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Evaluation of incorporation characteristics of mitoxantrone into unilamellar liposomes and analysis of their pharmacokinetic properties, acute toxicity, and antitumor efficacy*

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Summary. Mitoxantrone (MTO) was incorporated into small unilamellar liposomes by formation of a complex between the anticancer drug and negatively charged lipids. The complex was formed at a 2:1 molar ratio between the lipids and MTO, with phosphatidic acid (PA) being the strongest complex-forming lipid. Weaker complexes and lower incorporation rates of MTO resulted when liposomes containing dicetylphosphate, phosphatidyl inositol, phosphatidyl serine, phosphatidyl glycerol, oleic acid, and tridecylphosphate were used. Thus, all further experiments were performed with PA-MTO liposomes that contained 0.1–3 mg MTO/ml and had mean vesicle sizes of 40–150 nm, depending on the drug concentration and the method of liposome preparation. In vitro incubations of free and liposomal MTO with human plasma showed that the drug is slowly transferred from the liposome membranes to the plasma proteins. For liposomal MTO a transfer rate of 48% was determined, whereas 75.8% of free MTO was bound to the plasma proteins. The organ distribution of the two preparations in mice showed that higher and longer-lasting concentrations of liposomal MTO were found in the liver and spleen. The terminal elimination halflives in the liver were 77 h for liposomal MTO and 14.4 h for free MTO. In the blood, slightly higher concentrations were detected for liposomal MTO, which also had slower biphasic elimination kinetics as compared with the free drug. Drug distribution in the heart was not significantly different from that in the kidneys. The LD_{25} of PA-MTO liposomes in mice was 19.6 mg/kg and that of free MTO was 7.7 mg/kg. The antitumor effects of PA-MTO liposomes were evaluated in murine L1210 leukemia, in various xenografted human tumors, and in methylnitrosourea-induced rat mammary carcinoma. Generally, the liposomal application form was more effective and less toxic than the free drug. The cytostatic effects were dependent on the tumor model, the application schedule, and the drug concentration. At doses that were toxic when free MTO was used, the liposomal preparation produced strong antitumor effects in some cases. In summary, the incorporation of MTO into liposomes changes the drug's plasma-binding properties, alters its organ distribution, reduces its acute toxicity, and increases its cytostatic efficiency in various tumor models. The liposomal PA-MTO complex represents a new application form of MTO that has advantageous properties.

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Introduction

Mitoxantrone (MTO) is a new anthracenedione derivative that exhibits high antitumor activity in experimental tumor models as well as in human tumors [25, 29]. Phase I and II clinical trials have shown that it is valuable when given alone or in combination with other antitumor drugs for the treatment of breast cancer, acute leukemia, and lymphoma ([5, 9, 16, 21, 23, 28, 31, 46, 50; reviewed in [43, 44]). MTO is less cardiotoxic than the related drug doxorubicin, and its dose-limiting toxicity is myelosuppression [12]; its other nonthematological side effects are less severe and occur less frequently than those produced by anthracyclines. In addition, it has been shown to have antiviral, antibacterial, antiprotozoal, and immunological properties [44]. MTO exerts its effect by DNA intercalation [13, 26] and, possibly, by electrostatic interactions with membranes [8]. Recently it was found that MTO and other intercalating drugs displace chromatin proteins binding to nucleic acids [3]. Cell-damaging effects caused by lipid peroxidation following MTO administration have been found to be less pronounced than those observed after treatment with anthracyclines [48].

Many different strategies are followed to improve the therapeutic value of antitumor drugs. Among various approaches to develop more effective and less toxic drug

preparations, the incorporation of the drugs into liposomes represents a promising possibility. With the objective of enhancing the therapeutic index and reducing unwanted toxic side effects, many antitumor drugs have been incorporated into liposomes. Promising results were found using doxorubicin, whose entrapment into liposomes reduced its cardiotoxicity and increased its antitumor activity [2, 24, 30, 35]. Following the incorporation of other antitumor compounds into liposomes, such as methotrexate [22], arabinosyl cytosine (ara-C) [32], and *cis*-diamminedichloroplatinum(II) [45] reductions in toxicity and preservation of or increases in antitumor activity have been observed.

However, low entrapment efficiencies and the leakage of the small and mainly water-soluble drugs entrapped within the inner liposomal volume hampers their stability and, consequently, their clinical application. Therefore, efforts were made to incorporate the antitumor drugs into the lipid membrane of the liposomes. To achieve this, lipid-soluble derivatives of the parent antitumor drugs were synthesized, mainly by their modification with either long-chain alkyls or fatty acids. In the L1210 leukemia model, the incorporation of lipophilic cisplatin compounds into membranes of multilamellar liposomes resulted in antitumor effects comparable with those obtained using free cisplatin [27]. Significantly higher cytostatic effects for such liposomes have been observed against liver metatases of M5076 reticulosarcoma [33], probably due to the natural targeting of the liposomes to the mononuclear phagocyte system.

Using lipophilic derivatives of 5-fluoro-2′-deoxyuridine (FUdR) that had been incorporated into unilamellar liposomes, we could show that the liposome-associated drug derivatives were active against mammary carcinoma 13/C, Lewis lung carcinoma, and L1210 leukemia at concentrations significantly lower than those required when unmodidfied FUdR was used [47]. Following the same rationale, we found that various lipid-soluble derivatives of ara-C that had been incorporated into liposomes displayed superior antitumor effects in L1210 leukemia and B16 melanoma [37, 41]. A clinical pilot study in which patients suffering from relapsed acute myelogenous leukemia were treated with unilamellar liposomes containing N^4-oleyl-arabinosyl-cytosine showed that liposomal antitumor drug therapy is feasible and that promising results are obtained [42].

It is known that doxorubicin and related anthracyclines interact strongly with negatively charged membrane lipids [7, 20]. MTO is structurally related to the anthracyclines and possesses two secondary amino groups in its side chains that react as strong bases. Therefore, the formation of MTO complexes with negative charges on membranes can be expected.

Bearing these properties in mind, we incorporated MTO into small unilamellar liposomes that contained various lipophilic anionic compounds. We investigated their pharmacokinetic properties, their toxicity, and their antitumor effects in various tumor models.

Materials and methods

Preparation of phosphatidic acid/MTO liposomes. Small unilamellar liposomes were prepared by detergent dialysis as previously described [37, 38]. Briefly, soy phosphatidyl choline (SPC), cholesterol, phosphatidic acid (PA), and D,L-α-tocopherol as liposome membrane constituents, MTO-dihydrochloride (MTO · 2HCl), and sodium cholate were mixed in chloroform/methanol (1 : 1, v/v). The molar ratios of the lipids were 1 SPC : 0.2 cholesterol : 0.04–0.12 PA : 0.001 D,L-α-tocopherol. MTO · 2HCl was added to give final liposomal MTO concentrations of between 0.1 and 3 mg/ml and SPC concentrations of 20–40 mg/ml. Depending on the amount of MTO to be incorporated, PA was added in the molar range of 0.04 to 0.12 to yield the optimal complex ratio of 2 PA : 1 MTO. In some experiments the ratio between PA and MTO was increased to 3 : 1 and 4 : 1 to investigate whether higher MTO incorporation rates could be obtained. The ratio of sodium cholate to total membrane-forming lipids, including MTO, was 0.6–0.7.

After evaporation of the organic solvents, mixed micellar solutions were obtained in phosphate buffer (67 mM disodium hydrogen phosphate dihydrate and 67 mM potassium dihydrogen phosphate, pH 7.4). Liposome preparation was accomplished in two ways, depending on the volume: volumes of 10–30 ml were dialyzed with an equilibrium dialysis instrument as previously described [36], whereas for larger volumes of 100–500 ml, the capillary dialysis method described by Schwendener [38] was used.

The resulting PA-MTO liposomes were filtered through 0.45 μm sterile filters (Nalge, Rochester, N. Y.) and stored at 4° C. For plasma incubations and pharmacokinetic experiments, the PA-MTO liposomes were labeled with trace amounts of [^{14}C]-MTO · 2HCl (4.18 GBq/mmol, obtained through American Cyanamid Co., Wolfratshausen, FRG). Liposome size and population homogeneities were determined by laser light scattering as previously described [37]. The efficiency of MTO incorporation was determined either by UV-VIS spectroscopy or by the measurement of [^{14}C]-MTO, in either case by comparison of the drug concentration in the micellar solution with the final concentration in the liposomes.

Formation of MTO complexes using various negatively charged lipids and lipophilic acids. To find the lipophilic agent with which MTO most strongly associates, we used the following negatively charged compounds at a 2 : 1 molar lipid-to-drug ratio: PA, dicetyl phosphate (DCP), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl glycerol (PG), oleic acid, and tridecyl phosphate. Liposomes containing 1 mg MTO/ml were prepared as described above. The incorporation stability, that is, the amount of complexed MTO in proportion to the quantity of its lipophilic anionic counterpart, was determined by the elution of 0.5-ml liposome aliquots on a BioGel P6-DG column (1 × 10 cm; BioRad, Richmond, Calif.) using phosphate buffer (pH 7.4) as the eluent at 1 ml/min. The capacity of the anionic agents to form stable liposomal complexes with MTO was evaluated by spectroscopic determination (wavelength, 670 nm) of the drug concentration in the pooled liposome elution peak and then compared with the initial MTO concentrations before column chromatography.

Stability and spectral properties of the PA-MTO complex in liposomes. For the determination of possible leakage of MTO from the liposomal complex, freshly prepared PA-MTO liposomes and liposomes that had been stored at 4°–7° C for 6 months were chromatographed on a BioGel P6-DG column (2.5 × 12 cm) with 67 mM phosphate buffer (pH 7.4) at a flow rate of 1–2 ml/min. Aliquots of 0.1–1 ml liposomes containing 0.1–1 mg MTO/ml were applied to the column and elution was monitored at 670 nm in a Perkin Elmer Lambda 2 spectrometer equipped with a flow cell. For purposes of comparison, free MTO and empty PA liposomes were chromatographed under identical conditions (cf. Fig. 2). Absorption spectra for the lipsosomal PA-MTO complex and for MTO · 2HCl were recorded in the range of 500–700 nm. The concentration of MTO was 3.1×10^5 M (MTO · 2HCl).

In vitro interaction of PA-MTO liposomes and free MTO with human plasma. PA-MTO liposomes and MTO · 2HCl, both at 0.1 mg/ml and

trace-labeled with [^{14}C]-MTO·2HCl, were incubated with human plasma at 37° C for periods ranging from 30 to 450 min. The incubations were done at a drug preparation: human plasma: phosphate buffer (pH 7.4) ratio of 1 : 1 : 3 (by vol.). Aliquots of 0.5 ml were analyzed at the given time points by high-pressure liquid chromatography (HPLC) to determine the stability of MTO on incubation with the plasma proteins. The HPLC runs were performed with a Pharmacia-LKB (Uppsala, Sweden) dual-pump instrument using the following parameters: BioRad TSK 40 (300 × 7 mm; BioGel, Richmond, Calif.) gel-filtration column, 67 mM phosphate buffer (pH 7.4) as elution medium at a flow rate of 0.75 ml/min, UV detection at 280 nm, and fraction collection (0.5 ml/min) for determinations of [^{14}C]-MTO activity with a Pharmacia LKB 1214 liquid scintillation counter. To illustrate the interactions of free and liposomal MTO (PA-MTO liposomes) with plasma proteins, the elution curves obtained from counts of the labeled drug were superimposed onto the UV absorption curves calculated from elution of the plasma proteins (cf. Fig. 3). In control experiments, empty liposomes incubated with plasma were chromatographed under identical conditions to confirm their separation from the proteins (data not shown).

For quantification of the binding of free MTO to human plasma, equilibrium dialysis experiments were performed as described elsewhere [40]. Briefly, 1 ml [^{14}C]-MTO·2HCl dissolved in phosphate buffer (pH 7.4) at a concentration of 25–500 mM was diayzed at 37° C against 1 ml human plasma. The dialysis cells (Dianorm, München, FRG) were separated by a 10,000-Da molecular-weight-cutoff cellulose membrane. Equilibrium was reached after 5 h, after which the samples were removed. The [^{14}C]-MTO activity of free and protein-bound MTO was determined by scintillation counting.

Organ distribution and pharmacokinetics. Radioactively labeled free or liposomal MTO (1 mg/kg body weight, 10–15 kBq [^{14}C]-MTO/animal) was injected i.v. into the tail vein of female ICR mice (20–25 g, 12–15 weeks of age). After periods ranging from 3 min to 5 days, groups of three mice were killed by cervical dislocation and the heart, liver, spleen, and kidneys were excised. Blood was collected with a Pasteur pipette after excision of the heart. Whole weighted organs or aliquots were digested with 2–4 ml Soluene 350 (Packard, Downers Grove, Ill.) tissue solubilizer at 40° C. Blood aliquots were solubilized with 2 ml of a 1 : 1 (v/v) mixture of Soluene 350 and isopropanol. After solubilization had been completed, the samples were bleached by the addition of 0.5 ml 30% hydrogen peroxide and radioactivity was counted after the addition of 10–15 ml Hionic-Fluor (Packard) scintillation cocktail. The plasma clearance half-times were calculated from fits that were obtained using a least squares parameter-estimation program (Rstrip, MicroMath, Salt Lake City, Utah). Statistical parameters were calculated using Student's distribution test.

Acute toxicity and myelosuppression. The acute toxicity of i.v. injected PA-MTO liposomes (1–4 mg/ml) and free MTO·2HCl (1 mg/ml) dissolved in phosphate buffer (pH 7.4) was evaluated in female NMRI mice (mean body weight, 32 g; age 15–20 weeks; BRL, Füllinsdorf, Switzerland). Groups of six animals per dose were treated with a single i.v. injection of either PA-MTO liposomes at 5.7, 7.2, 11.2, 13.7, 17.5 and 22 mg/kg or free MTO at 5, 7.5, 11, and 16 mg/kg. The animals were observed for a period of 21 days after drug administration. At intervals of 3–5 days, drug-induced changes in body weight were recorded. The myelosuppressive effect of the two MTO treatments was tested by the i.v. administration of 7.5 mg/kg MTO to groups of three female ICR mice (20–25 g, 12–15 weeks of age). Leucocytes were counted in a Coulter counter at 3 days after drug administration.

Cytostatic activity against L1210 leukemia. The antitumor activity of PA-MTO liposomes and free MTO against L1210 leukemia was determined, essentially as described elsewhere [37]. Briefly, a suspension of 1×10^5 viable L1210 cells from ascites of male donor BDF1 mice [(C57Bl/6 × DBA/2)F1, 20–25 g, 12–20 weeks of age; BRL, Füllinsdorf, Switzerland] was injected i.p. into BDF1 mice. The drugs were given to randomized groups of 5–6 animals as single i.p. injections on days 1, 3 or 5 after i.p. implantation of the tumor cells (single-dose i.p./i.p. schedule). The increase in the life span of treated animals (T) as compared with untreated control (C) animals was calculated as the ratio T/C of the median survival values expressed in percent. Statistical parameters were calculated using Student's distribution test. The mice were observed daily until their death or for 60 days; animals surviving for 60 days were considered to be cured.

Antitumor activity in human tumor xenografts. For in vivo experiments, we used 6- to 8-week-old female athymic nude mice of NMRI genetic background. The animals were housed in macrolon cages set in laminar flow rackets and were maintained under conditions described by Fortmeyer and Bastert [17].

Tumor slices averaging 3 × 3 × 0.5–1 mm in diameter were implanted s.c. into both flanks of the animals. Treatment was started after 3–6 weeks, by which time the median tumor diameter had reached 6 mm. Mice were randomly assigned to treatment groups and an untreated control group. Tumor growth was recorded weekly by measurement of two perpendicular diameters, with the product of the two diameters representing the tumor size. The antitumor effect was evaluated following maximal tumor regression (in non-regressing tumors, after 3–4 weeks). Data evaluation was performed using specifically designed software. Relative tumor size (RTS) values were calculated for each single tumor by dividing the tumor size on day X by that on day 0 at the time of randomization ($RTS = TS_x \times 100/TS_0$). Median RTS values were used for further evaluation. The tumor-doubling time (DT) in test and control groups was defined as the period required to reach an RTS of 200%. The effect of treatment was classified as complete remission (RTS on day 21 or 28, ≤10% of the initial value), partial remission (11%–50%), minimal regression (51%–75%), no change (76%–124%) or progression (≥125%). A tumor was considered to be sensitive if at least a minimal regression was achieved.

The effect of PA-MTO liposomes and free MTO·2HCl was studied simultaneously in five human tumors grown s.c. in serial passage in nude mice. Both compounds were injected at the maximally tolerated dose as defined by the LD_{20} (drug-related mortality at 21 days after the start of treatment and a median drug-induced body-weight loss of 10%) (cf. Tab. 4). The human origin of the tumors was confirmed by isoenzymatic and immunohistochemical methods. Tumor models were selected on the basis of their chemosensitivity and growth behaviour from a panel of 180 well-characterized, regularly growing xenografts [14, 15].

Cytostatic activity against methylnitrosourea-induced rat mammary carcinoma. Tumor induction and drug treatment were carried out as described by Berger et al. [4]. The mammary carcinomas were induced by three i.v. injections of methylnitrosourea (MNU) into the tail vein of female Sprague-Dawley rats (Institut für Versuchstierkunde, Hannover, FRG) on days 50, 71 and 92 respectively, following their birth. Beginning at 4 weeks after the first MNU injection tumor manifestation was assessed and tumor volumes were determined. Animals exhibiting a total tumor volume of >0.8 cm^3 were randomly allocated to experimental groups, and i.v. therapy given once weekly for 5 weeks was started immediately thereafter.

Results

Characteristics of the liposomal MTO complexes

A hypothetical representation of the interaction of MTO base with two molecules of PA and the subsequent integration of the complex into the lipid membrane of a liposome is shown in Fig. 1. The association of MTO with PA occurs in the aqueous micellar solution before liposome formation. Therefore, it is conceivable that the complex is statistically distributed over both of the membranes of the liposome (cf. Fig. 1; filled squares represent the complex in the liposome membrane). However, the exact mode of association between the acidic phosphohydroxy groups of PA and the pair of the basic secondary amino groups on the side chains of MTO remains to be determined. It is possi-

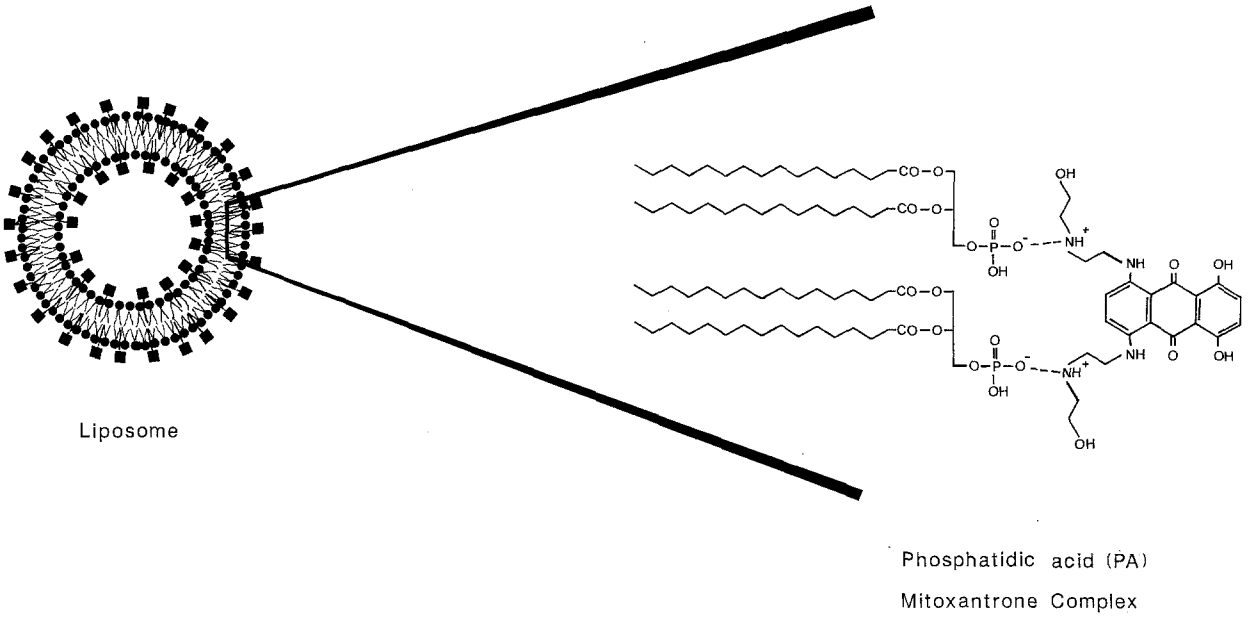

Fig. 1. Hypothetical view of the complex of MTO with two molecules of phosphatidic acid (PA) and its integration into the membrane of a liposome. *Filled squares* represent the complex in the liposome

Table 1. Association of MTO with negatively charged lipids and lipophilic acids[a]

Negatively charged compound	Relative strength of MTO association[b]	Percentage of incorporation[c]
Phosphatidic acid (PA)	1	95.5±3.8[d]
Dicetylphosphate (DCP)	0.89	85.9
Phosphatidyl inositol (PI)	0.85	81.75
Phosphatidyl serine (PS)	0.81	77.4
Phosphatidyl glycerol (PG)	0.74	71.2
Oleic acid	0.59	56.9
Tridecylphosphate	0.57	54.3

[a] The molar ratio of negatively charged complexing agent to MTO was 2:1

[b] The strength of MTO complexation to the negatively charged lipids was set in relation to that of PA, which produced the strongest complex among all compounds investigated

[c] The incorporation of MTO into liposomes containing the negatively charged compounds was determined spectroscopically at a wavelength of 670 nm in the pooled liposome fractions after column chromatography

[d] Mean ± SD of 3 determinations

ble that additional hydrophobic interactions with other membrane lipids attract the anthracenedione ring structure towards the hydrophilic-lipophilic interphase of the liposome membrane.

The association between various negatively charged lipids or lipophilic acids and MTO at a 2:1 molar ratio was set in relation to PA, which was found to be the strongest complex-forming anionic compound. As shown in Table 1, the lipophilic derivatives of phosphatidic acid, PA, and dicetyl phosphate (DCP) produced the highest rates of MTO incorporation into the liposomes, followed by the weakly acidic lipids phosphatidyl inositol (PI), phosphatidyl serine (PS), and phosphatidyl glycerol (PG). Long-chain fatty acids and alkylphosphates were the weakest anionic binding partners, yielding incorporation rates of about 50%. MTO could not be incorporated into positively charged stearylamine liposomes or into uncharged SPC liposomes. When these lipid compositions were used >90% of the drug was lost by dialysis during liposome formation and the remaining encapsulated MTO rapidly leaked out of the liposomes (data not shown). Thus, a lipophilic anionic molecule is a prerequisite for the stable association of MTO with the liposome membrane.

All anionic compounds formed complexes at a 2:1 molar ratio. The corresponding liposomes were unilamellar and had mean diameters of 40–70 nm. With the exception of tridecyl phosphate-MTO, all complexes yielded stable liposomes. Since all subsequent experiments were performed using PA-MTO liposomes, the physicochemical and biological properties of the other complexes were not further investigated. Depending on the method of preparation, the mean size of the PA-MTO liposomes differed. Liposomes prepared by the batch-dialysis method at volumes of 10–30 ml were homogeneous, exhibiting mean diameters of 50–60 nm and a variance of 0.2–0.3 (Variance is a parameter of liposome population homogeneity. Values of 0.2–0.5 represent highly homogeneous populations; less homogeneous and heterogenous populations show values of >0.5 [37]). Increasing the MTO concentration from 0.1 to 3 mg/ml had no effect on liposome size and homogeneity. Variation of the molar ratio of PA to MTO base from 0:1 to 4:1 resulted in a decrease in liposome size from 100 nm for the preparation without PA (empty liposomes) to 45–50 nm for PA:MTO at ratios of 2:1, 3:1, and 4:1. At a complex stoichiometry of 2:1 (cf. Fig. 1), stable PA-MTO liposomes with incorporation efficiencies of 92%–99% as compared with the drug concentration in the micellar solutions were obtained (cf. Table 1). The higher complex ratios of 3:1 and 4:1 did not increase the incorporation of MTO. Therefore, the 2:1 molar complex was used in all further preparations.

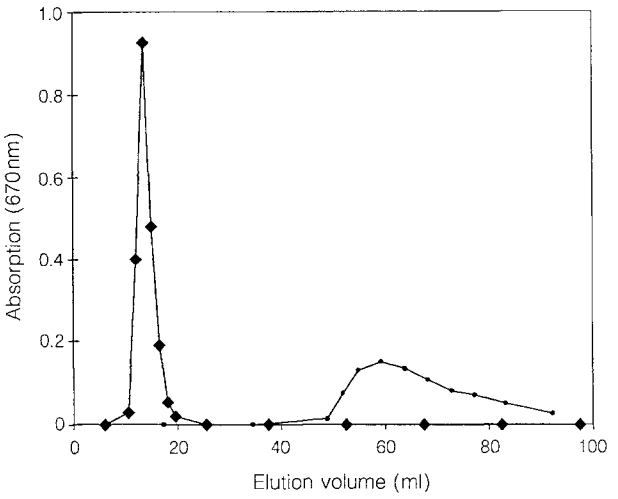

Fig. 2. Superimposed elution diagrams for PA-MTO liposomes (◆) and for free MTO (●). Liposome aliquots of 0.1–1 ml or free MTO were applied to a BioGel P6-DG column (2.5×12 cm) and eluted with phosphate buffer

Liposome batches (100–500 ml) that had been prepared by the capillary dialysis method were highly homogeneous but their size differed from that of liposomes prepared by the batch dialysis method. Their size also depended on the concentration of incorporated drug. At 0.1 mg MTO/ml, liposomes measuring 73 ± 10 nm ($n = 6$) in diameter and showing a mean variance of 0.28 ± 0.09 ($n = 6$) were obtained, whereas at 1 mg MTO/ml the size of liposomes ranged from 100–150 nm ($n = 2$), with a variance of 0.4–0.6 ($n = 2$). Thus, the higher concentration of PA-MTO incorporated produced larger and slightly less homogeneous liposomes. The difference in liposome size is probably caused by the different kinetics of removal of the detergent from the mixed micellar solutions.

The stability and efficiency of the incorporation of MTO into liposomes containing PA was investigated by column chromatography so as to separate uncomplexed MTO from the liposomes. Figure 2 shows superimposed diagrams of the elution of PA-MTO liposomes and of free MTO. PA-MTO liposomes that had been stored at 4°–7° C and chromatographed 6 months after their preparation exhibited an elution profile identical to that of the liposomes shown in Fig. 2. MTO that might have dissociated from the complex during storage was not detected. Empty reference PA liposomes were eluted at volumes identical to those of the PA-MTO liposomes (elution profiles not shown).

UV-VIS spectra for the liposomal PA-MTO complex recorded in 67mM phosphate buffer (pH 7.4) displayed a significant red shift of the drug absorption maximum from 660 nm for MTO · 2HCl to 672 nm for the lipsosomal PA complex. Concomitantly, an increase in relative absorbance was observed for the PA-MTO complex, similar to the effects observed following the interactions of MTO with DNA [3, 26]. Spectra recorded in organic solvents ($MeOH/CHCl_3$; 1 : 1, v/v) revealed no differences between free MTO and the PA-MTO complex, indicating that electrostatic interactions are predominantly responsible for the strong association with PA in aqueous media.

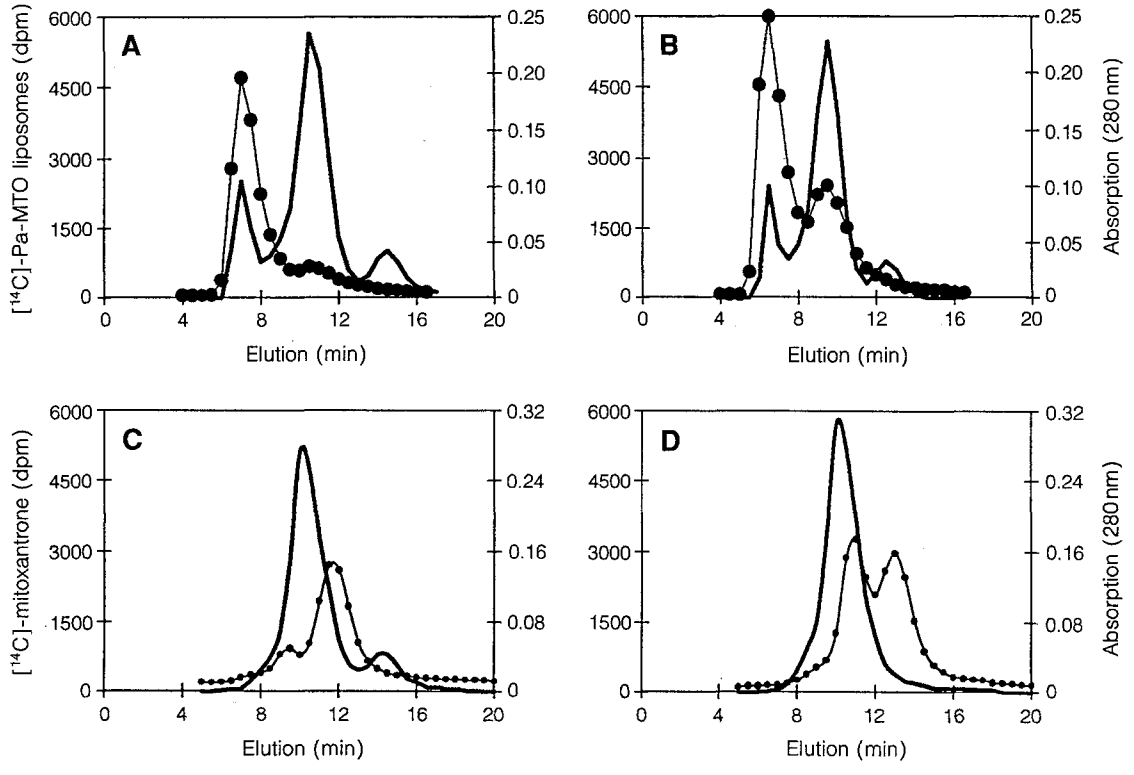

Fig. 3 A–D. HPLC elution diagrams for **A, B** liposomal MTO and **C, D** free MTO after incubation with human plasma at 37° C for different periods. **A** Elution of [^{14} C]-MTO liposomes (●——●) and plasma proteins (———) after 30 min incubation. **B** Elution of [^{14}C]-MTO liposomes after 240 min. **C** Elution of free [^{14}C]-MTO (●——●) and plasma proteins (———) after 30 min incubation. **D** Elution of free [^{14}C]-MTO after 240 min

In vitro interactions with human plasma

Figure 3 shows the interaction of liposomal MTO (panels A, B) and free MTO (panels C, D) at 30 (A, C) and 240 min (B, D) after incubation with human plasma at 37° C. The graphs illustrate the elution curves for [^{14}C]-MTO (filled circles) and the superimposed elution pattern of liposome and protein absorption detected at a wavelength of 280 nm (solid line). The transfer of MTO to the plasma proteins was calculated by forming the sums of the [^{14}C]-MTO counts under the elution peaks of the proteins (8–16 min retention time). The sum of the counts obtained in fractions collected at between 5 and 8 min were assigned to liposomal [^{14}C]-MTO. After 30 min incubation (panel A), 19.4% of the liposomal drug had bound to plasmaproteins; the corresponding value after 240 min was 34.5% (panel B), and that after 450 min was 48% (data not shown).

The corresponding elution curves for free MTO are shown in panels C and D. The quantitative binding of the drug to plasma proteins could not be calculated because distinct separation of free MTO could not be achieved with the HPLC column used. The two elution curves recorded after incubations for 30 (panel C) and 240 min (panel D) show a strong co-elution of [^{14}C]-MTO within the plasma protein peaks. The drug activities found under the plasma peaks after 240 min incubation were more pronounced and showed that free MTO interacts with plasma proteins distinctively differently than does liposomal MTO. The binding of free MTO to human plasma was quantitated in equilibrium dialysis experiments. In the concentration range of 0.025–0.5 mg MTO · 2HCl/ml (25–500 mM), the rate of binding to plasma proteins was 75.8% ± 2.9%. Thus, the complexation of MTO to PA liposomes reduces and delays its transfer and binding to plasma proteins.

Organ distribution and pharmacokinetic data

Figure 4 illustrates the time-dependent distribution of free MTO and liposomal drug in the blood, liver, and spleen of ICR mice after i. v. injection. The concentration of MTO in the heart was not significantly different from that in the kidneys following either mode of application. In the heart, decreasing amounts of free MTO ranged from 0.18 to 0.05 µg (0.9%–0.3% of the injected dose; time range, 0.05–24 h), whereas comparable amounts of liposomal drug ranged from 0.14 to 0.015 µg/organ (0.7%–0.08% of the injected dose; time range, 0.05–120 h). The elimination kinetics were comparable and did not reveal any of the cardiac accumulation of drug that is known to follow liposomal doxorubicin administration [2]. In the kidneys the values for free MTO were 2.7–0.74 µg/organ (13.5%–3.7%; time range, 0.05–24 h). For liposomal MTO, slightly lower values of 1.93–0.14 µg/organ (9.6%–0.7%; time range, 0.05–120 h) were found. Free drug was cleared from the kidneys in a biphasic fashion, with slow terminal elimination kinetics of 21.8 h, comparable with the $t_{1/2}\beta$ value calculated for liposomal MTO (cf. Table 2). The clearance of the two applications from the blood was found to display slightly different biphasic kinetics during

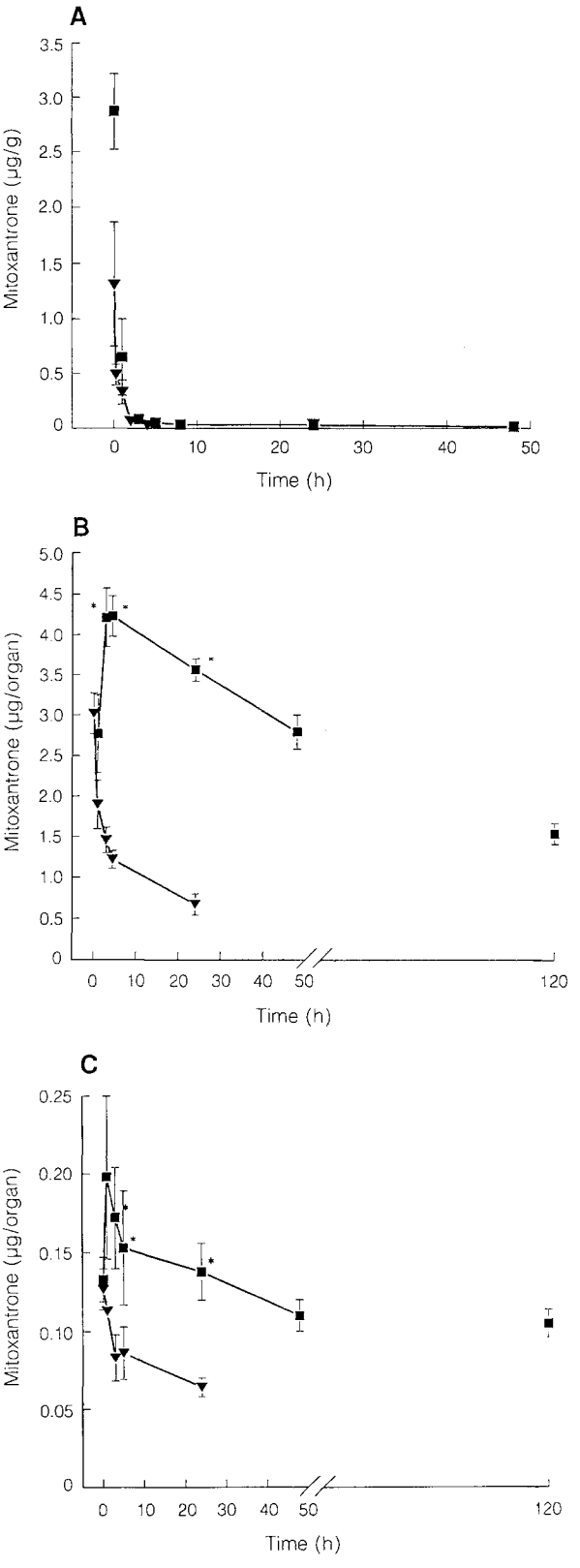

Fig. 4 A–C. Distribution of free (▼) and liposomal MTO (■) in **A** blood, **B** liver and **C** spleen after i. v. injection into ICR mice. MTO concentration was calculated from the [^{14} C]-MTO activities determined by scintillation counting. * P <0.05 for liposomal vs free MTO

Table 2. Pharmacokinetic data of free and liposomal MTO after i.v. injection in ICR mice

Organ	Elimination halflives[a]					
	Free MTO:			PA-MTO liposomes:		
	α (min)	β (h)	Range[b] (min)	α (min)	β (h)	Range[b] (min)
Blood	2.8	1.52	3–450	25.8	25	3–2,880
Liver	3.8	14.4	15–360	–	77	180–7,200
Spleen	120	48	3–1,440	–	25.1	60–7,200
Kidneys	6.6	21.8	3–1,440	24.3	25.1	3–7,200
Heart	22.1	41.2	3–1,440	29.9	41.2	3–7,200

[a] Half-lives were calculated by least-squares fitting

[b] Time ranges (min) from which the elimination half-lives were calculated

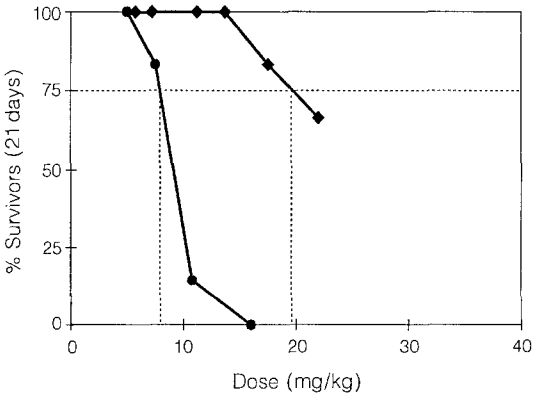

Fig. 5. Acute toxicity: survival curves for NMRI mice that were given a single i.v. injection of free MTO (●) and PA-MTO liposomes (◆). The period of observation was 21 days. The LD_{25} for free MTO was 7.7 mg/kg and that for PA-MTO liposomes was 19.6 mg/kg

the first 3 h. After liposomal MTO administration, a higher peak concentration and a slower rate of clearance were determined (cf. Fig. 4 and Table 2).

The difference in organ distribution was most prominent in the liver and spleen. In the liver, liposomal MTO accumulated at a concentration 2-fold that of free drug, showing a saturation effect at between 3 and 6 h after administration and reaching a peak level of 21% of the injected dose. Liposomally entrapped MTO was cleared from the liver at a very slow rate ($t_{1/2}\beta$, 77 h; cf. Table 2); after 5 days, 7.7% of the drug remained. Free MTO was also cleared in a biphasic fashion but at faster rates, showing a $t_{1/2}\beta$ value of 14.4 h, and without saturating the liver tissue; the amount of free drug remaining in the liver at 1 and 3 h after administration was 9.5% and 7.3% of the injected dose, respectively. Only 3.35% of the delivered dose of MTO remained in the liver after 24 h. In the spleen similar distribution values were found for the two application forms, with the concentration of liposomal drug being about 2-fold that of free MTO and persisting for about twice as long. Free MTO was cleared at rather slow rates from the spleen ($t_{1/2}\alpha$, 2 h; cf. Table 2), suggesting strong binding to splenic tissue.

Acute toxicity and myelosuppression

The acute toxicity of PA-MTO liposomes was compared with that of free drug in female NMRI mice. As shown in Fig. 5, LD_{25} values of 19.6 mg/kg for PA-MTO liposomes and 7.7 mg/kg for free MTO were obtained. The corresponding LD_{10} values were 16 and 6.5 mg/kg; thus, a 2.5-fold increase in the toxic concentration of the liposomal drug preparation resulted. The LD_{50} for PA-MTO liposomes could not be determined because the i.v. injection of large volumes was severely limited. The loss of weight in mice treated with free MTO was most prominent at 10 days after drug treatment. At the dosage of 7.5 mg MTO/kg the weight loss was 28% and 11 mg MTO/kg 37.5%, respectively. At the dosage of 16 mg MTO/kg all mice had died by day 10. Following the administration of PA-MTO liposomes, weight loss was clearly less pronounced: at dosages of up to 19 mg/kg, a loss of 12.5% of body weight resulted, whereas at 25 and 31 mg/kg, the reduction in body weight on day 10 was 31%. Generally, non-hematological toxic effects such as drug-induced weight loss, rough fur, and alopecia were less prominent following treatment with liposomally entrapped MTO.

The myelosuppressive effect of MTO was less pronounced when the liposomal form was injected. The sublethal dose of 7.5 mg/kg was given i.v. to groups of three mice, either in liposomes or as free drug. At 3 days after drug injection, the leucocyte count was $3{,}500 \pm 800/\mu l$ for the PA-MTO liposomes and $2{,}000 \pm 300/\mu l$ for the free drug; after 6 days these values were $4{,}800 \pm 2{,}000/\mu l$ and $2{,}300 \pm 800/\mu l$, respectively. The leucocyte count in untreated mice was $9{,}093 \pm 1{,}055/\mu l$. Thus, the myelosuppressive effect was reduced by about 50% when liposomal MTO was given.

Cytostatic activity against L1210 leukemia

The results of the treatment of L1210 leukemia in mice with the two application forms of MTO are summarized in Table 3. The cytostatic effect was evaluated using a single-dose schedule by which injections were given on day 1, 3, or 5 after tumor cell implantation (single-dose i.p./i.p. schedule). This protocol was chosen to evaluate whether the liposomal application of MTO exerted a stronger cytostatic effect that could possibly be due to a slow-release effect or to persistence of the drug in the peritoneal cavity.

Treatment with PA-MTO liposomes on day 1 or 3 was more effective, particularly at a dose of 8 mg/kg, at which not only higher T/C values were obtained, but also more 60-day survivors were recorded. A statistical difference was found only for the day-1 treatment with 8 mg/kg ($P = 0.1$). Single-dose treatment at 5 days after tumor inoculation was least effective for both drug preparations, most likely because the tumor burden was extremely high. The improved cytostatic effect of liposomal MTO at the onset of treatment may be attributed to the altered pharmacokinetic properties of the liposomal application. The free MTO dose of 8 mg/kg given as a bolus is comparable with

Table 3. Antitumor effect of PA-MTO liposomes against L1210 leukemia after a single i. p. dose on days 1, 3 or 5 after i. p. tumor implantation (single dose i. p./i. p. schedule)[a]

Drug preparation and day of treatment	Daily dose		Survival (days)	T/C[b] (%)	Survivors at 60 days (*n*)
	μmol/kg	mg/kg			
Free MTO[c]:					
Day 1	15.2	8	36 ± 2	414	0/5
	3.8	2	50.2±22	577	4/5
Day 3	15.2	8	44.6±23	512	3/5
	3.8	2	33 ±16	379	1/5
Day 5	15.2	8	27.2±18	312	1/5
	3.8	2	26 ±20	299	1/5
PA-MTO liposomes[d]:					
Day 1	15.2	8	60	689	5/5
	3.8	2	50.8±21	584	4/5
Day 3	15.2	8	49.6±23	570	4/5
	3.8	2	41.4±11	475	1/5
Day 5	15.2	8	26.4±12	303	0/5
	3.8	2	20.4± 6	234	0/5
Controls[e]	–	–	8.7± 2	100	0/7

[a] Ascites (0.2 ml) containing 1×10^5 viable L1210 cells was injected i. p. into BDF1 mice on day 0
[b] T/C, increase in the life span of drug-treated animals; ratio of the median survival for treated (T) and control (C) animals, expressed in percent
[c] MTO · 2HCl in phosphate buffer (pH 7.4)
[d] (1 mg/ml)
[e] Untreated control animals

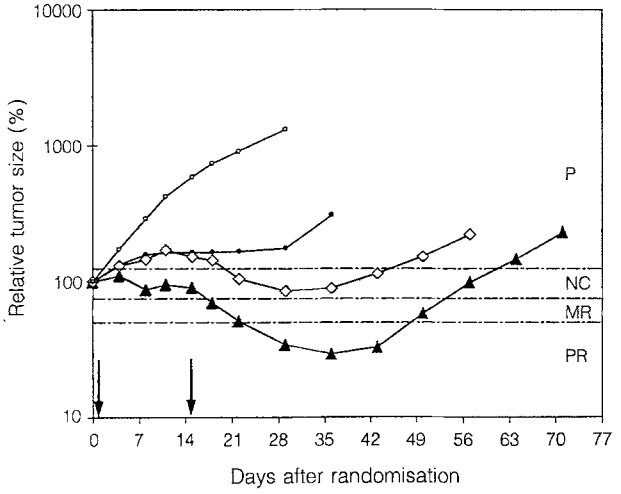

Fig. 6. Chemotherapy of large-cell lung carcinoma LXFL 529/10: survival curves after two i. v. treatments on days 1 and 15 after randomization. (○), Untreated controls; (◇), 4 mg/kg per day free MTO; (●), 4 mg/kg per day PA-MTO liposomes; (▲), 8 mg/kg per day PA-MTO liposomes; *arrows*, treatment days; *P*, progressive disease; *NC*, no change; *MR*, minor regression; *PR*, partial remission

the LD_{25} value of 7.7 mg/kg that was obtained in healthy mice (cf. Fig. 5). Given in the liposomal form, this dose of MTO was well tolerated by mice carrying L1210 leukemia, and a complete cure was obtained in all treated animals.

Antitumor activity in human tumor xenografts

Toxicity. The maximally tolerated dose (MTD) of free MTO in tumor-bearing nude mice was determined to be 4 mg/kg daily given i. v. on days 1 and 15. After 21 days, mortality was 15% (4/27 mice) and the median loss of body weight in treated animals was 11% (cf. Table 4). For PA-MTO liposomes, the MTD was found to be 8 mg/kg daily given i. v. on days 1 and 15, resulting in a mortality of 19% (5/26 mice) and a median body weight loss of 8% on day 21. Higher doses resulted in considerable toxicity for both MTO and PA-MTO liposomes.

Efficacy. MTO (4 mg/kg daily given i. v. on days 1 and 15) and PA-MTO liposomes (8 mg/kg daily given i. v. on days 1 and 15) were tested in five human tumor xenografts of different histologies. Three tumor lines (colorectal adenocarcinoma CXF 609, breast adenocarcinoma MAXF 401, and malignant melanoma MEXF 520) were resistant to both drugs. In two tumor lines, free MTO as well as PA-MTO liposomes effected tumor regressions. Breast cancer xenograft MAXF 449 showed a partial remission on day 17 that lasted for 15 weeks. No significant differences in the extent or duration of response were seen in animals treated with either form of MTO. Tumors in control animals grew progressively, showing a median

Table 4. Toxicity of MTO and PA-MTO liposomes in tumor-bearing nude mice

	Drug dose (mg kg^{-1} day^{-1})	Schedule (days)	Route	Mortality[a]		Median BWL[b] on day 21
				Day 14	Day 21	
Control	–	–	–	0/24 (0)	0/24 (0)	+ 2%
Free MTO	6	1,15	i.v.	5/12 (42%)	7/12 (58%)	–28%
	5	1,15	i.v.	5/20 (25%)	5/20 (20%)	–21%
	4	1,15	i.v.	3/27 (11%)	4/27 (15%)	–11%
PA-MTO liposomes	12	1,15	i.v.	5/6 (83%)	5/6 (83%)	–31%
	10	1,15	i.v.	6/12 (50%)	7/12 (58%)	–27%
	8	1,15	i.v.	3/26 (12%)	5/26 (19%)	– 8%
	6	1,15	i.v.	4/25 (16%)	4/25 (16%)	– 1%
	4	1,15	i.v.	3/25 (12%)	5/25 (20%)	0

[a] Drug-related toxicity
[b] Median body weight change as compared with the value at the start of treatment

Table 5. Influence of free MTO and PA-MTO liposomes on MNU-induced rat mammary carcinoma

Group	Animals (*n*)	Treatment[a] (mg/kg)	Median tumor volume[e]		Median number of tumors[b]		BWC[c]		Mortality[d]
			Week 3 (cm^3)	Week 6 (cm^3)	Week 3	Week 6	Week 3	Week 6	
I	10	MTO (2)	5.4 (54%)	5.7 (29%)	3.5 (1–6)	3 (1–6)	1%	–11.5%	50%
II	10	MTO (4)	3.2 (32%)	Tox[f]	3 (1–3)	Tox[f]	–7%	Tox[f]	100%
III	10	PA-MTO[g] (2)	3.5 (35%)	2.8 (14%)	2.5 (1–5)	3 (1–6)	–1%	1%	20%
IV	10	PA-MTO[g] (4)	0.7 (7%)	Tox[f]	2 (0–9)	Tox[f]	–12%	Tox[f]	100%
V	20	Controls	10 (100%)	20 (100%)	3 (3–5)	5 (3–6)	3%	7%	25%

[a] Treatment given i.v. once weekly for 5 weeks; the dose is shown in parentheses
[b] 95% confidence limits are shown in parentheses
[c] Body weight change
[d] Mortality at week 6
[e] Mean tumor volume of treated animals divided by that of controls
[f] Values not determined due to 100% mortality
[g] PA-MTO liposomes

tumor-doubling time of 22 days. Large-cell lung carcinoma LXFL 529 showed marginal sensitivity to MTO, as no change was observed (cf. Fig. 6). In contrast, PA-MTO liposomes given i.v. at a dose of 8 mg/kg daily on days 1 and 15 effected a partial tumor regression, resulting in a relative tumor size of 29% on day 36, after which slow regrowth was noted. The tumor-doubling time was 5.1 days in the control group as compared with 54.8 and 68.5 days in animals treated with free MTO and PA-MTO liposomes, respectively.

Antitumor activity against MNU-induced rat mammary carcinoma

The i.v. administration of free MTO (cf. Table 5) caused a significant tumor growth delay at week 6 when a dose of 2 mg/kg was given. Concomitantly, considerable toxicity as manifested by body weight decrease (11.5%) and mortality (50%) was observed. Higher doses (4 mg/kg) led to 100% mortality at the end of therapy, which did not enable meaningful growth-delay evaluation. PA-MTO liposomes were more active and less toxic in rats treated with 2 mg/kg as compared with the same dose of free drug. Again, when a higher dose was used (4 mg/kg) severe toxicity prevented assessment of tumor growth. A lower concentration (1 mg/kg) of the liposomal formulation, on the other hand, revealed activity similar to that observed at a dose of 2 mg/kg free MTO, indicating a plateau of anticancer activity at these dose levels (data not shown).

Discussion

MTO forms electrostatic complexes with several lipophilic anionic compounds. Among the compounds we investigated, PA formed the strongest complex at a 2:1 molar ratio, yielding the highest rate of stable incorporation of MTO into the liposome membranes. With the exception of tridecyl phosphate, all complexes with the lipophilic anionic compounds produced physically stable and homogeneous unilamellar liposomes. Presumably, the interaction of MTO with the anionic groups occurs through the electrostatic association of the two basic secondary amino groups of the MTO side chains.

Other investigators have prepared similar liposomal complexes with the antitumor drug doxorubicin [19, 30, 35], using PA, phosphatidyl glycerol or cardiolipin as the negatively charged lipid. These authors found that the nature of the amino group on the aminosugar moiety plays an important role in determining the strength and orientation of the interaction of the anthracyclines with negatively charged lipids [6, 7, 20]. Such liposomal doxorubicin preparations have been found to exert strong antitumor effects in different tumor models [2, 24, 30, 35]. In addition to their cytostatic activity, these preparations are significantly less cardiotoxic, which has encouraged their use in clinical trials [11, 19]. Comparably strong associations of basic drugs with negatively charged lipids in cell membranes or with liposomes have also been found using phenothiazines that exert some of their pharmacological effects by membrane interactions [10]. A preparation of chlorpromazine that was complexed to liposomes containing PA protected the drug from metabolic degradation in liver microsomes [39].

The in vitro interactions of liposomal MTO with human plasma in the present study showed that due to the strong complex formation with PA, MTO transfer and binding to plasma proteins was delayed. The use of different lipid compositions to prepare the liposomes might further reduce the transfer of drug to the proteins and lead to longer-lasting plasma levels of MTO [1, 18]. The organ-distribution experiments disclosed the most pronounced differences between free and liposomal MTO in the liver and spleen. The higher and longer-lasting concentrations of liposomal MTO found in these organs are most likely caused by the natural targeting of liposomes to the organs of the mononuclear phagocyte system (MPS). Liposomal MTO that is accumulated in these organs is released at slow rates, as was shown by the slow terminal clearance ($t_{1/2}\beta$, 77 h; cf. Table 2) of liposomal MTO from the liver. The clearance of liposomally entrapped MTO from the circulation was slower than that of the free drug [29]. This effect might be caused by the different kinetics of MTO binding to plasma proteins as determined in the in vitro incubation experiments (cf. Fig. 3).

In healthy mice, the PA-MTO liposome preparations displayed acute toxicity that was 2- to 3-fold lower than that of the free drug. In the L1210 leukemia tumor model, the liposomal drug preparation was effective when i.p.

implanted L1210 cells were treated by i.p. injection on days 1 or 3 after tumor cell implantation. These results suggest either a mechanism of slow release of the complexed drug from the liposomes or a slower but more effective cellular uptake of the released complex or intact liposomes by the tumor cells. Furthermore, it is conceivable that with liposomal application, the drug remains in the peritoneal cavity for longer periods, resulting in a higher cytostatic effect. The stronger antitumor effect obtained following a single dose of the liposomal preparation on day 1 reflects such a delayed mode of action. The opposite effect was obtained when the animals were treated once on day 5, where free MTO produced a slightly better effect. When treatment was begun this late, the tumor-cell burden had become too high to enable the achievement of a curative effect.

In nude mice bearing human tumor xenografts, the MTD for free MTO was 4 mg/kg daily given i.v. on days 1 and 15; for PA-MTO liposomes, a higher dose of 8 mg/kg daily was tolerated. Two of five xenografts tested at MTD levels demonstrated sensitivity to MTO. In LXFL 529, higher activity was observed for the liposomal formulation than for the free drug. In breast cancer MAXF 449, both preparations exerted the same effect. Three other tumor lines were resistant to both free MTO and PA-MTO liposomes. Thus, PA-MTO liposomes produced lower toxicity than did free MTO in tumor-bearing nude mice. At the higher dose of 8 mg/kg daily, an increase in the activity of the liposomal formulation was observed in xenografts that were sensitive to MTO.

The treatment of MNU-induced rat mammary carcinoma with the two application forms of MTO revealed higher anticancer activity and lower toxicity for the liposomal preparation. At a dose of 2 mg/kg, the tumor volumes and the T/C values were lower for PA-MTO liposomes. At the higher dose of 4 mg/kg, the liposomal application was more effective during the first 3 weeks of the experiment. After 6 weeks, however, both application forms were toxic, which led to the death of all treated animals.

In conclusion, we found that MTO can be incorporated into liposomes by the formation of a complex with negatively charged lipids, particularly with PA. The binding of MTO to plasma proteins was less pronounced when the drug was complexed to PA-liposomes. The organ distribution and pharmacokinetics of liposomal MTO were also different from those of the free drug, especially in the organs of the MPS. Using PA-MTO liposomes, we found an LD_{25} value that was approximately 2–3 times below that of the free drug. Also, depending on the tumor model and the application schedules used, cytostatic activities were obtained using the liposomal application form that were equivalent to, if not higher than, those determined for free MTO.

Thus, using liposomal MTO, we obtained advantageous pharmacokinetic and cytotoxic effects that were similar to those previously reported for other liposome-encapsulated drugs such as doxorubicin [2, 19, 24, 35] and methotrexate [22] of for lipophilic derivatives of cisplatin [27, 33] and cytosine arabinoside [37, 42]. The superior properties of the liposomal formulation of MTO enables the administration of higher-dose regimens. We have conducted a clinical phase I/II study of liposomal MTO in advanced breast cancer [34] and found the MTD to be 18 mg/m^2 as compared with 12–14 mg/m^2 for the free drug. Neutropenia was dose-limiting and thrombocytopenia as well as nonhematological side effects were rare. Partial responses were seen in 8 of 19 evaluable patients. The liposomal formulation of MTO may therefore represent an interesting new application for this anthracenedione compound. In addition to breast cancer therapy, the treatment of acute leukemias, lymphomas, and liver tumors or hepatic metastases may be more effective when the liposomal preparation is used. However, further preclinical studies are needed to elucidate the efficacy of liposomal MTO in other tumor models and to determine the cellular uptake mechanisms.

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References

1. Allen TM, Hansen C, Rutledge J (1989) Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. Biochim Biophys Acta 981: 27–35
2. Balazsovits JAE, Mayer LD, Bally MB, Cullis PR, McDonell M, Ginsberg RS, Falk RE (1989) Analysis of the effect of liposome encapsulation on the vesicant properties, acute and cardiac toxicities, and antitumor efficacy of doxorubicin. Cancer Chemother Pharmacol 23: 81–86
3. Bartkowiak J, Kapuscinski J, Melamed MR, Darzynkiewicz Z (1989) Selective displacement of nuclear proteins by antitumor drugs affinity for nucleic acids. Proc Natl Acad Sci USA 86: 5151–5154
4. Berger MR, Floride J, Schmähl D, Schreiber J, Eisenbrand G (1986) Estrogen-linked 2-chloroethylnitrosoureas: anticancer efficacy in MNU-induced rat mammary carcinoma, uterine activity in mice and receptor interactions. Eur J Cancer Clin Oncol 22: 1179–1191
5. Brito-Babapulle F, Catovsky D, Newland AC, Goldman JM, Galton DAG (1987) Treatment of acute myeloid leukemia with intermediate-dose cytosine arabinoside and mitoxantrone. Semin Oncol 14 [Suppl 1]: 51–52
6. Burke TG, Tritton TR (1985) Structural basis of anthracyline selectivity for unilamellar phosphatidylcholine vesicles: an equilibrium binding study. Biochemistry 24: 1768–1776
7. Burke TG, Sartorelli AC, Tritton TR (1988) Selectivity of the anthracyclines for negatively charged model membranes: role of the amino group. Cancer Chemother Pharmacol 21: 274–280
8. Burns CP, Haugstad BN, Mossman CJ, North JA, Ingraham LM (1988) Membrane lipid alteration: effect on cellular uptake of mitoxantrone. Lipids 23: 393–397
9. Coltman CA, Coltman TM, Balcerzak SP, Morrison FS, Von Hoff DD (1984) Mitoxantrone in refractory non-Hodgkin's lymphoma. A Southwest Oncology Group study. Semin Oncol 11 [Suppl 1]: 50–53
10. Conrad MJ, Singer SJ (1981) The solubility of amphipathic molecules in biological membranes and lipid bilayers and its implications for membrane structure. Biochemistry 20: 808–818
11. Delgado G, Potkul RK, Treat JA, Lewandowski GS, Barter JF, Forst D, Rahman A (1989) A phase I/II study of intraperitoneally administered doxorubicin entrapped in cardiolipin liposomes in patients with ovarian cancer. Am J Obstet Gynecol 160: 812–819
12. Drewinko B, Yang L-Y, Barlogie B, Trujillo JM (1983) Comparative cytotoxicity of bisantrene, mitoxantrone, ametantrone, dihydroxyanthracenedione, dihydroxy-anthracenedione diacetate, and doxorubicin on human cells in vitro. Cancer Res 43: 2648–2653

13. Durr FE (1984) Biologic and biochemical effects of mitoxantrone. Semin Oncol 11 [Suppl 1]: 3–10
14. Fiebig HH (1988) Comparison of tumor response in nude mice and in patients. In: Winograd B, Peckham MJ, Pineo HM (eds) Human tumor xenografts in anticancer drug development. Springer, Berlin Heidelberg New York London Paris Tokyo, pp 25–30
15. Fiebig HH, Löhr GW (1984) Wachstum menschlicher Malignome in der thymusaplastischen Nacktmaus. Med Welt 35: 52–58, 81–86
16. Ford JM, Panasci L, Leclerc Y, Margolese R (1987) Phase II trial of a combination of doxorubicin and mitoxantrone in metastatic breast cancer. Cancer Treat Rep 71: 921–925
17. Fortmeyer HP, Bastert G (1981) Breeding and maintenance of nu/nu mice and rnu/rnu rats. In: Bastert G, Fortmeyer HP, Schmidt-Mathiesen H (eds) Thymus aplastic nude mice and rats in clinical oncology. G. Fischer, Stuttgart New York, pp 25–38
18. Gabizon A, Papahadjopoulos D (1989) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 85: 6949–6953
19. Gabizon A, Sulkes A, Peretz T, Druckmann S, Goren D, Amselem S, Barenholz Y (1989) Liposome-associated doxorubicin: preclinical pharmacology and exploratory clinical phase I/II. In: Lopez-Berenstein G, Fidler IJ (eds) Liposomes in the therapy of infectious diseases and cancer. Alan R. Liss, New York, pp 391–407
20. Goormaghtigh E, Chatelain P, Caspers J, Ruysschaert JM (1980) Evidence of a specific complex between Adriamycon and negatively-charged phospholipids. Biochim Biophys Acta 597: 1–14
21. Grant Prentice H, Robbins G, Ma DDF, Ho AD (1984) Mitoxantrone in relapsed and refractory acute leukemia. Semin Oncol 11 [Suppl 1]: 32–35
22. Heath TD, Lopez NG, Piper JR, Montgomery JA, Stern WH, Papahadjopoulos D (1986) Liposome-mediated delivery of pteridine antifolates to cells in vitro: potency of methotrexate, and its alpha and gamma substituents. Biochim Biophys Acta 862: 72–86
23. Hiddemann W, Kreutzmann H, Straif K, Ludwig W-D, Mertelsmann R, Planker M, Donhuijsen-Ant R, Lengfelder E, Arlin Z, Büchner T (1984) High-dose cytosine arabinoside in combination with mitoxantrone for the treatment of refractory acute myeloid and lymphoblastic leukemia. Semin Oncol 14 [Suppl 1]: 73–77
24. Hoesel QCGM van, Steerenberg PA, Crommelin DJA, Dijk A van, Oort W van, Klein S, Douze JMC, Wildt DJ de, Hillen FC (1984) Reduced cardiotoxicity and nephrotoxicity with preservation of antitumor activity of doxorubicin entrapped in stable liposomes in the LOU/M Wsl rat. Cancer Res 44: 3698–3705
25. Johnson RK, Zee-Cheng RK-Y, Lee WW, Acton EM, Henry DW, Cheng CC (1979) Experimental antitumor activity of anthraquinones. Cancer Treat Rep 63: 425–439
26. Kapuscinski J, Darzynkiewicz Z, Traganos F, Melamed MR (1981) Interactions of a new antitumor agent, 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]-ethyl]amino]-9,10-anthracenedione, with nucleic acids. Biochem Pharmacol 30: 231–240
27. Khokhar AR, Al-Baker S, Krakoff IH, Perez-Soler R (1989) Toxicity and antitumor activity of *cis-bis*-carboxylato(*trans-R,R*-1,2-diaminocyclohexane) platinum(II) complexes entrapped in liposomes. Cancer Chemother Pharmacol 23: 219–224
28. Landys K, Borgstrom S, Andersson T, Noppa H (1985) Mitoxantrone as a first-line treatment of advanced breast cancer. Invest New Drugs 3: 133–137
29. Lu K, Savaraj N, Loo TL (1984) Pharmacological disposition of 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione dihydrochloride in the dog. Cancer Chemother Pharmacol 13: 63–66
30. Mayhew EG, Goldrosen MH, Vaage J, Rustum YM (1987) Effects of liposome-entrapped doxorubicin on liver metastases of mouse colon carcinomas 26 and 28. J Natl Cancer Inst 78: 707–713
31. Paciucci PA, Cuttner J, Holland JF (1984) Mitoxantrone as a single agent and in combination chemotherapy in patients with refractory acute leukemia. Semin Oncol 11 [Suppl 1]: 36–40
32. Patel KR, Baldeschwieler JD (1984) Treatment of intravenously implanted Lewis lung carcinoma with liposome-encapsulated cytosine arabinoside and non-specific immunotherapy. Int J Cancer 34: 415–420
33. Perez-Soler R, Khokhar AR, Lopez-Berenstein G (1987) Treatment and prophylaxis of experimental liver metastases of M5076 reticulosarcoma with *cis-bis*-neodecanato-*trans-R,R*-1,2-diaminocyclohexane platinum(II) encapsulated in multilamellar vesicles. Cancer Res 47: 6462–6466
34. Pestalozzi B, Schwendener RA, Sauter Chr (1990) Liposomal mitoxantrone (L-Mto) is effective in metastatic breast cancer (abstract). Proc Am Soc Clin Oncol 9: 26
35. Rahman A, Fumagalli A, Barbieri B, Schein P, Casazza AM (1986) Antitumor and toxicity evaluation of free doxirubicin and doxorubicin entrapped in cardiolipin liposomes. Cancer Chemother Pharmacol 16: 22–27
36. Richard B, Fabre G, Fabre I, Cano JP (1989) Excretion and metabolism of mitoxantrone in rabbits. Cancer Res 49: 833–837
37. Rubas W, Supersaxo A, Weder HG, Hartmann HR, Hengartner H, Schott H, Schwendener R (1986) Treatment of murine L1210 lymphoid leukemia and melanoma B16 with lipophilic cytosine arabinoside prodrugs incorporated into unilamellar liposomes. Int J Cancer 37: 149–154
38. Schwendener RA (1986) The preparation of large volumes of homogeneous, sterile liposomes containing various lipophilic cytostatic drugs by the use of a capillary dialyzer. Cancer Drug Deliv 3: 123–129
39. Schwendener RA (1988) Incorporation of chlorpromazine into bilayer liposomes for protection against microsomal metabolism and liver absorption. Eur J Drug Metab Pharmacokinet 13: 135–141
40. Schwendener RA, Weder HG (1978) The binding of chlorpromazine to bilayer liposomes. Biochem Pharmacol 27: 2721–2727
41. Schwendener RA, Schott H, Hartmann HR, Supersaxo A, Rubas W, Hengartner H (1987) Liposomen al Träger von lipophilen n Cytosinarabinosid- und Fluorodeoxyuridin-Derivaten. Onkologie 10: 232–239
42. Schwendener RA, Pestalozzi B, Berger S, Schott H, Hengartner H, Sauter C (1989) Treatment of acute myelogenous leukemia with liposomes containing N^4-oleyl-cytosine arabinoside. In: Lopez-Berenstein G, Fidler IJ (eds) Liposomes in the therapy of infectious diseases and cancer. Alan R. Liss, New York, pp 95–103
43. Shenkenberg TD, Von Hoff DD (1986) Mitoxantrone: a new anticancer drug with significant clinical activity. Ann Intern Med 105: 67–81
44. Smith IE (1983) Mitoxantrone (Novantrone): a review of experimental and early clinical studies. Cancer Treat Rev 10: 103–115
45. Steerenberg PA, Storm G, Groot G de, Claessen A, Bergers JJ, Franken MAM, Hoesel QGCM van, Wubs KL, Jong WH de (1988) Liposomes as drug carrier system for *cis*-diamminedichloroplatinum(II): II. Antitumor activity in vivo, induction of drug resistance, nephrotoxicity and Pt distribution. Cancer Chemother Pharmacol 21: 299–307
46. Stuart-Harris RC, Bozek T, Pavlidis NA, Smith IE (1984) Mitoxantrone: an active new agent in the treatment of advanced breast cancer. Cancer Chemother Pharmacol 12: 1–4
47. Supersaxo A, Rubas W, Hartmann HR, Schott H, Hengartner H, Schwendener RA (1988) The antitumor effect of lipophilic derivatives of 5-fluoro-2′-deoxyridine incorporated into liposomes. J Microencaps 5: 1–11
48. Vile GF, Winterbourn CC (1989) Microsomal lipid peroxidation induced by Adriamycin, epirubicin, daunorubicin and mitoxantrone: a comparative study. Cancer Chemother Pharmacol 24: 105–108
49. Wallace RE, Murdock KC, Angier RB, Durr FE (1979) Activity of a novel anthracenedione, 1,4-dihydroxy-5,8-bis{{[2-[(2-hydroxyethyl)amino]ethyl}amino}}-9,10-anthracenedione dihydrochloride, against experimental tumors in mice. Cancer Res 39: 1570–1574
50. Walters RS, Kantarjian HM, Keating MJ, Plunkett WK, Estey EH, Andersson B, Beran M, McCredie KB, Freireich EJ (1988) Mitoxantrone and high-dose cytosine arabinoside in refractory acute myelogenous leukemia. Cancer 62: 677–682