Cancer Chemother Pharmacol (1994) 35: 84–88

SHORT COMMUNICATION

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A new procedure for the preparation of liposomal doxorubicin: biological activity in multidrug-resistant tumor cells

Received: 8 October 1993 / Accepted: 1 June 1994

Abstract We describe a new procedure for the preparation of liposomal doxorubicin. Doxorubicin can be efficiently complexed to preformed or lyophilized cardiolipin-containing liposomes. Complex formation was performed by vigorous vortexing. As much as 96.8% of the initial drug quantity may be bound to those liposomes under optimal incubation conditions (4 h at 37 °C). The binding study showed the presence of two levels of specific binding (dissociation constants, 28 ± 8 μ*M* and 1.0 ± 0.3 m*M*). The drug is firmly integrated in the liposome-membrane lipid bilayer rather than binding at the surface. Cytotoxicity studies using tumor cells revealed efficient drug delivery using liposome-complexed doxorubicin. This new liposomal doxorubicin preparation reverses multidrug resistance in MCF-7/ADR and CH LZ cells at levels equivalent to that obtained with a previously described liposome-encapsulated doxorubicin preparation, showing that the drug is integrated as well in the liposome carrier and is transported as well into cells. Increased concentration of liposomes at the subcytotoxic level in liposome-complexed doxorubicin enhances drug cytotoxicity in multidrug-resistant CH LZ cells as compared with liposome-encapsulated drug. This new preparation for liposomal doxorubicin may be carried out immediately prior to clinical administration, offering advantages in terms of cost and stability.

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Key words Doxorubicin · Liposome · Drug resistance

Abbreviations *Dox* Doxorubicin · *Lip* liposomes · *Dox-Lip* liposomally encapsulated doxorubicin · *Dox+Lip* liposomally associated doxorubicin · *MDR* multidrug resistance

Introduction

Doxorubicin (Dox) has been proven to be one of the most effective single antitumor agents and has demonstrated activity in a wide spectrum of solid and hematological malignancies [4]. However, its effectiveness is limited by the potential for dose-limiting cardiotoxic effects [3, 4]. Thus, investigations have been focused on designing less cardiotoxic anthracycline formulations [16]. Encapsulation of Dox in liposomes has been shown to prevent chronic Dox cardiotoxicity in mice [1, 6] and dogs [5] and to enhance antitumor activity [11, 15]. A liposomal delivery system for Dox was found to reduce drug uptake in cardiac tissue. Although the use of liposomes as carriers of Dox has been extensively investigated [1, 5, 6, 10, 11, 14, 15, 20], development of liposomal Dox was hindered by formulation problems. Many preparations are difficult to manage at the industrial level, especially in terms of achieving a good encapsulation efficiency and preserving drug stability. In this report, we describe a new liposomal doxorubicin preparation that greatly simplifies the industrial preparation process and the clinical treatment practicality.

Materials and methods

Materials

Dox was purchased from Adria Laboratory. Egg phosphatidylcholine cholesterol and bovine cardiolipin were obtained from Sigma Chemical Company. Tissue-culture components were obtained from Gibco-BRL.

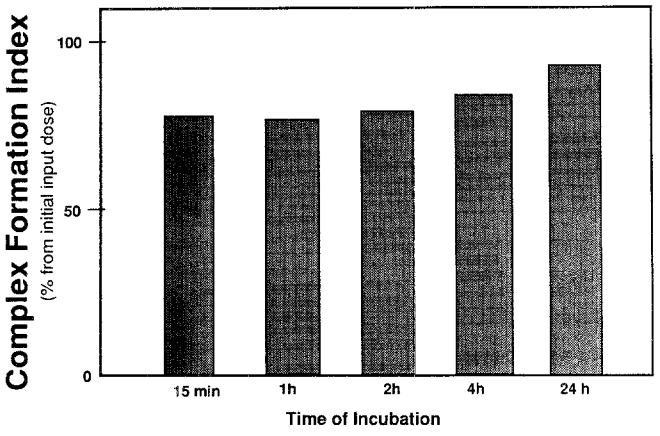


Fig. 1 Complex-formation efficiency is shown during incubation of Dox with cardiolipin-containing liposomes. Drug-association efficiency was determined as described in Materials and methods

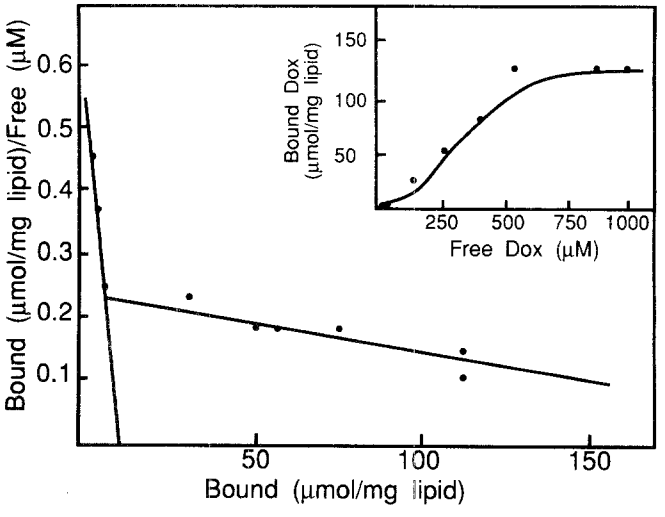
Liposome preparation

Small unilamellar vesicles were formed by mixing 19.1 μmol cardiolipin, 96.2 μmol phosphatidylcholine, and 64.6 μmol cholesterol (molar ratio, 2:10:7). After a thorough stirring, the mixture was evaporated to dryness in a 50-ml round-bottom flask using a rotary evaporator. The subsequent dried lipid film was resuspended in 10 ml sterile 0.9% NaCl solution. After a 30-min swelling time, the resulting suspension was sonicated (Heat System, W200f) in a fixed-temperature bath at 25 °C for 15 min. Liposomes (Lip) were then washed by extensive dialysis in sterile 0.9% NaCl solution. The final concentration of lipids was 15.6 μmol/ml or 11.3 mg/ml. Liposome suspensions were stored at 4 °C and used within 3 weeks of preparation. The lyophilisate of liposomes remains stable for at least 6 months.

Liposomal doxorubicin preparation

Complex formation and integration of Dox into the lipid bilayer was achieved by simple vortex mixing of a tube containing cardiolipin-liposomes and a doxorubicin solution prepared in 0.9% NaCl at 2 mg/ml. Vortex mixing was performed for 1 min and the mixture was kept at 25 °C for a 15-min period of incubation. The Dox concentration in liposomal Dox was determined following centrifugation of the preparation (70,000 g for 30 min) and by measurement of the fluorescence (470 nm, excitation; 580 nm, emission) of an aliquot of the subsequent supernatant. Liposome-encapsulated doxorubicin (Dox-Lip) was prepared as previously described elsewhere [15].

Fig. 2 Specific binding of Dox to cardiolipin liposomes is shown. *Insert:* Direct binding of Dox. Experiments were performed as described in Materials and methods. *Main chart:* Scatchard plots obtained from the above results. Each point is the average of two experiments performed in duplicate



Binding study

Binding of Dox to the cardiolipin-containing liposomes was measured by mixing 100 μl Lip into 400 μl Dox solution in an eppendorf tube. The mixture was immediately subjected to 15 s of vortexing and incubated at 25 °C for 1 h. Then the reaction medium was vigorously vortexed for 15 s and again incubated for 1 h at 25 °C. The tube contents were placed in a 10-ml centrifuge tube and subjected to centrifugation (70,000 *g* for 30 min). The concentration of free Dox in the preparation was determined by measuring the fluorescence of an aliquot of the subsequent supernatant. The dissociation constant (K_d) and binding capacity (B_{max}) were ascertained by Scatchard analysis.

Cell culture

Human breast cancer cells MCF-7/ADR were grown in Improved Modified Essential Medium (formula 88-0121 AJ, Gibco). Chinese hamster lung-fibroblast cells LZ were kindly provided by Dr. J. A. Belli (Galveston, Tex.) and were grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum and 2 m*M* L-glutamine. CH LZ and MCF-7/ADR cell lines have the multidrug-resistance phenotype and were established following a multistep selection process using Dox [2, 7].

Cytotoxicity assay

Drug cytotoxicity was determined by colony-forming assay after a 4-h cell treatment as previously described [17]. The IC_{50} value was defined as the concentration of drug resulting in 50% survival of the treated colonies as compared with controls.

Results

Formation of the complex liposome-Dox was evaluated relative to the time of the incubation at 25 °C after mixing of the two components (Fig. 1). A 15-min incubation period is sufficient to obtain a satisfactory complex formation index of 77%. Prolonged exposure increases slightly the formation of the complex until 92% of the drug is complexed after a 24-h incubation. When incubation was performed at 37 °C, nearly all of the drug (96.8% ± 1.3%) present in the incubation mixture is complexed to the cardiolipin-containing liposomes (data not shown). In addition, complex formation is very effective and nearly complete, even at a high dilution of both components (20 μg Dox/ml and 0.22 mg liposomes/ml.

To characterize the complex formation of Dox to the cardiolipin-containing liposomes, we studied the binding of the drug at increasing concentrations to a fixed quantity of liposomes (Fig. 2). Scatchard-plot analysis carried out at drug concentrations of up to 1.36 m*M* revealed two Dox binding sites in the liposomes: (1) a site corresponding to a K_d) value of 28 ± 8 μ*M* and a maximal binding (B_{max}) of

11.8 ± 3.5 μmol/mg lipids and (2) another site corresponding to a K_d value of 1 ± 0.3 m*M* and a B_{max} value of 235 ± 95 μmol/mg lipid. When lyophilisate liposomes were used under the same conditions, we observed the presence of two binding sites of similar binding parameters: (1) K_d = 3.0 ± 2.1 μ*M* and B_{max} = 12.4 ± 0.9 μmol/mg lipid and (2) K_d = 1.36 ± 0.15 m*M* and B_{max} = 288 ± 102 μmol/mg lipid (data not shown).

Clonogenic assays were performed to evaluate the toxicity of free Dox, cardiolipin-liposome-complexed Dox prepared as described in Materials and methods, and liposome-encapsulated Dox prepared as previously described [15] in MCF-7/ADR and LZ cells. Both cell lines are multidrug-resistant and express P-glycoprotein. MCF-7/ADR and LZ cells exhibit a 1,000- and 4,000-fold resistance to Dox, respectively, in terms of the IC_{50} as compared with their respective parental counterparts. The relative level of expression of P-glycoprotein as determined by flow cytometric analysis after immunochemical staining [17] was found to be 5.7 times higher in CH LZ cells than in MCF-7/ADR cells (data not shown).

Table 1 shows a comparison of the cytotoxicity of free Dox (Dox), encapsulated Dox (Dox-Lip), and the cardiolipin-liposome-complexed Dox (Dox + Lip) in CH LZ and MCF-7/ADR cells. In Dox + Lip I treatment, cells were exposed to cardiolipin-liposome-complexed Dox, which was prepared by mixing the drug with a concentrate of previously formed cardiolipin-liposomes. Dox + Lip II corresponds to cardiolipin-liposome-complexed Dox, which was prepared by mixing the drug into a cardiolipin-liposome lyophilisate. Dox-Lip and Dox + Lip exhibited comparable cytotoxicity against LZ cells and MCF-7/ADR and, thus a comparable drug-resistance-reversal capacity (factors of approximately 9 and 3 as compared with the free drug in terms of the IC_{50} in LZ and MCF-7/ADR cells, respectively). The cardiolipin-liposome concentration added to the drug was equal to that present at an equivalent Dox concentration in liposome-encapsulated Dox. When Dox + Lip are formulated at a higher Lip/Dox (w/w) ratio (12 instead of 7.5 as found in Dox-Lip), Dox + Lip exhibited increased cytotoxicity in CH LZ cells (IC_{50}, 9.1 ± 0.9 in lieu of 22 ± 4 for Dox-Lip). These IC_{50} concentrations contained a concentration of Lip corresponding to at least 90% cell survival.

Table 1 Reversal of multidrug resistance in CH LZ and MCF-7/ADR cells using liposome-associated doxorubicin[a] (*ND* Not determined)

	Lip/Dox ratio (w/w)	IC_{50} (μg/ml)	
		CH LZ	MCF-7/ADR
Dox		195 ±16	3.5±1.0
Lip		200 ±26	35 ±8.5
Dox-Lip	7.5	22 ± 4	1.1±0.009
Dox + Lip I	7.5	16 ± 3	1.3±0.16
Dox + Lip I	12	9.1± 0.9	ND
Dox + Lip II	7.5	18 ± 4	ND

[a] Cells were treated for 4 h with an increasing concentration of free doxorubicin (Dox), liposome-encapsulated doxorubicin (Dox-Lip), and liposome-associated doxorubicin prepared from liposome concentrate (Dox + Lip I) or liposome lyophilisate (Dox + Lip II). Cytotoxicity evaluated by colony-forming assay and IC_{50} values were determined from survival curves. Data represent mean values ±SD for two experiments in which duplicate cultures were assayed

Discussion

In this report we describe a new procedure for the preparation of liposomal Dox that offers the advantages of simplicity and efficiency and is suitable for drug delivery in a clinical setting. We have demonstrated that Dox may be complexed with cardiolipin-containing liposomes and may be bound efficiently to the liposomal carrier. Complex formation is achieved by simply mixing cardiolipin-containing liposome concentrates with a solution of Dox. The liposomal Dox may also be prepared by two additional methods: (1) by rehydrating lyophilized cardiolipin-containing liposomes with a solution of Dox and (2) by rehydrating with the buffered mixture of lyophilized liposomes and crystalline Dox. Due to the high association efficiency of Dox to these liposomes, it is not necessary to separate the free drug from the liposomal drug by dialysis or centrifugation.

Complex formation of the anthracycline drug to cardiolipin-containing liposomes is due to the high binding affinity of Dox to cardiolipin. The complex Dox-cardiolipin is strongly stabilized by an electrostatic interaction between two molecules of Dox and one molecule of cardiolipin and a stoichiometric interaction leading to a card-pack dimer formation [8]. The electrostatic interaction involves the protonated amino group of the sugar residue of Dox and the two negatively charged phosphates of cardiolipin. The second interaction is a ring-stacking interaction between adjacent anthraquinone chromophores [9]. Evidence suggests that Dox binds to the mitochondrial membranes [12, 13] and, more precisely, to cardiolipin, a characteristic phospholipid that is highly concentrated in the inner membranes of mitochondria [17]. Goormaghtigh et al. [7] brought evidence of a specific complex between Dox and cardiolipin and demonstrated the high affinity of the complex formation (association constant, 1.6 μM^{-1}).

In our study we determined two binding sites for Dox in cardiolipin liposomes, confirming a high-affinity binding similar to that of Dox to free cardiolipin. It is noteworthy that Nicolay et al. [12] also found two different binding sites in mitochondrial membrane preparations that exhibit dissociation constants of 40 and 200 μ*M*, respectively. As speculated by those authors [12], the two levels of specific binding observed here as well as in mitochondria and mitoplasts are the consequence of a difference in the apparent affinity of Dox for cardiolipin in the outer and the inner monolayers. Dox binding to cardiolipin liposomes as performed in this study appears higher than what would be expected for cardiolipin if it had been located only at the surface of liposomes. The Dox to-cardiolipin molar ratio may reach 1.75 after preparation as compared with 1.0–1.2 for the Dox-Lip formulation, whereas that for complete Dox binding to all of the cardiolipin molecules present in

the liposomes would be 2.0. This strong binding allows for the insertion and the combination of the drug in the membrane lipid bilayer. Hence, anthracycline is not situated at the surface of the liposome after complex formation but is rather integrated firmly in the liposomes.

Rahman et al. [15, 20] developed a liposomal Dox preparation using cardiolipin as one of the phospholipids in the liposome composition. Encapsulation of Dox in those liposomes has demonstrated antitumor activity at doses that caused fewer myocardial alterations [5] and has recently been shown to improve the therapeutic efficacy as compared with free Dox [20]. The procedure for liposomal Dox preparation presented in this report utilizes a completely different approach, since Dox is added and complexed to preformed cardiolipin-containing liposomes. It has recently been demonstrated that liposome-encapsulated Dox may modulate multidrug resistance in cancer cells [17]. Resistance to major classes of cytotoxic drugs may emerge in tumor cells from patients treated by chemotherapy. Therefore, multidrug resistance (MDR) may be one therapeutic obstacle in cancer treatment. The ability to increase Dox activity in MDR cells was due to the use of a liposomal carrier. We recently demonstrated that MDR reversal by liposomal Dox may be explained by an increase in cellular drug accumulation [17, 19] and by intracellular drug redistribution [19]. In addition, we showed that neither pretreatment of empty liposomes before drug treatment nor combined incubation of vincristine and empty liposomes modulates MDR in CHLZ cells, suggesting that liposomal encapsulated is needed to overcome MDR [19]. The capability to overcome MDR was studied when cardiolipin-liposome-complexed Dox was used in comparison with liposome-encapsulated Dox. Thus, the MDR-reversal ability bore witness to the integrity or stability of the liposome-cardiolipin-Dox complex.

Using two different MDR cell lines (the human breast carcinoma MCR-7/ADR and Chinese hamster lung-fibroblast LZ cell lines) we have demonstrated that cardiolipin-liposome-complexed Dox exhibits cytotoxicity similar to that of liposome-encapsulated Dox [17] at equivalent concentrations of drug and lipid and may modulate MDR as well. This result confirms that the liposomal carrier system complexed to the drug is responsible for the enhancement of Dox activity in MDR cells. Furthermore, the drug in a cardiolipin-liposome-complexed Dox form is integrated as well as that in liposome-encapsulated Dox. We demonstrated that the liposomes need to be associated with the drug for the latter to exert its MDR reversal effect [19]. However, in recent investigations we have shown that empty liposomes at high concentrations appear to be specifically cytotoxic to MDR cells [18] and may enhance the MDR-reversal effect of the liposomal Dox [19]. Dox + Lip may exhibit a higher MDR-reversal capacity by increasing the Lip concentration in Dox + Lip. Lip used at subcytotoxic concentrations may improve the activity of liposomal Dox in MDR cells, certainly by enhancing the effects of the mechanisms by which liposomal Dox modulates MDR, such as bypassing P-glycoprotein (Pgp)-associated drug efflux and altering intracellular drug trafficking [19]. However, from a pharmacological standpoint, lowering the lipid/drug ratio in a liposomal drug preparation is desirable to improve drug delivery to non-MDR cancer cells. Due to the possibility that liposomal Dox may be prepared just before its clinical administration, it is conceivable that clinicians may vary the drug/liposome ratio as a function of the type of cancer cells to be treated so as to obtain a more effective treatment.

Although the liposome-entrapped Dox preparations previously described, such as Dox-Lip [15, 20], are reliable in terms of drug-encapsulation efficiency, there are practical limitations. Dox-Lip preparation involves a two-step preparation process that includes complex formation of the drug with cardiolipin and then encapsulation into liposomes. For the removal of unencapsulated drug (35%–50% of the initial input), liposomal drug must be subjected to extensive dialysis over a period of 20 h [15], bringing the Dox-Lip preparation time to 2 days. In addition, Dox-Lip must be used within 3 weeks of its preparation because of drug leakage from liposome vesicles and drug oxidation.

In our study we illustrated the adaptability and versatility of the present method of making empty cardiolipin-containing liposomes and the effective and efficient internalization of the drug inside the liposomes because of the strong affinity of the specific lipid to Dox. The procedure of preparing liposomes presented herein greatly simplifies the industrial manufacturing process and the treatment of patients because of the potential bedside usefulness of the method.

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