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Research paper

Palmitoyl ascorbate-modified liposomes as nanoparticle platform for ascorbate-mediated cytotoxicity and paclitaxel co-delivery

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ABSTRACT

Ascorbate has multiple biological roles and chemical interactions, some of which differ between normal and cancerous tissues. Biological effects of ascorbate depend on concentration, route of exposure, and duration of exposure. High-dose ascorbate acts as a pro-oxidant in tissue fluids and delivers peroxide to tissues and fluids, which is then detoxified by erythrocytes and plasma catalase in normally perfused areas. We have previously shown that nanoparticles incorporating palmitoyl ascorbate (PA) targeted and killed cancer cells *in vitro*. Here, our studies provide additional indications of the importance of extracellular reactive oxygen species (ROS) in the anti-cancer-toxicity by PA-liposomes. Cell death *in vitro* can be blocked by catalase, superoxide dismutase, and the thiol reductant TCEP. Intracellullar iron may also play a role. Iron chelation by desferrioxamine inhibited cell death but EDTA did not. Further, the fluorescent marker of ROS production in cells indicated that the PA-liposomes caused an increase in ROS. Fluorescent microscopy of tumor sections taken at 3 h after injection of rhodamine-labeled liposomes demonstrated an increased accumulation of PA-liposomes compared to plain liposomes. However, the overall biodistribution of ^{111}In-labeled PA-liposomes was similar to plain liposomes. PA-liposomes provided substantial anti-tumor activity *in vivo* and enhanced the anti-cancer activity of liposomally encapsulated paclitaxel. Thus, nanoparticles incorporating PA provide a platform for enhancement of the anti-tumor activity of ascorbate.

1. Introduction

Ascorbate acts in numerous biological pathways fundamental to cellular function. Among its roles, ascorbate regulates hypoxic responses by enhancing the targeted destruction of hypoxia-inducible factor (HIF) subunits [1–3], inhibits cyclic adenosine monophosphate (cAMP) cell signaling by acting as a competitive inhibitor of adenylate cyclase, and down-regulates gene expression essential for cell cycle progression [4]. Pharmacologic concentrations of ascorbate have pro-oxidant properties, acting as a pro-drug that delivers hydrogen peroxide to tissue fluids [5]. Intravenous ascorbate treatment has been shown to slow tumor growth in numerous mouse tumor models [4,6,7]. Although, ascorbate has been studied for its anti-cancer activity for decades, recent pharmacokinetic and biochemical discoveries have propelled a new surge of interest.

Nanoparticles, such as liposomes and micelles, have been shown to enhance the delivery of incorporated drugs to tumors while minimizing delivery to certain critical non-target tissues [8]. They can be engineered to take advantage of the abnormally leaky vasculature typical of many tumors [9] to accumulate in their perivascular regions [10] through a process called the enhanced permeability and retention (EPR) effect [11]. Targeting nanoparticles to the tumor vasculature or tumor cells can dramatically increase cellular delivery of nanoparticles, even in the absence of increased accumulation of nanoparticles in the tumor as a whole [12,13]. Such nanoparticles are often designed to lengthen the time of exposure of tumors to therapeutic concentrations of anti-cancer agents, especially those with limited solubility or a short half-life in the circulation.

Recent pharmacokinetic studies have shown that i.v. ascorbate can generate plasma ascorbate concentrations of 5–20 mM that are not possible with oral supplementation [14,15]. High-dose oral supplementation generates peak plasma levels of ~200 μM [14], whereas normal dietary supplementation yields plasma ascorbate concentrations of ~20–90 μM [15]. The concentrations of ascorbate obtainable by i.v. treatment in humans [14] are capable of producing hydrogen peroxide *in vitro* [16] and in tissue fluids *in vivo* [5]. The high concentrations of ascorbate measured following i.v. ascorbate treatment in human patients [14,17] and rodents [5,7] are preferentially toxic to cancer cells compared to normal cells [7,16]. Reactive oxygen species (ROS) and intracellular metals are critical to the production of ascorbate-mediated anti-cancer

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toxicity since intracellular metal ion chelation and extracellular anti-oxidant treatment can prevent cell death [5,7].

Pharmacological concentrations of ascorbate have been shown to enhance the effects of multiple anti-cancer agents *in vitro* including paclitaxel (PCT), etoposide, 5-FU, cisplatin, doxorubicin, vincristine [7,18,19] but reduces anti-cancer properties of bortezomib (Velcade®) [20]. Ascorbate decreases non-specific toxicity of doxorubicin *in vivo* [21,22]. Treatment with dehydroascorbic acid (DHAA), the oxidized form of ascorbate, does not have these beneficial anti-cancer properties [23,24]. Yet another potential useful effect of high-dose ascorbate treatment is the depletion of glutathione [7,25] and ATP from cancer cells [7]. Ascorbate-mediated glutathione depletion has many potential uses in anti-cancer co-therapy regimens, and is being studied for the use with motexafin gadolinium and arsenic trioxide [25,26].

Clinical anti-cancer research on ascorbate had until recently been hindered by an incomplete understanding of ascorbate pharmacokinetics. Certain historically important clinical studies used intravenous plus oral supplements [27,28], while others used oral supplements alone [29,30]. Currently, clinical trials are reexamining intravenous ascorbate as an anti-cancer agent and as a co-therapy. A recent dose-finding phase I and pharmacokinetic study reaffirmed the safety of i.v. ascorbate, but concluded that the best uses of ascorbate might be in co-therapy regimens, since no objective responses were seen among the small group of patients in the study, even among the few receiving the higher doses of ascorbate [17]. The study hinted, but did not prove, that ascorbate at higher doses may be associated with slowed tumor growth and delayed mortality [17].

We have previously shown that the hydrophobized derivatives of ascorbate such as palmitoyl ascorbate (PA) can be stably loaded into liposomes. Moreover, these PA-modified liposomes can target and kill a variety of cancer cells *in vitro* [31]. Tumors exhibit abnormal local oxidation of extracellular ascorbate [32]. The oxidized ascorbic acid, DHAA, is transported into tumor cells by glucose transporter (GLUT) type-1 (GLUT-1) and type-3 (GLUT-3) [33,34]. However, DHAA has a half-life of only about 6 min at the physiologic pH [35]. Therefore, as the PA-liposomes circulate in the blood, it is possible that the small numbers of DHAA moieties that are generated from the ascorbate moieties decay before interacting with GLUT transporters. However, once the PA-liposome is within the solid tumor microenvironment, the abnormal abundance of superoxide anion should cause increased conversion of AA to DHAA and facilitate the interaction of the liposome with the GLUT transporters on the tumor cells.

Thus, the tumor-specific abnormal oxidation of ascorbate to dehydroascorbate in the tumor microenvironment followed by DHAA association with GLUT transporters provides a mechanism to enhance preferential association of ascorbate-decorated nanoparticles to cancer cells within the tumor microenvironment. Our aim here was to explore the mechanism of action of PA-liposomes as well as the capacity of PA-liposomes to target and treat tumors. These tumor studies were carried out using the subcutaneous breast cancer model with 4T1 tumor cell inoculation into immune-competent mice. The results indicate that PA added to a liposome formulation provides significant anti-tumor activity and increases the anti-tumor activity of encapsulated PCT.

2. Materials and methods

2.1. Materials

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Palmitoyl ascorbate (PA), paclitaxel (PCT), ethylenediaminetetraacetic acid (EDTA), desferrioxamine mesylate (DFO) and catalase were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) were from Invitrogen (Eugene, OR, USA). 4T1 (murine mammary carcinoma) and RAG (murine renal adenocarcinoma) 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).

2.2. Liposome preparation

PA (30 mol%) was incorporated in egg phosphatidylcholine/cholesterol (70:30) liposomes by the rehydration of lipid films prepared from requisite quantities of lipids [31]. Briefly, egg phosphatidylcholine, cholesterol and PA were aliquoted from stock solutions, and a lipid film was formed in a round bottom flask by solvent removal on a rotary evaporator. Where applicable, to facilitate liposome detection, PA-liposomes were fluorescently labeled by the incorporation of 0.5 mol% rhodamine-PE. The lipid film was then rehydrated with phosphate buffered saline, pH 7.4 sufficient to give a 10 mg/mL lipid final concentration. The preparation was bath-sonicated for 30 min followed by extrusion through 200 nm polycarbonate membranes. Where appropriate, liposomes were prepared with the addition of PCT to the lipid mixture followed by liposome preparation as described.

For biodistribution studies, liposomes labeled with ^{111}In were prepared by the addition of 0.5 mol% 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-diethylenetriaminepentaacetic acid (DTPA-PE) and 5 mol% of PEG_{2000}-PE. The liposome-DTPA-PE was supplemented with 1 M citrate buffer and incubated for 1 h with the ^{111}In-citrate complex 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.

Liposomal size (hydrodynamic diameter) was measured by the dynamic light scattering (DLS) using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL, USA). Amount of PA released from liposomes was studied at room temperature by dialysis method against 4L of 5 mM HBS, pH 7.4 using cellulose ester dialysis bag of MWCO 2000. After 24 h, amount of PA in liposomes was estimated by HPLC method as described below.

2.3. PA-liposome stability

PA-liposomes were stored at 4 °C in sealed vials purged with argon and monitored for PA content (as determined by HPLC) and size distribution by DLS. The amount of PA in liposomes was estimated by the reversed phase-HPLC. Liposomes were diluted with the mobile phase prior to 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 column was eluted with a 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.

2.4. Cell cultures

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

2.5. Cellular ROS detection

CM-H2DCFDA dye was used to detect ROS production. RAG cells were grown in Lab-Tek chamber plates and incubated with 25 μM of dye solution in PBS, pH 7.4 for 30 min. The cells were then incubated for 1 h with 200 μL of liposomes and washed three times with PBS. Cells were observed on a Nikon Eclipse 400 fluorescence microscope equipped with appropriate filters and a digital image capture device.

2.6. Cell death studies

4T1 cells were grown in 96-well plates (Corning, Inc., NY, USA) until 60–70% confluent. The cells were preincubated for 30 min with various ROS scavengers, a reducing agent and metal chelators before exposure to PA-liposomes (2.5 mM) 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 PBS and incubated for 24 h with complete medium. After 24 h, they were analyzed for viability using CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA).

To study the effect PEGylation on PA-liposomes induced cytotoxicity, 4T1 cells were incubated with different concentrations of PA-liposomes. After 1 h, cells were washed twice with PBS and incubated 24 h with completed medium. The cell viability was determined as described earlier.

2.7. Tumor localization studies

Approximately 10^5 4T1 cells were inoculated subcutaneously into the left flank of 6–8 weeks old female Balb/C mice. Twenty days after tumor inoculation, the mice were injected via tail vein with 0.2 mL of rhodamine-labeled liposome dispersions (3 mice per group). Tumors were harvested after 3 h, embedded in tissue freezing media and stored at −80 °C. Cryostat sections were made and examined with a Nikon Eclipse 400 fluorescence microscope equipped with appropriate filters and a digital image capture device. All animal studies were approved by the Ethics Committee of the Northeastern University.

2.8. Biodistribution studies

Twenty days after tumor inoculation in 9–12 week old female Balb/C, mice were injected via tail vein with 5 μCi of the radiolabeled liposome dispersions (5 mice per group). Mice were sacrificed at specific time points and selected organs were removed, weighed and the radioactivity quantified as mean CPM ± standard deviation using a Beckman 5500B gamma-counter.

2.9. Tumor growth reduction studies

Nine- to 12-week-old female Balb/C mice bearing 20-day-old tumors were injected via tail vein with different liposomal formulations. Normal saline was used for a control group. The tumor diameters were measured on alternate days with a vernier caliper in two dimensions. Individual tumor volumes (V) were calculated using the formula: $V = (L \times W^2)/2$, wherein length (L) is the longest diameter and width (W) is the shortest diameter perpendicular to length.

3. Results

3.1. Stable incorporation of PA into liposomes

PA was stably incorporated in liposomes at 30 mol%. Liposome size was 140.2 ± 15.0 and 146.6 ± 29.0 nm for plain and PA-liposomes, respectively. Loading with PCT or incorporation of 5 mol% PEG did not change size of PA-liposomes significantly ($P > 0.05$). PA-liposomes were stable for 1 week at 4 °C with no significant change ($P > 0.05$) in both PA content and size. The results of the *in vitro* dialysis study indicated that about 75 ± 2.91% of PA was associated with liposomes after 24 h.

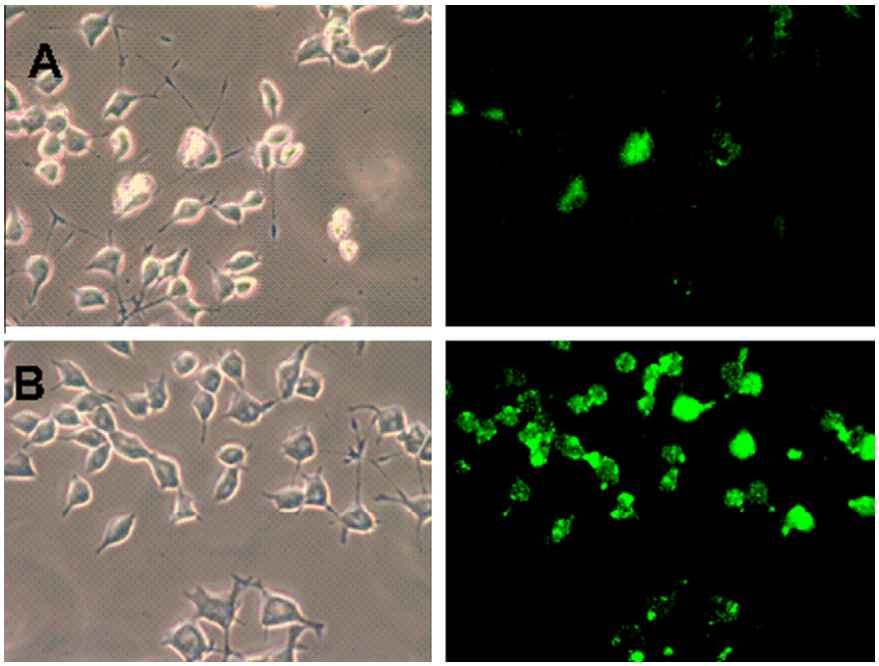

Fig. 1. Detection of ROS with CM-H2DCFDA by fluorescence microscopy in cancer cells. Left figure shows the bright field and right figure shows the fluorescent field of RAG cells treated with plain liposomes (A) and PA-liposomes (B). Magnification 40× objective. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. PA-liposomes allowed for enhanced production of ROS

Fluorescent microscopy of RAG cells *in vitro* using the fluorescent indicator of ROS, CM-H2DCFDA showed an enhanced production of ROS in cultured RAG cancer cells treated with PA-liposomes relative to cells treated with control plain liposomes (Fig. 1).

3.3. PA-liposomal killing of cancer cells unaffected by PEGylation and required extracellular ROS generation and intracellular metal ions

Modification of PA-liposomes with 5 mol% of PEG-PE did not result in any significant change in *in vitro* cytotoxicity at all concentrations tested (Fig. 2A).

Cell killing by PA-liposomes was prevented by catalase, SOD, TCEP, DFO, but not by EDTA (Fig. 2B). These results indicate that anti-cancer activity of PA-liposomes is inhibited by the absence of the superoxide or if the peroxide is detoxified by catalase. Thiol reduction by TCEP also inhibited PA-liposome-mediated cell death. In addition, cell death was prevented by the iron chelator DFO that is taken up by endocytosis, but not by EDTA, which acts extracellularly. This result indicates that extracellular peroxide and intracellular metal ions mediate the PA-liposome's toxicity. Reduction of anti-cancer toxicity of PA-liposomes by inhibition of ROS and chelation of metal ions indicates a mechanism of action that resembles those described for free (non-nanoparticle) ascorbate [7,16,36] and for palmitoyl ascorbate [36,37]. The mechanism through which extracellular ascorbate and intracellular iron exert this anti-cancer toxicity is unknown and is currently under investigation (see Fig. 3).

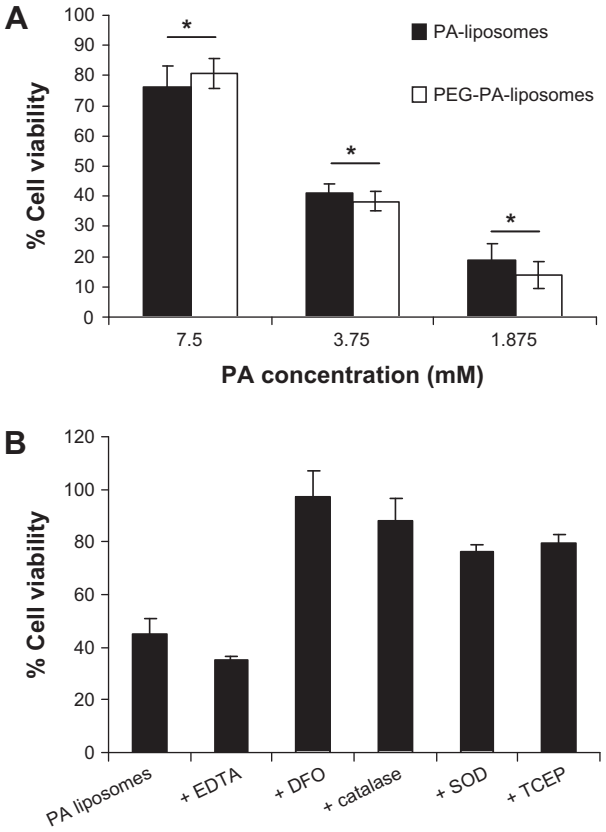

Fig. 2. *In vitro* cytotoxicity of PA-liposomes. Effect of PEGylation on PA-induced cytotoxicity ($^{*}P > 0.05$, $n = 3$, mean ± SD) (A), effect of combination of PA-liposome with ROS scavengers (catalase, SOD), a reducing agent (TCEP) and metal chelators (DFO, EDTA) on viability of 4T 1 breast cancer cells. $n = 3$, mean ± SD.

3.4. Enhanced tumor deposition of PA-liposomes

Tumor sections from mice, 3 h post injection, showed substantially increased accumulation of rhodamine-labeled PA-liposomes compared to plain liposomes. Previous *in vitro* research indicated that PA-liposomes preferentially associated with cancer cells compared to non-cancer cells in co-culture. Superoxide enhanced this association of PA-liposomes with cancer cells. TNF-alpha added to RAG cells in culture increased PA-liposome association and was inhibited by superoxide dismutase [31].

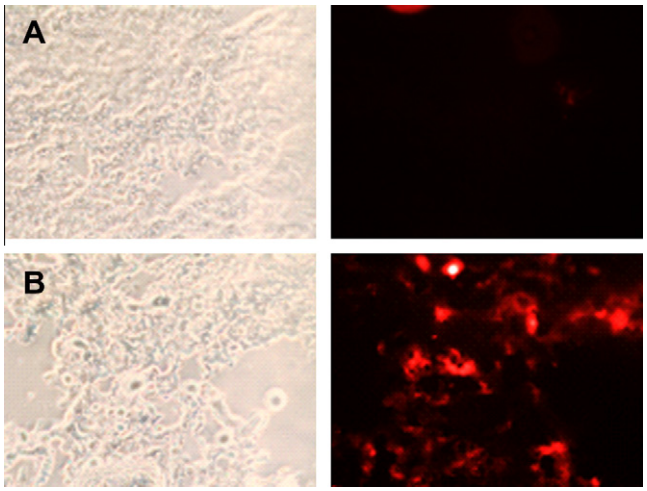

Fig. 3. Tumor localization of rhodamine-labeled liposomes by microscopy. Left figure shows the bright field and right figure shows the fluorescent microscopy of tumor sections from mice treated with plain liposomes (A) and PA-liposomes (B). Magnification 20× objective. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

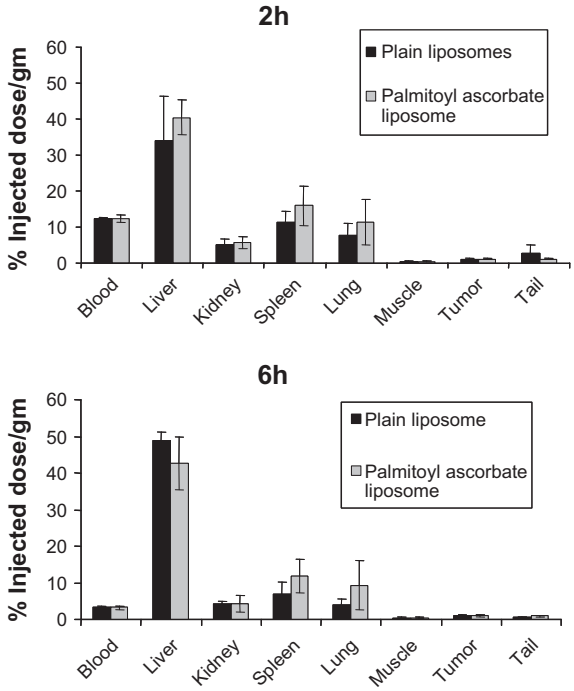

Fig. 4. Biodistribution of liposomes in 4T 1 tumor-bearing mice at 2 h and 6 h. ($n = 5$).

3.5. PA-liposome biodistribution

Accumulation of ^{111}In-labeled liposomes in tumor tissues was similar for control (plain liposomes) and PA-liposomes, which were deposited predominantly in the liver, spleen, and lungs (Fig. 4). Also there was no significant difference ($P > 0.05$) between accumulation of plain and PA-liposomes in any tissues.

3.6. Anti-cancer activity of PA-liposomes in vivo

PA-liposomes significantly limited tumor growth and enhanced the effect of PCT-encapsulated liposomes. At 20 mg/kg PA, remarkable tumor growth suppression was evident (Fig. 5). At 10 mg/kg PA, the effectiveness of combined PA and PCT also becomes evident (Fig. 6).

4. Discussion

Ascorbate has several anti-cancer properties, which could be of enormous benefit for cancer treatment. However, therapeutic uses of ascorbate are limited by rapid blood clearance [17]. Intravenous administration of ascorbate generates cytotoxic ascorbate concentrations for about an hour, and is generally repeated daily or several times per week [17]. Oral administration of ascorbate cannot generate the higher pharmacological concentrations seen with i.v. injection [14,15]. Research in animal models has consistently shown that high-dose injected ascorbate can slow tumor growth [4,6,7] and is consistent with recent preliminary findings in human patients [17]. The results shown here demonstrate that nanoparticle delivery of ascorbate, such as with liposomes, incorporating PA can deliver substantial anti-cancer toxicity. Nanoparticle platforms may be also able as well to overcome the delivery limits of free ascorbate and facilitate co-therapeutic delivery of anti-cancer drugs.

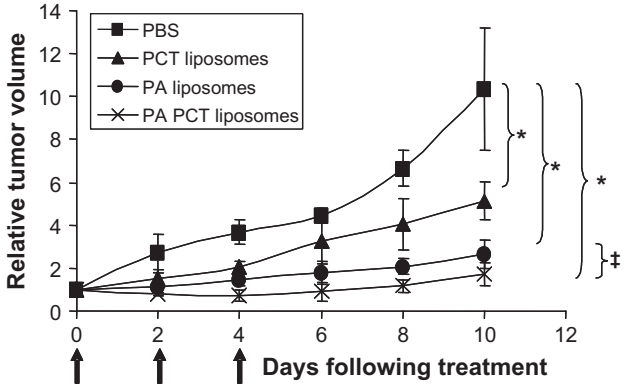

Fig. 5. Effect of i.v. administration of liposomal preparations on tumor growth in 4T1 tumor-bearing mice ($n = 5$, mean ± SD). Arrows indicate days of treatment. Doses: PA-liposomes (20 mg/kg of PA), PCT liposomes (3.4 mg/kg of PCT), PCT-loaded PA-liposomes (20 mg/kg of PA and 3.4 mg/kg of PCT). $^{*}P < 0.05$, $^{‡}P > 0.05$.

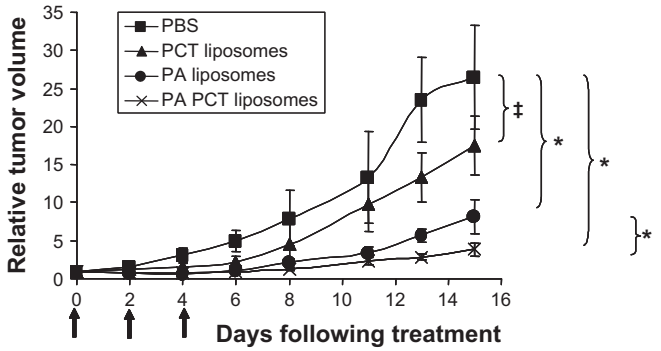

Fig. 6. Effect of i.v. administration of liposomal preparations on tumor growth in 4T 1 tumor-bearing mice ($n = 5$, mean ± SD). Arrows indicate days of treatment. Doses: PA-liposomes (10 mg/kg of PA), PCT liposomes (2 mg/kg of PCT), PCT-loaded PA-liposomes (10 mg/kg of PA and 2 mg/kg of PCT). $^{‡}P > 0.05$, $^{*}P < 0.05$.

These PA-modified nanoparticles were effective for killing tumor cells *in vivo* and provided significant anti-tumor activity. The mechanism of action of PA-liposomes is similar to that of free ascorbate. Inhibition of extracellular ROS or intracellular metal ions inhibited cell killing. In our *in vitro* cytotoxicity studies, the extracellular chelators failed to protect against PA-mediated toxicity. To explain the 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 [16]. 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, which efficiently detoxify hydrogen peroxide, ascorbate toxicity is completely inhibited in the presence of blood [5,16].

Fluorescent microscopy using ROS-sensitive markers confirmed the generation of ROS after treatment of cells with PA-liposomes. Our previously published work has shown preferential targeting and killing of a variety of cancer cells *in vitro* [31] and *in vitro* evidence indicated that TNF-alpha treatment increased the association of PA-liposomes with cancer cells. This increase in association was prevented by SOD, indicating a role of superoxide in the enhanced association of PA-liposomes with cancer cells. The precise mechanism by which ascorbate generates hydrogen peroxide in the extracellular medium is still unclear [38]. 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 [39].

Fluorescent microscopy of tumor sections taken at 3 h after injection of rhodamine-labeled liposomes clearly show an increased accumulation of PA-liposomes compared to plain liposomes. The overall tumor accumulation of these liposomes was relatively low, preventing meaningful quantitation of tumor accumulation from biodistribution experiments. Formulation changes may decrease non-specific uptake and clearance.

However, the anti-cancer activity of PA-liposomes could be substantial and greater than that of PCT liposomes without PA (Figs. 5 and 6). A co-treatment effect was clearly observed at a reduced dose of PA-liposomes (10 mg/kg). The potent anti-cancer effect of 20 mg/kg PA-liposomes concealed possible additive effect. PA-liposomes are quite toxic on their own, and at a larger dose, the effect of PA-liposomes does not leave any room for the additive effect.

5. Conclusions

PA-modified nanoparticles can provide a platform that enhances the anti-tumor properties of ascorbate. Particularly, PA-liposomes loaded with PCT can be more effective than plain PCT-loaded liposomes.

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References

[1] H.J. Knowles, R.R. Raval, A.L. Harris, P.J. Ratcliffe, Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells, Cancer Res. 63 (2003) 1764–1768.

[2] H. Lu, C.L. Dalgard, A. Mohyeldin, T. McFate, A.S. Tait, A. Verma, Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1, J. Biol. Chem. 280 (2005) 41928–41939.

[3] Y. Pan, K.D. Mansfield, C.C. Bertozzi, V. Rudenko, D.A. Chan, A.J. Giaccia, M.C. Simon, Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro, Mol. Cell. Biol. 27 (2007) 912–925.

[4] S. Belin, F. Kaya, G. Duisit, S. Giacometti, J. Ciccolini, M. Fontes, Antiproliferative effect of ascorbic acid is associated with the inhibition of genes necessary to cell cycle progression, PLoS One 4 (2009) e4409.

[5] Q. Chen, M.G. Espey, A.Y. Sun, J.H. Lee, M.C. Krishna, E. Shacter, P.L. Choyke, C. Pooput, K.L. Kirk, G.R. Buettner, M. Levine, Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo, Proc. Natl. Acad. Sci. USA 104 (2007) 8749–8754.

[6] Q. Chen, M.G. Espey, A.Y. Sun, C. Pooput, K.L. Kirk, M.C. Krishna, D.B. Khosh, J. Drisko, M. Levine, Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice, Proc. Natl. Acad. Sci. USA 105 (2008) 11105–11109.

[7] J. Verrax, P.B. Calderon, Pharmacologic concentrations of ascorbate are achieved by parenteral administration and exhibit antitumoral effects, Free Radic. Biol. Med. 47 (2009) 32–40.

[8] V.P. Torchilin, Targeted pharmaceutical nanocarriers for cancer therapy and imaging, AAPS J. 9 (2007) E128–E147.

[9] H. Hashizume, P. Baluk, S. Morikawa, J.W. McLean, G. Thurston, S. Roberge, R.K. Jain, D.M. McDonald, Openings between defective endothelial cells explain tumor vessel leakiness, Am. J. Pathol. 156 (2000) 1363–1380.

[10] F. Yuan, M. Leunig, S.K. Huang, D.A. Berk, D. Papahadjopoulos, R.K. Jain, Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft, Cancer Res. 54 (1994) 3352–3356.

[11] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, K. Hori, Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review, J. Control. Release 65 (2000) 271–284.

[12] D.W. Bartlett, H. Su, I.J. Hildebrandt, W.A. Weber, M.E. Davis, Impact of tumor-specific targeting on the biodistribution and efficacy of siRNA nanoparticles measured by multimodality in vivo imaging, Proc. Natl. Acad. Sci. USA 104 (2007) 15549–15554.

[13] D.B. Kirpotin, D.C. Drummond, Y. Shao, M.R. Shalaby, K. Hong, U.B. Nielsen, J.D. Marks, C.C. Benz, J.W. Park, Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models, Cancer Res. 66 (2006) 6732–6740.

[14] S.J. Padayatty, H. Sun, Y. Wang, H.D. Riordan, S.M. Hewitt, A. Katz, R.A. Wesley, M. Levine, Vitamin C pharmacokinetics: implications for oral and intravenous use, Ann. Intern. Med. 140 (2004) 533–537.

[15] M. Levine, C. Conry-Cantilena, Y. Wang, R.W. Welch, P.W. Washko, K.R. Dhariwal, J.B. Park, A. Lazarev, J.F. Graumlich, J. King, L.R. Cantilena, Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance, Proc. Natl. Acad. Sci. USA 93 (1996) 3704–3709.

[16] Q. Chen, M.G. Espey, M.C. Krishna, J.B. Mitchell, C.P. Corpe, G.R. Buettner, E. Shacter, M. Levine, Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues, Proc. Natl. Acad. Sci. USA 102 (2005) 13604–13609.

[17] L.J. Hoffer, M. Levine, S. Assouline, D. Melnychuk, S.J. Padayatty, K. Rosadiuk, C. Rousseau, L. Robitaille, W.H. Miller Jr., Phase I clinical trial of i.v. ascorbic acid in advanced malignancy, Ann. Oncol. 19 (2008) 1969–1974.

[18] C.M. Kurbacher, U. Wagner, B. Kolster, P.E. Andreotti, D. Krebs, H.W. Bruckner, Ascorbic acid (vitamin C) improves the antineoplastic activity of doxorubicin, cisplatin, and paclitaxel in human breast carcinoma cells in vitro, Cancer Lett. 103 (1996) 183–189.

[19] C.D. Chiang, E.J. Song, V.C. Yang, C.C. Chao, Ascorbic acid increases drug accumulation and reverses vincristine resistance of human non-small-cell lung-cancer cells, Biochem. J. 301 (Pt 3) (1994) 759–764.

[20] L. Catley, K.C. Anderson, Velcade and vitamin C: too much of a good thing?, Clin Cancer Res. 12 (2006) 3–4.

[21] K. Fujita, K. Shinpo, K. Yamada, T. Sato, H. Niimi, M. Shamoto, T. Nagatsu, T. Takeuchi, H. Umezawa, Reduction of adriamycin toxicity by ascorbate in mice and guinea pigs, Cancer Res. 42 (1982) 309–316.

[22] K. Shimpo, T. Nagatsu, K. Yamada, T. Sato, H. Niimi, M. Shamoto, T. Takeuchi, H. Umezawa, K. Fujita, Ascorbic acid and adriamycin toxicity, Am. J. Clin. Nutr. 54 (1991) 1298S–1301S.

[23] M.L. Heaney, J.R. Gardner, N. Karasavvas, D.W. Golde, D.A. Scheinberg, E.A. Smith, O.A. O'Connor, Vitamin C antagonizes the cytotoxic effects of antineoplastic drugs, Cancer Res. 68 (2008) 8031–8038.

[24] G.G. Meadows, H.F. Pierson, R.M. Abdallah, Ascorbate in the treatment of experimental transplanted melanoma, Am. J. Clin. Nutr. 54 (1991) 1284S–1291S.

[25] J. Dai, R.S. Weinberg, S. Waxman, Y. Jing, Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system, Blood 93 (1999) 268–277.

[26] A.M. Evens, P. Lecane, D. Magda, S. Prachand, S. Singhal, J. Nelson, R.A. Miller, R.B. Gartenhaus, L.I. Gordon, Motexafin gadolinium generates reactive oxygen species and induces apoptosis in sensitive and highly resistant multiple myeloma cells, Blood 105 (2005) 1265–1273.

[27] E. Cameron, L. Pauling, Supplemental ascorbate in the supportive treatment of cancer: prolongation of survival times in terminal human cancer, Proc. Natl. Acad. Sci. USA 73 (1976) 3685–3689.

[28] E. Cameron, L. Pauling, Supplemental ascorbate in the supportive treatment of cancer: reevaluation of prolongation of survival times in terminal human cancer, Proc. Natl. Acad. Sci. USA 75 (1978) 4538–4542.

[29] E.T. Creagan, C.G. Moertel, J.R. O'Fallon, A.J. Schutt, M.J. O'Connell, J. Rubin, S. Frytak, Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. A controlled trial, N. Engl. J. Med. 301 (1979) 687–690.

[30] C.G. Moertel, T.R. Fleming, E.T. Creagan, J. Rubin, M.J. O'Connell, M.M. Ames, High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy. A randomized double-blind comparison, N. Engl. J. Med. 312 (1985) 137–141.

[31] G.G. D'Souza, T. Wang, K. Rockwell, V.P. Torchilin, Surface modification of pharmaceutical nanocarriers with ascorbate residues improves their tumor-cell association and killing and the cytotoxic action of encapsulated paclitaxel in vitro, Pharm. Res. 25 (2008) 2567–2572.

[32] D.B. Agus, J.C. Vera, D.W. Golde, Stromal cell oxidation: a mechanism by which tumors obtain vitamin C, Cancer Res. 59 (1999) 4555–4558.

[33] D.B. Agus, S.S. Gambhir, W.M. Pardridge, C. Spielholz, J. Baselga, J.C. Vera, D.W. Golde, Vitamin C crosses the blood–brain barrier in the oxidized form through the glucose transporters, J. Clin. Invest. 100 (1997) 2842–2848.

[34] S.C. Rumsey, O. Kwon, G.W. Xu, C.F. Burant, I. Simpson, M. Levine, Glucose transporter isoforms GLUT 1 and GLUT 3 transport dehydroascorbic acid, J. Biol. Chem. 272 (1997) 18982–18989.

[35] R.C. Rose, A.M. Bode, Biology of free radical scavengers: an evaluation of ascorbate, Faseb J. 7 (1993) 1135–1142.

[36] G. Rosenblat, M.F. Graham, A. Jonas, M. Tarshis, S.Y. Schubert, M. Tabak, I. Neeman, Effect of ascorbic acid and its hydrophobic derivative palmitoyl ascorbate on the redox state of primary human fibroblasts, J. Med. Food 4 (2001) 107–115.

[37] N. Miwa, H. Yamazaki, Y. Nagaoka, K. Kageyama, Y. Onoyama, I. Matsui-Yuasa, S. Otani, S. Morisawa, Altered production of the active oxygen species is involved in enhanced cytotoxic action of acylated derivatives of ascorbate to tumor cells, Biochim. Biophys. Acta 972 (1988) 144–151.

[38] A. Carr, B. Frei, Does vitamin C act as a pro-oxidant under physiological conditions?, Faseb J 13 (1999) 1007–1024.

[39] B. Frei, S. Lawson, Vitamin C and cancer revisited, Proc. Natl. Acad. Sci. USA 105 (2008) 11037–11038.