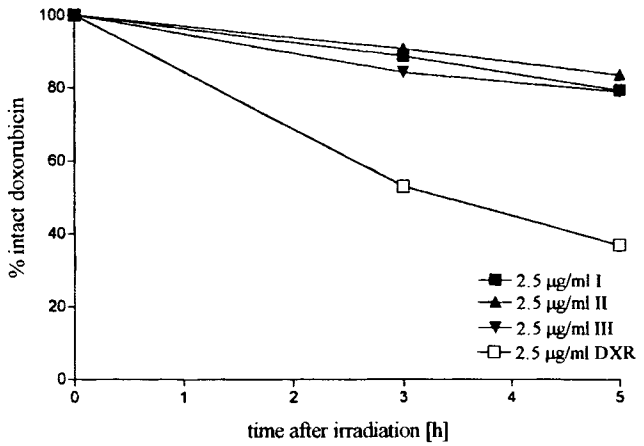

Fig. 6. Degradation curves of three different liposomal preparations of doxorubicin (I, II and III, containing ~35, 7 and 0.5 mg/ml intraliposomal doxorubicin, respectively) at 2.5 μg/ml doxorubicin in liposomal suspension, in comparison to the degradation of 2.5 μg/ml doxorubicin (DXR) in bulk solution.

DISCUSSION

This study demonstrates that DXR, when remote loaded into liposomes by the ammonium sulfate gradient, is protected from photodegradation by UV-A light, while DXR present in bulk solution is photodegradable in inverse relationship to its concentration. There are four main factors which may contribute to the differences between DXR in Doxil and DXR in bulk solution, with regard to stability top hotodegradation by UV light at the measured concentration range of 10^{-4}–10^{-5}M:

(i) Protection by the liposome lipid bilayer.

(ii) The very high concentration of DXR in the intraliposomal aqueous phase which is much above the measured concentration. The concentration-factor is equal to the ratio of *total solution volume/liposome trapped volume* (18).

(iii) DXR "gelation"/aggregation inside the liposomes as sulfate salt (10,20).

(iv) The low pH of the intraliposomal aqueous phase generated by the ammonium sulfate gradient (10).

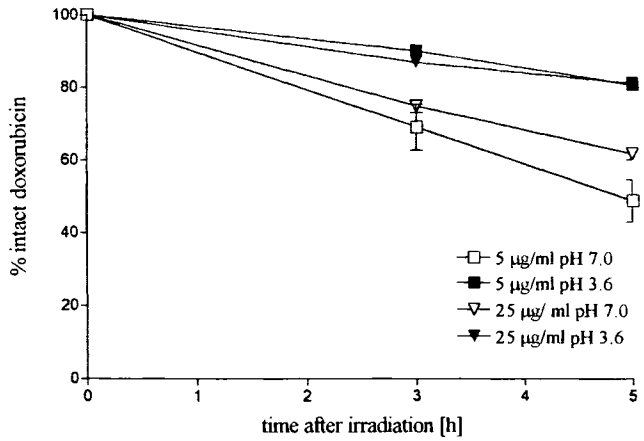

Fig. 7. Degradation of 5 and 25 μg/ml doxorubicin in Hepes buffered saline at pH 7.0, in comparison to the degradation of doxorubicin in acetic buffered saline at pH 3.6.

The first factor can be discarded as DXR in the internal aqueous phase of liposomes has normal absorbance even inside a bilayer of ~4 nm composed of components which do not absorb UV-A light (366 nm). On the other hand, even if we assume that dissolved oxygen is mediating the degradation of DXR molecules, the liposomal bilayer should not act as a barrier, since the solubility of dissolved oxygen in lipids or membranes is four times higher than in aqueous solutions (21). However, a liposome bilayer composed of high phase transition lipids, such as in Doxil, can protect encapsulated DXR from many of the photoenhancers present in biological fluids. In this regard, we have shown that Doxil circulates for prolonged periods of time in the organism without undergoing drug leakage over a period of several days (22).

Our studies indicate that the high intraliposomal concentration has a major contribution. Calculations based upon the trapped volume of these liposomes as spheres show that the range of intraliposomal calculated DXR concentration is from the low end of ~0.5 mg/ml to the high end of ~35 mg/ml. Thus, the lowest intraliposomal DXR concentration was at least 10 times higher than the highest concentration of DXR in bulk solution (50 μg/ml). Already at 50 μg/ml bulk concentration, DXR photodegradation occured at zero order kinetics and at a slow rate (Fig. 1d). Important support to this explanation is obtained from the experiment showing that releasing DXR of Doxil formulation brings it to the identical level of degradation, as when DXR is in bulk solution. It seems therefore that the actual concentration of DXR in the liposome compartment and not the measured DXR concentration, determines its rate and kinetic order of degradation. The direct contribution of DXR gelation (precipitation) is unlikely, since reduction of intraliposomal drug concentration to a soluble physical state did not increase sensitivity to UV-A light. Likewise, ammonium sulfate had no protective effect on UV-A-induced degradation.

The intraliposomal low pH is another factor which contributes to the increased stability of DXR in Doxil. It is well established that chemical stability of DXR in bulk solution is optimal at pH 3.6 (19,23). The ammonium sulfate concentration in Doxil formulation is 250 mM. Even at the highest level of DXR loaded in the liposomes, there is rather a large residual excess of sulfate in the liposomal aqueous phase and therefore the pH will remain acidic.

What is the mechanism of protection of liposomal DXR from photodegradation? Our results show that photodegradation is not a bimolecular reaction and therefore is not dependent on the collision between DXR molecules (or their reactive species). The proposed mechanism of degradation suggests a photooxidative process with or without direct involvement or interaction between excited DXR (or DXR derivatives) and oxygen (or singlet excited state oxygen). Such a process may be affected by the state of aggregation or by the polarity of the DXR molecules in close proximity. Both may reduce the photodegradation either due to reduction in availability of oxygen reactive species to DXR, or due to increased rate of recombination between DXR reactive species which "break" and stop the photooxidative reaction. The inverse relationship of photodegradation with DXR bulk concentration supports the explanation of neutralization of DXR reactive species by recombination.

It can be concluded that the internal microenvironment of liposomes, which differs from the bulk solution (i.e., low pH) and the actual concentration of the agent have a large effect

on the stability of the agent. When the agent encapsulation does not reduce therapeutic efficacy, as in the case of Doxil (24), the improved photostability may be another added value to liposomal formulations.

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