Pharmaceutical Research, Vol. 25, No. 11, November 2008 (© 2008)
DOI: 10.1007/s11095-008-9674-4

Research Paper

Surface Modification of Pharmaceutical Nanocarriers with Ascorbate Residues Improves their Tumor-Cell Association and Killing and the Cytotoxic Action of Encapsulated Paclitaxel *In Vitro*

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Received May 20, 2008; accepted June 19, 2008; published online July 11, 2008

Purpose. To evaluate the potential of ascorbate as a novel ligand in the preparation of pharmaceutical nanocarriers with enhanced tumor-cell specific binding and cytotoxicity.
Methods. Palmitoyl ascorbate was incorporated into liposomes at varying concentrations. A stable formulation was selected based on size and zeta potential measurements. A co-culture of cancer cells with GFP expressing non-cancer cells was used to determine the specificity of palmitoyl ascorbate liposome binding. Liposomes were fluorescently labeled to facilitate analysis by flow cytometry and fluorescence microscopy. The cytotoxic action of palmitoyl ascorbate liposomes against a variety of cell types was assayed using a standard metabolic assay. The cytotoxic effect of a low dose of paclitaxel incorporated in palmitoyl ascorbate liposomes on various cell lines was also determined.
Results. Palmitoyl ascorbate liposomes associated preferentially with various cancer cells compared to non-cancer cells in a co-culture model. Palmitoyl ascorbate liposomes exhibited anti-cancer toxicity in numerous cancer cell lines. Furthermore, ascorbate liposomes enhanced the effectiveness of encapsulated paclitaxel compared to paclitaxel encapsulated in 'plain' liposomes.
Conclusions. Surface modification of liposomes with ascorbate residues represents a novel way to target and kill certain types of tumor cells and additionally can potentiate the effect of paclitaxel delivered by the liposomes.

KEY WORDS: ascorbate; cancer; liposomes; nanocarriers; targeting.

INTRODUCTION

While the importance of ascorbate as a dietary supplement has been recognized for many years, there has recently been renewed interest in the physiology of ascorbate and in the role of ascorbate in the prevention and treatment of cancer. Harnessing the anti-cancer properties of ascorbate has not been simple. The activity of ascorbate depends on multiple parameters including the delivery route, concentration, and timing of cell exposure. Recent pharmacokinetic studies have shown that the route of delivery of ascorbate is critical. Intravenous ascorbate delivery provides far higher serum concentrations of ascorbate than oral delivery (1). This pharmacokinetic reality was not recognized when the early trials of ascorbate in cancer therapy were performed and led to critical differences in methodology between studies. Some studies used IV plus oral ascorbate (2), whereas others used oral ascorbate alone (3, 4).

The high concentrations of ascorbate attainable following intravenous ascorbate treatment produce hydrogen peroxide in extracellular fluids *in vitro* (5) and *in vivo* (6). Recently, high dose ascorbate has been shown to have marked cytotoxicity towards several types of tumor cells *in vitro* (5). Intravenous delivery of high dose ascorbate is believed to kill cancer cells through the extracellular generation of significant hydrogen peroxide in the interstitial fluid of tissues, distant from the erythrocytes that scavenge it (5). 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 abnormal accumulation of ascorbate by tumor cells (7) since glucose transporters GLUT-1, GLUT-3, and GLUT-4 can transport DHAA, but not ascorbate (8–10).

In addition to direct anti-cancer toxicity, ascorbate has been shown to increase the effectiveness of various anti-cancer treatments *in vitro*. Ascorbate improved the effectiveness of doxorubicin, cisplatin, and paclitaxel in breast cancer cells (11). Ascorbate has been shown to increase the effectiveness of vincristine on non-small cell lung cancer cells, reversing their vincristine resistance (12). The effectiveness of motexafin gadolinium can be enhanced by the glutathione depletion caused by ascorbate (13). Ascorbate has also been studied in clinical trials for the enhancement of the effectiveness of arsenic trioxide in patients with multiple myeloma (14). Although such studies have helped confirm the enor-

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mous potential for ascorbate in the treatment of cancer, the *in vivo* application of ascorbate is currently limited by the need for very high plasma concentrations to achieve therapeutic levels in the tumor.

The need for high doses of ascorbate for anti-cancer toxicity is due largely to the strict physiologic regulation of ascorbate combined with the limited chemical stability of native ascorbate. As a result, several hydrophobized derivatives of ascorbate have been explored as stable alternatives (15, 16). Such hydrophobized derivatives retain the antioxidant effects of ascorbate (15) and are more stable in biological milieu. Moreover, hydrophobized derivatives of ascorbate offer the potential for the formulation of ascorbate-bearing pharmaceutical nanocarriers (16, 17). Pharmaceutical nanocarriers are known to accumulate in the tumor by the enhanced permeability and retention effect (18–20) and it is therefore reasonable to assume that incorporation of ascorbate into such nanocarriers could serve to improve the delivery of ascorbate to the tumor thereby reducing the need for high doses of ascorbate.

The abnormal local oxidation of ascorbate in tumors also presents a potential targeting mechanism for nanocarriers such as liposomes, micelles, and polymersomes. Ascorbate-mediated targeting of nanocarriers might be particularly effective in targeting cancers like renal cell carcinoma that have activated the hypoxia induced pathways regulated by the HIF transcription factors. Activation of hypoxic response pathways in cancer cells leads to upregulated production of reactive oxygen species (ROS) and of GLUT-1 and GLUT-3. Mutation or silencing by methylation of the VHL gene is common in renal cell carcinomas. This silencing of the VHL gene allows the activation of the HIF-1and HIF-2 transcription factors, which in turn induce a set of genes that includes angiogenesis promoting factors, growth factors, glucose transporters 1 and 3 as well as environmental regulating proteins (21). HIF activation, including activation caused by loss of VHL, also leads to increased production of ROS. This occurs through an increased production of NAD(P)H oxidases, which in turn allow continued HIF-2α expression and thus HIF-2 activity (22). The surface modification of pharmaceutical nanocarriers with ascorbate might therefore serve to improve the binding and retention of the nanocarriers in renal tumors and other cancers characterized by HIF activation.

In this study we sought to test the hypothesis that pharmaceutical nanocarriers bearing ascorbate residues on the surface will show enhanced tumor cell specific association and toxicity. To this end, we used liposomes as they are pharmaceutical nanocarriers ideally suited to surface modification with various ligands. To facilitate the incorporation of ascorbate residues on the surface of liposomes, we used palmitoyl ascorbate, a commercially available hydrophobized ascorbate derivative. We determined the tumor cell binding and cytotoxicity and of palmitoyl ascorbate liposomes in a variety of tumor cell lines and studied the use of palmitoyl ascorbate liposomes as carriers for the anticancer drug paclitaxel.

MATERIALS AND METHODS

Palmitoyl ascorbate was purchased from Sigma Aldrich, Milwaukee, MI. All lipids were purchased from Avanti Polar Lipids, Alabaster, AL. A2780 cells were obtained from Rutgers University, NJ and all other cell lines were obtained from American Type Culture Collection (ATCC), Manassas, VA.

Liposome Preparation and Characterization

Palmitoyl ascorbate at various molar ratios (see Table I) was incorporated in egg phosphatidylcholine/cholesterol (70:30) liposomes by the rehydration of lipid films prepared from requisite quantities of lipids. Briefly, egg phosphatidylcholine, cholesterol and palmitoyl ascorbate (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, palmitoyl ascorbate liposomes were fluorescently labeled by the incorporation of 0.5% rhodamine-PE. The lipid film was then rehydrated with PBS sufficient to give 10 mg/ml lipid final concentration. The preparation was bath-sonicated for 30 min followed by extrusion through 100 nm polycarbonate membrane to give the final product. Where appropriate, liposomes were prepared as above but with the addition of paclitaxel (0.4 mM final concentration) to the lipid mixture followed by liposome preparation as described. Size and zeta-potential measurements were made on a Beckman Coulter N4 Plus particle sizer and a Brookhaven Zeta Sizer, respectively.

Flow Cytometry

Mouse embryo yolk sac cells expressing GFP (C166-GFP) were co-cultured with various tumor cell types at a 1:1 ratio. 200 μl of rhodamine labeled-liposome preparation was added per flask of co-cultured cells in 5 ml of medium and incubated for the indicated time. Cells were washed, then trypsinized, and finally resuspended in 800 μl of 10% paraformaldehyde in PBS. The fixed cells were then analyzed on a BD FACSCalibur flow cytometer. Data shown were derived from three separate experiments.

Fluorescence Microscopy

RAG cells were seeded in eight-well culture slides and allowed to grow to 50% confluency. Cells were then incubated overnight in medium containing 0.5% serum. TNF-α was added to a final concentration of 100 ng/ml followed by incubation for 5 min. Twenty microliters of liposome formulation was added and incubated for 10 min. Cells were then washed three times with PBS and mounted in fluoromount medium. One control lacked TNF-α, while

Table I. Physical Characterization of Palmitoyl Ascorbate Liposomes

Palmitoyl ascorbate (mol%)	Size distribution (nm)	Zeta potential (mV)
0	155.8±1.5	−10.96±1.97
1	135.6±66.3	−21.79±2.06
5	150.0±35.5	−49.5±1.92
10	146.3±51.9	−72.98±3.91
20	140.4±36.2	−84.29±1.97
30	146.6±29.0	−83.0±1.41

another was treated with TNF-α plus superoxide dismutase (SOD), added to a final concentration of 10 U/μl 2 min after the addition of TNF-α. Cells were observed on a Nikon Eclipse 400 fluorescence microscope equipped with appropriate filters and a digital image capture device.

Cytotoxicity Measurements

Cells were grown in 96 well plates until 40–50% confluent, followed by a 1-h exposure to liposomes containing varying concentrations of palmitoyl ascorbate. The cells were washed, incubated for 24 h in complete medium, and then analyzed for viability using the methyl tetrazolium salt (MTS) based CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI).

RESULTS

Palmitoyl Ascorbate can be Stably Incorporated into Liposomes

Incorporation of palmitoyl ascorbate into liposomes at concentrations ranging from 1 to 30 mol% yielded liposome preparations with uniform size and no evidence of precipitation upon standing at room temperature for up to 1 week, indicating that at these ratios, palmitoyl ascorbate was stably incorporated into the liposome preparations. Zeta-potential measurements revealed that incorporation of palmitoyl ascorbate imparted a negative surface charge to the liposomes. Table I summarizes the data obtained for liposome size and zeta-potential. For further experiments 30 mol% palmitoyl ascorbate liposomes were chosen.

Palmitoyl Ascorbate Liposomes Exhibit Preferential Association with Certain Types of Cancer Cell Lines

In order to evaluate the ability of ascorbate nanocarriers to specifically recognize cancer cells, we designed a co-culture assay wherein a cancer cell line (target) is grown in culture with a non-cancer cell line (non-target), then treated with labeled nanocarriers. Significant preferential association of nanocarriers with the cancer cells compared to non-cancer cells indicates targeting. The mouse embryo yolk sac endothelial cell line C166 engineered to express green fluorescent protein (GFP) was used as a representative non-cancer cell line that was easy to identify in a mixed population by virtue of the GFP fluorescence. Fig. 1A shows representative data of mouse renal carcinoma RAG cells co-cultured with C166-GFP cells and treated with fluorescently labeled liposomes. The two populations were resolved based on fluorescence intensity in the green channel (*y*-axis of depicted scatter plots) as shown. The events in region R3 indicate the green

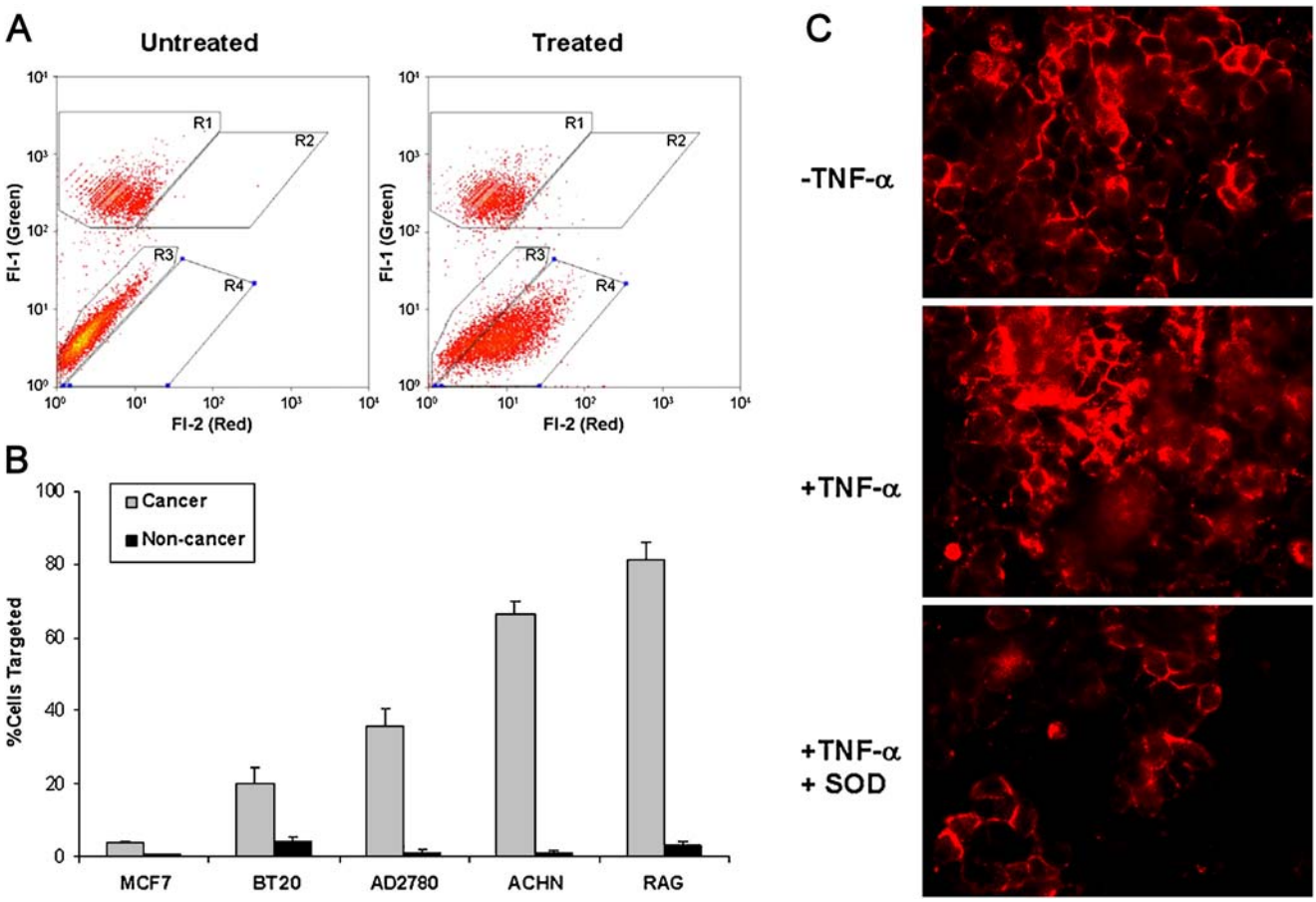

Fig. 1. Cancer cell specific association of palmitoyl ascorbate liposomes. **A** Representative flow cytometry data from co-culture targeting assay. Data shown are from an experiment with RAG cells co-cultured with the C166-GFP cells. *Upper panel* shows untreated co-culture and lower panel shows co-culture treated with rhodamine-labeled PA-liposomes. **B** Percent targeting of cancer cell lines in co-culture assay with non cancer endothelial cells. **C** Influence of TNF-α on the binding of palmitoyl ascorbate liposomes to RAG cells.

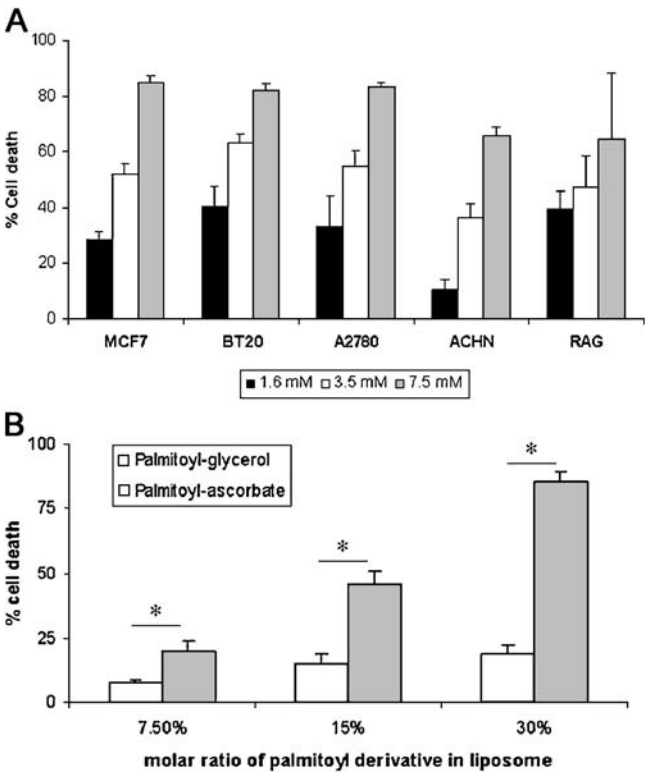

Fig. 2. Cytotoxic effect of palmitoyl ascorbate liposomes: **A** on indicated cancer cell lines at indicated palmitoyl ascorbate concentrations, **B** on MCF7 cells compared to liposomes prepared with non-ascorbate modified palmitoyl residues (*P<0.01 where n=8).

fluorescent C166-GFP cells and the events in region R1 show the non green fluorescent RAG cell line. Upon treatment with red fluorescent rhodamine-labeled nanocarrier it is clear that the RAG population acquires an appreciably higher red fluorescent signal (x-axis of plot). The percent of the population in regions R1 and R3 that shifted into regions R2 and R4 respectively were calculated in three independent experiments to give the values for the percent of cells targeted (Fig. 1B). Similar experiments were conducted using human ovarian carcinoma A2780, human breast tumor MCF7 and BT20 and human renal adenocarcinoma ACHN cells. Under the conditions of the co-culture assay, palmitoyl ascorbate liposomes associated preferentially with all the cancer cell lines (Fig. 1B). This preferential association was most evident in the renal cell carcinoma cell lines ACHN and RAG.

To determine whether tumor environment could influence the association of the ascorbate nanocarriers with tumor cells, we studied the effect of TNF-α on the interaction of our nanocarriers with tumor cells. TNF-α exposure during ascorbate liposome treatment appeared to enhance the association of ascorbate liposomes with the renal cell carcinoma RAG cells (Fig. 1C) as well as ACHN cells (not shown).

Palmitoyl Ascorbate Liposomes are Toxic to a Variety of Cancer Cell Lines

Palmitoyl ascorbate liposomes were found to have appreciable dose-dependent toxicity towards a variety of tumor cell types (Fig. 2A). Human ovarian carcinoma A2780, breast tumor cell lines MCF7 and BT20, renal adenocarcinoma ACHN, as well as the mouse renal carcinoma line RAG were tested for sensitivity to PA-liposome mediated toxicity. The toxicity of the liposome formulation was verified to be due to the presence of ascorbate residues. Liposomes prepared with a non-ascorbate palmitoyl analog (palmitoyl glycerol) showed little cytotoxicity in a representative tumor cell line (MCF7) (Fig. 2B).

Incorporation of a Non-toxic Dose of Paclitaxel into Palmitoyl Ascorbate Liposomes Significantly Improves Toxicity in a Variety of Cancer Cell Lines

The effect of palmitoyl ascorbate liposomes on the action of encapsulated anticancer drugs was studied using paclitaxel as a model drug. Paclitaxel was incorporated in palmitoyl ascorbate liposomes to give an effective concentration of 0.4 mM. The 0.4 mM dose of paclitaxel was non-toxic when incorporated in plain liposomes (Fig. 3). Incorporation of paclitaxel into palmitoyl ascorbate liposomes significantly increased the toxicity over that of empty palmitoyl ascorbate liposomes in the cell lines tested, indicating that even such a low dose of paclitaxel can contribute to toxicity when incorporated in an ascorbate nanocarrier.

DISCUSSION

Ascorbate is an anti-oxidant at low concentrations, a pro-oxidant at high concentrations, can provide anti-cancer-toxicity *in vitro*, and can enhance the effectiveness of various anti-cancer treatments *in vitro*. Nevertheless, effectively harnessing these properties for *in vivo* application has been difficult. Historically, the studies designed to measure the *in vivo* and *in vitro* anti-cancer effects of ascorbate have also been complicated by methodological differences that were at the time thought to be unimportant, yet are now known to be critical. For example, *in vivo* studies require intravenous as opposed to oral administration of ascorbate to produce elevated serum ascorbate concentrations in rodents (6) as well as in people (23). The results of *in vitro* studies can be influenced by cell culture

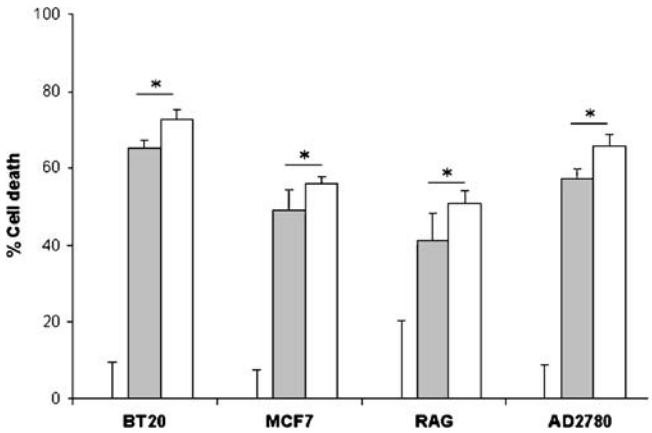

Fig. 3. Effect of palmitoyl ascorbate liposomes on the cytotoxicity of paclitaxel. *Black bars* indicate paclitaxel in plain liposomes (only error bars are visible as the average percentage of cell death was very low), *grey bars* indicate empty PA-liposomes, *white bars* indicate paclitaxel in PA-liposomes. (*P<0.01 where n=8).

conditions such as the nutrient profile of the cell culture media (24), cell density, the length of time since cell passage (15), and other factors. Even the genetic background of the mice can be critical, as was observed in ascorbate-deficient Gulo$^{-/-}$ mice (25, 26).

We believe that pharmaceutical nanocarriers prepared with hydrophobized ascorbate derivatives represent a solution to some of the difficulties in utilizing ascorbate to mediate tumor specific toxicity. Ascorbate converted to DHAA is relatively unstable, with a half-life of about 6 min at physiologic pH but becomes increasingly stable in decreasing pH (27). 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 (1) and so limit the duration of exposure of the tumor to high concentrations of ascorbate. However, anchoring ascorbate on 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, tumor microenvironment conditions may act on the ascorbate nanocarriers to enhance their association with the cancer cells.

TNF-α, PDGF, EGF, and IL-1B can increase the production of reactive oxygen species such as superoxide (24). TNF-α is known to be produced in the tumor environment and as such can contribute to increased levels of ROS in the tumor environment. Our data show that TNF-α enhanced the association of ascorbate liposomes with cancer cells and that the presence of SOD which removes ROS reduced this increase in cell association mediated by TNF-α. While the precise mechanism underlying these observations still requires investigation, these data suggest that biochemical factors in the tumor microenvironment could be expected to favor the association of ascorbate nanocarriers with tumor cells.

The physical architecture of a solid tumor (leaky vasculature, poor lymphatic drainage and high interstitial pressure) can also be expected to improve the accumulation of ascorbate nanocarriers in the tumor. As an ascorbate nanoparticle circulates in the blood, the small numbers of DHAA molecules that might be generated on its surface would likely decay before interacting with GLUT transporters on the surface of erythrocytes. However, once within the interstitial fluids of solid tumor, an ascorbate nanocarrier would be exposed to an abnormal abundance of superoxide anion, which will cause an increase in the rate of conversion of AA to DHAA on the nanocarrier. This increase in the concentration of DHAA on the carrier surface should facilitate the interaction of the carrier with the GLUT transporters on the tumor cells. Additionally, the slow and erratic fluid flow through the convoluted microvessels of tumors should enhance the interactions between the carrier and the tumor cells allowing for the improved retention of the nanocarrier and its cargo in the tumor environment.

The ascorbate-modified liposomes described in this study clearly retained the toxicity of ascorbate towards tumor cells. The liposome size of about 150 nm is ideally suited for *in vivo* applications and should allow for selective accumulation in solid tumors by virtue of the well-documented enhanced permeability and retention effect. Based on the preferential association of ascorbate nanocarriers with cancer cells in our co-culture experiments it is reasonable to expect that our nanocarrier formulation will preferentially associate with tumor cells within a solid tumor.

CONCLUSIONS

Taken together, the data suggest that surface modification of liposomes with ascorbate residues represents an effective way to target and kill certain types of tumor cells and that incorporation into ascorbate liposomes might potentiate the effect of anticancer agents. Ascorbate nanocarriers offer the potential for improved action over the use of free ascorbic acid for anti cancer therapy *in vivo*.

ACKNOWLEDGEMENTS

This research is based on a hypothesis originated and proposed by Anthony R. Manganaro. Funding was provided by Anthony R. Manganaro.

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