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RESEARCH PAPER

# Palmitoyl Ascorbate Liposomes and Free Ascorbic Acid: Comparison of Anticancer Therapeutic Effects Upon Parenteral Administration

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## ABSTRACT

***Purpose*** To evaluate and compare anticancer therapeutic effect of palmitoyl ascorbate liposomes (PAL) and free ascorbic acid (AA).

***Methods*** Liposomes incorporating palmitoyl ascorbate (PA) were prepared and evaluated for PA content by HPLC. To elucidate mechanism of action of cell death *in vitro*, effect of various $H_2O_2$ scavengers and metal chelators on PA-mediated cytotoxicity was studied. Effect of various combinations of PAL and free AA on *in vitro* cytotoxicity was evaluated on 4T1 cells. *In vivo*, PAL formulation was modified with polyethylene glycol; effect of PEGylation on *in vitro* cytotoxicity was evaluated. Biodistribution of PEG-PAL formulation was investigated in female Balb/c mice bearing murine mammary carcinoma (4T1 cells). *In vivo* anticancer activity of PEG-PAL (PEG-PAL equivalent to 20 mg/kg of PA injected intravenously on alternate days) was compared with free AA therapy in same model.

***Results*** PEG-PAL treatment was significantly more effective than free AA treatment in slowing tumor growth.

***Conclusions*** Nanoparticle formulations incorporating PA can kill cancer cells *in vitro*. The mechanism of PA cytotoxicity is based on production of extracellular reactive oxygen species and involves intracellular transition metals.

**KEY WORDS** ascorbic acid · cancer · liposomes · palmitoyl ascorbate

## ABBREVIATIONS

| | |
|---|---|
| AA | ascorbic acid |
| DHAA | dehydroascorbic acid |
| DFO | desferrioxamine mesylate |
| DMEM | Dulbecco's Modified Eagle Medium |
| DTPA-PE | 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid |
| EDTA | ethylenediaminetetraacetic acid |
| FBS | fetal bovine serum |
| GLUTs | glucose transporters |
| HIF | hypoxia-inducible factors |
| PA | palmitoyl ascorbate |
| PAL | palmitoyl ascorbate liposomes |
| $PEG_{2000}$-PE | 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (poly (ethylene glycol))-2000] ($PEG_{2000}$-PE) |
| PL | plain liposomes |
| ROS | reactive oxygen species |
| SOD | superoxide dismutase |
| TCEP | tris(2-carboxyethyl) phosphine hydrochloride |

## INTRODUCTION

Studies using animal tumor models have demonstrated that free ascorbic acid (AA) therapy produces peroxide in the

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extracellular fluid of tumors (1,2) and significantly slows the growth of numerous aggressive tumor types (1,3,4). Clinical studies have shown that AA injection is very safe for screened patients (5–7), and may offer clinical benefit by improving quality of life (8). Clinical trials are currently underway to rigorously evaluate the potential of injected AA to extend survival of cancer patients, an effect suggested by results from older clinical study relying on use of historical control patient populations rather than control treatment populations (6,7). Currently, many cancer therapy investigators are looking for ways to enhance the anticancer activities of AA while retaining the excellent safety profile.

Palmitoyl ascorbate (PA)-containing liposomes (PAL) and AA have each been the focus of anticancer therapy research. However, these treatments have not been compared previously for relative effectiveness *in vivo*. Nanoparticle drug carriers such as liposomes alter the pharmacokinetics of incorporated drugs. Appropriately designed nanoparticles increase the delivery of incorporated drugs to tumors while limiting the delivery to normal tissues (9). In our experiments, PAL have been used to deliver ascorbate moieties to tumor tissues.

This present study addresses the question of whether PAL can produce anticancer activity equal to or greater than the activity of AA therapy, which has been reported to decrease tumor growth rates by about 40% in numerous rodent models (1,3,4). Although PAL are being used to deliver ascorbate, the effects of PAL cannot be expected to be identical to those of injected free AA due to differences in delivery dynamics and biochemical behavior. PA is an acylated derivative of AA known to have anticancer properties and to produce a spectrum of reactive oxygen species (ROS) similar to, but broader than, AA (10). PA incorporates readily into nanoparticles, including micelles and liposomes at high molar concentrations (11,12). Furthermore, PAL have been shown to have substantial anticancer activity and to increase the effectiveness of encapsulated paclitaxel *in vivo* (12).

AA has certain chemical and biological activities that are magnified by cancer cells and their surrounding microenvironments. The reaction of AA with tumor-generated ROS causes abnormal local oxidization of AA, forming products, including dehydroascorbic acid (DHAA), which can associate with the glucose transporters (GLUTs) and enter tumor cells (13). DHAA can be transported by glucose transporters including GLUT-1, GLUT-3, and GLUT-4 (14–16). Tumor cells can accumulate high intracellular AA concentrations through this mechanism (13). Rapid influx of DHAA into cancer cells can lead to glutathione and ATP depletion as DHAA is converted into AA (4,17). AA counteracts the effects of tumor-secreted factors and microenvironment stresses on cellular hypoxic signal regulation by overcoming inhibition of the enzyme that marks hypoxia-inducible factors (HIF) for destruction (18,19). AA normalizes hypoxic signaling in cancer cells as well as in normoxic stromal cell types including immune cells (20–22). By contrast, our present liposome formulation containing PA exerts pro-oxidative effects outside the cell, but is not expected to deplete glutathione or alter HIF regulation. PAL are expected to accumulate and persist in tumor tissues and thereby prolong the exposure of tumor tissues to ROS. AA has multiple anticancer properties dependent on location, concentration, and duration of exposure. However, the comparative effectiveness of PAL and injected free AA on tumor growth has not been determined previously *in vivo*.

## MATERIALS AND METHODS

### Materials

Lipids were purchased from Avanti Polar lipids (Alabaster, AL). L-ascorbic acid (AA), palmitoyl ascorbate (PA), ethylenediaminetetraacetic acid (EDTA), catalase and desferrioxamine mesylate (DFO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was from Invitrogen (Eugene, OR, USA). 4T1 (murine mammary carcinoma) and MCF-7 (human mammary carcinoma) cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cell culture media, heat-inactivated fetal bovine serum (FBS) and concentrated solution of sodium pyruvate and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA). For *in vivo* studies, 9–12-week-old Balb/C female mice were purchased from Charles River Laboratories (Wilmington, MA). The animals were allowed free access to food and water. $In^{111}$ was obtained from Perkin-Elmer (Boston, MA).

### Liposome Preparation

PA (30 mol%) was incorporated in liposomes composed of egg phosphatidylcholine/cholesterol (70:30 mol%) by the rehydration of lipid films (11,12). Briefly, a lipid film was formed from aliquots of stock solutions of egg phosphatidylcholine, cholesterol, and PA in a round-bottom flask by solvent removal on a rotary evaporator. The lipid film was hydrated with 10 mM phosphate-buffered saline (PBS), pH 7.4, to give a final lipid concentration of 10 mg/ml, followed by bath sonication for 30 min and then extrusion through 200 nm polycarbonate membranes. Wherever applicable, 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (poly (ethylene glycol))-2000] ($PEG_{2000}$-PE) was added to the lipid mixture followed by liposome preparation as described.

For animal studies, liposomes were labeled with $^{111}$In by the addition of 0.5 mol % 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (DTPA-PE) and 4 mol% of $PEG_{2000}$-PE. The DTPA-PE-containing liposome was supplemented with 1 M citrate buffer. The $^{111}$In-citrate complex was incubated with liposomes for 1 h at room temperature and dialyzed overnight against 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered saline (HBS), pH 7.4, at 4°C to remove free label. Plain liposomes (PL) were prepared similarly but without PA. Liposomes were characterized for size (hydrodynamic diameter) by the dynamic light scattering using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). The amount of PA in liposomes was estimated by the reversed phase-HPLC. Liposomes were diluted with the mobile phase before application onto the HPLC column. A D-7000 HPLC system equipped with a diode array and fluorescence detector (Hitachi, Japan) and Spherisorb ODS2 column, 4.6 mm×250 mm (Waters, Milford, MA, USA) was used. The mobile phases consisted of phosphate buffer (20 mM, pH 2.5) /acetonitrile/methanol mixture (5:40:55% v/v) at 1.0 ml/min. PA was detected at 254 nm. Injection volume was 50 µl. All samples were analyzed in triplicate.

### Cell Culture

All cell lines were grown in DMEM cell culture medium supplemented with 10% fetal bovine serum, 50 µg/ml streptomycin, and 50 units/ml penicillin at 37°C, 5% $CO_2$.

### *In Vitro* Cytotoxicity of PAL

4T1 cells were seeded in 96-well plates (Corning, Inc., NY, USA) and grown until 60–70% confluent. Before exposure to PAL (2.5 mM), the cells were incubated for 30 min with various ROS scavengers, metal chelators, and a reducing agent at the following concentrations: catalase, 1200 U/ml; SOD, 300 U/ml; TCEP, 500 µM; EDTA, 1 mM; DFO, 500 µM. After 1 h, cells were washed twice with sterile PBS and incubated for 24 h with complete medium. After 24 h, they were analyzed for viability using CellTiter 96® $AQ_{ueous}$ Non-radioactive Cell Proliferation Assay (Promega, Madison, WI).

### Comparison of Cytotoxic Effect of Various Combinations of PAL and Free AA *In Vitro*

Cells were grown in 96-well plates (Corning, Inc, NY) until 60–70% confluent, followed by an hour exposure to varying concentrations of PAL (1.875 mM or 3.75 mM of PA), free AA solution (5 mM or 10 mM), combination of PAL (1.875 mM of PA) with free AA solution (10 mM), or combination of PAL (3.75 mM of PA) with free AA (5 mM). At the end of formulation exposure, each well was washed with DMEM and incubated with complete medium for 24 h, and cell viability was analyzed as described above.

### Effect of PEGylation on Cytotoxic Effect of PAL *In Vitro*

4T1 cells were grown in 96-well plates (Corning, Inc., NY) until 60–70% confluent. The cells were exposed for an hour to varying concentrations (0, 1, 3 or 6 mol%) of $PEG_{2000}$-PE containing PAL. The cells were washed with DMEM and incubated with complete medium for 24 h, and cell viability was analyzed as described above.

### Biodistribution Studies

Approximately $10^5$ 4T1 cells in PBS were inoculated subcutaneously into the left flank in 9–12-week-old female Balb/C mice. Twenty days after tumor inoculation, the radiolabeled liposomal formulations (5$\mu$Ci, 5 mice per group) were injected via the tail vein. Mice were sacrificed at specific time points, and selected organs were removed and weighed, and the radioactivity quantified as mean CPM ±standard deviation using a Beckman 5500B gamma-counter. The accumulated radioactivity per gram of tissue was calculated.

### Gamma Scintigraphy of 4T1 Tumor-Bearing Mice with $^{111}$In-Labelled Liposomes

For tumor visualization, mice bearing 5- to 10-mm tumors were injected with various $^{111}$In-labelled liposomal formulations (approx. 70 µCi/mouse) via the tail vein. At different time points post-injection the mice were anesthetized with a 10:1 vol/vol mixture of xylazine and ketamine intraperitoneally. The accumulation of $^{111}$In-liposomes in tumors was visualized using a Picker SX-300 (Picker International Inc., OH) and 3 mm pin-hole collimator. The images were acquired and displayed using Gamma 600 program. The visual interpretation of the images was based on activity in the zone corresponding to the site of the tumor relative to the background.

### Tumor Growth Analysis

Approximately $10^5$ 4T1 cells in PBS were inoculated in 9–12-week-old female Balb/C mice by subcutaneous injection into the left flank. Treatment was started when tumors first appeared (~1 mm). PEG-PAL formulation equivalent to 20 mg/kg of PA were administered via the tail vein on alternate days. Fresh AA solution was prepared for each experiment and adjusted to pH 7.0 with NaOH and

administered intraperitoneally daily at dose of 1 g/kg. Sterile PBS pH 7.4 was used as control.

The tumor diameters were measured on alternate days with a vernier caliper in two dimensions. Individual tumor volumes (V) were calculated using the formula: $V = (L \cdot X \cdot W^2)/2$, where length (L) is the longer dimension and width (W) is the shorter dimension perpendicular to length.

### Data Analysis

Results are presented as mean values±standard deviation (S.D.). Comparisons between the groups were made using Student's *t*-test. With more than two groups, ANOVA was used to compare results. All statistical analysis was performed using SPSS, version 16.

## RESULTS

### Characterization of PAL

PA was incorporated in liposomes at 30 mol% as analyzed by HPLC. Liposome size was 142.2±17.0 and 148.6± 25.0 nm for PL and PAL, respectively. Incorporation of $PEG_{2000}$-PE (1–6 mol%) did not change size of PAL significantly. *In vitro* cytotoxicity of PAL required extracellular ROS generation and intracellular metal ions. Cell killing by PAL was prevented by catalase, SOD, TCEP, DFO, but not by EDTA (Fig. 1).

PL showed <5% cytotoxicity (data not shown). Thus, the absence of the superoxide or detoxification of the peroxide by catalase inhibited anticancer activity of PAL. Thiol reduction by TCEP prevented PAL-mediated cell death. Cell death was inhibited by the iron chelator DFO that is taken up by endocytosis, but not by EDTA, which acts extracellularly.

Reduction of anticancer toxicity of PAL by ROS inhibitors and metal chelators indicates a mechanism of action that resembles those described for free AA (4,23,24) and for PA (10,24). The mechanism through which extracellular ascorbate and intracellular iron exert this anticancer toxicity is unknown and is currently under investigation.

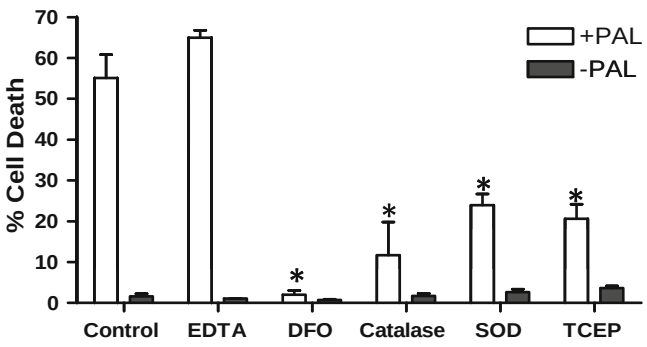


**Fig. 1** Effect of ROS scavengers (catalase, SOD), a reducing agent (TCEP), and metal chelators (DFO, EDTA) with or without PAL (2.5 mM) on viability of 4T1 breast cancer cells. * Statistically significant when compared with PAL at $P < 0.05$. ($n = 3$, ±S.D.).

Treatment of cancer cells with PAL followed by or simultaneous to AA enhanced anticancer activity compared to either treatment alone. Cell viability was measured following exposure of cancer cells to PAL alone, AA alone, PAL simultaneously with AA, PAL followed by AA or AA followed by PAL. Two concentration ratios were studied: 1.875 mM PAL: 10 mM AA, and 3.75 mM PAL: 5 mM AA. PAL was significantly cytotoxic *in vitro* compared to free AA. Co-therapy with PAL simultaneously with AA or PAL prior to AA caused significantly enhanced cell killing compared to either therapy alone. AA followed by PAL provided the same percent cell killing as PAL alone. The results are similar at either PA: AA concentration ratio (Fig. 2). Cells treated with PL at equivalent lipid concentrations without PA exhibited <5% cytotoxicity (data not shown).

### PA-Liposomal Killing of Cancer Cells Unaffected by PEGylation

In order to determine whether PEG would inhibit cytotoxicity of PAL *in vitro*, liposome formulations containing 0, 1, 3, and 6 mol% $PEG_{2000}$-PE were prepared containing PA at either, 1.85 mM, 3.7 mM, or 7.4 mM. Treatment of 4T1 cells with all PAL formulations caused decreased cell viability. The percent of cells killed in the 0% PEG formulations increased with increasing PA content (Fig. 3). The PEGylation of PAL (i.e PEG-PAL) did not affect cell viability for any PA concentration tested (Fig. 3).

### PAL Biodistribution

Accumulation of $^{111}$In-labeled liposomes in tumor tissues was similar for plain liposomes modified with 4 mol% $PEG_{2000}$-PE (PEG-PL) and PAL modified with 4 mol% $PEG_{2000}$-PE (PEG-PAL), which showed predominant deposition in the liver, spleen, and lungs (Fig. 4).

The distribution of PEG-PAL liposomes in tumor-bearing mice was studied using $^{111}$In-labeled liposomes visualized using a gamma camera. The distribution of PEG-PL and PEG-PAL was visualized at different time points post-injection. Both formulations showed accumulation in tumors. Tissue accumulation of PEG-PL and PEG-PAL were similar (Fig. 5).

### Effect of Various Formulations on Tumor Growth *In Vivo*

*In vivo* anti-tumor effects of injected free AA and PEG-PAL were compared in the 4T1 mouse mammary tumor model.

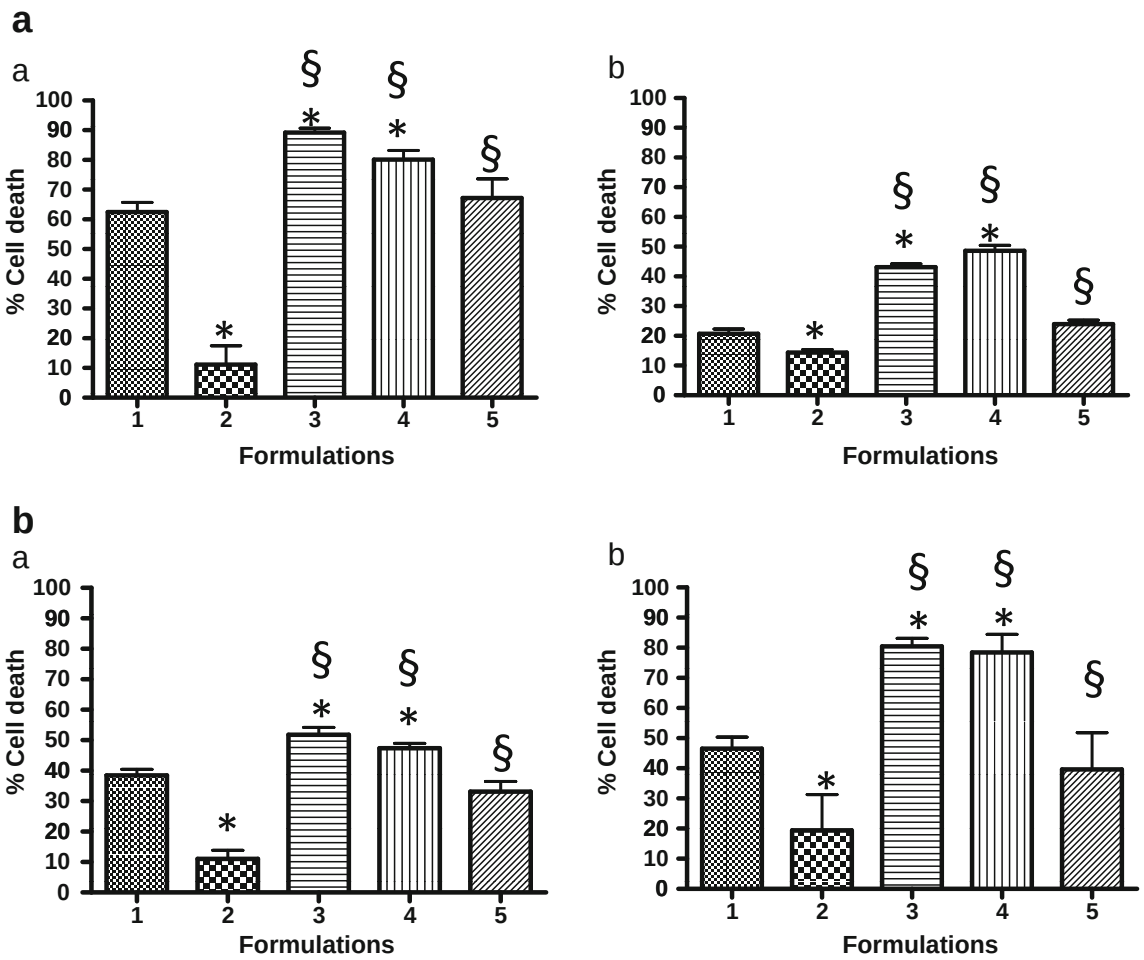


**Fig. 2** *In vitro* cytotoxicity of 4T1 cells (**a**) and MCF-7 cells (**b**) treated with either PAL or AA alone or in combination. Data shown are for 3.75 mM PAL and 5 mM AA (**a**) and 1.875 mM PAL and 10 mM AA (**b**). Formulations: PAL, **1**; AA, **2**; PAL and AA together, **3**; PAL followed by AA, **4**; AA followed by PAL, **5**. * Statistically significant when compared with PAL at $P < 0.05$. § Statistically significant when compared with AA at $P < 0.05$. ($n = 3$, ±S.D.).

Doses of free AA were selected as reported in (1,3,4), and PAL dose was selected based on our preliminary results. Our initial studies with mice injected with 4 g/kg of free AA showed signs of toxicity (data not shown); hence, 1 g/kg dose was selected for subsequent studies. The anti-tumor effectiveness of free AA injected at 1 g/kg was compared to the effectiveness of PEG-PAL and control injections. PEG-PAL significantly ($P < 0.05$) slowed growth of tumors, whereas injected free AA did not, at the end of the treatment (Fig. 6).

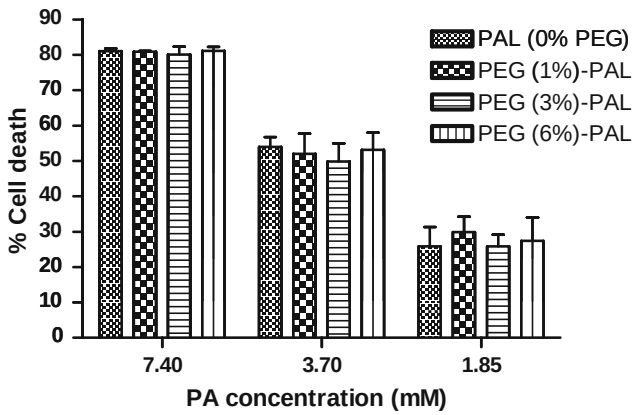


**Fig. 3** Effect of PEG on PAL *in vitro* cytotoxicity at different PA concentration ($n = 3$, ±S.D.).

## DISCUSSION

The fundamental question addressed by this research is whether liposomal PA therapy could equal or surpass the effectiveness of injected AA therapy. The results show that PA liposomal formulations provided improved anticancer effectiveness compared to free AA. Furthermore, in these studies, the liposomal PA formulations were injected on alternate days at a much lower dose (20 mg/kg of PA) compared to AA (injected daily at 1 g/kg). In addition, there was no evidence of

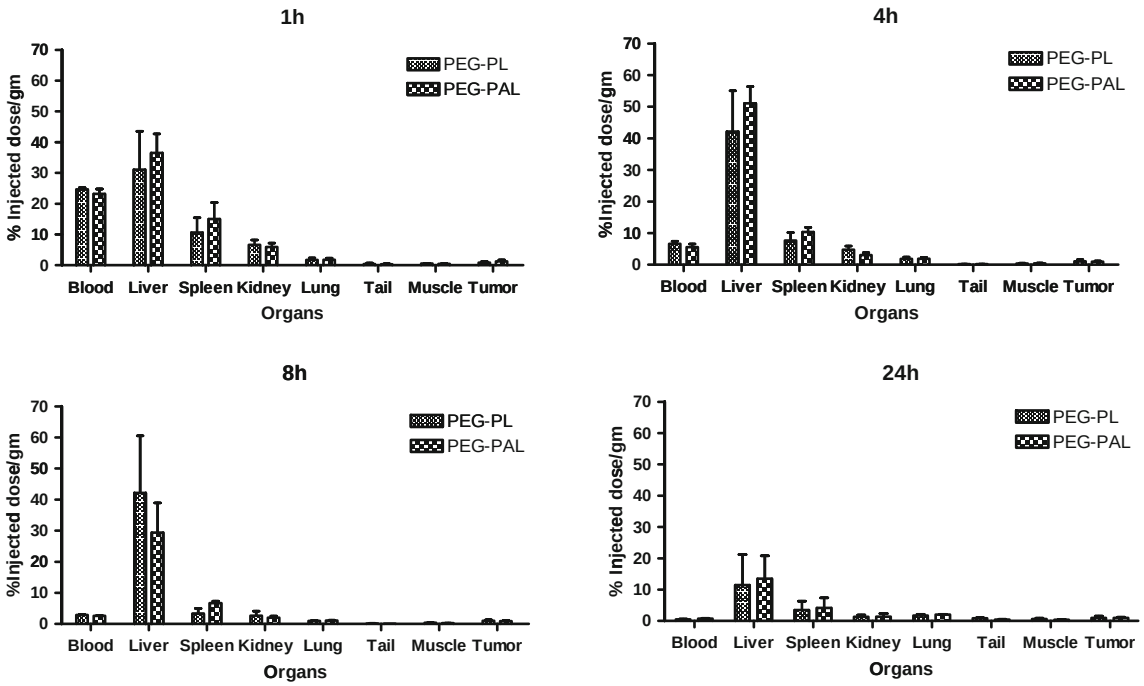


**Fig. 4** Biodistribution of liposomes in 4T1 tumor-bearing mice at different time points ($n = 5$, ±S.D.).

toxicity in the PAL-treated mice. It is also not yet clear what the maximum tolerated dose of PAL might be, as toxicity was not observed at the dose studied.

Our *in vitro* studies confirmed that the major anticancer effects of PAL related to extracellular ROS generation and required intracellular metal ions, similar to the mechanisms of ROS-mediated anticancer toxicity described for AA (2,23) and for PA (10). Several important differences distinguish the effects of ascorbate treatment and PAL treatment. PAL were detected in mouse tumors for at least 24 h after injection (12,25), whereas high concentrations of AA have been shown by others to be cleared from

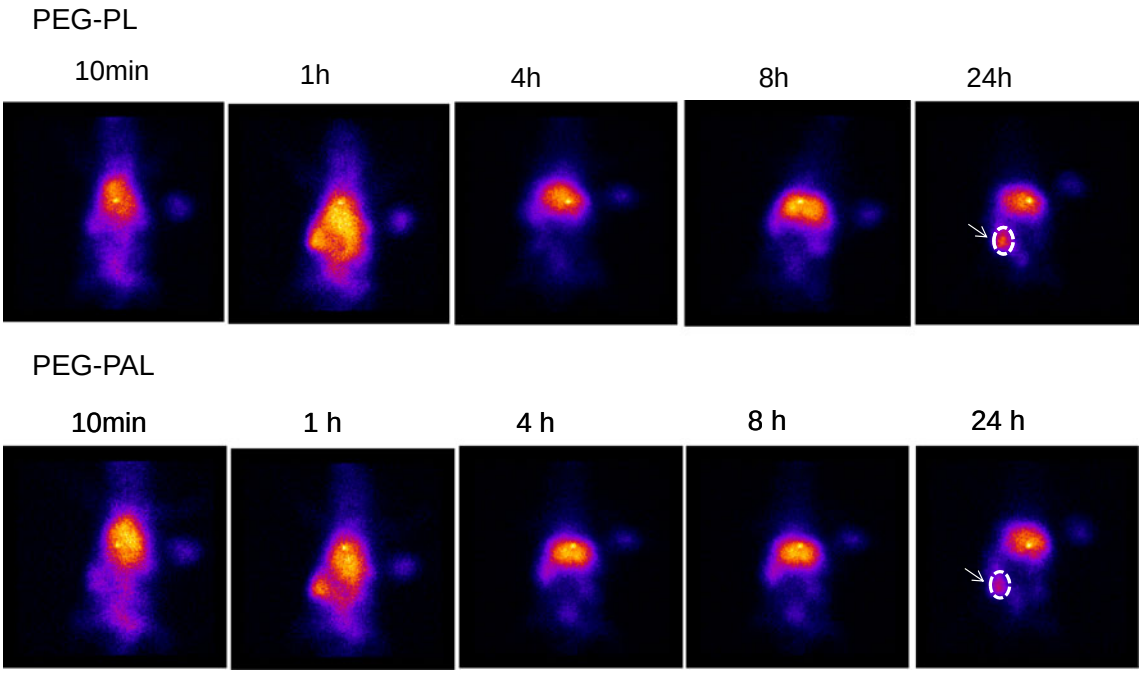


**Fig. 5** Whole-body gamma images of 4T1 tumor-bearing mice, receiving $^{111}$In-labelled liposomal preparations, taken at times post-injection. Calibration control was kept on right side of mouse.

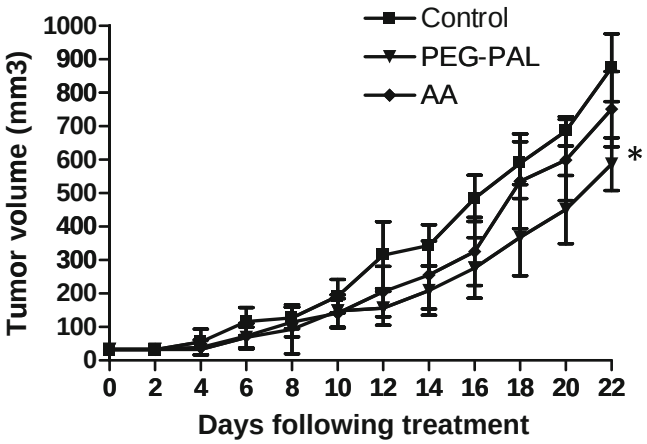


**Fig. 6** Effect of i.v. administration of PEG-PAL and i.p administration of free AA on tumor growth in 4T1 tumor-bearing mice ($n = 5$, ±S.D.). * statistically significant when compared with control at $P < 0.05$ ($n = 3$, ±S.D.).

circulation within hours (2,26). Injected AA causes a spike in serum ascorbate concentrations, resulting in serum ascorbate concentrations of 5 to 20 millimolar, far higher than the 200 micromolar concentration possible from oral ascorbate delivery (26,27). AA can be expected to penetrate farther into tumor tissues than liposomal PA due to size and diffusion differences. Within the tumor microenvironment, AA is oxidized to DHAA, which is taken up at high concentrations by tumor cells through GLUT family glucose receptors (13). Subsequent intracellular conversion of the high concentrations of DHAA to AA depletes GSH and ATP from cancer cells (4,17). The formulations of PAL tested here are not expected to replicate the effects of AA either in ascorbate loading of cells or the resulting energy depletion. However, the mechanisms of DHAA association with glucose receptors may be relevant to association of some PAL formulations with cancer cells.

Our previous studies indicated that PAL association with RAG renal carcinoma cells *in vitro* was enhanced by treatment of these cells with TNF, which increases superoxide production (11). This effect of TNF on RAG-PAL association is blocked by removing superoxide with SOD (11). The mechanism of action of PAL is similar to that of free ascorbate. The cell killing was inhibited by extracellular ROS scavengers or intracellular metal ions chelators (Fig. 1). The extracellular chelators failed to protect against PA-mediated toxicity. To explain the failure of extracellular chelators, Chen *et al.* suggested the existence of extracellular metalloprotein catalysts present in the serum that could participate in hydrogen peroxide production by ascorbate (23). They also demonstrated that the generation of hydrogen peroxide by ascorbate *in vivo* is possible only in extracellular fluids. Ascorbate toxicity is completely inhibited in the presence of blood (2,23), since red blood cells show both catalase and glutathione peroxidase activities, which efficiently detoxify hydrogen peroxide. For *in vivo* studies, we have coated liposomes with PEG, since it was shown that PEGylation prolongs the circulation times of liposomes in the blood (28). Addition of PEG to PAL formulations did not alter PAL-induced *in vivo* cytotoxicity (Fig. 3). The mechanism of PA-mediated cytotoxicity is due mainly to generation of ROS, and thus the PEGylation did not interfere with this mechanism.

Previous research has indicated that PAL enhanced the activity of encapsulated paclitaxel (12). In addition, data presented here indicate that treatment of cancer cells with PAL followed by or simultaneous with AA enhances anticancer activity compared to either treatment alone. In contrast, treatment with AA prior to addition of PAL does not show enhancement of activity (Fig. 2). This finding may have implications for timing in potential co-therapy regimens designed to incorporate PAL plus infused pharmacological intravenous ascorbate concentrations. Such co-therapy regimens have not yet been tested *in vivo*.

Tumors are often highly vascularized, but have poor fluid circulation dynamics. Tumor vasculature is characteristically convoluted, poorly formed, and leaky (29). Poor perfusion combined with elevated interstitial fluid pressures within tumors decreases drug delivery and speeds drug removal. Conversely, the vascular properties of tumors enhance nanoparticle drug delivery through a process often referred to as the enhanced permeability and retention effect (EPR), whereby untargeted nanoparticles escape the tumor vasculature to accumulate heterogeneously in perivascular regions (25,30). Nanoparticles can be engineered with targeting moieties to increase the delivery of therapeutic compounds to tumor cells and decrease the percentage that accumulates in non-target tissues. Targeting moieties can increase delivery of nanoparticle cargo to tumor cells even when overall tumor localization dynamics resemble those of untargeted delivery (31,32). Interestingly, nanoparticles tend to accumulate in some organs that are common sites of metastatic disease, including the liver and lungs. Certain PAL formulations have shown anticancer targeting properties (11,12), but the overall biodistribution of the PEG-PAL formulation used here resembled that of untargeted liposomes (Fig. 4).

The results presented here indicate that liposomal PA at a much lower dose (liposomes equivalent to 20 mg/kg of PA injected intravenously on alternate days) was more effective than free AA (1 g/kg daily by intraperitoneal injection) at slowing tumor growth. The current PA liposomal formulations slowed tumor growth but were unable to induce tumor regression or cure in the 4T1 breast cancer model at the doses tested. Higher PAL doses may be possible, as toxicity was not observed with the regimen studied. Maximum tolerated dose has not yet been determined. There are several questions which have not

yet been addressed by this research. Among these questions is whether PAL would be effective *in vivo* in co-therapy regimen with injected ascorbate. Additionally, an anticancer regimen using PAL with cytotoxic drugs or targeted therapies should be investigated. Ascorbate has been shown to enhance the *in vitro* activity of numerous anticancer drugs including paclitaxel, etoposide, 5-FU, cisplatin, and doxorubicin (33). Ascorbate injections are used clinically to enhance activity of arsenic trioxide therapy (34). Our research suggests that PAL may be a valuable addition to anticancer treatment regimens.

## ACKNOWLEDGMENTS & DISCLOSURES

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