Acta Oncologica Vol. 33, No. 7, pp. 779–786, 1994

CLINICAL STUDIES OF LIPOSOME-ENCAPSULATED DOXORUBICIN

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Initial clinical studies with doxorubicin entrapped in the bilayer of phosphatidylglycerol-rich liposomes were hindered by the avid reticuloendothelial system (RES) uptake and by drug leakage from circulating liposomes. In contrast, recent tests of a doxorubicin formulation of polyethyleneglycol-coated liposomes (Doxil) in cancer patients indicate that the drug pharmacokinetic properties are significantly altered, with a prolonged distribution half-life of approximately 2 days. Plasma fractionation studies show that nearly all the drug measured in plasma is in liposome-encapsulated form. The dose of Doxil has been escalated from 25 to 60 mg/m^2. Stomatitis is the most significant toxicity, and skin toxicity, in the form of hand-foot syndrome, may complicate the repeated administration of Doxil. A number of objective antitumor responses in a variety of malignancies have been observed, indicating that Doxil is an active antitumor compound. Polyethyleneglycol-coated liposomes show a distinct advantage over previous liposome formulations directed at the RES and appear to be a promising drug delivery system for doxorubicin.

One of the most encouraging and prolific areas in the liposome-anticancer drug field is the work with anthracyclines. A large body of preclinical pharmacology is available from the literature (reviewed in ref. 1), and a number of clinical trials are ongoing (reviewed in ref. 2). The administration of doxorubicin (DOX) in liposome-encapsulated form has been advocated as a means of reducing the cardiac damage induced by anthracyclines (1,2). Preclinical investigations from several laboratories have established that the use of DOX encapsulated in a variety of liposome formulations results in a substantial decrease of drug uptake by the heart (3–5) and in a significant attenuation or prevention of the characteristic cardiomyopathy resulting from exposure to anthracyclines (6–8).

Thus, one approach in the clinical development of liposomal DOX is to enable the administration of greater cumulative doses of DOX without risk of cardiotoxicity. Phase I and II clinical studies have been reported for various formulations of liposomal DOX (9–12), and mitoxantrone (13). In all of these studies, the dose-limiting toxicity has been myelosuppression, much alike that of free DOX. Although some of these studies suggest a reduced cardiotoxicity of liposomal anthracyclines in humans, the present information is as yet inconclusive on this issue.

While buffering the drug toxicity is an important element of a drug delivery approach, the key part of any strategy in cancer chemotherapy is to enhance the exposure of tumor cells to the drug. Recent studies in this field have demonstrated that specific types of liposomes, also referred to as stealth or sterically stabilized liposomes (reviewed in ref. 14), can circulate in the blood for prolonged periods of time without being trapped in the reticulo-endothelial system (RES). The best example is given by liposome formulations containing a small fraction of a polyethylene-glycol (PEG)-derivatized phospholipid which have been shown to alter dramatically the pharmacokinetic properties of encapsulated DOX in rodents and dogs, leading to long distribution half-lives and small volumes of distribution (15–16).

In this article, we summarize our clinical and pharmacokinetic studies in patients treated with two formulations of liposomal DOX: a 'first generation' liposome carrier of

Received 11 November 1993.
Accepted 18 May 1994.
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Presented at the 18th International Congress of Chemotherapy, Stockholm, Sweden, June 27–July 2, 1993.

 ISSN 0284-186X

short circulation time (abbreviated as L-DOX); and, a 'second generation' liposome of long circulation time (referred to as Doxil), shown to enhance the accumulation of DOX in experimental tumors. Part of the results discussed here have been presented in detail in previous reports (9,17–18).

Material and Methods

Formulation. The general aspects of the formulations used are described in Table 1. L-DOX was prepared in our laboratory as described previously (9). The Doxil formulation (19), also known as DOX-SL[1], was provided by Liposome Technology Inc. (Menlo Park, CA). The studies reported here were done with a frozen storage form of Doxil. Treatment was administered through the IV route either by slow bolus (Doxil, 25 and 50 mg/m^2 only) or by rapid ($\sim$1 h) drip infusion (Doxil, 60 mg/m^2, and all L-DOX dose levels). The dose of L-DOX and Doxil is based on the doxorubicin content.

Patients. The criteria of patient selection were those generally adopted for phase I clinical trials. Pretreatment evaluation and follow up included physical examination and Karnofsky's performance status, chest x-ray, ECG, and left ventricle ejection fraction, complete blood counts before each treatment and weekly thereafter, complete biochemistry panel before and after each treatment course, and tests to document the extent of malignant disease. Grading of toxicity was according to WHO recommendations (19).

Pharmacokinetics of DOX. Blood was collected in Vacutainer tubes with K_3-EDTA anticoagulant and kept at 5°C. Plasma was separated by centrifugation usually within 1 h and not later than 24 h after blood collection, and stored at -20°C. Fractionation of liposome-associated DOX and plasma determination of DOX and its metabolites was done as previously described (16–18).

Imaging studies. These were done by radiolabeling deferoxamine-containing liposomes with ^{111}In using an ^{111}In-oxine complex (Amersham). Further details on the labeling and on the patient imaging technique have been previously reported (17).

Results and Discussion

Clinical studies with DOX entrapped in PG-PC-Chol liposomes

The entrapment of DOX in the liposome bilayer is significantly enhanced by the presence of negatively-charged phospholipids such as PG (20). In animal studies, the administration of DOX entrapped in PG-PC-Chol

Table 1

Comparison of DOX liposomes: a 'first generation' carrier (L-DOX) versus a 'second generation' stealth type carrier (Stealth-DOX)

	L-DOX	Stealth-DOX[a]
Liposome type	RES-directed, Short circulation time	Sterically-stabilized, Long circulation time
Lipid composition (approximate molar ratio)	PC:PG:Chol 50:20:30	HPC:PEG-DSPE:Chol 55:5:40
T_m of matrix lipid (PC,HPC)	< 0°C	52°C
Surface charge in: Low ionic strength High ionic strength	 Strongly negative Negative	 Negative Almost no charge
Size (diameter in nm)	200–500	< 100
Number of lamellae	Oligolamellar	Mostly unilamellar
Location of drug	Membrane-associated	Aqueous interior phase
Drug/phospholipid molar ratio	0.05–0.07	0.1–0.25
Release of drug upon dilution	Fast and K_p dependent	None
Storage form	Liquid, liophilized	Liquid, frozen
Plasma pharmacokinetics[b]: 1st $t_{1/2}$ 2nd $t_{1/2}$	 5 min 12 h	 3 h 45 h

[a] Doxil is a stealth-DOX type of formulation.
[b] From refs. (17) and (18).
Abbreviations: PC = egg phosphatidylcholine; PG = egg-derived phosphatidylglycerol; Chol = cholesterol; HPC = hydrogenated soybean phosphatidylcholine; PEG-DSPE = polyethylene-glycol-distearoyl-phosphatidylethanolamine.

liposomes (L-DOX) results in decreased cardiac exposure to the drug, decreased cardiotoxicity, and decreased systemic toxicity (20). Thus, the mouse LD_{50} of L-DOX is approximately twice that of free DOX. Liposome-entrapped DOX is rapidly cleared from plasma by the RES, leading to an increased deposition of drug in the liver and spleen (21). This may account for the superior antitumor effect of the liposome formulation over free drug when tested against tumors infiltrating liver and spleen. Liposome-entrapped DOX and free DOX were equally effective against bone marrow-residing leukemia cells. However, the antitumor activity of liposome-entrapped DOX was inferior to that of free DOX when tumor cells are inoculated in extra-RES compartments (22). Despite this limitation, the important pharmacologic advantages of this PG-containing formulation were attractive enough to justify clinical testing.

L-DOX was tested in cancer patients within the frame of a phase I clinical-pharmacokinetic study (9,17). A total of 35 patients were entered to this study (Table 2). Because of our prior preclinical experience, preference for accrual was given to patients with primary or secondary neoplastic liver disease. Only 5 patients did not have neoplastic involvement of the liver. The maximal tolerated dose (MTD) was 100 mg/m^2 as judged by WHO toxicity criteria. Above this dose, grade 4 toxicity, in the form of myelosuppression and stomatitis, was observed in 2 out of 3 patients receiving 120 mg/m^2. The MTD of 100 mg/m2 points at a somewhat reduced toxicity of the liposomal drug considering that the MTD for the free drug is 75 mg/m^2 (23). However, these results are still inferior to the observations in rodents of a twofold decrease in the toxicity of doxorubicin (21). In patients receiving repeated courses of L-DOX, there was no indication of cumulative toxicity or cardiotoxicity, although, due to rapid tumor progression, most patients received a relatively low cumulative dose of the drug (only 2 patients received a cumulative dose >400 mg/m^2). Regarding anti-tumor activity, one partial response in a hepatoma patient with a 5-fold decrease in the α-fetoprotein titer was observed.

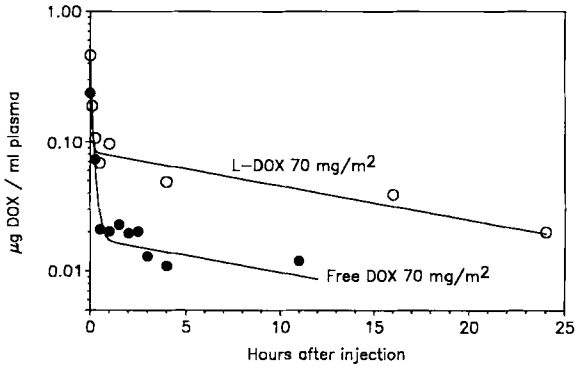

Fig. 1. Plasma clearance of doxorubicin in two patients suffering from hepatocellular carcinoma and receiving 70 mg/m^2 of either L-DOX (O) or free DOX (●). Treatment was administered by a 1 h intravenous infusion. The time points are calculated from the end of the infusion. With free DOX, drug levels were not detectable beyond the 11 h time point.

The pharmacokinetics of L-DOX was examined in cancer patients within the frame of this phase I study. The pharmacokinetic parameters were of a similar order of magnitude to those reported for free DOX (17). Fig. 1 depicts the plasma concentration/time curves of doxorubicin, after administration of free DOX and L-DOX to two hepatoma patients at the same dose level (70 mg/m^2). It may be seen that the two clearance curves are very similar although L-DOX resulted in slightly higher drug levels at the end of infusion and throughout the terminal elimination phase. Although not shown here, doxorubicinol was generally readily detectable within less than 1 h

Table 2

L-DOX clinical study: patient characteristics

Number of patients (male/female)		35 (20/15)
Age in years — median (range)		58 (22–75)
Primary tumor site		
Colon and rectum cancer		16
Liver (hepatoma)		12
Soft tissue sarcoma		2
Melanoma		1
Small cell lung cancer		1
Breast cancer		1
Pancreatic cancer		1
Stomach cancer		1
Liver involvement (primary or secondary)	Yes/No	30/5
Previous chemotherapy — Yes/No		26/9
Previous treatment with DOX — Yes/No		8/27
Number of L-DOX courses:		
Total number		75
Median number per patient (range)		2 (1–7)

Table 3

Relative decrease of liposome-associated drug in plasma in patients with extensive liver involvement receiving L-DOX

Diagnosis	Dose (mg/m^2)	% DOX in liposome-associated form at peak plasma concentration[a]	Liver involvement[b]
Colon cancer	100	100	0
Colon cancer	85	100	I
Colon cancer	120	100	0
Pancreatic cancer	85	81	II
Hepatoma	100	71	II
Hepatoma	120	62	Lobectomy
Colon cancer	120	55	III

[a] Measurement of drug level in plasma at the end of infusion of L-DOX.
[b] Grading of liver involvement with tumor (ref. 33): 0 = no hepatic involvement; I = < 25% hepatic replacement with tumor; II = 25–75% hepatic replacement; III = > 75% hepatic replacement.

after end of infusion, suggesting that L-DOX rapidly becomes bioavailable (18).

One important observation of this study was the reduced tolerance to treatment with liposomal DOX in patients with extensive tumor involvement of the liver (9,17). This was apparently due to a delay in the clearance of liposome-associated drug by the affected liver followed by extensive leakage of the drug from circulating liposomes. Thus, in 7 patients in whom we calculated the fraction of circulating drug released from liposomes, the results suggested a positive correlation between the degree of liver involvement, and the amount of free drug released (Table 3). To minimize the amount of DOX leaking from PG liposomes, the rate of RES-mediated clearance must be substantially faster than the rate of leakage. The rate of drug leakage is quite constant and dependent on the physicochemical characteristics of the formulation (23). In contrast, the rate of liposome uptake by the RES is difficult to predict and may be influenced by species differences, by a variety of physiopathological conditions, and by a saturation effect related to the lipid dose (25).

The PG-PC-Chol formulation was labeled with ^{111}In to study the liposome biodistribution in a subgroup of cancer patients within the frame of the phase I clinical trial of L-DOX. These liposomes had similar physicochemical characteristics to L-DOX, except that they were devoid of the drug. We found that the ^{111}In-labelled liposomes were rapidly cleared by liver and spleen. However, no label uptake was noticed in intrahepatic tumor nodules (Fig. 2).

Clearly, the PG-PC-Chol liposome carrier system has two important drawbacks: leakage of the bilayer intercalated drug, and lack of tumor targeting. To prevent drug leakage and reduce the toxicity of DOX, the vesicles are to be quickly cleared from the circulation by the RES. Further, accumulation of the liposome-associated drug in RES

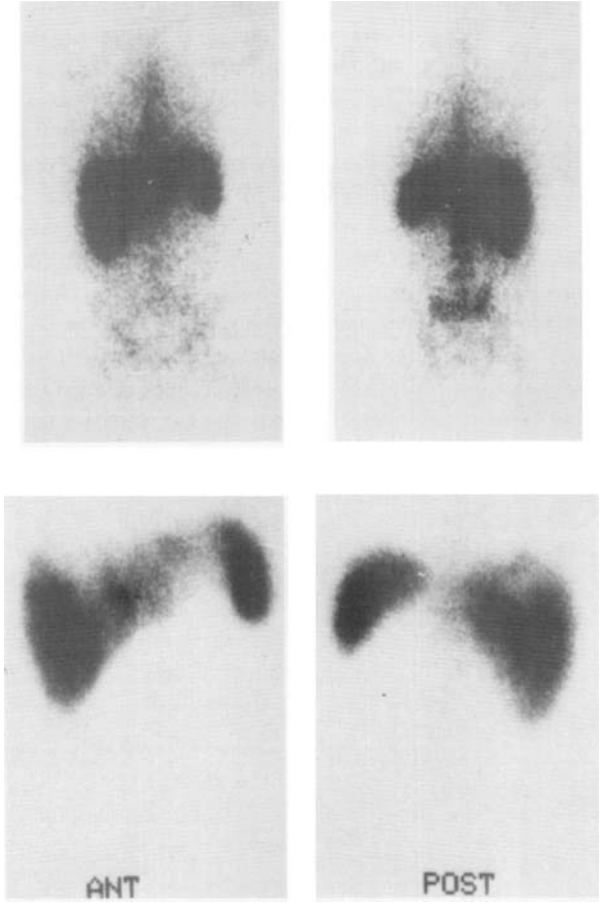

Fig. 2. Whole body scintigraphy with ^{111}In-labelled liposomes in a hepatoma patient. Upper panel: Image taken 5 min after injection. Note rapid biodistribution of the label to the RES with prominent uptake in liver and spleen, and faint uptake in the skeletal bone marrow. The area of the heart is labeled by liposomes remaining in circulation. Lower panel: Image taken 2 h after injection. Filling defects corresponding to tumor nodules can be recognized in the left lobe of the liver.

Table 4

Doxil clinical study: patient characteristics

Number of patients (male/female)		23 (9/14)
Age in years median (range)		59.5 (33–73)
Primary tumor site		
Breast cancer		7
Non small cell lung cancer		4
Ovarian cancer		4
Mesothelioma (peritoneal/pleural)		2(1/1)
Soft tissue sarcoma		1
Sebaceous gland cancer		1
Cancer of unknown origin		1
Renal cell cancer		1
Colorectal cancer		1
Gall bladder cancer		1
Liver involvement (primary or secondary)	Yes/No	5/18
Previous chemotherapy—Yes/No		16/7
Previous treatment with DOX—Yes/No		7/16
Number of Doxil courses		
Total number		102
Median number per patient (range)		3 (1–10)

may in fact reduce drug availability to extrahepatic as well as intrahepatic tumors (17). The complexity and lability of this system was not foreseen by murine models.

Clinical studies with long-circulating DOX liposomes

Two important developments in the design of DOX liposomes were critical in overcoming the obstacles mentioned above: drug loading methodology enabling the encapsulation of DOX in the water phase of preformed liposomes; and, use of liposome components leading to improved drug retention, steric stabilization, and prolonged circulation times. The former is exemplified by methods to generate proton gradients that drive DOX into the liposome water phase (26,27). The latter depends on various factors such as the presence of high T_M phospholipids (distearoyl-PC, hydrogenated PC) to achieve stable drug retention (16,28) and some specific lipids (monosialoganglioside, phosphatidylinositol, PEG-derivatized phosphatidylethanolamine) to inhibit opsonization and RES uptake (15,29,30). Progress made along these lines has resulted in one formulation of doxorubicin encapsulated in PEG-coated liposomes referred to in 'Material and Methods' as Doxil.

We have treated 23 cancer patients with a frozen storage form of Doxil at dose levels between 25 and 60 mg/m^2, every 3 to 4 weeks (Table 4). The first group of 15 patients were treated within the frame of a study aimed at examining the pharmacokinetics of Doxil (18) and included 8 patients who received 25 mg/m^2 Doxil as first dose with escalation to 50 mg/m^2 in the second dose, and 7 patients who received 50 mg/m^2 as first dose of Doxil. The second group of 8 patients received 60 mg/m^2 Doxil within the frame of a phase I study to investigate tolerance and clinical effects. Altogether, 102 courses (8, 45, and 49 courses at 25, 50, and 60 mg/m^2 respectively) of Doxil were given. Treatment was generally well tolerated. In five instances, there was an immediate reaction characterized by facial flushing and shortness of breath which was resolved within minutes by discontinuing or reducing the rate of injection. These reactions did not occur when the rate of injection was maintained or resumed at below 5 ml/min. Nausea occurred frequently, generally in a delayed (i.e., 24 h post treatment) and mild fashion (WHO grades 1 and 2), extending for several days. Vomiting was reported only in sporadic cases. The most severe side-effect was stomatitis, occurring 7 to 14 days after treatment in 13/23 patients at doses of 50 mg/m^2 to 60 mg/m^2. Grade 3–4 stomatitis requiring dose reduction was observed in four patients, two at 50 mg/m^2 and two at 60 mg/m^2. Myelosuppression in the form of leukopenia/neutropenia was generally mild (grade 1 or 2) and always afebrile. Nadir WBC counts below the normal limit of 4 000 μl were observed on day 14 after injection in less than 50% (6/15) of patients dosed with 50 mg/m^2 Doxil. As seen in Table 5, the median nadir WBC following the first course of Doxil 60 mg/m^2 was 2500 μl, with a trend to slightly lower values (2000 μl) when all courses are considered. No significant thrombocytopenia or anemia related to Doxil were observed at any dose level. Following the 25 mg/m^2 dose of Doxil, there was neither myelosuppression nor stomatitis. In five patients, a desquamating dermatitis, affecting mainly palms of hands and soles of feet and equivalent to the Hand-Foot Syndrome (31), was noticed after 3 or more courses of Doxil. Three of these patients developed moderate to severe reactions, requiring dose modification. This reaction

Table 5

Myelosuppressive effect of Doxil at 60 mg/m² [a]

Cell counts × $10^3/\mu l$ blood	1st course	All courses
Median WBC nadir (range)	2.5 (0.9–5.0)	2.0 (0.9–3.9)
Median granulocyte nadir (range)	1.6 (0.5–3.2)	1.2 (0.4–2.5)
Median platelet nadir (range)	220 (33–296)	100 (33–226)

[a] Number of patients, 8; Number of courses, 49; Median number of courses per patient (range), 7 (1–10); Number of patients/courses in whom a dose reduction took place (−25%) due to grade 3–4 toxicity, 4/5.

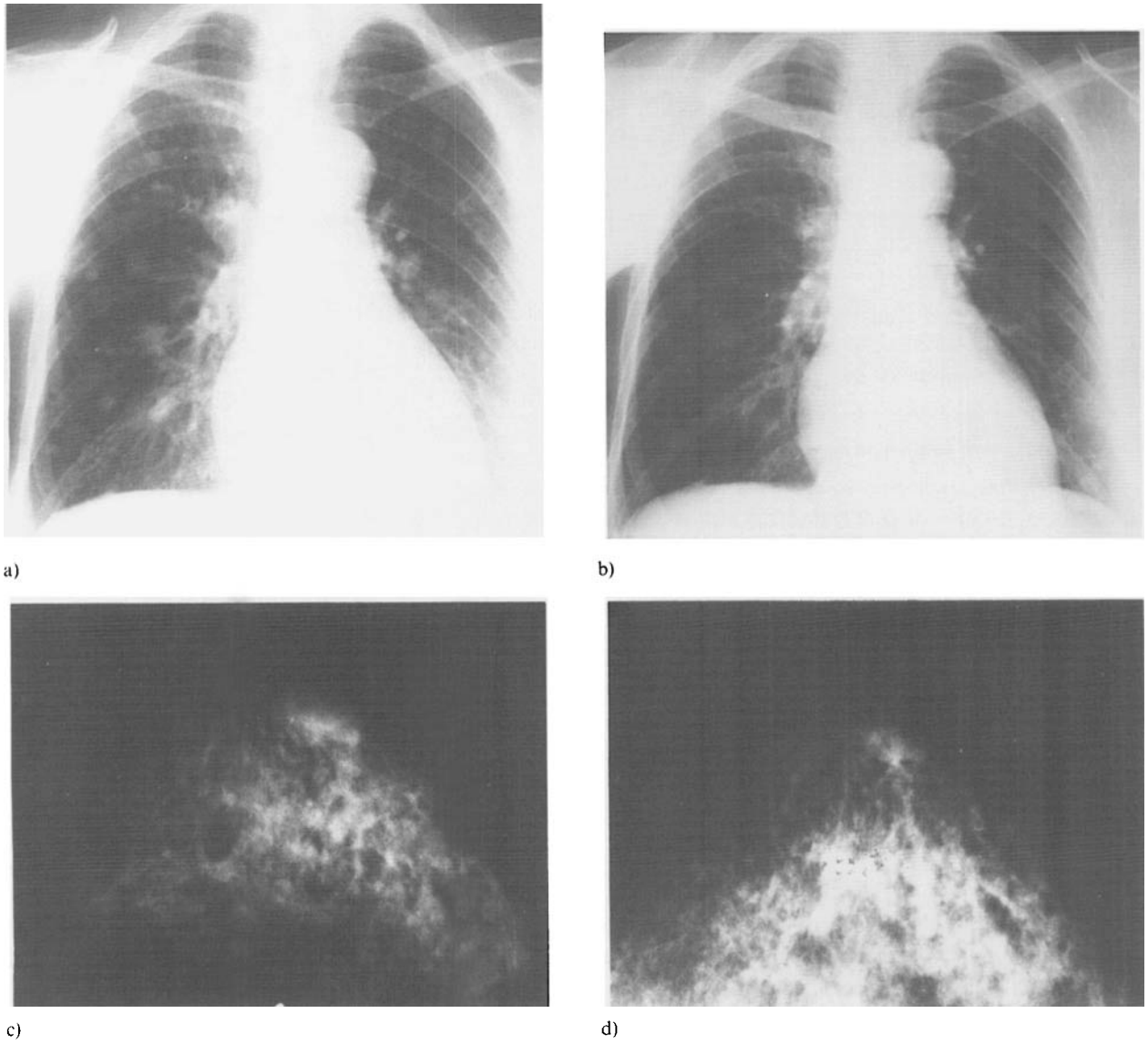

Fig. 3. 54-year-old patient suffering from metastatic breast cancer in lungs, proven by fine needle aspiration, and contra-lateral breast. This patient was previously treated with chemotherapy including epirubicin. An anti-tumor response to Doxil was documented in both sites of metastatic disease, lungs and breast. Response to treatment was considered partial since CT-scan of the chest revealed residual lung nodules. She remained without any evidence of relapse and without any maintenance therapy during 9 months after completion of 10 courses of Doxil, when regrowth of the pulmonary hilar lymph nodes was noticed by CT-scan. Upper panel left: Status after right mastectomy. Pretreatment chest x-ray shows multiple metastatic nodules in both lungs. Upper panel right: After 10 courses of Doxil (60 mg/m²), chest x-ray shows complete resolution of metastatic disease. Lower panel left: On mammography, a pretreatment left cranco-caudal view shows an irregular lobulated mass in the central portion of the breast. Lower panel right: Posttreatment shows resolution of the mass. Physical examination was consistent with the disappearance of a palpable mass.

waned and gradually healed within a 2- to 3-week period, and may be related to a cumulative toxic effect of Doxil on the skin. The Hand-Foot Syndrome is a known side-effect of various chemotherapy regimens and has also been reported with continuous infusion of doxorubicin (32).

The median cumulative dose of Doxil administered was 150 mg/m^2 (range, 50–600). There were no clinical or angiocardiographic (decrease of the left ventricle ejection fraction) signs of cardiac toxicity in 8 patients receiving 5 or more courses of Doxil, including a patient who received a cumulative dose of 235 mg/m^2 Doxil after a prior dose of 540 mg/m^2 free DOX, and 5 patients who received 450 to 600 mg/m^2 cumulative dose of Doxil. Altogether, dose reduction for grade 3–4 (moderate to severe) toxicities was required in 4/15 patients (8/45 courses) at 50 mg/m^2 and 4/8 patients (5/49 courses) at 60 mg/m^2. A phase I study to define the MTD is currently ongoing with an improved reformulation of Doxil in liquid storage form.

Regarding anti-tumor activity, we observed three objective anti-tumor responses (breast cancer, renal cell cancer, non-small cell lung cancer) at the highest dose given, 60 mg/m^2, with 'times to treatment failure' of 18, 7, and 4 months respectively. The most dramatic and long-lasting response was observed in the patient with metastatic breast cancer and is described in Fig. 3. In the patient with renal cell cancer, there was a partial regression of the lung metastases but, later on, the skeletal metastatic disease progressed. In the patient with non-small cell lung cancer (adenocarcinoma type), the lung and liver lesions shrank significantly and the C.E.A. level dropped from 462 to 93 ng/ml, but a new paravertebral mass developed 4 months after initiation of treatment. Stable disease was noticed in two additional patients (peritoneal carcinomatous spread most probably of ovarian origin; lung metastases of rectal cancer) for periods of 10 and 16 months respectively. Two additional responses (ovarian cancer, peritoneal mesothelioma) observed at 50 mg/m^2 are difficult to evaluate since these patients received previously a course of free doxorubicin.

Fig. 4 shows the plasma clearance curves of doxorubicin in two representative patients receiving 25 mg/m^2 (Fig. 4A) and 50 mg/m^2 (Fig. 4B) of free DOX and Doxil, separated by a 3-week rest period. Free doxorubicin clears very rapidly and distributes extensively to body tissues, while Doxil is slowly cleared from circulation and has a very small volume of distribution. The plasma concentration/time curve of free DOX is clearly biexponential, with an initial phase of extremely short half-life (5 min), while that of Doxil is quasi-monoexponential or biexponential with most of the drug being cleared from plasma with a slow half-life of 45 h. Parameters such as volume of distribution and clearance are decreased by more than two orders of magnitude using Doxil. Based on this delayed clearance, the frequency of administration of Doxil was gradually moved from 3 to 4 weeks. Another important

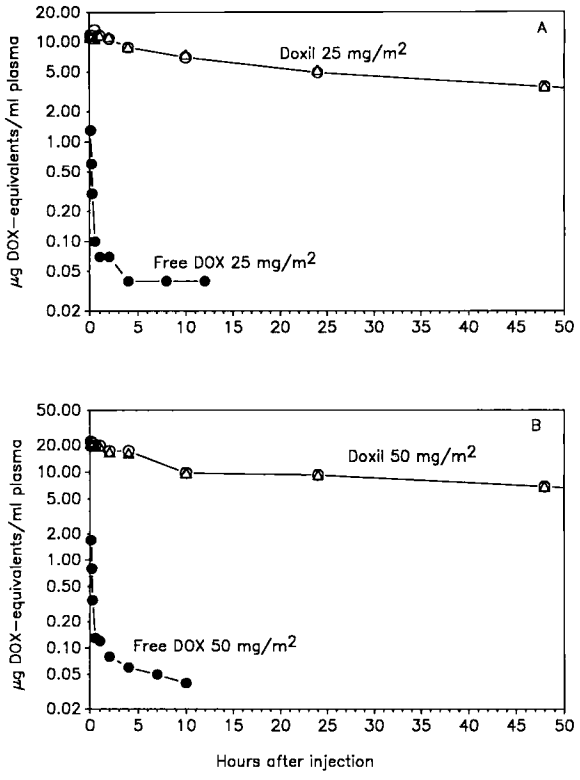

Fig. 4. Plasma clearance of Doxil and free DOX. Treatment administered by slow bolus injection (5–15 min). ○ = total DOX level in plasma following Doxil treatment; △ = liposome-associated DOX level in plasma following Doxil treatment; ● = total DOX level in plasma following free DOX treatment. With free DOX, drug levels were not detectable beyond the 10 h time point. A) 44-year-old male patient suffering from peritoneal mesothelioma received free DOX (25 mg/m^2) followed 3 weeks later by Doxil (25 mg/m^2). B) 47-year-old female suffering from ovarian carcinoma received free DOX (50 mg/m^2) followed 3 weeks later by Doxil (50 mg/m^2).

point of Doxil pharmacokinetics, illustrated in Figure 4, is the fact that practically all of the circulating drug (circles) was accounted for by liposome-associated drug (triangles). Doxorubicin metabolites in plasma were either undetectable or present in negligible amounts. This is consistent with stable retention of the drug in liposomes, resulting in delayed bioavailability and metabolism. These observations suggest that, in the case of Doxil, the pharmacokinetics of doxorubicin is controlled by its liposome carrier.

Concluding remarks

The clinical studies summarized here clearly indicate that the shift from a 'first generation' formulation of liposomal DOX (L-DOX, RES-directed liposome with bilayer-intercalated DOX), to a 'second generation' stealth-type formulation (Doxil, long-circulating liposome with water phase-entrapped DOX) leads to substantial changes in the drug pharmacokinetics. Further clinical studies are needed to determine whether these changes will

modify significantly the clinical activity of the drug, at the level of antitumor efficacy or toxicity. Because of the reduced clearance of Doxil and the likelihood of an altered tissue distribution pattern, treatment with Doxil may result in a greater dose intensity than an equivalent dose of free DOX. The current studies suggest that Doxil is reasonably well tolerated at 60 mg/m^2 every four weeks and shows appreciable antitumor activity.

ACKNOWLEDGEMENTS

Work supported in part by Liposome Technology Inc. (Menlo Park, CA, USA), and the Robert Szold fund for Applied Science (Jerusalem, Israel). A. Gabizon is the recipient of a research career award of the Israel Cancer Research Fund.

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