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RESEARCH PAPER

Palmitoyl Ascorbate-Loaded Polymeric Micelles: Cancer Cell Targeting and Cytotoxicity

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ABSTRACT

Purpose To evaluate the potential of palmitoyl ascorbate (PA)-loaded micelles for ascorbate-mediated cancer cell targeting and cytotoxicity.

Methods PA was incorporated in polyethylene glycol-phosphatidyl ethanolamine micelles at varying concentrations. The formulations were evaluated for PA content by RP-HPLC. A stable formulation was selected based on size and zeta potential measurements. A co-culture of cancer cells and GFP-expressing non-cancer cells was used to determine the specificity of PA micelle binding. *In vitro* cytotoxicity of the micellar formulations towards various cancer cell lines was investigated using a cell viability assay. To elucidate the mechanism of action of cell death *in vitro*, the effect of various H_2O_2 scavengers and metal chelators on PA-mediated cytotoxicity was studied. The *in vivo* anti-cancer activity of PA micelles was studied in female Balb/c mice bearing a murine mammary carcinoma (4T1 cells).

Results PA micelles associated preferentially with various cancer cells compared to non-cancer cells in co-culture. PA micelles exhibited anti-cancer activity in cancer cell lines both *in vitro* and *in vivo*. The mechanism of cell death was due primarily to generation of reactive oxygen species (ROS).

Conclusions The anti-cancer activity of PA micelles associated with its enhanced cancer cell binding and subsequent generation of ROS.

KEY WORDS cancer · micelles · nanocarriers · palmitoyl ascorbate · targeting

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INTRODUCTION

It is now recognized that the controversial history of ascorbate in the treatment of cancer was due to the different routes of administration used in clinical trials. Some studies used IV plus oral ascorbate (1), whereas others used oral ascorbate alone (2,3). Later, it was shown that higher serum concentrations are achievable with IV administration (4). The blood concentrations reached after daily oral administration of doses of ≥400 mg is 60–100 μM. In contrast, IV infusions of ascorbate can reach plasma concentrations up to 20 mM, 200 times greater than that achieved orally (4). Further, the high concentrations of ascorbate obtained following IV ascorbate treatment produce hydrogen peroxide in extracellular fluids both *in vitro* (5) and *in vivo* (6) and is believed to be a significant factor for ascorbate-mediated cell death.

Tumors often display abnormal local oxidation of ascorbate as a result of elevated levels of superoxide produced by stromal and/or tumor cells (7). Oxidation of ascorbate to dehydroascorbic acid (DHAA) in tumor tissues allows elevated accumulation of ascorbate by tumor cells (7), since the glucose transporters GLUT-1, GLUT-3, and GLUT-4 can transport DHAA, but not ascorbate (8–10). In addition to its direct anti-cancer toxicity, ascorbate has been shown to increase the effectiveness of various other anticancer treatments both *in vitro* and *in vivo* (11–15).

However, the *in vivo* application of ascorbate is currently limited by the need for very high plasma concentrations to achieve therapeutic levels in tumors. The limited chemical stability and strict physiologic regulation of the native ascorbate demands the high doses of ascorbate necessary for its anti-cancer activity. As a result, several more stable hydrophobized derivatives of ascorbate that retain the antioxidant effects of ascorbate have been explored (16,17). Additionally, these derivatives have been considered for incorporation in formulations of ascorbate-bearing pharmaceutical nanocarriers (17,18).

Palmitoyl ascorbate (PA) is a stable alternative to free ascorbate and can be incorporated into pharmaceutical nanocarriers, such as liposomes and micelles, that are known to accumulate in tumors by the enhanced permeability and retention (EPR) effect (19). It was therefore proposed that such nanocarriers will deliver high doses of ascorbate to tumors and eliminate the need for high doses of free ascorbate. Previously, we have shown that surface modification of liposomes with PA residues could target and kill certain types of tumor cells and potentiate the effect of the anticancer drug paclitaxel delivered by these liposomes *in vitro* (20). These results showed that treatment with TNF-α (known to produce reactive oxygen species (ROS)) (21) enhanced the association of PA liposomes with cancer cells and that the presence of superoxide dismutase (SOD), which removes ROS, reduced the increase in cell association mediated by TNF-α *in vitro*, suggesting a role for specific biochemical factors in the tumor's microenvironment that favor the association of ascorbate nanocarriers with tumor cells.

We have recently shown that micelles prepared from polyethylene glycol-phosphatidyl ethanolamine (PEG-PE) conjugates can be efficiently loaded with anti-cancer drugs and accumulate well in experimental tumors via the EPR effect (22–24). Here, we report on studies of PA-loaded phospholipid micelles as a potential system for the delivery of ascorbate for the treatment of cancer. Being poorly soluble in nature, PA is an ideal candidate for loading into PEG-PE micelles. Our aim here was to evaluate the potential of PA micelles to target and kill cancer cells via ascorbate mediate cytotoxicity (See scheme in Fig. 1). We investigated cancer cell targetability and the cytotoxic activity and of PA micelles *in vitro* against various cancer cell lines. In order to elucidate the mechanism of cytotoxicity, we studied the effect of different ROS scavengers and metal chelators on the cytotoxicity of PA micelles. Finally, the antitumoral activity of PA micelles was investigated in a 4T1 tumor-bearing mouse model.

MATERIALS AND METHODS

1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (poly(ethylene glycol))-2000] (PEG_{2000}–PE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh–PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). PA, ethylenediaminetetraacetic acid (EDTA), deferoxamine mesylate (DFO) and catalase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was from Invitrogen (Eugene, OR, USA). The A2780 cell line was obtained from Rutgers University (NJ, USA). All other cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture media, heat-inactivated fetal bovine serum (FBS), and concentrated solutions of sodium pyruvate and penicillin/streptomycin stock solutions were purchased from Cellgro (Herndon, VA, USA).

Micelle Preparation

Various amounts of PA (Table I) were incorporated in PEG_{2000}–PE micelles by the lipid film hydration method. Briefly, PA (10 mg/ml in methanol) was added to a PEG_{2000}-PE solution in chloroform. The organic solvents were removed by rotary evaporation and freeze-drying. The film was hydrated with phosphate-buffered saline (PBS), pH 7.4 at room temperature, and vortexed for 5 min to give a final lipid concentration of 10 mg/ml. For

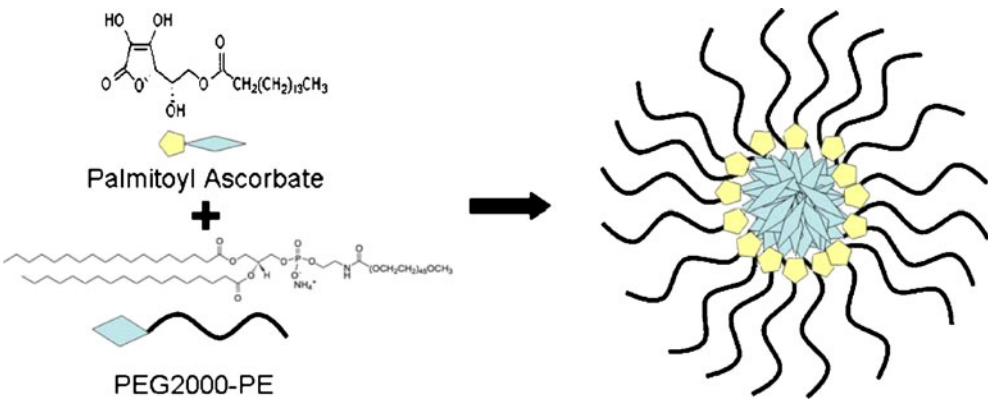

Fig. 1 Schematic representation of PA micelles.

Table I Size Distribution and Zeta Potential of Various Formulations of PA Micelles[a]

PA concentration (mM)	Size distribution (nm)	Zeta potential (mV)
0	15.7 ± 5	0.00
1	20.5 ± 7	−36.64 ± 0.98
2	25.4 ± 5	−35.7 ± 3.46
4	25 ± 7	−33.42 ± 1.43
6	530 ± 80	−35.90 ± 3.79

[a] The values are shown as mean ± S.D., $n = 3$

in vitro cell interaction studies, 0.5 mol% of the fluorescent probe, Rh-PE, was added to the micelle ingredients during micelle preparation.

Characterization of Micelles

Micelle Size Distribution

The micelle size (hydrodynamic diameter) was measured by dynamic light scattering (DLS) using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). The micelle suspensions were diluted with the deionized, distilled water until a concentration providing a light scattering intensity of 5×10^4 to 1×10^6 counts/sec was achieved. The particle size distribution of all samples was measured in triplicate.

Zeta Potential Measurement

The zeta potential of the micelle formulation was measured using phase analysis light scattering (PALS) with a Brookhaven Zeta Sizer (Brookhaven, NY, USA). For each sample, zeta potential measurements were repeated five times.

Micelle Stability

The PA micelles were stored at 4°C in vials sealed with argon and were monitored for PA content as determined by HPLC (see below) and size distribution by DLS. The micelle suspension was allowed to equilibrate at room temperature. For size analysis, the micelles were gently vortexed, and aliquot was diluted with deionized water and analyzed by DLS.

The amount of PA in the micellar phase was measured by reversed-phase HPLC. The PA micelles were centrifuged at 12,500 rpm for 5 min, and then supernatant was withdrawn and diluted with the mobile phase prior to application to the HPLC column (since the mobile phase contains acetonitrile, micelles are disrupted, and free PA is determined). The 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 column was eluted with phosphate buffer (20 mM, pH 2.5) /acetonitrile/methanol (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.

At the end of one week, the *in vitro* cytotoxicity of PA micelles was also investigated with 4T1 murine mammary carcinoma cells as described below in cytotoxicity assay.

Cell Cultures

All cell lines were maintained in DMEM cell culture medium at 37°C, 5% CO_2. DMEM media were supplemented with 10% FBS, 50U/ml-penicillin, and 50 µg/ml streptomycin.

Flow Cytometry of Co-cultured Cells

Mouse embryo yolk sac cells expressing GFP (C166-GFP) were co-cultured with various tumor cell types in a 1:1 ratio. Two-hundred µl of a rhodamine-labeled micelle preparation were added per flask of co-cultured cells in 5 ml of medium and incubated for 1 h. Cells were washed, trypsinized, and finally resuspended in 800 µl of 10% paraformaldeyhde in PBS. The fixed cells were analyzed on a BD FACS Calibur flow Cytometer (BD Biosciences, San Jose, CA, USA). Data shown were derived from three separate experiments.

Cytotoxicity Assays

Cells were grown in 96-well plates (Corning, Inc., NY, USA) until 60–70% confluent, followed by a one-hour exposure to micelles containing varying concentrations of PA. After an hour, cells were washed with DMEM and further incubated for 24 h with complete medium. After 24 h, cell viability was assayed using CellTiter 96® AQ_{ueous} Non-radioactive Cell proliferation Assay (Promega, Madison, WI, USA). PA micelles producing 50% inhibition of cell viability (IC50) were calculated using GraphPad Prism 4.

In Vivo Studies

Approximately 10^5 4T1 cells were inoculated in 6–8-week-old female Balb/C mice by subcutaneous injection into the left flank. Twenty days after tumor inoculation, mice were injected with a micellar formulation equivalent to 15 mg/kg of PA via the tail vein at days 0, 2 and 4. Normal saline was used for the control group. The tumor volumes were monitored on alternate days with a vernier caliper. The tumor volume was measured with perpendicular measurements (a and b, where a > b) using the formula $[a \times b^2] / 2$.

Data Analysis

Data are reported as mean±standard deviation. Comparisons between the groups were made using Student's *t*-test and with more than two groups, ANOVA was used to compare results. The $p<0.05$ values were considered statistically significant. All statistical analysis was performed using SPSS, version 16.

RESULTS

Stable Incorporation of PA into PEG-PE Micelles

PA was incorporated into the PEG_{2000}-PE micelles at various concentrations ranging from 1 mM to 6 mM (Table I). The micelle size observed was 20–25 nm at PA concentration up to 4 mM. Zeta-potential measurements indicated that the PA incorporation into micelles introduced a negative surface charge to the micelles. The formulation found to be most stable was with 4 mM of PA, since no signs of precipitation were seen after storage at 4°C for up to 1 week. For further experiments, 4 mM PA micelles were used.

The PA micelles were stable for 1 week at 4°C with no significant change ($P>0.05$) in PA content based on HPLC and size. Also, there was no significant change ($P>0.05$) in *in vitro* cytotoxicity (i.e., the calculated IC50 value) of PA micelles with 4T1 cells at the end of 1 week (Table II).

Selective Targeting of Cancer Cells with PA Micelles

To determine the targetability of PA micelles towards a cancer cell line, we developed a co-culture assay (20). Briefly, the cancer line was grown in culture with the non-cancer cell line (non-target). A preferential association of PA micelles with the cancer cells implies selective targeting. The mouse embryo yolk sac endothelial cell line C166, designed to express green fluorescent protein, was used as a representative non-cancer cell line that was easy to identify in a mixed population by virtue of the GFP fluorescence. As an additional control, NIH3T3 murine fibroblasts cells were also co-cultured with C166-GFP cells. Figure 2a shows the cell population data of mouse mammary carcinoma 4T1 cells co-cultured with C166-GFP cells and treated with fluorescently labeled micelles. The position of two cell populations in the graph is based on the fluorescence intensity in the green channel. The events in region R1 suggest non-green fluorescent 4T1 cell line, and the events in region R2 show the green fluorescent C166-GFP cells. After the treatment with red fluorescent rhodamine-labeled PA micelles, the 4T1 population showed a significantly higher red fluorescence signal. The percent of the population in regions R1 and R2 that shifted into regions R3 and R4, respectively, were calculated in three independent experiments to give the values for the percent of cells targeted. Similar experiments were performed using human renal adenocarcinoma ACHN cells, human breast cancer tumor MCF-7 and BT-20 cells and mouse renal cell carcinoma RENCA and RAG cell lines (Fig. 2b). Under the conditions of the co-culture assay, PA micelles associated preferentially with all cancer cell lines. Also, as an additional control, association of PA micelles was studied with NIH3T3 murine fibroblasts cells. Statistically significant difference ($P<0.05$) in PA micelle uptake was observed when compared between all cell lines. Also, there was significant difference ($P<0.05$) in uptake of PA micelles when compared between all cancer cell lines and NH3T3 fibroblasts. It is worth noting that plain micelles showed less than 10% association with both cancer and non-cancer cell lines (data not shown).

Table II IC50 Values of PA Micelles with Different Cell Lines[a]

Cell lines	IC50 (mM)
BT20	0.52 ± 0.01
MCF-7	0.51 ± 0.01
RAG	0.74 ± 0.32
RENCA	0.37 ± 0.11
ACHN	0.91 ± 0.02
A2780	0.28 ± 0.08
4T1	0.28 ± 0.15
NIH3T3	13.46 ± 2.5
4T1[b]	0.19 ± 0.05

[a] The values are shown as mean±S.D., $n=3$

[b] The IC50 value of the PA micelles after 1 week of storage at 4°C

Selective Killing of Cancer Cells with PA Micelles: Role of ROS

PA micelles exhibited toxicity towards a variety of cancer cell lines (Table II). The significant difference in IC50 value was observed between BT20, MCF-7, RAG, RENCA, A2780, 4T1 (cancer-cell lines) and NIH3T3 as representative non-cancer cell line. The toxicity of the micelles was due to the ascorbate residue, since plain micelles showed no toxicity (data not shown).

The ROS scavengers, catalase and SOD protected against PA micelle-mediated cell death (Fig. 3). Thiol reduction by TCEP also inhibited PA micelle-mediated cell death. Pre-incubation with DFO, a cell-permeant metal chelator, protected the cancer cells exposed to PA micelles. In contrast, the cell-impermeant chelator, EDTA failed to protect against PA-induced toxicity. Note that catalase, SOD, TCEP, DFO and EDTA were not toxic to cells (data not shown).

Suppression of Tumor Growth Rate with PA Micelles

To assess the effectiveness of PA micelles on tumor growth, we administered PA micelles IV (3 alternate doses at

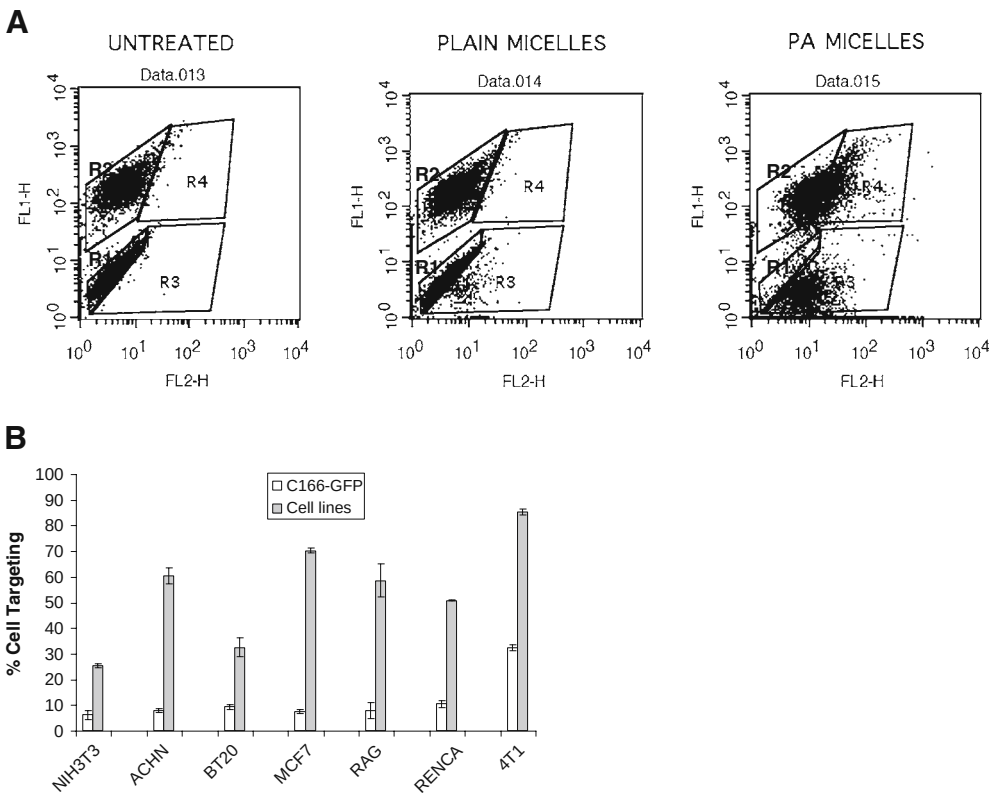

Fig. 2 Representative flow cytometry data from a co-culture targeting assay. Data shown are for 4T1 cells co-cultured with the C166-GFP cells and treated with micellar formulations (**A**). Percent PA micelles targeting of cancer and non-cancer cells in a co-culture assay ($n=3$, ± S.D.) (**B**). Statistically significant when compared for each cell line with C166-GFP cells at $P<0.05$. Similarly, each cancer cell line showed statistically significant uptake compared with NIH3T3 fibroblasts at $P<0.05$.

15 mg/Kg) to 4T1 tumor-bearing mice. The PA micelles significantly reduced the rate of tumor growth in 4T1 tumor-bearing mice (Figure 4).

DISCUSSION

IV administration of ascorbate generates cytotoxic ascorbate concentrations for about an hour, and is generally repeated daily or several times per week (25). Ascorbate, when converted to dehydroascorbic acid (DHAA), is relatively unstable, with a half-life of about 6 min at physiologic pH, but becomes increasingly stable as pH decreases (26). Free ascorbate in blood is converted to DHAA and is scavenged by GLUT receptors on erythrocytes. Plasma levels of ascorbate fall rapidly after IV treatment (4) and so limit the duration of exposure of a tumor to high concentrations of ascorbate. Oral administration does not generate the pharmacological concentrations of ascorbate seen with IV injection (4,27). However, work reported in animal models has consistently shown that high dose ascorbate injection can slow tumor growth (15,28,29).

Our hypothesis of modification of nanocarriers such as liposomes and micelles with hydrophobized derivatives of

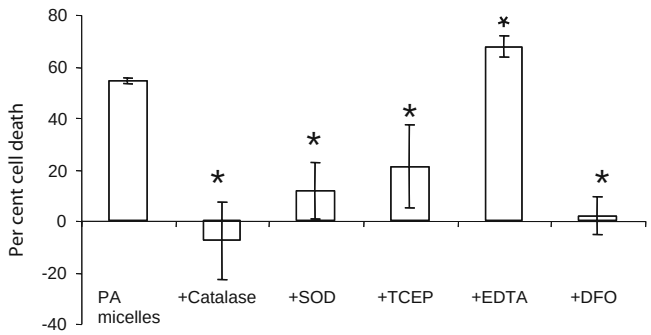

Fig. 3 4T1 cells were preincubated for 30 min with different ROS scavengers, a reducing agent (TCEP) and metal chelators (DFO, EDTA) before exposure to PA micelles (1 mM) at the following concentrations: catalase, 1200U/ml; SOD, 300U/ml; TCEP, 500 μM; EDTA, 1 mM; DFO, 500 μM. After 1 h cells were washed twice with PBS and reincubated in fresh medium for 24 h. Cell viability was assessed by the MTT assay. Statistically significant when compared between PA micelles and PA micelles after treatment at $P<0.05$. ($n=3$, ± S.D.).

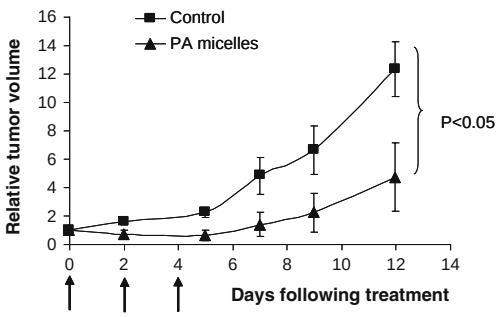

Fig. 4 Effect of IV administration of PA micelles (15 mg/Kg) on tumor growth in 4T1 tumor-bearing mice ($n=5$). Arrows indicate days of treatment.

ascorbate such as PA is ideally suited for *in vivo* applications and will allow for selective accumulation in solid tumors by virtue of the EPR effect (23). Loading PA in nanocarriers is expected to significantly change the pharmacokinetics of ascorbate with respect to the tumor and should extend the duration of exposure of tumor cells to ascorbate effects. In addition, the tumor microenvironmental conditions may act on the ascorbate nanocarriers to enhance their association with the cancer cells. Nanoparticle platforms may be able to overcome delivery limits of free ascorbate as well as to facilitate co-therapy delivery.

We have repeatedly shown that PEG-PE micelles can by efficiently loaded with various poorly soluble anti-cancer drugs such as paclitaxel (24,30), camptothecin (31,32), and porphyrin (33), and can successfully accumulate and deliver loaded drugs to tumors by passive targeting (34) via EPR effect or active targeting when modified with cancer-specific antibody (30). PA, being extremely hydrophobic, is an ideal candidate for loading into micelles; hence, we investigated PEG-PE micellar nanocarriers as a carrier for delivery of poorly soluble PA to tumors.

It was found that PA can be loaded into micelles up to 4 mM with no significant change in size compared to plain micelles. At 6 mM of PA, there was significant increase ($P< 0.05$) in size of micelles; some precipitation of free non-incorporated PA was also observed in micellar solution. Hence, 4 mM PA micelles were chosen to use for further studies. PA micelles were found to be stable at 4°C for 1 week with no significant change in size of micelles. This indicated that PA was still incorporated in micelles and did not precipitate out of the micellar solution. Since PA is prone to oxidative degradation (35), we also anlayzed the stability of micelle- incorporated PA. Each time before HPLC analysis, the micelles were centrifuged at 12, 500 rpm for 5 min to separate free PA (since it is poorly soluble, it will precipitate out if not incorporated in micelles; however, such precipitation was not seen) form micellar solution. The result of the HPLC analysis indicated that there was no significant change in PA content at the end of one week. This also resulted in no change in *in vitro* cytotoxicity of micelles with 4T1 cells at the end of 1 week.

We have previously shown that PA-loaded liposomes can selectively target and kill cancer cells and thus can serve as an alternative to ascorbate-mediated anti-cancer activity. Also, the toxicity is due to ascorbate residue, since liposomes prepared with non-ascorbate palmitoyl analog (palmitoyl glycerol) showed little cytotoxicity in a representative tumor cell line (MCF7) (20). To investigate the selective binding of PA micelles with cancer cells, micelles were labeled with 0.5 mol% of Rh-PE. We have previously shown that Rh-PE can be efficiently incorporated in micelles and can be used to study micelle to cell interaction *in vitro* (24,32,34,36). The data shown here demonstrate that nanocarriers such as phospholipid micelles incorporating PA can specifically bind to cancer cells and deliver substantial anti-cancer cell toxicity. Significant difference in IC50 value was obtained when compared to BT20, MCF-7, RAG, RENCA, A2780, 4T1 (cancer-cell lines) and NIH3T3 (non-cancer cell line). Additionally, PA micelles are effective in reducing the tumor growth rate *in vivo*, providing anti-tumor activity. The greater sensitivity of cancer cells to ascorbate could be due to a higher basal status of intracellular ROS (37–39) and/or low antioxidant status in various cancer cell lines (40–42).

The contribution of ROS in ascorbate-mediated cell death and the role of H_2O_2 scavengers have been confirmed in some studies (5,15). Chen *et al.* reported that ascorbate at pharmacologic concentrations acts as a pro-oxidant, generating H_2O_2-dependent cytotoxicity toward a variety of cancer cells *in vitro* without adverse effects on normal cells (5). H_2O_2 scavengers were completely protective against ascorbate-mediated cell death, and a protective effect of membrane permeant metal chelators has been reported (15). The precise mechanism by which ascorbate generates hydrogen peroxide in the extracellular medium is still unclear (43). Ascorbate does not readily react with oxygen to produce reactive oxygen species, but it readily donates an electron to redox-active transition metal ions. These reduced metals can react with oxygen to produce superoxide ions which, in turn, may dismutate to produce H_2O_2 (44). The *in vitro* evidence in our previous publication indicates that TNF-alpha treatment increases the association of PA liposomes with cancer cells and is prevented by SOD, indicating the role of superoxide in enhancing an association of PA liposomes with cancer cells (20). Our *in vitro* results support the idea that ascorbate induces the production of extracellular ROS, since the ROS scavengers were protective against PA micelle-mediated cytotoxicity. In our *in vitro* cytotoxicity data, the extracellular chelators failed to protect against PA-mediated toxicity. On the other hand, preincubation with DFO, a cell-permeative metal chelator, had a protective effect against PA micelle toxicity. Similar results were obtained by others (15,45). To explain failure of extracellular chelators, Chen *et al.* have suggested the existence of extracellular metalloprotein catalysts present in the serum that could participate in hydrogen peroxide production by ascorbate (5). It was also demonstrated that the generation of hydrogen peroxide by ascorbate *in vivo* is possible only in extracellular fluids because red blood cells exhibit both catalase and glutathione peroxidase activities. Ascorbate toxicity is completely inhibited in the presence of blood, which efficiently detoxifies hydrogen peroxide (5,6).

Free ascorbic acid suppressed tumor growth after daily IP administration in TLT-tumor bearing mice at a dose of 1 g/Kg (15) and at a dose of 4 g/Kg in nude mice bearing either Ovcar5, Pan02, and 9 L tumors (29). We, here, show

that the PA micelles with much lower dose of 15 mg/kg were effective in reducing tumor growth rate in 4T1 tumor-bearing mice and thus represent a viable alternative to free ascorbate for anti-cancer therapy. Thus, PA micelles can be considered as a potential alternative to ascorbic acid for treatment of cancer. Further studies are currently in progress to compare *in vivo* toxicity of PA micelles with free ascorbic acid in tumor-bearing mice.

CONCLUSIONS

The results clearly indicate that PA-loaded micelles represent an effective way to target and kill several types of cancer cells. The mechanism of cell death is primarily due to generation of ROS.

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