Reduced Toxicity and Superior Therapeutic Activity of a Mitomycin C Lipid-Based Prodrug Incorporated in Pegylated Liposomes

Alberto A. Gabizon,[1,3] Dinah Tzemach,[1] Aviva T. Horowitz,[2] Hilary Shmeeda,[1] Jerry Yeh,[4] and Samuel Zalipsky[4]

Abstract

Purpose: A lipid-based prodrug of mitomycin C [MMC; 2,3-(distearoyloxy)propane-1-dithio-4′-benzyloxycarbonyl-MMC] was designed for liposome formulation. The purpose of this study was to examine the *in vitro* cytotoxicity, pharmacokinetics, *in vivo* toxicity, and *in vivo* antitumor activity of this new lipid-based prodrug formulated in polyethylene glycol–coated (pegylated) liposomes.

Experimental Design: MMC was released from the MMC lipid–based prodrug (MLP) by thiolytic-induced cleavage with a variety of thiol-containing reducing agents. MLP was incorporated with nearly 100% efficiency in cholesterol-free pegylated liposomes with hydrogenated phosphatidylcholine as the main component and a mean vesicle size of ~90 nm. This formulation was used for *in vitro* and *in vivo* tests in rodents.

Results: *In vitro*, the cytotoxic activity of pegylated liposomal MLP (PL-MLP) was drastically reduced compared with free MMC. However, in the presence of reducing agents, such as cysteine or *N*-acetyl-cysteine, its activity increased to nearly comparable levels to those of free MMC. Intravenous administration of PL-MLP in rats resulted in a slow clearance indicating stable prodrug retention in liposomes and long circulation time kinetics, with a pharmacokinetic profile substantially different from that of free MMC. *In vivo*, PL-MLP was ~3-fold less toxic than free MMC. The therapeutic index and absolute antitumor efficacy of PL-MLP were superior to that of free MMC in the three tumor models tested. In addition, PL-MLP was significantly more active than a formulation of doxorubicin in pegylated liposomes (DOXIL) in the M109R tumor model, a mouse tumor cell line with a multidrug-resistant phenotype.

Conclusions: Delivery of MLP in pegylated liposomes is a potential approach for effective treatment of multidrug-resistant tumors while significantly buffering the toxicity of MMC.

Mitomycin C (MMC) is an antibiotic isolated from *Streptomyces caespitosus*, with a wide spectrum of antineoplastic activity (1, 2). Its mechanism of toxicity has been associated with DNA cross-linking, formation of monoadducts with DNA, and free radical–induced DNA strand breaks (3–5). Activation of MMC is proposed to occur as a result of enzymatic reduction of the quinone moiety by either a one-electron pathway to a semiquinone or by a direct two-electron reduction pathway to a hydroquinone (6, 7). MMC is a highly potent drug with tumor cell inhibitory concentration (IC_{50}) values usually in the submicromolar range. Resistance to MMC in a panel of human colon cancer cell lines is best correlated with deficiency of diphtheria toxin-diaphorase and not related with expression of the P-glycoprotein efflux pump (8). MMC is a poor substrate for P-glycoprotein (9, 10) and retains activity against many of the cell lines with P-glycoprotein-mediated multidrug resistance (MDR) phenotype (11, 12). In this context, we have reported significant *in vitro* and *in vivo* activity against MDR tumors using mitomycin A, a potent methoxy analogue of MMC (13). Although MMC remains an optional component of the chemotherapeutic approach to the treatment of disseminated cancers of the lung, breast, and gastrointestinal tract (14), its clinical efficacy is modest and often disappointing in these diseases. The reasons stem partly from its problematic toxicity profile characterized by protracted damage to bone marrow and other tissues, requiring long dose intervals despite the fact that the drug is rapidly cleared and metabolized (14).

Long-circulating liposomes obtained by polyethylene glycol surface coating, also called pegylated or STEALTH liposomes, provide important pharmacologic advantages in cancer drug delivery due to their ability to extravasate in tissues with increased microvascular permeability and thereby concentrate in tumors (15). In addition, liposomes often reduce acute and delayed side effects of anticancer drugs as a result of slow drug

Authors' Affiliations: [1]Shaare Zedek Medical Center, [2]Hadassah Medical Center, and [3]Hebrew University-Faculty of Medicine, Jerusalem, Israel; and [4]ALZA Corporation, Mountain View, California

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Requests for reprints: Alberto A. Gabizon, Oncology Institute, Shaare Zedek Medical Center, P.O. Box 3235, 91031 Jerusalem, Israel. Phone: 972-2-6555036; Fax: 972-2-6521431; E-mail: alberto@md.huji.ac.il.

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release and changes in tissue distribution, thus sparing sensitive tissues (16). As a highly potent cytotoxic compound with a problematic toxicity profile, MMC would be an attractive candidate for pegylated liposomal delivery were it not for its rapid trans-lipid bilayer diffusion that prevents stable entrapment in liposomes. Moreover, long-circulating liposomes impose stringent requirements on the drug loading and leakage characteristics to reach tumors with an almost intact drug payload. It is a formidable challenge to retain the drug payload in the blood stream during prolonged periods of circulation, thereby taking full advantage of the pharmacokinetic and biodistribution benefits of the pegylated liposomal carriers (17). In early experiments, we attempted to encapsulate MMC in liposomes either passively in buffer solution or by encapsulation of cyclodextrin-MMC complexes in liposomes, but did not obtain stable formulations due to rapid leakage of MMC resulting in an *in vivo* half-life in rodents of only up to 1 hour.[5] An alternative approach to delivering MMC via long-circulating liposomes was to design a lipophilic prodrug that would be retained in liposomes, but could gradually release active MMC upon exposure to appropriate environmental conditions. Attachment of drugs to bilayer-compatible lipids can ensure prolonged association with a liposome (18, 19), where an acceptable release rate is determined by the proper choice of the linkage. We have prepared a lipophilic MMC prodrug conjugate where the drug is attached to 1,2-distearoyl glycerol lipid via a cleavable dithiobenzyl linker (Fig. 1A; refs. 20, 21). This conjugate (known as JNJ-27548547, ALZA Corporation, Mountain View, CA) is highly lipophilic and compatible with liposomal bilayers. Upon thiolytic cleavage of the disulfide-substituted benzyl urethane, MMC is released and becomes bioavailable (22). We hypothesized that the kinetics of liposome localization in tumor tissue will be faster than the kinetics of prodrug thiolytic activation *in vivo*, thus enabling selective tumor drug delivery in the prodrug form and reduced systemic toxicity. In this report, we present our pharmacologic studies of a pegylated liposomal formulation carrying this MMC lipid-based prodrug (PL-MLP) in rodents.

Materials and Methods

Liposome preparation. A mixture of hydrogenated soybean phosphatidylcholine, methoxy-polyethylene glycol (~MW = 2,000 Da)-distearoyl-phosphatidylethanolamine, and the prodrug conjugate [2,3-bis(distearoyloxy)propane-1-dithio-4′-benzyloxycarbonyl-MMC] at a molar ratio of 90:5:5, respectively, was prepared and solubilized in tertiary butanol. The batch size ranged between 500 to 1,600 μmol phospholipid. This mixture was lyophilized to yield a large surface area, fine powder cake, and then resuspended in an aqueous (pH 6.7) buffer containing HEPES (15 μmol/L), dextrose (4.5%), and NaCl (0.09%) at a phospholipid concentration of 50 to 100 μmol/mL. The liposome suspension was serially extruded under high pressure through polycarbonate membranes from 1 μm pore size down to 0.05 μm pore size. MLP concentration in the liposome suspension was measured as MMC equivalents by absorbance at 360 nm after liposome solubilization in a 20-fold volume of isopropanol. A standard curve of free MMC in isopropanol was used as reference because spectrum and absorbance of free MMC were identical to MLP in isopropanol. Phospholipid concentration in the liposome suspension was measured by a phosphate assay and liposome size was measured by dynamic laser scattering as described previously (23). The final product was a translucid, light-blue liposome suspension with mean vesicle size of 80 to 90 nm, a drug (MMC equivalents)/phospholipid ratio of ~18 μg/μmol, and a concentration of 0.5 mg/mL MMC equivalents after adjustment with further dilution in buffer. Input and output drug/phospholipid ratios were similar, indicating that encapsulation efficiency of MLP was very high. Some drug loss occurred apparently during extrusion, concomitantly with lipid loss. There was no decrease in drug concentration after extensive dialysis of the final product, indicating that there was no free drug present and that the liposome-entrapped drug was constitutively and stably incorporated in the liposome membrane. The PL-MLP formulation was stable for at least 6 months after preparation with no change in particle size and no drug leakage.

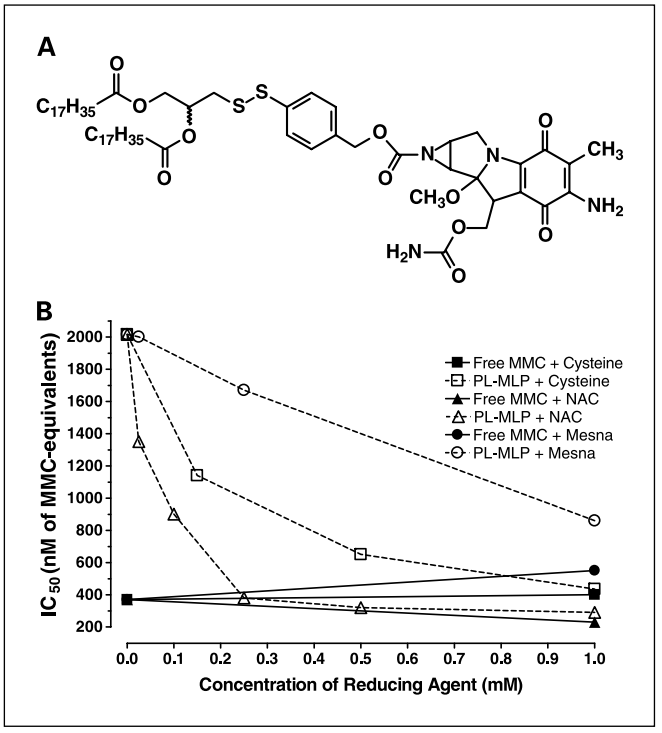


Fig. 1. *A,* structure of MLP, 2,3-(distearoyloxy)propane-1-dithio-4′-benzyloxycarbonyl-MMC (JNJ-27548547). *B,* enhancement of *in vitro* cytotoxicity of PL-MLP by reducing agents. IC_{50} values (MMC equivalents) as determined in M109 tumor cell line after 72 hours of continuous exposure to free MMC or PL-MLP with or without reducing agents.

In vitro *studies*. Mouse carcinoma M109 cells (24) were seeded in 96-multiwell plates. After overnight incubation, drugs were added for a total continuous exposure time of 72 hours. Cell growth was evaluated colorimetrically with methylene blue staining and growth rates and IC_{50} values were calculated as described previously (25).

Pharmacokinetic study. Sprague-Dawley rats were injected i.v. with free MMC or PL-MLP. An aliquot of rat plasma (0.1 mL) was denatured by 10-minute incubation with methanol (0.9 mL) followed by centrifugation at 3,000 rpm for 10 minutes. The supernatant was analyzed for the presence of MMC and MLP by reverse-phase high-performance liquid chromatography. The system consisted of a Supelco C-8, 4.6 mm × 5 cm column, a mobile phase consisted of a gradient of 10 mmol/L ammonium phosphate (pH 7.0) and methanol, and UV detection at 360 nm. The injection volume, flow rate, and run time were respectively 40 μL, 1 mL/min, and 15 minutes per sample. Control plasma samples of noninjected rats were added to MMC and MLP to obtain standard curves. MMC and MLP were clearly separated with retention times of 2.60 and 10.85 minutes, respectively. Plasma concentration data were expressed as MMC equivalents and analyzed for calculation of pharmacokinetic variables of free MMC and PL-MLP

[5] A. Gabizon et al., unpublished data.

using PK-Analyst software (Salt Lake City, UT) with monoexponential (concentration = $Ae^{-\alpha \times \text{time}}$, where A and α are the variables found by the model) or biexponential (concentration = $Ae^{-\alpha \times \text{time}} + Be^{-\beta \times \text{time}}$, where A, α, B, and β are the variables found by the model) models of clearance. The following parameters are presented in Table 1: peak plasma concentration (C_{max}), half-life ($t_{1/2}$), area under the curve, clearance (calculated as dose / area under the curve), and volume of distribution at steady-state (calculated as dose × mean residence time / area under the curve). The monoexponential model was used for free MMC, which was detected only during a single phase of distribution/elimination. The biexponential model was used for analyzing the clearance of PL-MLP. Pharmacokinetic analysis was based on the mean values of plasma levels from four rats for each treatment group.

In vivo *tumor models*. Eight-week-old BALB/c female mice were obtained from Harlan (Jerusalem, Israel) and housed in a specific pathogen-free animal facility with food and water *ad libitum*. The animal studies were approved by the Hebrew University Ethics Committee for Animal Care and Experimentation. All tumor types tested (M109, M109-R, and C26) were syngeneic models in BALB/c female mice. Besides PL-MLP prepared as described above, doxorubicin (Teva Phamaceuticals, Israel), MMC (Kyowa, Japan), and pegylated liposomal doxorubicin (DOXIL, ALZA) were used in these studies.

The mouse M109 lung carcinoma and its multidrug-resistant (MDR) subline, M109R (23), were inoculated into the mouse hind footpad at 10^6 cells per mouse. Tumors growing in the footpad were easily detectable and could be accurately measured. The footpad thickness at its maximal diameter was measured with calipers to an accuracy of ±0.1 mm. Normal footpad thickness is 1.5 mm. Treatment was begun by i.v. injections when the average footpad thickness reached 2.0 to 2.5 mm, ~5 to 7 days after inoculation. Mice were labeled with marker colors enabling individual follow-up of tumor growth and mouse weight. When footpad thickness exceeded 5 mm, mice were sacrificed. Mice were followed up for at least 3 months after inoculation with 2 to 3 weekly measurements of footpad thickness and weekly body weight. Results were plotted as median footpad thickness of each experimental group. In addition, the probability of remaining alive with footpad tumor <5 mm diameter was plotted as event-free survival curves and analyzed for statistical significance by the log-rank test (Prism, GraphPad Software, San Diego, CA). Death or tumors measuring >5 mm were considered as "events" in this analysis.

C26 colon carcinoma (26) was inoculated i.p. at 10^6 cells per mouse. Five days later, mice were treated by i.v. drug injection. Mice were followed up for survival by 3 weekly inspections. Statistical analysis of survival analysis was done by the log-rank test with Prism software.

In some of the therapeutic experiments, cysteine or *N*-acetyl-cysteine (NAC) were injected (s.c. or i.p.) daily for 2 or 3 consecutive days beginning 48 hours after injection of PL-MLP in an attempt to enhance prodrug release from liposomes accumulated in the tumor site. The lag time between the injections of PL-MLP and the reducing agent was meant to avoid premature prodrug release from circulating liposomes. For both reducing agents, a round dose of 5 mg/mouse/d was used. There were no toxic effects associated with cysteine or NAC injections.

Results

In vitro cytotoxicity

Using the M109 carcinoma mouse cell line, we found that PL-MLP was substantially (5- to 6-fold) less cytotoxic than free MMC (Fig. 1B), indicating that most of the prodrug was not activated in the standard conditions of a 72-hour assay in culture medium. However, upon addition of reducing agents, such as cysteine or NAC, the cytotoxic activity of the liposomal prodrug formulation was substantially increased by a factor of as much as 6-fold for concentrations of the reducing agent between 0.25 to 1 mmol/L (Fig. 1B). In contrast, the activity of free MMC was not affected by these thiols indicating that the reducing agent effect was related to MLP decomposition and release of active MMC from the liposomes. The effects of cysteine and NAC on the cytotoxicity of PL-MLP were much

Table 1. Compiled results of therapeutic studies with free MMC and PL-MLP in the M109 footpad tumor model

Treatment* (dose, schedule)	Median time to tumor >5 mm or death (d)†	*n*	%Cures‡	%Toxic deaths§
Untreated	19.5 (16-25)	52	0	0
1. Free MMC, 2 mg/kg (day 7)	27	10	20	0
2. Free MMC, 5 mg/kg (day 7)	31.5	21	5	0
3. Free MMC, 2 mg/kg ×3 (days 7, 14, 21)	34	10	0	0
4. Free MMC, 6 mg/kg (day 5)	49	20	15	5
5. Free MMC, 6 mg/kg ×2 (days 5, 12)	29	10	0	100
6. Free MMC, 4 mg/kg ×3 (days 7, 14, 21)	46	10	0	90
1. PL-MLP, 2 mg/kg (day 7)†	27	20	0	0
2. PL-MLP, 5 mg/kg (day 7)	32	21	0	0
3. PL-MLP, 2 mg/kg ×3 (days 7, 14, 21)	34	10	0	0
4. PL-MLP, 4 mg/kg ×3 (days 7, 14, 21)	44	10	0	0
5. PL-MLP, 6 mg/kg ×2 (days 5, 12)	61	10	0	0
6. PL-MLP, 6 mg/kg ×3 (days 5, 12, 19)	62	20	30	0

NOTE: BALB/c f mice were inoculated with 10^6 M109 cells in the right hind footpad in six separate experiments. Median follow-up, 78 days (35-104) after tumor injection. Mice with tumor >5 mm were removed from follow-up and sacrificed.

*All treatments were given i.v.

†Median time calculated from day of tumor injection. Range of median times is shown when more than one experiment was done at the same dose schedule.

‡Cures are defined as tumor-free animals at end of follow-up period.

§Toxic deaths are defined as dead animals during follow-up period with tumor <5 mm.

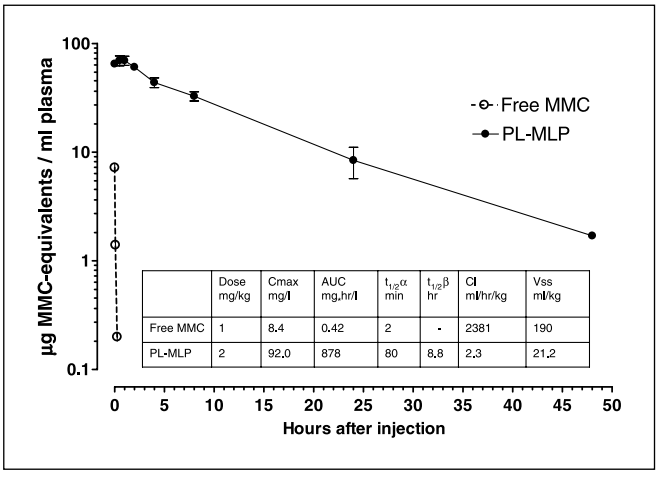
more prominent than those of other reducing agents, such as mesna (Fig. 1B) and lipoic acid (not shown). Of note, the choice of a cholesterol-free formulation was based on previous observations indicating that cysteine-induced prodrug activation is significantly reduced when cholesterol is present in the PL-MLP formulation (20). Based on these results, we decided to proceed to *in vivo* experiments using PL-MLP with and without exogenous administration of cysteine or NAC.

Pharmacokinetics

We examined the plasma levels of MMC and MLP in rats after i.v. injection of PL-MLP and free MMC. As seen in Fig. 2 and its inset table, free MMC was rapidly cleared from plasma and levels were already undetectable as soon as 0.5 hour after injection. As a result, we could not determine its second elimination phase and the analysis is limited to monoexponential modeling of the first distribution phase. In contrast, PL-MLP was cleared very slowly following a biexponential curve, with a short initial distribution phase and a longer and slower second distribution phase accounting for most of drug clearance from plasma. No detectable levels of MMC were found in rats injected with PL-MLP. The pharmacokinetic data for liposomal prodrug show clearly that the parameters are consistent with the typical pharmacokinetics of long-circulating liposomal systems, as indicated by the large area under the curve and slow clearance with a long $t_{1/2}$ (8-9 hours), and by the small volume of distribution. This indicates that cleavage and leakage in plasma of the liposomal prodrug are negligible and most of the drug is cleared in intact prodrug form together with liposomes. Hence, it is safe to assume that the initial tissue distribution of MMC prodrug parallels that of the liposome carrier.

In vivo toxicity

Free MMC was lethal at a single dose of 10 mg/kg in BALB/c mice within 11 days after injection (Fig. 3A-B). In contrast, PL-MLP was given at a weekly dose of 10 mg/kg for 2 or 3 consecutive weeks reaching cumulative doses of 20 or 30 mg/kg without toxic effects (weight loss or deaths) for 30 days



	Dose mg/kg	Cmax mg/l	AUC mg,hr/l	$t_{1/2}\alpha$ min	$t_{1/2}\beta$ hr	Cl ml/hr/kg	Vss ml/kg
Free MMC	1	8.4	0.42	2	-	2381	190
PL-MLP	2	92.0	878	80	8.8	2.3	21.2

Fig. 2. Pharmacokinetics of free MMC and PL-MLP in rats. Each group consisted of four rats. Dose per rat: free MMC, 1 mg/kg i.v.; PL-MLP, 2 mg/kg i.v. Inset table, pharmacokinetic parameters of PL-MLP and free MMC in rats. Equation variables for PL-MLP using a biexponential model: $A = 26.7 \pm 12.1$; $B = 65.4 \pm 13.1$; $\alpha = 0.54 \pm 0.44$; $\beta = 0.0789 \pm 0.0151$ (coefficient of determination = 0.996).

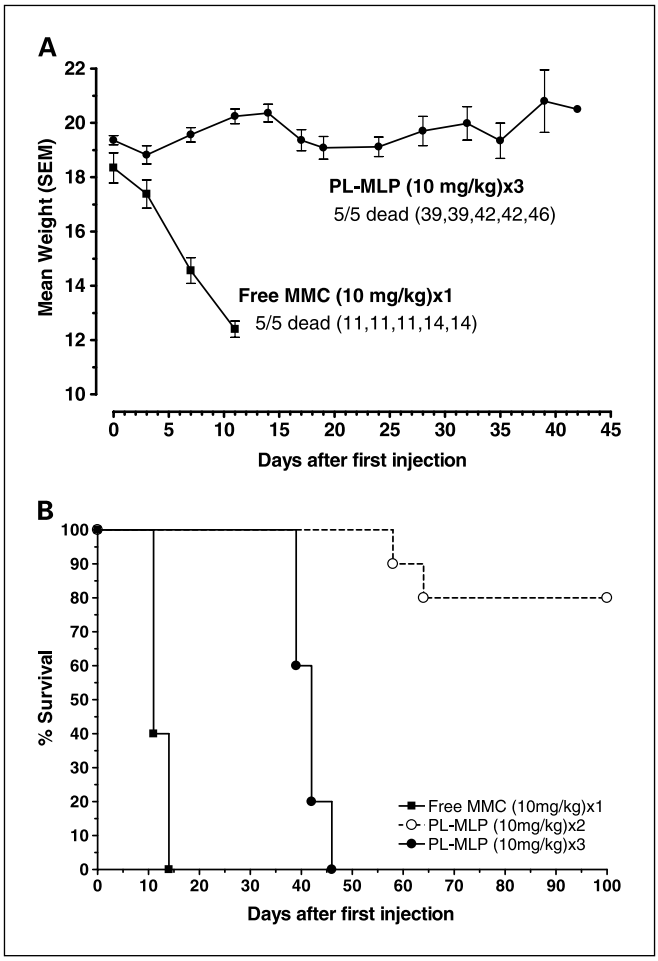


Fig. 3. Toxicity of free MMC and PL-MLP in BALB/c f mice. *A,* weight curve. *B,* mortality. Treatment given i.v. at a dose of 10 mg/kg: free MMC, day 1; PL-MLP (×3), days 1, 8, and 15; PL-MLP (×2), days 1 and 8. Number of mice: free MMC, 5; PL-MLP (×3), 5; PL-MLP (×2), 10. Log-rank test: free MMC versus PL-MLP (×3), $P = 0.002$; PL-MLP (×3) versus PL-MLP (×2), $P < 0.0001$.

(Fig. 3A-B). With further follow-up, all mice receiving 30 mg/kg died. A small number of delayed deaths also occurred in the group receiving 20 mg/kg during the 100-day follow-up period (Fig. 3B). Remarkably, toxic deaths in mice receiving PL-MLP were not preceded by weight loss in contrast to free MMC that caused major weight loss before toxic death (Fig. 3A). From these and other observations obtained during the course of therapeutic studies, we concluded that the maximal tolerated doses of free MMC and PL-MLP are 5 to 6 mg/kg (single dose) and 16 to 18 mg/kg (in two or three split doses of 8 or 6 mg/kg, respectively), respectively, thus indicating that PL-MLP is ~3-fold less toxic than free MMC.

In vivo antitumor efficacy

Therapeutic studies were carried out in three mouse tumor models: mouse M109 carcinoma, its MDR subline M109R, and mouse C26 carcinoma. The mouse tumors were inoculated into syngeneic BALB/c recipients.

M109 model. The results of a series of therapeutic experiments in the M109 footpad tumor model (Table 1) indicate that there was no significant difference between free and liposome-associated prodrug when the total dose is limited to

the maximal tolerated dose level of free MMC (~6 mg/kg). However, when we gave one or two additional injections of PL-MLP, raising the total dose to 12 or 18 mg/kg, its therapeutic activity improves significantly above that of free MMC. This is manifested by a greater delay in tumor growth and a number of complete tumor regressions (cures) with no evidence of toxic deaths. In fact, a combined analysis of the results presented in Table 1 indicates that both forms of treatment resulted in roughly the same number of cures (7%), but free MMC resulted in 23% toxic deaths whereas PL-MLP caused none. Thus, the therapeutic index of PL-MLP is significantly greater than that of free MMC. The reduction in toxicity seen with PL-MLP is clearly a critical factor in enabling an improvement in therapeutic index over free MMC. A dose effect on the therapeutic activity of PL-MLP is also evident in Table 1. Systemic administration of cysteine with PL-MLP, as well as with free MMC, did not affect significantly the antitumor activity in the *in vivo* M109 model (data not shown).

M109R model. This MDR tumor is highly resistant to doxorubicin with an IC_{50} of ~5 μmol/L (23), ~50-fold greater than the IC_{50} for the drug-sensitive M109. Indeed, previous *in vivo* experiments had shown that free doxorubicin is totally inactive in this tumor model (13). In the M109R model, the activity of PL-MLP was at least equivalent to that of free MMC after a single dose of 8 mg/kg with 4 of 10 cures and no toxic deaths for PL-MLP, versus 2 of 10 cures and 3 of 10 toxic deaths for free MMC. However, increasing the dose of PL-MLP with a second injection of 8 mg/kg resulted in 6 of 10 cures and no toxic deaths, whereas a second injection of 8 mg/kg free MMC resulted in 10 of 10 toxic deaths (data not shown), pointing again at an improvement of therapeutic index with the liposomal formulation.

In another experiment, with the M109R tumor, we tested the antitumor activity of PL-MLP versus pegylated liposomal doxorubicin[6] (PL-DXR), administering in both cases two injections of 10 mg/kg each versus no treatment (Fig. 4). For the sake of comparison, the best result obtained in this model with free MMC is plotted in the same figure. One group of mice treated with PL-MLP also received cysteine 48 hours after each dose of PL-MLP. As seen in Fig. 4, PL-MLP resulted in major suppression of tumor growth with 73% cures (i.e., 11 of 15 mice were tumor-free after 90 days of observation) and a significant delay in tumor growth in those mice developing tumors. In contrast, PL-DXR caused a slight delay in tumor growth with no cures. There was no significant difference whether or not cysteine was added to PL-MLP treatment. PL-DXR was given at a total dose of 20 mg/kg bordering the maximal tolerated dose (27), therefore ruling out the possibility that the difference in therapeutic outcome was related to underdosing of PL-DXR. PL-MLP was given at a total dose of 20 mg/kg slightly exceeding its maximal tolerated dose (18 mg/kg) because this experiment was run before we had information on the delayed toxic deaths of PL-MLP (see section above). However, within the 3-month observation period, there were no toxic deaths among the tumor-free mice treated with PL-MLP.

C26 model. This tumor is relatively more resistant (~6-fold) *in vitro* to doxorubicin than the M109 tumor and less resistant (~9-fold) than the M109R tumor. The *in vivo* antitumor activity of free doxorubicin against the C26 tumor is negligible (28). In contrast, PL-DXR is a very active agent in this model, achieving significant survival prolongation and cures (28), suggesting that drug delivery factors may overcome partially the *in vivo* drug resistance. In the current experiments (Fig. 5A), the survival curve obtained with PL-MLP was superior to that of free MMC, which had minimal activity in this model, and untreated groups. When the results of three separate experiments done in this tumor model are analyzed, a dose effect was clearly evident when the dose of PL-MLP is increased from 4 to 8 mg/kg resulting in longer median survival and cures in 9 of 20 of the animals as opposed to only 1 of 10 for free MMC at its maximal tolerated dose (6 mg/kg; data not shown). The addition of NAC (Fig. 5A) to PL-MLP improved the antitumor activity of PL-MLP slightly but not significantly. When the therapeutic effects of free

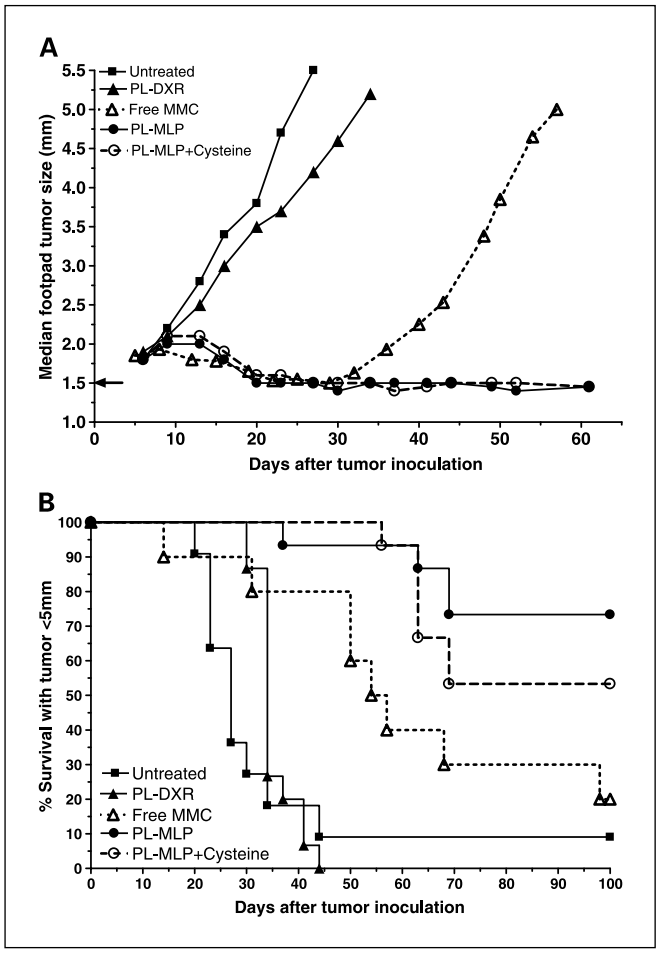


Fig. 4. Therapeutic effect of PL-MLP with and without cotreatment with cysteine in the M109R tumor model and comparison to free MMC and PL-DXR (DOXIL). *A,* tumor growth curve (*arrow,* upper normal limit of footpad size). *B,* probability of survival with tumor <5 mm. Schedule: 10^6 M109R cells in the right hind footpad of BALB/c f mice. Free MMC i.v., 8 mg/kg, on day 5 after tumor inoculation; PL-DXR i.v., 10 mg/kg, on days 5 and 12 after tumor inoculation; PL-MLP i.v., 10 mg/kg, on days 5 and 12 after tumor inoculation. Cysteine s.c., 5 mg/mouse, on days 7, 8, 9, 14, 15, and 16 after tumor inoculation. Number of mice: untreated, 11; free MMC, 10; PL-DXR, 15; PL-MLP, 15; PL-MLP + cysteine, 15. Statistical analysis (log-rank test): untreated versus PL-DXR, $P > 0.1$; untreated versus free MMC, $P < 0.0278$; untreated versus PL-MLP and PL-MLP + cysteine, $P < 0.0001$; free MMC versus PL-MLP, $P = 0.0031$; PL-DXR versus PL-MLP and PL-MLP + cysteine, $P < 0.0001$.

[6] Pegylated liposomal doxorubicin is marketed under the commercial names of DOXIL and CAELYX.

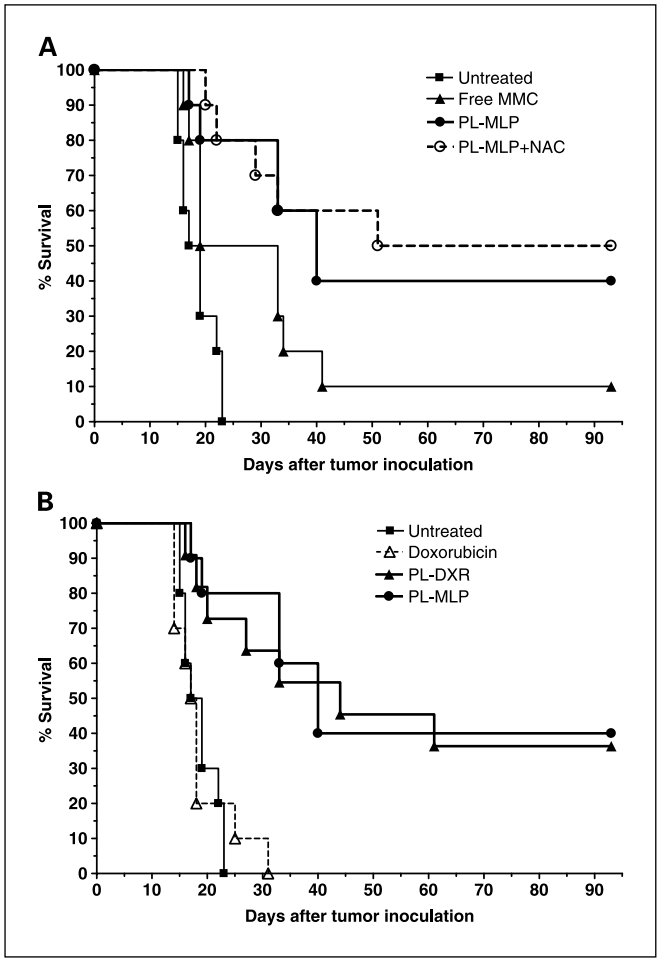


Fig. 5. Therapeutic effect of PL-MLP with and without cotreatment with NAC in the C26 tumor model and comparison with free MMC, free doxorubicin, and PL-DXR (DOXIL). *A,* survival curves of mice treated with free MMC and PL-MLP with or without NAC. *B,* survival curves of mice treated with free doxorubicin, PL-DXR, and PL-MLP. Schedule: 10^6 C26 cells i.p. in BALB/c f mice. Free MMC i.v., 6 mg/kg, on day 5 after tumor inoculation; PL-MLP i.v., 8 mg/kg, on day 5 after tumor inoculation; NAC i.p., 5 mg/mouse, on days 7 and 8 after tumor inoculation; free doxorubicin i.v., 10 mg/kg, on day 5 after tumor inoculation; PL-DXR i.v., 10 mg/kg, on day 5 after tumor inoculation. Number of mice: untreated, 10; free MMC, 10; PL-MLP, 10; PL-MLP + NAC, 10; free doxorubicin, 10; PL-DXR, 11. Statistical analysis (log-rank test): untreated versus free MMC, $P = 0.0197$; untreated versus PL-MLP and PL-MLP + NAC, $P < 0.0001$; free MMC versus PL-MLP and PL-MLP + NAC, $P = 0.0202$; free doxorubicin versus PL-DXR, PL-MLP, and PL-MLP + NAC, $P < 0.0001$.

doxorubicin, PL-DXR, and PL-MLP are compared in the C26 model (Fig. 5B), it was found that the activity of PL-DXR and PL-MLP are comparable and superior to those of free doxorubicin, which was totally inactive.

Discussion

Pegylated liposomes represent an attractive, drug delivery–based approach to improve the therapeutic index of a number of cytotoxic drugs whose potential exploitation in therapy is limited when administered in their native free form due to toxicity or pharmacokinetic factors (29, 30). The ability of these long-circulating nanoparticles to accumulate preferentially in tumors is well documented (31, 32) and is credited to the enhanced permeability and retention effect associated with the properties of the tumor vasculature and its limited lymphatic drainage, as also shown for macromolecules (33, 34). The present study shows the pharmacologic and therapeutic advantages of a liposomal formulation of MMC prodrug over free MMC and its potential application to the treatment of doxorubicin-resistant tumors. PL-MLP is a novel drug-carrier system combining a double strategy: delivery with a long-circulating liposome carrier and a prodrug approach in which the prodrug activation step is immediately followed by drug release from the liposome carrier.

Our approach relies on the tumor-homing ability of pegylated liposomes as well as on the thiolytic release of the cytotoxic agent, MMC, from the lipid-based prodrug. In this context, it is pertinent to note that various reductive/thiolytic factors are overexpressed in various tumor types (35). Most notable among such factors are thioredoxin, thioredoxin reductase, and protein disulfide isomerase with their abundant plasma membrane localization and secretion (36), all potentially contributing to the local thiolytic conditions in tumors. Furthermore, MMC thiolytically liberated from MLP is a poor substrate of molecular efflux pumps that contribute to MDR (8–12).

The *in vitro* tests point to enhanced cytotoxicity of PL-MLP in the presence of reducing agents (Fig. 1B), which must result from a large increase in the bioavailable drug fraction upon thiolytic cleavage. This also indicates that, after prodrug cleavage, MMC retention in liposomes is negligible. The pharmacokinetic data for liposomal MMC prodrug obtained here are consistent with the typical STEALTH liposome pharmacokinetics with a major change of several orders of magnitude from free MMC pharmacokinetics. Because pharmacokinetic studies did not provide any evidence of significant drug release in plasma, *in vivo* prodrug activation and release must be a slow process occurring in tissues where long-circulating liposomes are mostly deposited. These tissues include tumor, liver, spleen, and skin (37). Indeed, our *in vivo* therapeutic studies show that PL-MLP has substantial antitumor activity (Figs. 4 and 5; Table 1), indicating that MMC became bioavailable in tumor tissue at a pharmacologically relevant rate.

The transformation of the lipophilic prodrug into MMC will be immediately followed by drug dissociation from the carrier given the rapid diffusion of MMC across lipid membranes. Therefore, the rate-limiting step for bioavailability in such a system is actually the activation of prodrug rather than the release of drug from the liposome. In the current system, any thiol-containing agent can cleave the dithiobenzyl linker and generate free MMC (22, 38). Although thiols are ubiquitously present in biological systems (39), these are mostly polar molecules (e.g., glutathione and cysteine) or macromolecules (e.g., albumin). Their access to the prodrug located in the polyethylene glycol-shielded, high phase transition–temperature (T_m) lipid bilayer of intact hydrogenated soybean phosphatidylcholine–based liposomes is probably quite slow and limited. This may explain the need for high concentrations of NAC or cysteine to obtain *in vitro* cytotoxicity with PL-MLP. Liposome composition is clearly a factor determining the rate of prodrug activation by reducing agents because we have shown previously that the inclusion of cholesterol in the formulation reduces prodrug activation and *in vitro* cytotoxicity of PL-MLP (20). However, the mechanism of *in vivo* drug

release from intact liposomes is still unclear. The high *in vitro* concentrations of cysteine or NAC leading to prodrug thiolytic activation *in vitro* are not achievable under any physiologic conditions in plasma or extracellular fluids. It is also unknown whether the reducing activity of the tumor interstitial fluid will be sufficient to trigger thiolytic activation of the MMC prodrug. Complementary mechanisms based on liposome breakdown by tissue phospholipases often present at high levels in tumor interstitial fluid (40), and/or liposome breakdown by lysosomal enzymes following liposome cell uptake, will increase exposure of the prodrug to reducing agents and accelerate drug release. Regardless of the mechanisms involved, significant *in vivo* therapeutic activity of PL-MLP was shown here in various tumor models in the absence of any exogenous cysteine or NAC. The lack of significant enhancement of antitumor activity of PL-MLP when large doses of reducing agents were coadministered may be related to the difficulty in achieving the high concentrations of these rapidly cleared agents in the tumor interstitial fluid required for thiolytic activation of MLP. Obviously, it is still possible that the use of more intensive dose regimes or more effective reducing agents may lead to a significant *in vivo* effect.

Efforts at developing drug delivery systems for MMC date back to the early 1980s when MMC was conjugated to high molecular weight dextran, resulting in sustained release and reduction of toxicity (41). After systemic administration, these polymeric MMC derivatives were found to accumulate in tumors and to have a greater antitumor effect than free MMC (42). Other types of polymer conjugates have also been examined for delivery of MMC in experimental systems with similarly positive results (43, 44). In addition, delivery of lipophilic MMC prodrugs by means of liposomes or emulsions has also attempted in the past (45, 46); however, to our knowledge, this is the first study combining a lipophilic prodrug with thiolytic release and a pegylated liposomal carrier that provides a clear advantage over earlier liposome formulations in terms of stability and circulation time. Although polymeric conjugates and liposomal formulations of cytotoxic drugs have seldom been compared head to head, one important difference lies in the fact that polymers usually release the drug after tumor cell uptake and breakdown in the endosomal compartment (33), whereas in the case of liposomes, especially for the pegylated ones, drug is released in the tumor extracellular fluid (32, 37).

A recent report on other long-circulating MMC carriers based on phospholipid vesicles with surface-coupled hyaluronic acid points to substantial enhancement of therapeutic activity when compared with free MMC (47) in tumor models expressing hyaluronic acid receptors lending further support to the liposome approach for efficient delivery of this drug. The data presented here support the use of long-circulating liposomal carriers for reducing toxicity and improving the therapeutic index of MMC, an approach already shown to have positive value for anthracyclines (30, 48). The apparent activity of our MMC-releasing formulation in MDR tumors, as shown here, represents a potential added value over pegylated liposomal doxorubicin. Further studies in human tumor xenografts will be required to determine the overall spectrum of activity of PL-MLP and its comparative activity to other therapeutic options for MDR tumors.

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