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Liposomal formulation and antitumor activity of 14-*O*-palmitoyl-hydroxyrubicin*

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Summary. The 14-*O*-palmitoyl ester of 3′-deamino-3′-hydroxydoxorubicin was synthesized to study the liposomal formulation and biological activity properties conferred by the attachment of a lipophilic group to position 14 of the anthracycline molecule. The entrapment efficiency of 14-*O*-palmitoyl-hydroxyrubicin in multilamellar vesicles composed of dimyristoylphosphatidyl choline and dimyristoylphosphatidyl glycerol was >99%. In addition, the stability of liposomes containing 14-*O*-palmitoyl-hydroxyrubicin was >99% at 14 days as assessed by the amount of drug leaking out of the liposomes and the absence of crystals of free drug in the liposome pellet. Esterification at position 14 did not significantly decrease the potency of the parent compound 3′-hydroxydoxorubicin. Liposome-entrapped 14-*O*-palmitoyl-hydroxyrubicin was significantly more active than doxorubicin against two murine tumor models. Against ip L-1210 leukemia, liposome-entrapped 14-*O*-palmitoyl-hydroxyrubicin injected i.p. into mice at doses of 60 and 80 mg/kg resulted in a %T/C value (median survival of treated/control animals $\times 100$) of >600, with 3–4 of 6 animals being cured, whereas in the same experiments, doxorubicin injected at the optimal dose (10 mg/kg) resulted in a %T/C value of 340, with 1 of 6 animals being cured. In animals bearing liver metastases of M-5076 reticulosarcoma, liposome-entrapped 14-*O*-palmitoyl-hydroxyrubicin showed significant antitumor activity when given on a three-i.v.-injection schedule of 20 mg/kg on days 4, 8, and 12 (%T/C, 175), whereas doxorubicin injected at optimal doses of 6–8 mg/kg on the same days was devoid of antitumor activity (%T/C, 129–133). These results indicate that esterification at position 14 enhances the affinity of this type of compounds for lipid bilayers without negatively affecting their biological activity.

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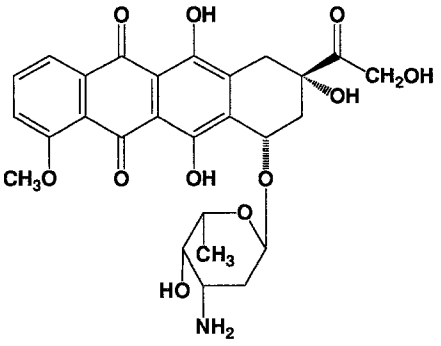
Introduction

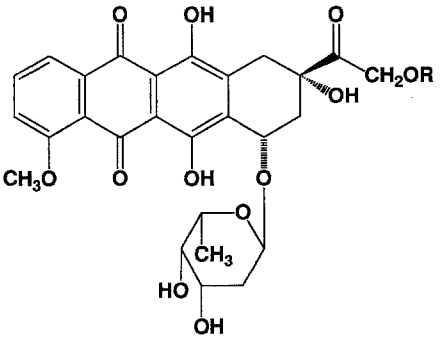
During the last decade, the use of liposomes as carriers of the antitumor agent doxorubicin has been extensively explored by many different investigators [1–6, 8, 12–14, 22, 24]. Encapsulation of doxorubicin in liposomes has been shown to decrease the cardiotoxic potential of this drug in mice [1, 5] and dogs [8] and to increase its antitumor activity against different experimental tumor models [4, 13, 14]. Different liposomal formulations of doxorubicin are currently being tested in humans, with encouraging results. Phase I studies have shown that the dose-limiting toxicity of free and liposomal doxorubicin in humans is myelosuppression [2, 3, 6, 22]. However, liposomal doxorubicin tends to cause less gastrointestinal toxicity and alopecia than does free doxorubicin. A phase II study of liposomal doxorubicin in patients with metastatic breast carcinoma has demonstrated antitumor activity at least comparable with that of free doxorubicin [23]. In addition, no episode of cardiotoxicity has thus for been observed. However, further studies are needed to evaluate fully the cardiotoxic potential of liposomal doxorubicin in humans.

From the beginning the development of liposomal doxorubicin was hampered by cumbersome formulation problems, most of them stemming from doxorubicins limited affinity for lipid bilayers and, hence, its need to be encapsulated in the aqueous space. In addition, once doxorubicin is in the intraliposomal aqueous space, it tends to leak out of the lipid vesicles. By creating a pH gradient across the liposome membrane, other investigators have managed to increase the encapsulation efficiency of doxorubicin to virtually 100% [11]. Notwithstanding this significant improvement, the "in situ" encapsulation procedure is not simple and remains to be optimized.

For several years, we have advocated the use of analogs rationally designed for incorporation within the lipid bilayers of liposomes rather than compounds originally developed for parenteral administration in water solutions as a means of overcoming some of the liposome-formulation problems associated with drugs such as doxorubicin [16, 19]. In addition, this approach has the advantage of



1 - Doxorubicin



2 - R = H; Hydroxyrubicin
3 - R = $CO(CH_2)_{14}CH_3$

Fig. 1. Molecular structures of doxorubicin (*1*), 3′-deamino-3′-hydroxydoxorubicin (*2*), and 14-*O*-palmitoyl-hydroxyrubicin (*3*)

offering a great deal of flexibility in the chemical design of the compounds, thus enabling the introduction into the candidate compounds of modifications that not only may improve the affinity of the compounds for the liposome membranes but also may favorably alter their therapeutic index. The attachment of lipophilic groups, including phospholipid moieties, is a logical approach for increasing the affinity for lipid membranes that has successfully been used in different types of compounds, including analogs of cisplatin [23], cytosine arabinoside [9], muramyl dipeptide [15], and tumor necrosis factor [25].

Lipophilicity in itself does not guarantee a satisfactory entrapment of anthracyclines in liposomes [17]. There is a clear relationship between drug structure and liposome entrapment in this family of compounds. We have previously identified subtle changes in the sugar and aglycone portions of the anthracycline molecule that can dramatically and unexpectedly increase the liposome entrapment and, more generally the affinity of these compounds for lipid membranes [19]. As a logical continuation of our efforts to define further the structural requirements of this family of compounds for optimal liposome entrapment, we decided to determine whether the attachment of a lipophilic group would increase the liposome-entrapment properties of these compounds as has been shown for other molecules. For that prupose, we synthesized as a prototype the 14-*O*-palmitoyl ester of the doxorubicin analog 3′-deamino-3′-hydroxydoxorubicin (Fig. 1, compound 2), which has shown significant antitumor activity and a lack of cross-resistance with doxorubicin [10]. The present report describes the liposome formulation and the antitumor activity obtained using this compound.

Materials and methods

Chemicals. Palmitoyl chloride was purchased from Pfaltz and Bauer, Inc. Dimyristoyl phosphatidyl choline (DMPC) and dimyristoyl phosphatidyl glycerol (DMPG) were obtained from Avanti Polar Lipids (Pelham, Ala.).

Cell lines. L1210 cells were obtained from the DCT Tumor Repository, National Cancer Institute (Frederick, Md.) and maintained in BDF_1 mice by weekly intraperitoneal passage. M5076 cells were obtained from Dr. I. Fidler from the Department of Cell Biology, M. D. Anderson Cancer Center, maintained in C57BL/6 mice as an ascitic tumor, and transplanted every 3–4 weeks.

Animals. BDF_1 and C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, Mass.) and kept in cages housing five animals each. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Synthesis of 7-0-(2,6-dideoxy-α-L-lyxo-hexopyranosyl)-14-O-palmitoyl-adriamycinone. The substrate 7-*O*-(2,6-dideoxy-α-L-lyxo-hexopyranosyl)-adriamycinone (108.0 mg, 0.195 mmol) [10] was dissolved in dry pyridine (3 ml). Palmitoyl chloride (92 mg, 0.335 mmol) was added, and the reaction was stirred at room temperature and monitored by thin-layer chromatography (TLC) using toluene-acetone (1 : 1, v/v)[1]. After 90 min, another portion of palmitoyl chloride (55 mg, 0.20 mmol) was added. After 2 h, the solution was poured into water to stop the reaction and extraction with dichloromethane was carried out. The organic layer was sequentially washed once with a 10% solution of hydrochloric acid, once with a saturated solution of sodium bicarbonate, and twice with water. The solution was then dried over sodium sulfate overnight. Filtration and evaporation under diminished pressure at 50° C led to the formation of a red, thick oil.

Purification by column chromatography on silica gel 60 (22 g, 230- to 400-mesh; E. Merck, Darmstadt, FRG) using 50 ml 2 : 1 (v/v) and then 1 : 1 (v/v) toluene-acetone as an eluant yielded 59 mg 7-*O*-(2,6-dideoxy-α-L-lyxo-hexopyranosyl)-14-*O*-palmitoyl-adriamycinone (Fig. 1, compound 3) as a red solid (yield, 32.4%). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 200 and 50 MHz, respectively, by an IBM NR/200 AF spectrometer: mp, 180°–182° C; $[\alpha]^{25}_D$ +139° C ± 1.6 (*C* CH_3OH-$CHCl_3$, 1 : 1) ^{13}C NMR (50 MHz, $CDCl_3$) δ 206.4 (C-13), 186.9, 186.5 (C-5, 12), 173.2 (C = *O* ester), 161.0 (C-4), 156.2, 155.7 (C-6, 11), 135.6, 135.4, 133.9, 133.7 (C-2, 6a, 10a, 12a), 120.8 (C-4a), 119.7 (C-1), 118.4 (C-3), 111.5, 111.3 (C-5a, 11a), 101.1 (C-1), 77.3 (C-9), 71.0, 69.4, 67.1, 65.7 (×2) (C-3′, 4′, 5′, 7, 14), 56.6 (OMe), 35.2, 33.9, 33.7, 32.5, 31.9, 29.6 (intensity, >5-fold), 29.4, 29.3, 29.1, 24.9, 24.7, 22.6 (C-8, 10, 2′, CH_2-palmitoyl chain), 16.7 (C-6′), 14.1 (CH_3-palmitoyl chain). Compound 3 is virtually insoluble in water but is soluble in methanol and chloroform.

Liposome-formulation studies. Multilamellar vesicles containing compound 3 were prepared as previously described [17, 18], with slight modifications. In brief, a mixture of DMPC, DMPG, and compound 3 at a 10.5 : 4.5 : 1 weight ratio was dissolved in chloroform. The organic solvent was evaporated in a rotavapor at 40° C. Multilamellar vesicles containing compound 3 were obtained by hydrating the lipid film with 0.9% NaCl in water at a final concentration of 0.5 mg/ml at room temperature. Complete dispersion of the red lipid film into the aqueous phase was obtained within a few minutes by mild hand-shaking of the hydration mixture.

[1] TLC was performed on precoated plastic sheets (0.22 mm) and glass plates (0.25 mm) using silica gel 60F-254 (E. Merck, Darmstadt FRG). For the detection of components, the plates were sprayed with 2 M sulfuric acid and subsequently heated

The entrapment efficiency was determined by measurement of the amount of compound 3 in the aqueous milieu followed by assessment of the presence of drug crystals in the liposome pellet obtained after centrifugation of the liposome suspension at 30,000 *g* for 45 min. The amount of drug solubilized in the aqueous phase was measured by UV spectrophotometery at a wavelength of 250 nm. The percentage of entrapment efficiency (% EE) was calculated by the following equation:

$$\% \text{ EE} = \frac{\text{Drug in liposome suspension} - \text{drug in supernatant}}{\text{Drug in liposome suspension}} \times 100.$$

In the absence of crystals of free drug, the % EE value is an accurate parameter of the actual incorporation of drug into the lipid vesicles. In the presence of free-drug crystals, the % EE value indicates the amount of drug that has actually been incorporated into the liposomes plus the amount that has remained unsolubilized in the aqueous milieu after liposome formation.

The stability of the liposomes containing compound 3 was assessed by measuring at different time points after liposome formation the amount of drug leaking from the vesicles, determining the vesicle-size profile, and assessing the presence of crystals of free drug in the liposome pellet obtained after centrifugation of the liposome suspension. Liposomes were sized in a Coulter counter and channelizer (Coulter Electronics, Hialeah, Fla.).

Antitumor activity studies. The antitumor activity was tested in two murine tumor models: ascitic L-1210 leukemia and M-5076 reticulosarcoma. Liposome-entrapped compound 3 was compared with doxorubicin. Experiments were performed using previously determined higher nontoxic doses of both compounds. Free compound 3 was not used due to its lack of water solubility.

BDF_1 mice in groups of six to eight animals each were inoculated i. p. with 1×10^6 L-1210 cells on day zero. Drugs were injected i. p. on day 1. The results were expressed as % T/C values (median survival of treated animals/median survival of control animals $\times 100$). Experiments were terminated on day 60; animals that were alive at this time were considered to be cured. C57BL/6 mice in groups of six to eight animals each were inoculated i. v. with 2×10^4 M-5076 cells on day zero. Drugs were injected i. v. on days 4, 8, and 12. The Results were expressed as % T/C values.

Results

Characteristics of multilamellar vesicles containing 14-O-palmitoyl-hydroxyrubicin

The entrapment efficiency of 14-*O*-palmitoyl-3′-hydroxyrubicin in multilamellar liposomes was >99% as assessed by the method described above; this was expected due to the compound's lack of water solubility. However, since no free-drug crystals were detected in the liposome pellet using optic microscopy, we concluded that all of the drug must have been incorporated into the lipid bilayers of the vesicles. This was confirmed by fluorescent microscopy; all liposomes were intensely fluorescent and no fluorescent crystals that might correspond to precipitates of free drug were seen. The particle size was 1–5 μm as assessed by Coulter counter. The stability of the liposome preparation over 14 days at 4° C was >99% as determined by measuring the amount of nonentrapped drug in the aqueous milieu. In addition, no change in the liposome-size profile was observed, and no crystals of free drug were detected in the liposome pellet.

Table 1. Antitumor activity of 14-*O*-palmitoyl-hydroxyrubicin entrapped in liposomes against L1210 leukemia[a]

Compound	Dose (mg/kg)	% T/C (cures)	
		Experiment 1	Experiment 2
L-Palmitoyl-hydroxyrubicin			
	40	211 (2/6)	>600 (3/6)
	60	>600 (3/6)	>600 (4/6)
	80	–	>600 (3/6)
Doxorubicin	10	337 (0/6)	340 (1/6)

[a] Tumor inoculation was carried out i. p. (1×10^6 cells). Treatment was given i. p. on day 1

Table 2. Antitumor activity of 14-*O*-palmitoyl-hydroxyrubicin entrapped in liposomes against M-5076 reticulosarcoma[a]

Compound	Dose (mg/kg)	Schedule (days)	% T/C
L-Palmitoyl-hydroxyrubicin			
	20	4, 8, and 12	175
	35	4, 8, and 12	Toxic
Doxorubicin	8	4, 8, and 12	129
	6	4, 8, and 12	133

[a] Tumor inoculation was carried out i. v. (20,000 cells). Treatment was given i. v.

Antitumor activity of 14-O-palmitoyl-hydroxyrubicin entrapped in multilamellar vesicles

Tables 1 and 2 show the antitumor activity observed for liposome-entrapped compound 3. Against L1210 leukemia, the optimal dose of the tested preparation was 60 mg/kg. At this dose, entrapped compound 3 was significantly more active than the optimal dose of 10 mg/kg doxorubicin [%T/C, >600 (4/6 animals cured) vs 340 (1/6 animals cured)]. Against M-5076 reticulosarcoma, entrapped compound 3 given at a dose of 20 mg/kg on days 4, 8, and 12 showed significant antitumor activity, whereas doxorubicin (8 and 6 mg/kg on the same days) was basically inactive in this tumor model (%T/C, 175 vs 133). Higher doses of compound 3 (35 mg/kg on days 4, 8, and 12) were toxic.

Discussion

Our results indicate that the esterification of anthracyclines at position 14 with a long aliphatic group markedly enhances the affinity of these compounds for lipid bilayers. The 14-*O*-palmitoyl ester (Fig. 1, compound 3) of hydroxyrubicin (compound 2) was used as a prototype compound with the above modification. This compound showed optimal entrapment efficiency in multilamellar vesicles, and its association with the lipid vesicles remained stable for 14 days. By contrast, we have previously reported that the entrapment efficiency of both doxorubicin (compound 1) and hydroxyrubicin in liposomes of the same composition and size as those used in the present study is around 50% with the other 50% of the drug getting

dissolved in the aqueous solution during the process of liposome formation. Attempts to formulate doxorubicin in liposomes have been more successful when the drug has been encapsulated in the aqueous milieu of unilamellar vesicles [12].

In a previous study, we screened a series of lipophilic anthracyclines to investigate the structure/lipid-affinity relationship in this family of compounds. We observed that upon their exposure to a water solution in the presence of phospholipids, most lipophilic anthracyclines tend to precipitate in the aqueous phase rather than being incorporated within the lipid bilayers. However, we identified two specific modifications that enhance the affinity of such compounds for lipid membranes in general and, thus, their incorporation into liposomes: demethoxylation at position 4 of the aglycone and halogenation at position 2′ of the sugar [17]. The modification described in the current study, esterification at position 14 of the aglycone, constitutes a third, more conventional, type of chemical modification that enhances the affinity of this family of compounds for lipid membranes.

When tested in two different murine tumor models at optimal doses, liposome-entrapped 14-*O*-palmitoyl-hydroxyrubicin showed markedly higher antitumor activity than did doxorubicin. A comparison with the liposomal doxorubicin formulations currently undergoing clinical trials could not be performed. Esterification of hydroxyrubicin at position 14 did not significantly reduce the potency of the compound. The optimal dose of hydroxyrubicin given as a single i.p. or i.v. injection in mice ranges between 25 and 75 mg/kg [21]. The optimal dose of compound 3 lay within the same range (60 mg/kg).

Part of the superior antitumor activity of liposome-entrapped compound 3 may be explained by the observation that both tumor models are particularly suited for exploitation of the pharmacologic advantages of liposome entrapment of antitumor agents. The large liposomes used in the present study are cleared slowly from the peritoneal cavity, thus enabling a prolonged exposure of the L-1210 cells to effective concentrations of the agents entrapped in these vesicles [26]. After their i.v. inoculation, M-5076 cells form multiple tumor colonies in the liver parenchyma [18]. Because of this anatomical preference and their phagocytic activity [7], these tumor cells are naturally targeted by large multilamellar vesicles. Enhanced antitumor activity for liposomal doxorubicin as compared with free doxorubicin against this tumor model has previously been reported [13].

However, part of the superior antitumor activity observed with liposome-entrapped compound 3 may be attributable to the use of hydroxyrubicin instead of doxorubicin as a precursor for the esterification reaction. Deamination at position 3′ results in a partial overcoming of multidrug resistance. We have previously reported that hydroxyrubicin exerts significant in vitro and in vivo antitumor activity against tumor cells that express the multidrug-resistant phenotype and are resistant to doxorubicin [20]. Therefore, the significant antitumor activity observed for liposome-entrapped compound 3 against M-5076 reticulosarcoma might have been partly related to hydroxylation at position 3′.

The liposomal formulation of compound 3 reported herein differs substantially from the liposomal doxorubicin formulations currently undergoing clinical trial. The liposomal formulation developed for compound 3 consists of multilamellar vesicles with a mean diameter of 2–3 μm, whereas the liposomal doxorubicin formulations consist of large unilamellar vesicles measuring 0.1–0.2 μm in diameter. In addition, compound 3 is associated with the lipid bilayers inside the liposomes, whereas doxorubicin is encapsulated in the aqueous space. Since large and small liposomes may have different spectra of potential applications as a result of differences in their pharmacokinetics and organ distribution and since the biological profile of hydroxyrubicin differs from that of doxorubicin, the liposomal formulation described in the present report does not appear to be just an alternative to the currently available liposomal doxorubicin formulations but represents a substantially different antitumor agent in terms of its potential applications.

In summary, esterification of hydroxyrubicin (compound 2) at position 14 resulted in a compound exhibiting optimal liposome-formulation characteristics and impressive antitumor activity after both i.p. and i.v. administration. As compared with the liposomal doxorubicin formulations currently undergoing clinical trials, compound 3 is incorporated within the lipid bilayers of large vesicles (2–3 μm in diameter) rather than being solubilized in the inner aqueous space of small vesicles (0.1 μm in diameter). Studies of the cardiotoxicity and antitumor of this compound as compared with liposomal doxorubicin in multidrug-resistant tumor models are indicated to define better the clinical potential of this liposomal formulation.

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