Cancer Chemother Pharmacol (2010) 66:43–52
DOI 10.1007/s00280-009-1132-4

ORIGINAL ARTICLE

Improved therapeutic activity of folate-targeted liposomal doxorubicin in folate receptor-expressing tumor models

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Received: 18 July 2009 / Accepted: 7 September 2009 / Published online: 25 September 2009

Abstract
Purpose The folate receptor (FR) is overexpressed in a broad spectrum of malignant tumors and represents an attractive target for selective delivery of anti-cancer agents to FR-expressing tumors. Targeting liposomes to the FR has been proposed as a way to enhance the effects of liposome-based chemotherapy.
Methods Folate–polyethylene glycol–distearoyl–phosphatidyl–ethanolamine conjugate was inserted into pegylated liposomal doxorubicin (PLD). The therapeutic activity of folate-targeted (FT-PLD) and non-targeted (PLD) pegylated liposomal doxorubicin was tested in two human tumor models (KB, KB-V) and in one mouse ascitic tumor model (FR-expressing J6456) by the i.v. systemic route in all models, and by the i.p. intracavitary route in the ascitic tumor model only.
Results Consistent with previous studies, PLD was clearly superior to free doxorubicin in all tumor models. When targeted and non-targeted liposome formulations were compared, FT-PLD was more effective than PLD in the KB and KB-V xenograft models, and in the J6456 intra-cavitary therapy model. The therapeutic effect was dose-dependent in the KB model and schedule-dependent in the J6456 intra-cavitary therapy model. In some experiments, toxic deaths aggravated by folate-depleted diet were a major confounding factor. In a non-FR expressing J6456 model, FT-PLD was as active as PLD indicating that its activity is not limited to FR-expressing tumors.
Conclusion Folate-targeting confers a significant albeit modest therapeutic improvement to PLD in FR-expressing tumor models, which appears particularly valuable in intracavitary therapy. The potential clinical added value of this approach has yet to be determined.

Keywords Liposome · Folate receptor · Targeting · Doxorubicin · Tumor model · Cancer therapy

Electronic supplementary material The online version of this article (doi:10.1007/s00280-009-1132-4) contains supplementary material, which is available to authorized users.

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Introduction

Pegylated liposomal delivery of doxorubicin has long become an acceptable therapeutic option in a variety of human cancers [1]. Its toxicity profile is safer than that of conventional doxorubicin treatment with an impressive risk reduction in cardiac toxicity [2]. In addition, the clinical antitumor activity of pegylated liposomal doxorubicin (PLD) is at least comparable to that of doxorubicin in breast cancer and multiple myeloma and probably

superior to doxorubicin in ovarian cancer and Kaposi sarcoma [1]. The most frequently used formulation is a version of PLD known commercially as Doxil or Caelyx. Pegylation significantly improves the stability and prolongs the circulation time of liposomes [3]. The latter is critical for liposomal deposition in tumors since liposome circulation time is positively correlated with their accumulation in tumor tissue [4]. However, pegylated liposomes are seldom taken up by tumor cells in vitro and in vivo. Coupling of an appropriate ligand to the liposome surface may enable liposome tumor cell binding and internalization, a process that may enhance the potency of the delivered drug.

Conceivably, these targeted liposomes may have a greater therapeutic efficiency than non-targeted liposomes for specific tumors. This rationale is the basis for the thrust in this field as reflected in the study of a number of research groups with antibody-targeted liposomes directed against nucleosome epitopes [5], cell-surface gangliosides [6], Her2 receptor [7], EGF-receptor [8], transferrin receptor [9], and others. We chose here folate as an attractive tumor targeting ligand since it is a small non-toxic molecule whose receptor is over-expressed in many tumor types [10]. Folate receptor binding enables endocytosis of ligand-conjugated carriers [11, 12] and is being actively exploited for development of targeted drugs in cancer [13].

Ligand-binding to the folate receptor results in an endocytosis-driven process transporting the ligand to non-lysosomal endosomal vesicles [14]. When multivalent binding takes place in the cell surface, as in the case of folate-targeted nanoparticles such as liposomes, the ensuing endocytosis process appears to involve lysosomes [15] although the exact pathway and kinetics are less well-known than for folate monovalent conjugates. For more than a decade, folate targeting of liposome-encapsulated drugs has been studied as a means of enhancing tumor selectivity. While folate targeting does not enhance overall liposome accumulation tumors [16, 17], intra-tumor distribution does appear to be modified, at least in ascitic tumors, with more liposome material found in tumor cells and less in the surrounding fluid as compared to non-targeted liposomes [17]. Similar observations have been made for anti-Her2-targeted liposomes [18].

In recent years, formulation of targeted liposomes has been simplified by the use of post-insertion techniques in which a ligand–PEG–lipid conjugate is grafted on the surface of pre-formed liposomes by micellar insertion [19–21]. We report here on the results of therapeutic studies with folate-targeted (FT-) PLD in various models of folate receptor-expressing tumors using formulations incorporating the ligand micellar post-insertion technique.

Materials and methods

Liposome formulation

The PLD formulation used in these studies was Doxil™, a product of Johnson&Johnson, marketed in Israel by Janssen-Cilag (Shefayim, Israel). 3H-cholesterol radiolabeled liposomes were prepared as described previously [22]. 3H-cholesterol-hexadecyl ether (3H-CHE) was obtained from Amersham (Buckinghamshire, England). Other liposome components were as reported by Shmeeda et al. [22].

Folate conjugate insertion in liposomes

Folate-derivatized PEG (3350)-DSPE was prepared as described previously [23] and provided by Alza Corp. (Mountain View, CA). Ligand post-insertion was achieved by incubation of PLD with folate–PEG–DSPE conjugate at 45°C for 2 h, at a ligand molar ratio of 0.5% of phospholipid concentration in PLD (based on phosphorus content). Liposomes were then centrifuged at 3,000 rpm for 10 min to remove non-incorporated ligand. The percentage of ligand incorporation was determined spectrophotometrically by measuring folate at 285 nm after liposome solubilization in 3% SDS. Typically, more than 80% ligand became associated with liposomes, no precipitate was observed, and no drug leakage occurred during the ligand insertion procedure. However, as a matter of caution, both PLD and FT-PLD were filtered through a Dowex cation exchange resin to remove any possible contamination with free drug, and the concentration of liposomal doxorubicin was subsequently measured as described previously [22].

Tumor cell lines

Four tumor cell lines were used in this study. The FR-expressing KB and KB-V human cell lines have been described previously in the context of liposome cell binding studies [23]. The mouse J6456 lymphoma has been used to test the therapeutic efficacy of PLD in a lymphoma model [24]. More recently, a FR-expressing subline of J6456 (J6456-FR) was developed and used in vivo to test for targeting of folate-targeted liposomes [22]. Except for the parental FR-negative J6456, all other cell lines were grown in folate-depleted RPMI medium (Beyt Haemek, Israel) with 10% bovine serum (GIBCO). KB-V was not grown in the presence of vinblastine or colchicine as recommended by some investigators [25], a factor that may have contributed to a reduction in the level of multidrug resistance of this cell line as observed in our laboratory (data not shown).

Animal tumor models

The animal studies were approved by the Hebrew University Ethics Committee for Animal Care and Experimentation. The Hebrew University is an AAALAC International accredited institute.

- KB and KB-V models: 1 million tumor cells suspended in 50 µl were inoculated in the right hind footpad of 8–10 week old female nude mice (Harlan, Israel) using a 30 g-needle and a 0.3 ml syringe. Mice were put on a folate-free diet (Harlan Teklad, Madison, WI) 4–7 days prior to tumor inoculation and throughout the treatment period. The standard, folate-enriched, diet was resumed 7 days after last treatment. The footpad thickness (normal footpad diameter in tumor-free mice ~1.5 mm) was measured twice per week with high precision calipers to an accuracy of 0.1 mm. Treatment was given by tail vein injection and started when the footpad reached ~2.0 mm average diameter with a range between 1.8 and 2.4 mm. Mice were marked for individual follow-up with footpad measurements ×2/week, body weight ×1/week, and cage inspection for dead or distressed animals ×5/week. Mice with footpad diameter exceeding 5 mm were euthanized. In our experience, when footpad tumors grow to >5 mm despite treatment, the chance of any tumor regression is nil.
- J6456 ascitic model: 1 million tumor cells suspended in 0.2 ml were inoculated i.p. into 8–10 week old female BALB/c mice (Harlan, Israel). Treatment was given either by i.v. (systemic therapy) or i.p. (regional intracavitary therapy) injection on day 7 post-tumor inoculation. When the FR-expressing J6456 tumor was assayed, mice were put on a folate-free diet (Harlan Tekled) 4–7 days prior to tumor inoculation and throughout the treatment period. The standard, folate-enriched diet was resumed 7 days after last treatment. When the non-FR expressing parental J6456 cell line was tested, a standard diet was used all along. Follow-up was by cage inspection for dead or distressed animals ×5/week and body weight ×1/week.

Statistical analysis

In the KB and KB-V models, two analyses were performed as done in previous studies with solid tumors [26]: *tumor growth curve by mean or median size*, and, *event-free survival curve*.

(i) The mean or median footpad diameter of each experimental group was plotted against time after tumor inoculation. Euthanized mice with tumors >5 mm were included in the mean/median calculation past the euthanasia date using the last tumor measurement through the end of the experiment. However, mice found dead prior to developing a 5-mm tumor were excluded from mean/median calculation past the death date. This is because the course of tumor growth is unpredictable had toxic death not occurred.

(ii) For an analysis of the combined impact of treatment on tumor size and on toxicity, Kaplan–Meier event-free survival probability curves were obtained for each experimental group. 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 (>3 mm in the KB-V model) were considered as ''events'' in this analysis. By providing a median time to treatment failure (equivalent to median to event), this analysis results in a more clinically meaningful evaluation of the differences in outcome between the various treatment groups as compared to the commonly used analysis of differences in tumor size by time point.

In the J6456 models, survival curves were plotted and analyzed by the log-rank test using Prism software. In this model, survival is well-correlated with the tumor burden of the animal, provided there is no evidence of treatment-related toxicity [24].

Results

In vitro binding of folate-targeted liposomes to FR-expressing tumor cells

Pegylated liposomes incorporating a radiolabel (H3-cholesteryl-oleate) were grafted with the folate ligand and incubated with KB cells for 3–48 h. The uptake of targeted liposomes was about 10-fold greater than that of non-targeted liposomes with most of the specific uptake occurring during the first 3 h of incubation (see supplementary material, Fig. S1). In subsequent experiments, the doxorubicin uptake of KB and J6456-FR cells exposed to FT-PLD and PLD was tested. A major increase of drug uptake was noticed when FT-PLD is compared to PLD, the former reaching similar cellular levels to those observed with free drug. When two formulations of FT-PLD prepared by micellar insertion of the ligand were compared to PLD, we found a consistent increase (~10-fold) in the amount of doxorubicin associated with J6456-FR cells (see supplementary material, Fig. S2A–B).

Therapeutic activity in the human KB tumor model

Treatment was started in the first week after tumor inoculation (days 5–7) when tumors reached an average size of

2 mm. Free doxorubicin was not effective in the KB tumor model (Table 1, see also supplementary material, Fig. S3A–B) and was not tested further. Using a low dose (3 weekly injections of 5, 2.5, and 2.5 mg/kg), we found a small but not statistically significant therapeutic advantage of FT-PLD over PLD. Both treatments were able to significantly delay tumor growth (Fig. 1a, b). When the dose was raised to three weekly injections of 8, 5, and 5 mg/kg, FT-PLD conferred a significant therapeutic advantage over PLD with approximately 90% cures (Fig. 1c, d). For both types of treatment, there was a clearly improved outcome when the high dose regime is compared to the low dose regime. A repeat experiment with the high dose regime (data not shown) resulted in a large number of toxic deaths (8/10 in the PLD group and 3/9 in the FT-PLD group) preventing a meaningful analysis of net differences in therapeutic efficacy between PLD and FT-PLD. Yet, these experiments suggest that PLD is more toxic and has a lower therapeutic index than FT-PLD in mice fed folate-depleted diet.

Therapeutic activity in the human KB-V tumor model

In our experience, in vivo KB-V inoculation often results in delayed tumor growth. Therefore, treatment was started in the 2nd week (day 12) after tumor inoculation to allow for tumors to reach an average size of 2 mm. As seen in Fig. 2a, PLD and FT-PLD were both effective against the KB-V tumor. No significance difference between PLD and FT-PLD was obtained, but the analysis was hindered by a large number of toxic deaths. Because of the confounding toxicity factor and the slow growth rate of KB-V tumor, toxic deaths were censored and the results were reanalyzed lowering the tumor size event threshold to 3 mm. In this new analysis, FT-PLD was more effective than PLD at a statistically significant level (Fig. 2b).

Therapeutic activity in the J6456 ascitic tumor model

Systemic (i.v.) therapy

In mice under normal diet, the activity of FT-PLD and PLD were comparable and greatly superior to that of free doxorubicin in the FR-expressing J6456 tumor (Fig. 3a). In the FR-negative J6456 tumor model, FT-PLD and PLD were also equally effective (Fig. 3b). The FR-negative J6456 tumor behaves more aggressively than the FR-positive J6456 tumor, as indicated by the shorter median survival of untreated mice inoculated with the former (19 days) in comparison to the latter (28 days) (Table 1). This probably accounts for the smaller gain in survival for treated mice when Fig. 3b is compared to Fig. 3a.

When the treatment was administered in mice under folate-depleted diet, toxic deaths were observed, notably in the PLD group, resulting in a significantly longer survival for FT-PLD (Fig. 4a). The experiment was repeated with a supplement of folic acid to PLD treated mice, equivalent to the amount of folate present in FT-PLD. The results of this

Table 1 Summary of time-to-event and survival medians

Tumor model (Figure)[a]	Median time-to-event or survival				P^c (PLD versus FT-PLD)
	Days after tumor inoculation (*N*)				
	Untreated	Doxorubicin	PLD	FT-PLD	
KB (Fig. S3)	25 (6)	28.5 (10)	–	–	
KB (Fig. 1b)	20 (7)	–	23 (9)	30 (9)	ns
KB (Fig. 1d)	19 (11)	–	114 (11)	>120 (11)	0.0260
KB-V (Fig. 2a)	61 (6)	–	114 (11)	>150 (11)	ns
KB-V (Fig. 2b)	35 (6)	–	121 (11)	>150 (11)	0.0225
J6456-FR (Fig. 3a)	28 (10)	22.5 (10)	50 (10)	49.5 (10)	ns
J6456 (Fig. 3b)	19 (7)	–	27 (10)	27 (9)	ns
J6456-FR (Fig. 4a)	25 (10)	–	12 (10)	50.5 (10)	<0.0001
J6456-FR (Fig. 4b)	21 (6)	–	56 (9)[b]	54 (9)	ns
J6456-FR (Fig. 5a)	32 (7)	33.5 (10)	>96 (10)	>96 (10)	ns
J6456-FR (Fig. 5b)	25 (15)	–	85.5 (20)	>110 (20)	0.0158
J6456-FR (Fig. 5c)	22.5 (6)	–	35 (11)	39 (10)	ns

[a] For further experimental details, see Figure legends

[b] PLD co-injected with free folic acid

[c] Statistical analysis of PLD versus FT-PLD by log rank test. Significant differences, if detected, always favored FT-PLD treatment

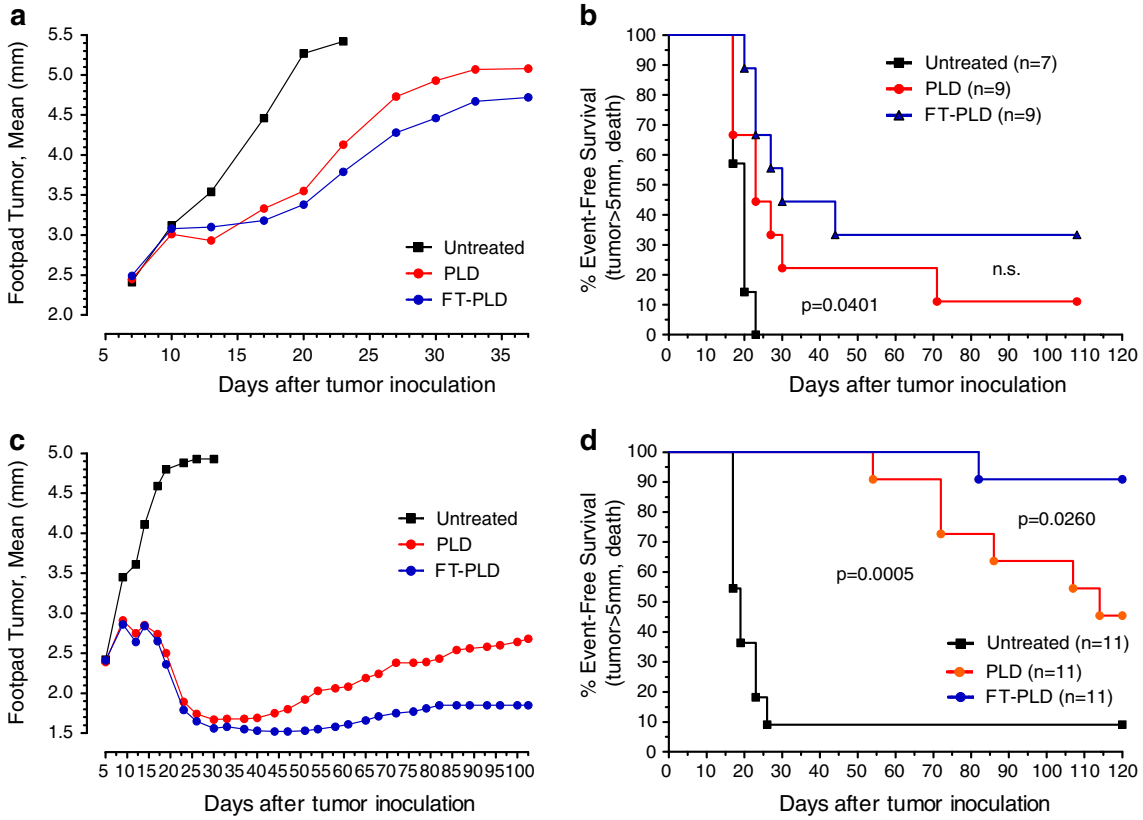


Fig. 1 Therapeutic effect of PLD and FT-PLD in KB tumor model. *Curves* depict tumor growth (**a** and **c**), and time to event (defined as 5-mm tumor or death) (**b** and **d**). **a**, **b** Mice on folate-depleted diet treated i.v. with PLD or FT-PLD at multiple doses of 5, 2.5, and 2.5 mg/kg on days 7, 14, and 21 after tumor inoculation, respectively. Log rank test: Untreated versus PLD, $P = 0.0401$; Untreated versus FT-PLD, $P = 0.0008$; PLD versus FT-PLD, ns. **c**, **d** Mice on folate-depleted diet treated i.v. with PLD or FT-PLD at multiple doses of 8, 5, and 5 mg/kg on days 5, 14, and 23 after tumor inoculation, respectively. Log rank test: Untreated versus PLD, $P = 0.0005$; Untreated versus FT-PLD, $P < 0.0001$; PLD versus FT-PLD, $P = 0.0260$

experiment show that the toxicity of PLD was greatly decreased, with no appreciable differences in the anti-tumor efficacy of PLD and FT-PLD treated mice (Fig. 4b).

Thus, we could not detect a significant gain of efficacy with i.v.-administered FT-PLD over PLD in the ascitic J6456-FR model, although we observed that PLD appears to be more toxic than FT-PLD in mice fed folate-depleted diet.

Intracavitary (i.p.) therapy

In past studies, we have shown that i.p.-administered FT-PLD results in a major increase of drug levels in ascitic tumor cells when compared to PLD, and a significant decrease in plasma levels, leading to the assumption that FT-PLD could increase efficacy and decrease toxicity by increasing tumor exposure and reducing systemic exposure to doxorubicin. Figure 5 shows the results of three therapeutic studies in the J6456-FR ascitic tumor model. When treatment was given on day 5 post-tumor inoculation (Fig. 5a), both forms of liposomal treatment (PLD and FT-PLD) were extremely and equally effective with nearly all animals seemingly cured (110 days tumor-free), and a major therapeutic improvement over the conventional free drug in agreement with results observed with the systemic therapy model. When the treatment was started on day 7 post-tumor inoculation (Fig. 5b), FT-PLD performed better than PLD in two separate experiments nearly doubling the number of cures (15/20 as opposed to 8/20 tumor-free mice at end of experiment). We analyzed the results combining both experiments (Fig. 5b) to achieve more statistical power, and were able to confirm the superior efficacy of FT-PLD over PLD on day 7 with statistical significance ($P = 0.0158$, Table 1). Both forms of treatment appeared slightly less effective than in day 5 (Fig. 5a), although the loss of efficacy for PLD appeared greater than for FT-PLD. When treatment was postponed to day 10 after tumor inoculation (Fig. 5c), the efficacy of both forms of treatment dropped considerably and no differences were observed when comparing PLD to FT-PLD, although the outcome of treated mice was still significantly better than when untreated.

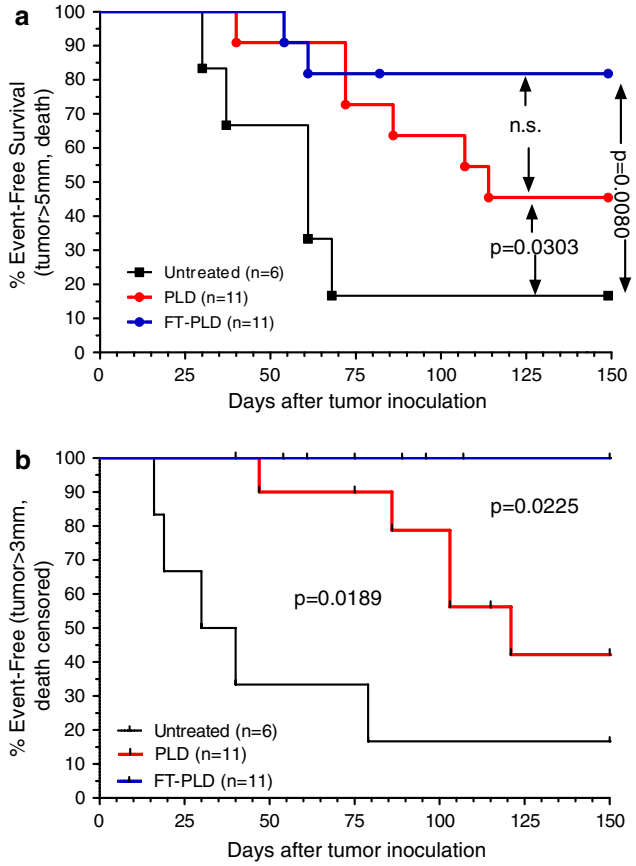


Fig. 2 Therapeutic effect of PLD and FT-PLD in KB-V tumor model. Mice on folate-depleted diet treated i.v. with PLD or FT-PLD at multiple doses of 8, 5, and 5 mg/kg on days 12, 19, and 26 after tumor inoculation, respectively. **a** Time to event (5-mm tumor or death). Log rank test: Untreated versus PLD, $P = 0.0303$; Untreated versus FT-PLD, $P = 0.0080$; PLD versus FT-PLD, ns. **b** Time to event (3-mm tumor, deaths censored). Log rank test: Untreated versus PLD, $P = 0.0189$; Untreated versus FT-PLD, $P < 0.0001$; PLD versus FT-PLD, $P = 0.0225$

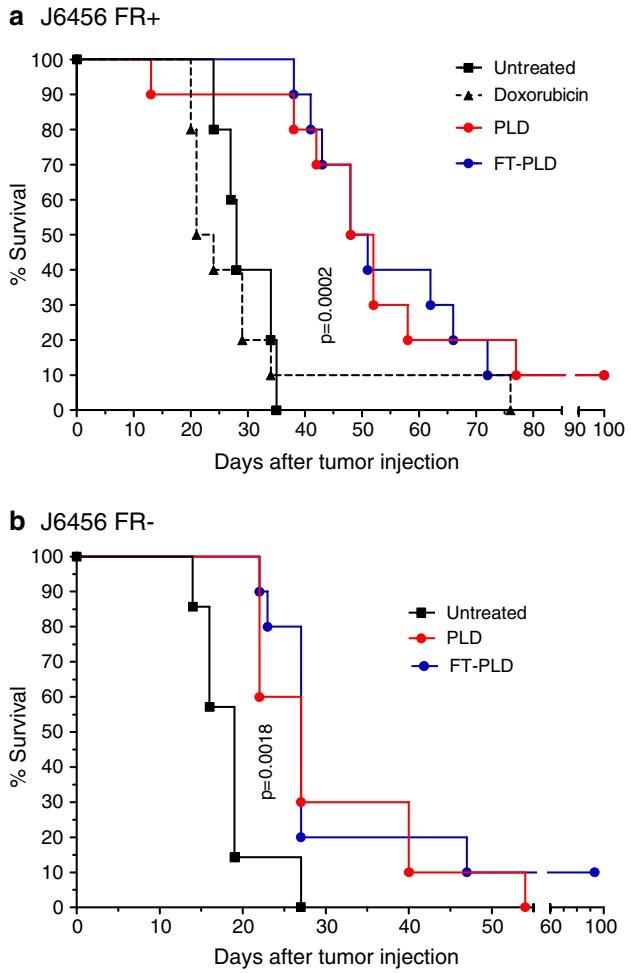


Fig. 3 Comparative therapeutic effect of systemic (i.v.) treatment with PLD and FT-PLD in the J6456-FR (FR+) and J6456 (FR−) ascitic tumor models. Mice on standard diet treated i.v. with free doxorubicin, PLD or FT-PLD at a single dose of 10 mg/kg on day 7 after tumor inoculation. **a** (J6456-FR) Survival time. Log rank test: Untreated versus free doxorubicin, ns; Untreated versus PLD, $P = 0.0002$; Untreated versus FT-PLD, $P < 0.0001$; PLD versus FT-PLD, ns. **b** (J6456) Survival time. Log rank test: Untreated versus PLD, $P = 0.0018$; Untreated versus FT-PLD, $P = 0.0012$; PLD versus FT-PLD, ns

In the i.p. therapy model, there was no apparent lethal toxicity of either treatment under folate-depleted diet, neither PLD nor FT-PLD. All deaths appeared to be cancer-related with characteristic abdominal swelling upon animal inspection.

Discussion

PLD is a non-targeted nanoparticulate formulation of doxorubicin approved for clinical use and shown to be therapeutically superior to free doxorubicin in nearly all experimental animal tumor models [27]. Its improved efficacy over free drug is also clearly evident in our results here. In most instances, PLD passively accumulates in the tumor interstitial fluid and gradually releases doxorubicin without any significant interaction with tumor cells [28]. Indeed, microscopic observations with gold- or fluorescent-labeled non-targeted PLD-like liposomes in experimental tumors indicate that their distribution is limited to the extracellular fluid and tumor-infiltrating macrophages [18, 29, 30].

Conceivably, intracellular delivery of the drug cargo by FT-PLD into FR-expressing tumor cells could provide a more potent anti-tumor effect than the gradual extra-cellular drug release from PLD, even if it does not increase the overall amount of drug deposited in the tumor [17]. There is one report suggesting that the therapeutic effect of a formulation of FT-PLD is greater than that of its non-targeted counterpart in the KB tumor model, but this study may be tainted by toxic deaths given the high cumulative dose of doxorubicin injected (60 mg/kg) and the high animal death

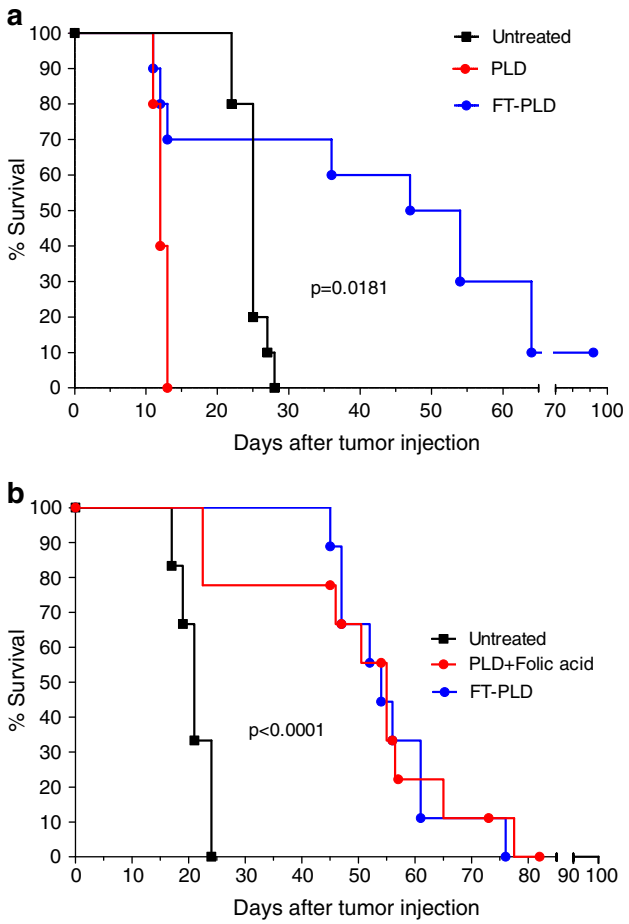


Fig. 4 Therapeutic effect of systemic (i.v.) treatment with PLD and FT-PLD in the J6456-FR ascitic tumor model. Mice on folate-depleted diet treated i.v. with PLD or FT-PLD at a single dose of 10 mg/kg on day 7 after tumor inoculation. In **b**, the PLD group received a supplementary dose of folic acid (see "Materials and methods" section). **a** Survival time. Log rank test: Untreated versus PLD, $P < 0.0001$; Untreated versus FT-PLD, $P = 0.0181$; PLD versus FT-PLD, $P < 0.0001$. **b** Survival time. Log rank test: Untreated versus PLD + folic acid, $P < 0.0001$; Untreated versus FT-PLD, $P < 0.0001$; PLD + folic acid versus FT-PLD, ns

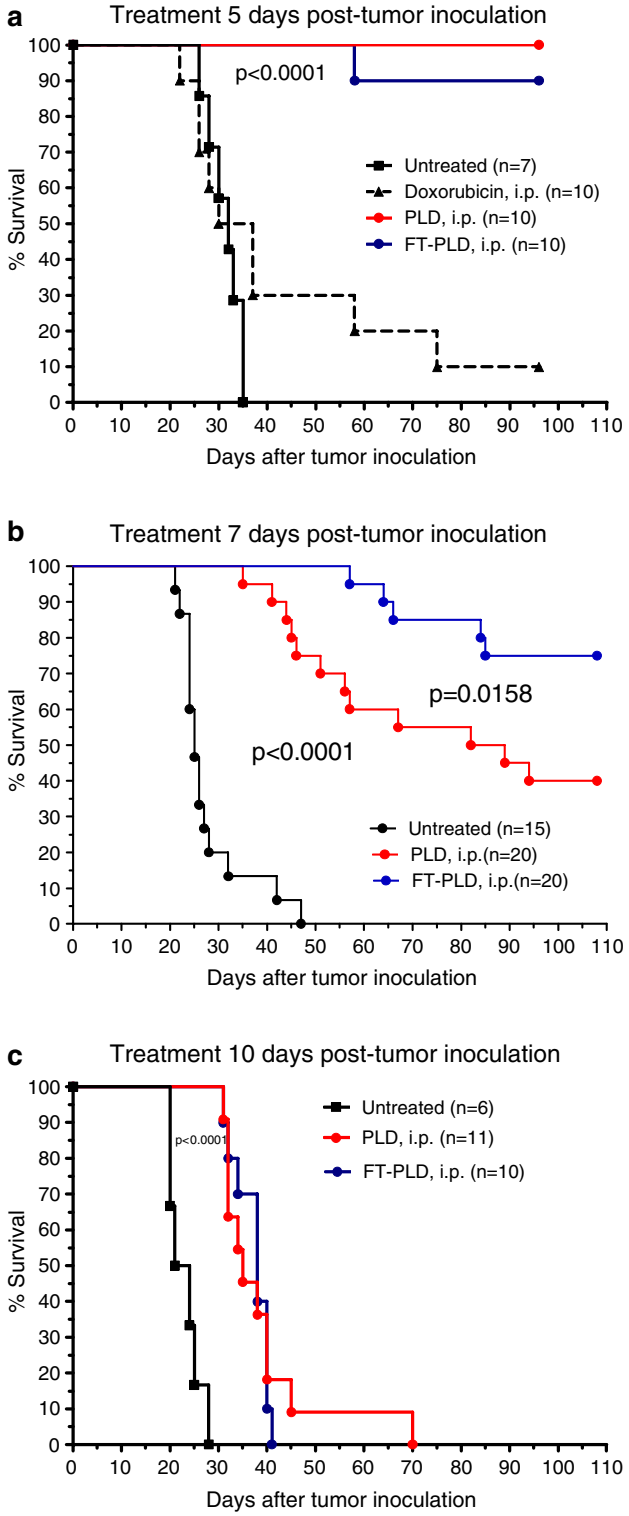
rate reported [31]. In an earlier report, we failed to observe any superiority of FT-PLD over PLD in the FR-expressing M109 and M109R mouse tumor models [15].

The most direct approach for a practical application of targeted liposomes is to design a formulation by insertion of the targeting ligand into an existing, ready-to-use, non-targeted formulation. This approach enables flexibility regarding the choice of targeting ligand and liposomal drug and spares the need for manufacturing a liposomal formulation anew. By limiting the placement of the ligand conjugate to the outer leaflet of liposomal membrane, it also results in a more relevant formulation design and economical use of the conjugate. Micellar insertion of lipophilic PEG-derivatized ligands was first reported by Zalipsky et al. [21], where conjugates of oligosaccharide- and peptide–PEG–lipids were prepared and introduced on the surface of preformed liposomes. This method has been successfully used by us and other investigators [15, 19, 32], and recently reviewed in detail [33]. It results in stable formulations that retain the ligand for long periods in circulation as shown for folate and antiHer2 scFv conjugates [17, 34]. In this study, we have used the micellar insertion method to prepare a formulation of folate-targeted liposomal doxorubicin (FT-PLD) based on commercially available DOXIL™ (PLD), and examine its therapeutic activity in mouse tumor models.

Overall, our current results (Table 1) point to a small but significant therapeutic advantage for the targeted formulation. An exception is the i.v. systemically treated J6456-FR lymphoma model in which any advantage in outcome appears to be related to a decreased toxicity of FT-PLD (Figs. 3, 4). A reassuring note is that FT-PLD was as active as PLD in the FR-negative J6456 model, thereby indicating that targeting does not incur in any loss of activity when tumors lack the targeted receptor.

In Winn assay-like experiments, FT-PLD results unequivocally in much stronger tumor inhibition than PLD [15, 35], raising the question of in vivo factors that minimize the difference between FT-PLD and PLD. Although FT-PLD is cleared slightly faster from plasma than PLD [17], it is unlikely that this minor difference will significantly hinder the potential in vivo activity of the targeted formulations. Since PLD is highly stable in plasma and enters tissue in nearly intact form, it follows that the key events are those taking place in the tumor environment. Several pathways of interaction of targeted liposomal drugs with the tumor milieu are possible.

- Direct interaction of liposomes with individual tumor cells followed by drug delivery into the intracellular compartment.
- Gradual leakage of liposomal drug in the tumor interstitial fluid with subsequent drug redistribution by diffusion to tumor cells and other surrounding cells, including vascular endothelium. Leakage may occur as a predictable kinetic process due to gradual collapse of the proton gradient of PLD, or after damage to the liposomal membrane by phospholipases present in the extracellular medium.
- Liposome uptake and lysosomal degradation by tumor-infiltrating macrophages and other phagocytic cells, resulting in cytotoxic damage to phagocytic cells of the supporting tumor stroma and leading indirectly to inhibition of tumor growth. Macrophage liposomal uptake may also be followed by drug regurgitation and redistribution by diffusion into tumor cells, although there is no experimental evidence supporting this process.



◀ **Fig. 5** Schedule-dependency of the therapeutic effect of intracavitary (i.p.) treatment with free doxorubicin, PLD and FT-PLD in the J6456-FR ascitic tumor model. Mice on folate-depleted diet treated i.p. with free doxorubicin, PLD or FT-PLD at a single dose of 10 mg/kg on day 5 (**a**), day 7 (**b**), or day 10 (**c**) after tumor inoculation. **a** (day 5) Survival time. Log rank test: Untreated versus free doxorubicin, ns; Untreated versus PLD, $P < 0.0001$; Untreated versus FT-PLD, $P < 0.0001$; PLD versus FT-PLD, ns. **b** (day 7) Survival time. Log rank test: Untreated versus PLD, $P < 0.0001$; Untreated versus FT-PLD, $P < 0.0001$; PLD versus FT-PLD, $P = 0.0158$. This panel depicts a pooled analysis of two identical experiments. **c** (day 10) Survival time. Log rank test: Untreated versus PLD, $P < 0.0001$; Untreated versus FT-PLD, $P < 0.0001$; PLD versus FT-PLD, ns

The use of targeted liposomes should theoretically tilt the respective contribution of each of these processes in favor of the first one: direct interaction with tumor cells. However, since most liposomes are found in the perivascular space of tumors [30] and their sheer size makes penetration through the tumor tissue unlikely as shown for large polymers [36], direct tumor cell uptake may be a relatively minor fraction of the injected liposome dose. This is likely to limit the therapeutic advantage of targeted over non-targeted liposomal drugs in solid tumors.

The intracavitary approach should give a clear advantage to FT-PLD over PLD, as predicted by the ~17-fold greater tumor cell uptake observed in this model [22]. Indeed, we were able here to achieve a significant improvement in survival and cure rate if we adequately timed the therapeutic intervention (Table 1; Fig. 5). The failure to improve survival at a late stage derives probably from the extra-peritoneal seeding of the tumor (liver, lungs, etc.). These organ metastases will probably be more effectively reached with PLD which results in ~14-fold higher plasma levels than FT-PLD after i.p. injection [22]. This may cancel out any advantage of FT-PLD within the ascitic tumor cell compartment. Overall, the i.p. administration of a folate-targeted liposomal formulation, such as FT-PLD, appears to be an attractive complement to systemic therapy in the management of FR-expressing ascitic malignant tumors such as the case of serous ovarian carcinoma. Moreover, as noted above, the fact that FT-PLD is at least as effective as PLD against FR-negative tumors (Fig. 3b) is reassuring when considering the possibility of heterogeneous tumors with regard to FR expression.

In several experiments with mice fed folate-depleted diet, a number of acute or delayed toxic deaths occurred. Toxic deaths were more frequent in mice treated with non-targeted liposomes suggesting that the folate conjugate present in folate-targeted liposomes may provide a source of folate and minimize toxicity. This hypothesis is supported by the fact that a supplement of folate, equivalent to the amount of folate conjugate, protected folate-depleted diet-fed mice treated with PLD from toxicity (Fig. 4b). The fact that toxicity was not observed when PLD was given by the i.p. route may be related to a reduction in systemic exposure to the drug due to some degree of peritoneal uptake of liposomes.

One important aspect of the targeted approach with PLD is the fact that doxorubicin is an amphipathic molecule that can enter tumor cells by diffusion through cell membranes

without requiring a targeted vehicle for cell access. Indeed, in vitro exposure to FT-PLD results in no substantial advantage in drug uptake over free doxorubicin despite a large advantage over PLD as shown here (see supplementary material Fig. S2A) and in other studies [35, 37]. The liposome targeted approach using specific ligands to cancer cells is particularly of value when delivering drugs that are highly cell-impermeable. In these cases, the drug cannot exert its antitumor activity unless a vehicle transports the drug into the intracellular compartment. The potential of liposomes to enable internalization and pharmacologic activity of cell-impermeable drugs has been recognized long ago for drugs such as methotrexate-gamma-aspartate [38], 5-fluoroorotate [39], and *N*-(phosphonacetyl)-L-aspartic acid (PALA) [40], that were referred to as "liposome-dependent drugs." A recent study from our laboratory with zoledronic acid, another cell-impermeable drug and a potent inhibitor of farnesyl-pyrophosphate synthase, has indicated that folate-liposomal targeting of zoledronic acid results in a major increase of drug uptake and in a ~100-fold increase of in vitro cytotoxicity when compared to the free drug [41]. A similar claim can be made for the delivery of plasmid DNA and oligonucleotides by folate-targeted liposomes [16, 42, 43]. Future studies with selected approaches such as the use of cell-impermeable drugs may help to further define the role of ligand-targeted liposomal systems in cancer therapy.

Acknowledgment Supported by the Israel Cancer Research Fund and by Alza Corporation (Mountain View, CA).

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