Comparison of Vitamin E Derivatives α-TEA and VES in Reduction of Mouse Mammary Tumor Burden and Metastasis

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A novel nonhydrolyzable ether derivative of RRR-α-tocopherol, RRR-α-tocopherol ether acetic acid analog [2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA)], and a hydrolyzable ester derivative RRR-α-tocopheryl succinate (vitamin E succinate; VES) inhibited BALB/c mouse 66cl-4-GFP mammary tumor cell growth *in vitro* and *in vivo*. Treatment of 66cl-4-GFP cells in culture with α-TEA or VES induced dose-dependent DNA synthesis arrest and apoptosis and inhibited colony formation. Liposomal formulations of α-TEA delivered orally or by aerosol significantly reduced subcutaneous 66cl-4-GFP tumor burden and metastasis to lung and lymph nodes. Liposomal formulations of VES delivered by aerosol significantly reduced tumor burden and lung metastasis, but not lymph node metastasis. Unlike α-TEA, VES was ineffective in reducing tumor burden and metastasis to lungs and lymph nodes when administered orally. Analyses of tumor sections showed that α-TEA delivered by either method significantly reduced tumor cell proliferation as measured by Ki67, and increased apoptosis as measured by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL), whereas VES delivered by aerosol reduced tumor cell proliferation and increased apoptosis, but not significantly. In summary, the nonhydrolyzable ether vitamin E derivative α-TEA was effective in reducing tumor burden and metastasis when delivered either by aerosol or orally, whereas the hydrolyzable ester vitamin E derivative VES was effective only when delivered by aerosol. Exp Biol Med 229:954–963, 2004

Key Words: vitamin E analog α-TEA; RRR-α-tocopheryl succinate (VES); metastasis; antitumor agents; syngeneic mouse mammary cancer model

Introduction

Our laboratory has been investigating the antitumor properties of natural and synthetic vitamin E compounds, with major emphasis on using human epithelial breast cancer cells in culture to analyze the cellular, molecular, and biochemical events involved in the ability of a succinate ester derivative of vitamin E (RRR-α-tocopherol), RRR-α-tocopheryl succinate (vitamin E succinate; VES) to induce DNA synthesis arrest, differentiation, and apoptosis (1–10). Vitamin E succinate has been shown by this laboratory and others to be a potent inhibitor of epithelial cancer cell growth, inducing human breast, prostate, lung, colon, cervical, and endometrial cancer cells to undergo apoptosis in culture, but not normal human mammary epithelial cells or normal prostate epithelial cells (1–3, 7–9).

Although VES has proven to be a potent anticancer agent *in vitro* and has provided important insights into anticancer signaling pathways, its basic structure has the potential of compromising its potency *in vivo*; namely, the ester linkage can be hydrolyzed by cellular esterases, yielding vitamin E (RRR-α-tocopherol) and succinic acid, neither of which exhibit anticancer properties (1, 9).

In an effort to overcome the potential problem of cellular esterases hydrolyzing the ester-linked succinate moiety of VES and rendering VES ineffective as an anticancer agent, our laboratory has developed a non-hydrolyzable ether analog of RRR-α-tocopherol, namely, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chro-

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man-6-yloxyacetic acid (RRR-α-tocopheryloxyacetic acid or RRR-α-tocopherol ether-linked acetic acid analog [α-TEA]), that exhibits similar anticancer properties to VES in cell culture (11).

Like VES, the parent compound for making α-TEA is natural vitamin E (RRR-α-tocopherol). α-TEA differs from VES in that it has an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head by an ether linkage, whereas VES has a succinic acid moiety linked by an ester linkage at this site (12). α-TEA, like VES, is a potent anticancer agent and does not induce apoptosis in normal human mammary epithelial cells (11–13). Expectations were that the ether derivative would be more stable and a better *in vivo* anticancer agent.

BALB/c mammary tumor cell line 66cl-4-GFP, which was originally derived from a spontaneously arising mammary adenocarcinoma and subsequently was stably transfected with the enhanced green fluorescent protein (EGFP), was used in these studies because this cell line, when transplanted into BALB/c mice, exhibits an aggressive tumor with metastasis to lungs and lymph nodes (12). Liposomal formulation of each compound was chosen for this study because α-TEA and VES are lipids and insoluble in water, and α-TEA/peanut oil formulation delivered orally was ineffective (12). Liposome formulations of α-TEA and VES can be administered several ways, including aerosol or gavage. Delivery of lipophilic chemotherapeutic agents to mice by aerosol increased drug concentrations in the lungs and other organs compared with intramuscular or oral administration, and this method of drug delivery has been shown to be highly effective against pulmonary metastasis of melanoma and osteosarcoma in mice (14–16).

We compared the anticancer properties of α-TEA and VES *in vitro* and *in vivo*. *In vitro*, both vitamin E derivatives were effective anticancer agents, inducing DNA synthesis arrest and apoptosis as well as inhibiting colony formation. *In vivo*, α-TEA was superior to VES, significantly reducing tumor burden and metastasis regardless of method of delivery.

Materials and Methods

VES and α-TEA. Vitamin E succinate (formula weight = 530.8) was purchased from Sigma Chemical Co. (St. Louis, MO). Synthesis of α-TEA (formula weight = 488.8) was performed by one of the authors (J.A) and has been described in detail (12).

66cl-4-GFP Murine Mammary Tumor Cell Line. 66cl-4 cells were derived from a spontaneous mammary tumor in a BALB/cfC3H mouse and isolated as a 6-thioguanine-resistant clone (17, 18). These cells were stably transfected with an expression vector of the enhanced green fluorescence protein (EGFP: pEGFP-N1 from BD Biosciences Clontech Laboratories, Inc. Palo Alto, CA) and transfected cells sorted with fluorescent-activated cell sorter (FACS) twice to obtain a population of brightly fluorescing cells. 66cl-4-GFP cells have been shown to be highly metastatic, with approximately 40% of animals developing visible macroscopic metastases and 100% of animals developing microscopic metastases detectable with fluorescent microscopy in the lungs 26 days following subcutaneous injection of 2×10^5 tumor cells into the inguinal area (12). 66cl-4-GFP cells were maintained as monolayer cultures, and *in vitro* experiments were performed as previously described (12).

Determination of DNA Synthesis by Incorporation of Tritiated Thymidine. Effects of α-TEA and VES on inhibition of DNA synthesis of 66cl-4-GFP cells were determined by tritiated thymidine incorporation as described previously (6, 19). Briefly, 66cl-4-GFP cells at 2.0×10^4 cells in 0.2 ml volume/well in 96 well plates were cultured with 2.5, 5, 10, or 20 μg/ml of α-TEA (2.6, 5.1, 10.2, 20.5 μ*M*, respectively) or VES (2.3, 4.7, 9.4, 18.8 μ*M*, respectively) for 24 hrs. During the last 6 hrs of incubation, cultures were pulsed with 0.5 μCi tritiated thymidine/well, cells were harvested, and tritiated thymidine uptake was measured using a Beckman LS5000TD liquid scintillation counter (Beckman Coulter, Fullerton, CA). Percent DNA synthesis arrest was determined by comparing the tritiated thymidine uptake (cpm) of treatment groups to tritiated thymidine uptake (cpm) of untreated controls.

Determination of Apoptosis by Morphological Evaluation of 4′,6-Diamidino-2-Phenylindole (DAPI)-Stained Nuclei. Apoptosis was determined using previously published procedures and criteria (2, 12). Data are reported as percentage of apoptotic cells per cell population (i.e., number apoptotic cells/total number of cells counted). For each sample, three different microscopic fields were examined and 200 cells counted at each location for a minimum of 600 cells counted per slide. Apoptotic data are presented as mean ± SD for three independent experiments.

Colony Forming Assay. Effects of α-TEA and VES on colony formation of 66cl-4-GFP cells were determined as previously described (20). Briefly, 66cl-4-GFP cells were seeded in 35 × 10 mm tissue culture plates (Nunclon, Rochester, NY) at increasing cell numbers ranging from 5×10^2 to 1×10^5 cells/plate. Cells were allowed to adhere overnight, then treated with 1.25, 2.5, 5, or 10 μg/ml α-TEA or VES or untreated for 10 days. After 10 days, media were removed; cells were washed in phosphate-buffered saline (PBS) and stained with 0.1% methylene blue in PBS. Plating efficiency was determined by dividing the number of colonies present after 10 days in the untreated plate by the number of cells seeded. The surviving fraction of treated samples was determined as number of colonies present divided by number of cells seeded × plating efficiency.

BALB/c Mice. Female BALB/cJ mice at 6 weeks of age (~25 g body weight) were purchased from Jackson Labs (Bar Harbor, ME) and allowed to acclimate for 1 week. Mice were housed, 5/cage, given water and standard lab chow (Harlan Teklad #2018 Global 18% Protein Rodent Diet; Madison, WI) *ad libitum* at the Animal Resource

Center at the University of Texas at Austin and maintained in an environment of 74 ± 2°F with 30%–70% humidity and a 12-hour alternating light-dark cycle. Guidelines for the humane treatment of animals were followed as approved by the University of Texas Institutional Animal Care and Use Committee.

Tumor Cell Inoculation. 66cl-4-GFP cells were harvested by trypsinization, collected by centrifugation, and resuspended at a density of 2×10^5 cells/100 μl in McCoy's media, containing no supplements. Mice were injected with 2×10^5 cells/100 μl in the inguinal area at a point equal distance between the fourth and fifth nipples on the right side using a 25-gauge needle.

Mice, 10/group, were placed in 6 groups (liposome/aerosol control, liposome/gavage control, liposomal α-TEA/aerosol, liposomal VES/aerosol, liposomal α-TEA/gavage, and liposomal VES/gavage) so that the average tumor volume for all groups was closely matched. Each group had an average tumor volume/group of 0.569 mm^3, 0.450 mm^3, 0.519 mm^3, 0.375 mm^3, 0.613 mm^3, and 0.681 mm^3, respectively, at the start of treatments, which were begun 9 days following tumor cell inoculation. Tumors were measured using calipers every other day, and volumes were calculated using the following formula: volume (mm^3) = [width (mm^2) × length (mm)]/2 (21). Body weights were determined weekly.

Preparation of Liposomal α-TEA and VES. An α-TEA or VES/liposome ratio of 1:3 (w/w) was determined empirically to be optimal by methods previously described (12, 14). To prepare the α-TEA or VES/lipid combinations, the components were first brought to room temperature. The lipid [1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC); Avanti Polar-Lipids, Inc., Alabaster, AL], at a concentration of 120 mg/ml, was dissolved in tertiary-butanol (Fisher Scientific, Houston, TX) and then sonicated to obtain a clear solution. α-TEA or VES at 40 mg/ml was dissolved in tertiary-butanol and vortexed until all solids were dissolved. The lipid DLPC and α-TEA or DLPC and VES were then combined in equal amounts (v:v) to achieve the desired ratio of 1:3 α-TEA or VES/liposome, mixed by vortexing, frozen at –80°C for 1–2 hrs, and lyophilized overnight to a dry powder prior to storing at –20°C until needed.

Aerosol and Gavage Delivery. Aerosol was administered to mice as previously described (12, 14). Briefly, an air compressor (Easy Air 15 Air Compressor; Precision Medical, Northampton, PA) producing a 10 L/min airflow was used with an AeroTech II nebulizer (CIS-US, Inc., Bedford, MA) to generate aerosol. Particle size and stability of α-TEA and VES liposomes after discharge from the AeroTech II nebulizer was determined using an Anderson Cascade Impactor. The mass median aerodynamic diameter was approximately 2 μm for both vitamin E compounds. Also, it was determined that the formulations were stable; namely, no chemical/physical alterations occurred throughout the 15-min nebulization process. (Note: Once resuspended in water, the liposomal formulations appear to be very stable for hours before or after aerosolization.)

Mice were placed in plastic cages (7 × 11 × 5 in.) with a sealed top in a safety hood. Aerosol entered the cage via a 1-cm accordion tube at one end and was discharged at the opposite end, using a one-way pressure release valve. Mice were exposed to aerosol until all liposomal α-TEA, liposomal VES, or liposome only (control) was aerosolized (~15 mins). Aerosol treatments were conducted once a day, 7 days per week, for a total of 21 days. High-performance liquid chromatography analyses were conducted on α-TEA liposomes recovered from aerosol collected with an All Glass Impinger (Ace Glass Co., Vineland, NJ). An estimate of the amount of aerosolized α-TEA delivered/mouse/treatment was derived from the following formula (12): delivered drug dose = drug concentration (μg/liter) × duration of drug delivery in minutes × estimated percentage of aerosolized drug deposited in the respiratory tract, which includes the nose, trachea, and lungs (30%). On the basis of this formula, we estimate that 36 μg of α-TEA or VES were deposited in the respiratory tract of each mouse each day. Thus, for the 21-day treatment period, we estimate that each mouse received 756 μg of α-TEA or VES from liposomal aerosol delivery (12).

For gavage treatments, lyophilized preparations of liposomal α-TEA, liposomal VES, or liposome only were brought to room temperature and reconstituted by adding 2.5 ml distilled water to achieve the final desired concentration of 32 mg/ml α-TEA or VES. (Note: As mentioned above, once resuspended in water, the liposomal formulations are stable for several hours.) Treatments were vortexed vigorously immediately prior to administration by gavage, 100 μl/mouse at two different times each day, ~8 hours apart. Particle size range of α-TEA and VES liposomes delivered orally was determined to be 4–10 μm. The total amount of α-TEA, VES, or liposome control administered by gavage was 200 μl/mouse per day (final concentration, 6.4 mg α-TEA or VES/mouse/day). Gavage treatments were given twice per day, 7 days/week, for a total of 21 days. Thus, for the 21-day treatment period, each mouse received 134 mg of α-TEA or VES via gavage delivery; however, we do not know the bioavailability of α-TEA or VES delivered by this method.

Lung and Lymph Node Metastasis. Macroscopic metastases in all five lung lobes were counted visually at time of sacrifice (21 days after treatment initiation). Fluorescent microscopic lung metastases were counted as described previously, using a Nikon fluorescence microscope (TE-200; ×200 magnification; Ref. 12). For analyses of lung tissue, left lung lobes were flattened and the entire surface (top and bottom) scored for fluorescent green microscopic metastases. For analyses of axillary and brachial lymph nodes, the tissues were flattened and scored for fluorescent green microscopic metastases. Fluorescent microscopic metastases were scored by size into three size groupings: <20 μm, 20–50 μm, and >50 μm. On the basis

of a typical 66cl-4-GFP tumor cell size of 10–20 μm in diameter, the <20-μm grouping is thought to represent solitary cells; the 20–50-μm grouping two to five cells; and the >50-μm grouping microscopic metastases of greater than two to five cells.

Ki-67 Staining for Detection of Proliferation *In Vivo*. Tumors were collected at the time of sacrifice, 21 days post–treatment initiation. Deparaffanized sections (5-μm) of tumor tissue were used to assess proliferation using antibody to the Ki-67 antigen that is a nuclear antigen expressed in proliferating cells and serves as an indicator of the number of cells undergoing active cell division. Briefly, endogenous peroxidase activity was blocked using a 3% H_2O_2 solution for 10 mins, followed by washing with PBS. Rabbit serum (10% v/v in PBS) was applied to sections in order to block nonspecific antibody binding. Sections were incubated with primary Ki-67 antibody (rat-anti-mouse Ki-67 antibody; DAKO Corp., Carpinteria, CA; 1:200 dilution) overnight at 4°C. After primary antibody incubation, slides were incubated with biotinylated rabbit-anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 30 mins at room temperature. Tissue sections were then incubated with avidin-biotin complex (ABC-HRP; Vector Laboratories) for 30 mins at room temperature. Immunoreactivity was visualized via incubation with di-aminobenzidine dihydrochloride. Slides were lightly counterstained with hematoxylin. Ki-67 positive stained cells were counted in five separate 400× microscopic fields per tumor sample.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Biotin Nick-End Labeling (TUNEL) Assay for Detection of Apoptosis *In Vivo*. Deparaffinized sections (5 μm) of tumor tissue were used to assess apoptosis using reagents supplied in the ApopTag *In Situ* Apoptosis Detection kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Nuclei that stained brown were scored as positive for apoptosis, and those that stained blue were scored as negative. At least 16 microscopic fields (×400) were scored per tumor. Data are presented as the mean ± SE number of apoptotic cells counted in at least eight separate tumors from each group.

Statistical Analyses. Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor analysis of variance (ANOVA) using SPSS (SPSS Inc., Chicago, IL). Difference in number of fluorescent microscopic metastases/group, Ki-67 stained cells/group, and TUNEL positive cells/group were determined using the two-tailed Mann-Whitney rank test using Prism software version 3.0 (Graphpad, San Diego, CA). A level of $P < 0.05$ was regarded as statistically significant.

Results

VES- and α-TEA-Inhibited DNA Synthesis in 66cl-4-GFP Cells *In Vitro*. 66cl-4-GFP cells were treated with VES or α-TEA to determine the ability of each to inhibit DNA synthesis (Fig. 1A). Cells treated with 2.5, 5, 10, or 20 μg/ml VES or α-TEA exhibited 9%, 14%, 30%, and 54% and 11%, 17%, 34%, and 82% reductions in DNA

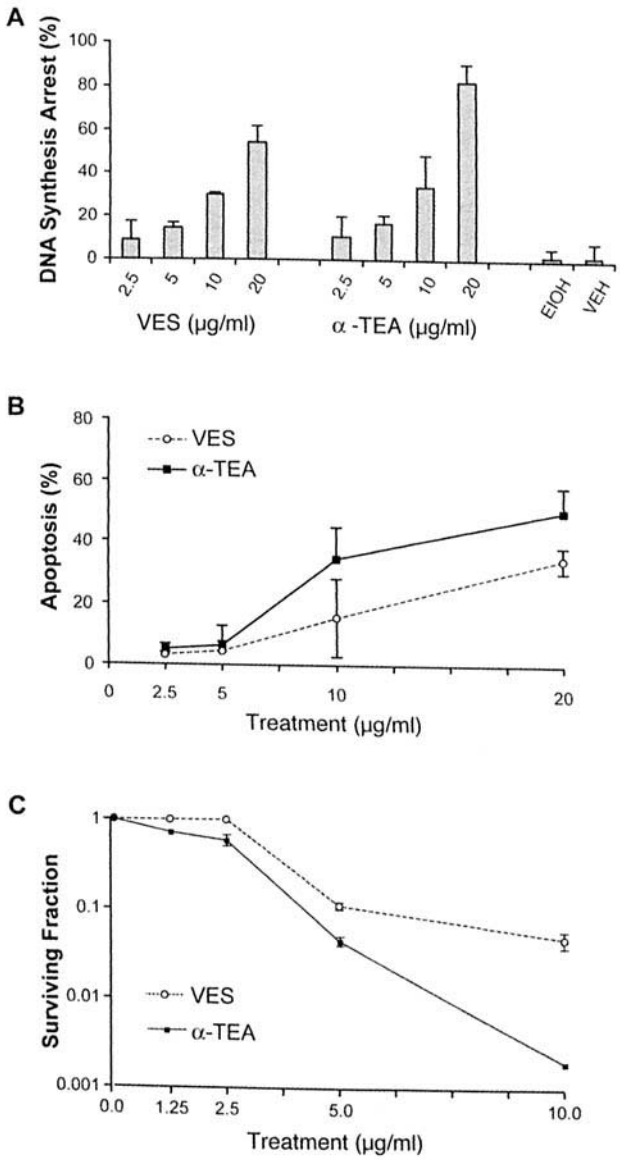

Figure 1. Documentation of VES and α-TEA induced DNA synthesis arrest and apoptosis and inhibition of colony formation. (A) Murine mammary cells were treated with varying concentrations of VES or α-TEA, equivalent amounts of ethanol (EtOH), or succinic acid + ethanol (VEH) in highest treatment dose or untreated and cultured for 24 hrs. Percent DNA synthesis arrest was determined by comparing the tritiated thymidine uptake (cpm) of treatment groups to tritiated thymidine uptake (cpm) of untreated controls. Data are mean ± SD of three independent experiments. (B) Murine mammary cancer cells were treated with varying concentrations of VES, α-TEA, or controls and cultured for 3 days. Apoptosis was measured by analyses of morphology of nuclei of DAPI-stained cells. Data are depicted as mean ± SD of three independent experiments. (C) Murine mammary cells were seeded at varying concentrations in tissue culture plates and treated with varying concentrations of VES or α-TEA for 10 days. Survival fractions were determined as number of colonies present divided by number of cells seeded × plating efficiency. Data are mean ± SD of two independent experiments.

synthesis after 24 hrs of treatment when compared with untreated controls, respectively (Fig. 1A). Vehicle (VEH) and ethanol (EtOH) controls exhibited 2% DNA synthesis arrest when compared with untreated control.

VES- and α-TEA-Induced Apoptosis in 66cl-4-GFP Cells, *In Vitro*. 66cl-4-GFP mammary cancer cells were treated with VES or α-TEA, and apoptosis was assessed by analyses of DAPI-stained cells for condensed nuclei and fragmented DNA. The level of apoptosis of 66cl-4-GFP cells treated for 3 days with 2.5, 5, 10, or 20 μg/ml VES or α-TEA was 3%, 5%, 16%, and 34%, and 5%, 6%, 34%, and 50% apoptosis, respectively (Fig. 1B). The levels of apoptosis induced by α-TEA and VES were not statistically different when compared pairwise by dose. However, percent apoptosis increased linearly with increasing dose of each agent, and linear regression analyses of the data showed that percent apoptosis increased more steeply with dose of α-TEA than dose of VES. An indicator-variable approach was used to test the null hypothesis of equal slopes, which was rejected ($P = 0.002$). Vehicle- and ethanol-treated controls exhibited 2%–3% apoptosis (data not shown).

VES and α-TEA Inhibited Colony Formation of 66cl-4-GFP Cells. In this study, the plating efficiency for 66cl-4-GFP cells varied between 24% and 40%. The surviving fraction of 66cl-4-GFP cells after treatment with 1.25, 2.5, 5, or 10 μg/ml of VES or α-TEA was 1.0 ± 0.0, 1.0 ± 0.0, 0.1 ± 0.01, and 0.05 ± 0.01 and 0.72 ± 0.01, 0.58 ± 0.12, 0.045 ± 0.007, and 0.002 ± 0.0 after 10 days of treatment, respectively (Fig. 1C).

Liposomal Formulations of VES and α-TEA Delivered by Aerosol Decreased 66cl-4-GFP Tumor Burden. Mean tumor volumes of control animals treated with liposomes by aerosol for 21 days were significantly higher than mean tumor volumes of animals treated by aerosol with liposomal formulations of either VES or α-TEA ($P < 0.001$; Fig. 2A). There were no differences in the mean tumor volumes between mice receiving the VES or α-TEA treatments (Fig. 2A). On the basis of previous experiments (12), we estimate that 36 μg of α-TEA or VES were deposited in the respiratory tract of each mouse each day. Thus, for the 21-day treatment period, we estimate that each mouse received 756 μg of α-TEA or VES from liposomal aerosol delivery.

Liposomal Formulations of α-TEA Delivered by Gavage but Not Liposomal Formulations of VES Delivered by Gavage Reduced 66cl-4-GFP Tumor Burden. Mean tumor volumes of mice treated by gavage with liposomal formulated α-TEA were significantly lower than that of control mice ($P < 0.001$; Fig. 2B). Vitamin E succinate incorporated into liposomes and delivered by gavage was not effective in reducing primary tumor burden when compared with control animals ($P < 0.10$; Fig. 2B). Although mice received 6.4 mg/day for 21 days of treatment by gavage for a total of 134.4 mg, we do not know the bioavailability of α-TEA or VES delivered by this method.

Liposomal Formulations of α-TEA and VES Delivered by Aerosol Suppressed 66cl-4-GFP Lung and Lymph Node Metastasis in BALB/c Mice. At sacrifice, all five lung lobes and axillary and brachial lymph nodes were examined visually for macroscopic metastases. The α-TEA aerosol treatment groups contained one animal (10%; total of one metastases) and the VES aerosol treatment group contained three animals (30%; total of five metastases) exhibiting macroscopic lung metastases, respectively, whereas the aerosol control group contained five animals (50%; total of 17 metastases) exhibiting macro-

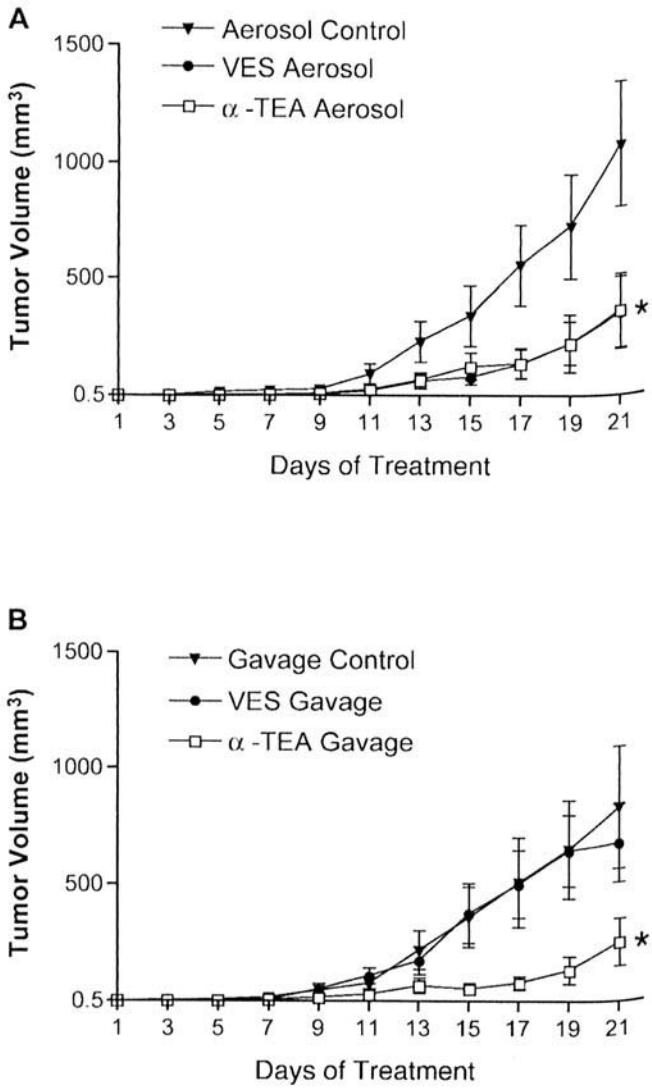

Figure 2. Comparisons of effects of liposomal formulated α-TEA or VES delivered by either aerosol (A) or gavage (B) on tumor burden. 66cl-4-GFP cells (2×10^5/mouse) were injected into the inguinal area at a point equidistant between the fourth and fifth nipples. Nine days after tumor cell injection, treatments were initiated and administered daily for a total of 21 days. Tumor volume/mouse was determined at 2-day intervals. Tumor volumes (mm^3) are depicted as mean ± SE. (A) Mice were treated with liposomal formulated α-TEA (80 mg/cage/day), VES (80 mg/cage/day), or liposome control by aerosol. (B) Mice were treated with liposomal formulated α-TEA (6.4 mg/mouse/day), VES (6.4 mg/mouse/day), or liposome control by gavage. * Designates a significant reduction in tumor burden in comparison with control ($P < 0.001$).

Table 1. 66cl-4-GFP Mammary Cancer Cell Macroscopic Lung Metastasis in BALBb/c Mice Receiving Liposomal Formulated α-TEA or VES by Aerosol or by Gavage

Delivery/Treatments	No. animals/group with macroscopic lung metastases[a]	Total no. macroscopic lung tumor foci[b]
Aerosol/liposomal control	5/10	17
Aerosol/liposomal VES	3/10	5
Aerosol/liposomal α-TEA	1/10	1
Gavage/liposomal control	7/10	12
Gavage/liposomal VES	5/10	7
Gavage/liposomal α-TEA	0/10	0

[a] Macroscopic lesions in all five lung lobes for each animal in all treatment groups were counted visually at the time of sacrifice.
[b] Data are expressed as the total number of macroscopic lung tumor foci observed in the 10 mice in each treatment group.

scopic lung metastases (Table 1). The number of mice with macroscopic metastases in the aerosol α-TEA and VES treatment groups was not significantly different from control or from each other, but in terms of total numbers of macroscopic lung tumor foci, the α-TEA group was significantly lower than control ($P < 0.048$). For oral treatments, both the number of mice with macroscopic lung metastases and the total number of tumor foci observed in the α-TEA treatment group were statistically lower than control or VES ($P < 0.003$ and $P < 0.03$; and $P < 0.02$ and $P < 0.015$, respectively). No macroscopic metastases were observed in the lymph nodes of any of the aerosol/liposomal groups: control, α-TEA, or VES (data not shown).

Use of a Nikon fluorescence microscope permitted measurement of green fluorescing microscopic metastases into three size groupings (small, <20 μm; medium, 20–50 μm; and large, >50 μm; Fig. 3A). Because the tumor cells are ~10–20 μm in diameter, the microscopic metastases scored as <20 μm most likely represent single cells. This analysis showed a decrease in microscopic lung metastases between the aerosol control group and both the α-TEA and VES aerosol treatment groups. The mean ± SE of total lung microscopic metastases in the α-TEA (31.2 ± 2.7; $n = 10$) and the VES (43.9 ± 5.3; $n = 10$) treatment groups in comparison to aerosol control (73.4 ± 8.8; $n = 10$) were significantly reduced ($P < 0.0001$ and $P < 0.009$, respectively; Fig. 3A). Of interest, lung microscopic metastases in the VES aerosol–treated animals were significantly lower than control in only one of the size categories of microscopic metastases; that is, the medium-sized lesions ($P < 0.007$), whereas the α-TEA aerosol–treated animals showed significant decreases in all three size categories. P values for α-TEA aerosol versus VES aerosol for mean number of small, medium, and large microscopic lung metastases are 0.02 versus 0.07, 0.0001 versus 0.007, and 0.0008 versus 0.15, respectively.

Mice treated with α-TEA aerosol had a lower number of axillary and brachial lymph node microscopic metastases,

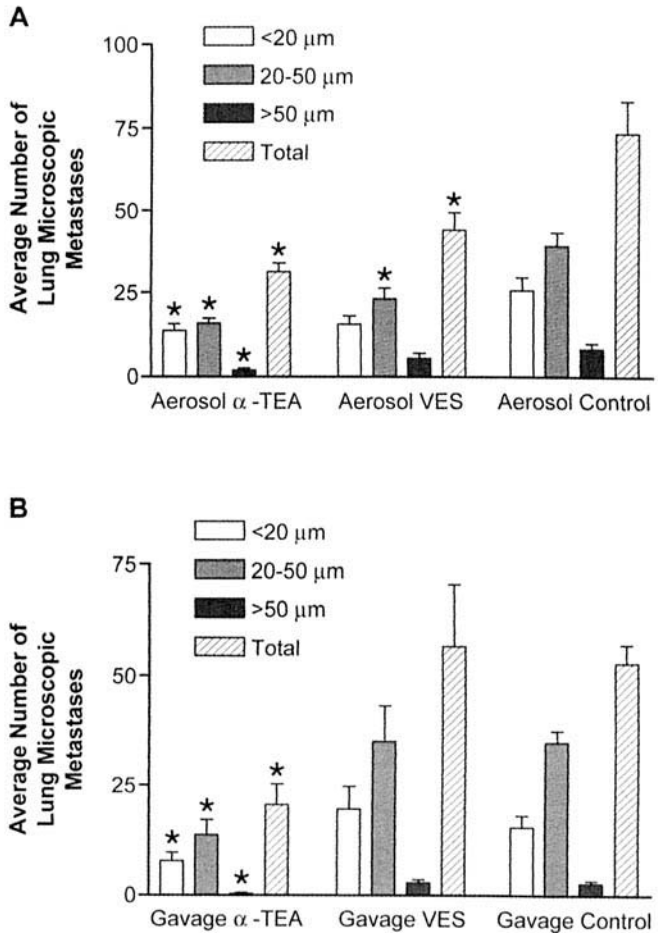

Figure 3. Comparison of effects of α-TEA or VES on lung microscopic metastases. The number of fluorescent microscopic metastases on the surface (top and bottom) of flattened left lung lobes from mice treated with liposomal formulated α-TEA, VES, or liposome control delivered by aerosol (A) or by gavage (B) were determined. Data are depicted as average ± SE of total number of lung microscopic metastases or number of metastases in each size category ($n = 10$ for each treatment group). * Designates a significant reduction in metastatic lesions in comparison with appropriate control.

whereas mice treated with VES aerosol showed no significant decrease in microscopic metastases found in lymph nodes (Fig. 4A). More specifically, α-TEA aerosol–treated mice had a mean ± SE of 1.4 ± 0.5 microscopic metastases per lymph node as compared with a mean ± SE of 6.0 ± 1.1 microscopic metastases found in lymph nodes from control animals ($P < 0.0001$). In contrast, VES aerosol–treated mice had a mean ± SE of 4.4 ± 1.2 microscopic metastases per lymph node in comparison with control mice ($P < 0.19$). Of additional interest, 48% of lymph nodes in mice treated with α-TEA aerosol were free of micrometastases, in comparison with 4% in control mice and 20% in VES aerosol–treated mice.

Liposomal Formulations of α-TEA Delivered by Gavage Suppressed 66cl-4-GFP Lung and Lymph Node Metastasis in BALB/c Mice, Whereas Liposomal Formulations of VES Delivered by Gavage

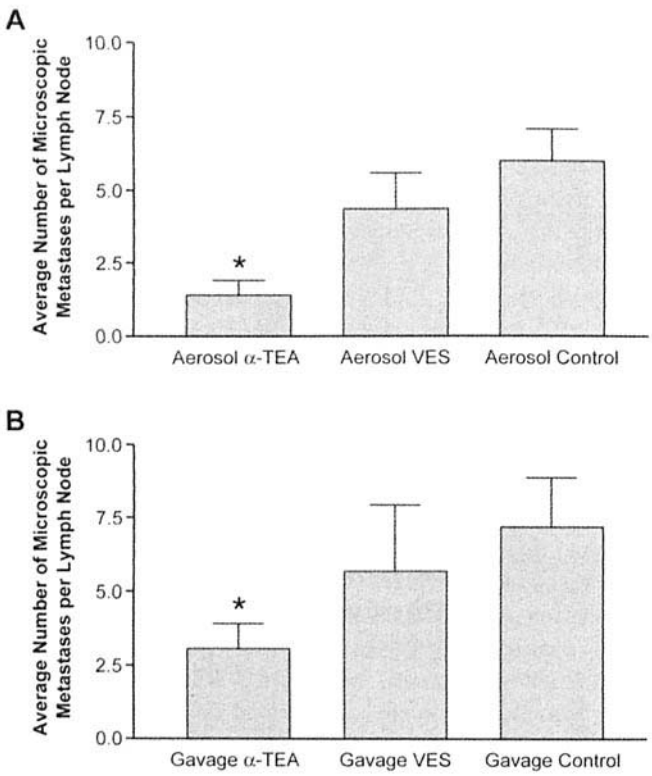

Figure 4. Comparison of effects of α-TEA or VES on microscopic metastases in lymph nodes. The number of fluorescent microscopic metastases on the surface of flattened axillary and brachial lymph nodes from mice treated with liposomal formulated α-TEA, VES, or liposome control delivered by aerosol (A) or by gavage (B) were determined. Number of lymph nodes/treatment group examined were aerosol α-TEA ($n = 21$), aerosol VES ($n = 15$), aerosol control ($n = 23$), gavage α-TEA ($n = 22$), gavage VES ($n = 22$), and gavage control ($n = 20$). Data are depicted as average ± SE of number of microscopic metastases/lymph node. * Designates a significant reduction in metastatic lesions compared with control.

Did Not. At sacrifice, all five lung lobes and axillary and brachial lymph nodes were examined visually for macroscopic metastases. There were no macroscopic lung metastases in the α-TEA gavage treatment group (0/10; 0%; 0 metastases), 5/10 (50%; total of 7 metastases) in the VES gavage treatment group, and 7/10 (70%; total of 12 metastases) in the gavage control group (Table 1). No macroscopic metastases were found in the lymph nodes of any animals from any of the gavage/liposomal treatment groups.

Use of a Nikon fluorescence microscope permitted measurement of green fluorescing microscopic metastases in lung tissue into three size groupings as discussed above. This analysis showed a decrease in the total number of microscopic lung metastases between the α-TEA gavage–treated mice, but not the VES gavage–treated mice in comparison with the gavage control animals. More specifically, the mean number of total microscopic metastases in the α-TEA gavage treatment group (21.5 ± 4.9; $n = 10$) and the VES gavage treatment group (57.4 ± 13.4; $n = 10$) in comparison with the gavage control group (52.7 ± 4.2; $n = 10$) was significantly reduced ($P < 0.0006$) for the former but not for the latter ($P < 0.74$; Fig. 3B). P values for α-TEA gavage versus VES gavage for mean number of small, medium, and large microscopic lung metastases are 0.05 versus 0.92, 0.0004 versus 0.49, and 0.0004 versus 0.90, respectively.

Mice treated with α-TEA by gavage had a lower number of microscopic metastases found in the axillary and brachial lymph nodes in comparison with control mice (Fig. 4B), whereas mice treated with VES by gavage exhibited no significant decrease in microscopic metastases found in lymph nodes in comparison with control mice (Fig. 4B). More specifically, α-TEA and VES gavage-treated mice had a mean ± SE of 3.0 ± 0.8 and 5.7 ± 2.2 microscopic metastases/lymph node; respectively, in comparison with a mean ± SE of 7.1 ± 1.7 microscopic metastases/lymph nodes in control animals ($P < .05$ and $P < 0.32$, respectively; Fig. 4B). Of additional interest, 32% of lymph nodes from mice treated with α-TEA by gavage were free of micrometastases, versus 23% in VES-treated and 15% in control mice.

Liposomal Formulations of VES or α-TEA Delivered by Aerosol or Gavage Did Not Exhibit Toxicity. No differences in mean body weights and no adverse side effects were found among any of the treatment or control groups (data not shown).

Inhibition of Cell Proliferation by VES and α-TEA *in Vivo*. Tumor sections from each of the treatment groups were examined by immunohistochemistry for proliferation status using the nuclear Ki-67 antigen expressed in proliferating cells as a biomarker. Tumors from mice treated with α-TEA via aerosol or gavage had mean ± SE of 151 ± 27.7 and 107.8 ± 27.2 Ki-67 positive cells/field, respectively, in comparison with tumors from aerosol and gavage control mice that had mean ± SE of 253.8 ± 34.7 and 241.1 ± 45.9 Ki-67 positive cells/field ($P < 0.05$ and $P < 0.03$, respectively; Fig. 5A and B). Ki-67 staining of tumors from mice treated with VES via aerosol or gavage showed no significant decrease in proliferation in comparison with tumors from corresponding control animals, with a mean ± SE of 167.8 ± 47.1 and 258.5 ± 26.8 Ki-67 positive cells/field ($P < 0.4$ and $P < 0.97$, respectively; Fig. 5A and B). There was no significant difference between VES and α-TEA in the mean number of Ki-67 positive cells observed when they were delivered by aerosol ($P < 0.96$).

Induction of Apoptosis by α-TEA and VES *in Vivo*. Tumors from treatment and control mice were taken at the completion of 21 days of treatment. Tumor sections from each of the treatment groups were examined by immunohistochemistry for apoptosis using TUNEL. No differences were found in the location of apoptotic cells in tumor sections from mice treated with α-TEA or VES delivered by aerosol or gavage. Tumors from mice treated with α-TEA delivered by aerosol or gavage had a mean ± SE of 1.54 ± 0.37 and 1.31 ± 0.31 apoptotic cells/field, respectively, whereas tumors from aerosol and gavage

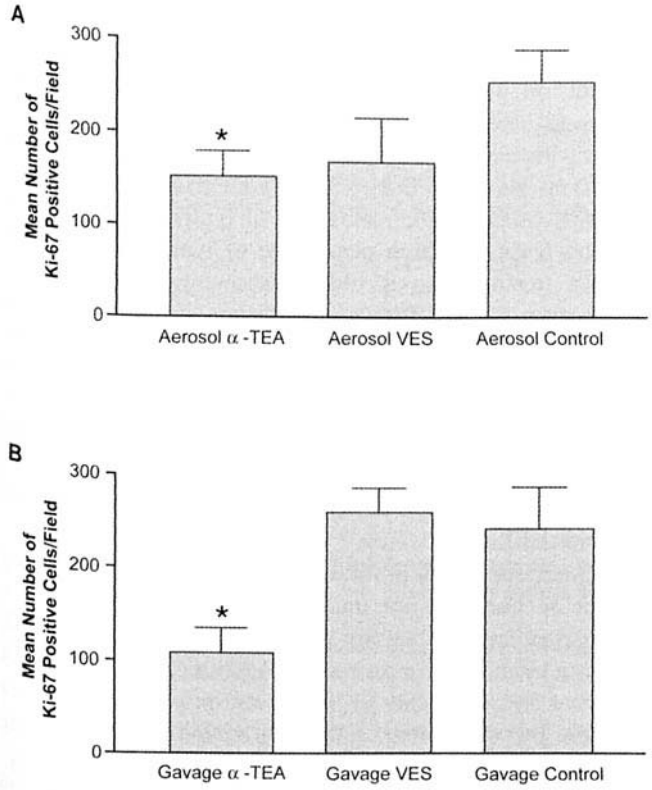

Figure 5. α-TEA, but not VES, significantly inhibited primary tumor cell proliferation. Comparisons of Ki-67 positive cells in tumor sections (5 μm) obtained from mice treated with liposomal formulated α-TEA, VES, or liposome control delivered by aerosol (A) or by gavage (B) are depicted. Number of tumors/treatment group examined were aerosol α-TEA (n = 8), aerosol VES (n = 8), aerosol control (n = 9), gavage α-TEA (n = 8), gavage VES (n = 8), and gavage control (n = 9). Five separate sections of each slide/tumor were scored for Ki-67 positive cells. Data are depicted as mean ± SE Ki-67 positive cells/field. * Designates a significant reduction in proliferating cells in comparison with control.

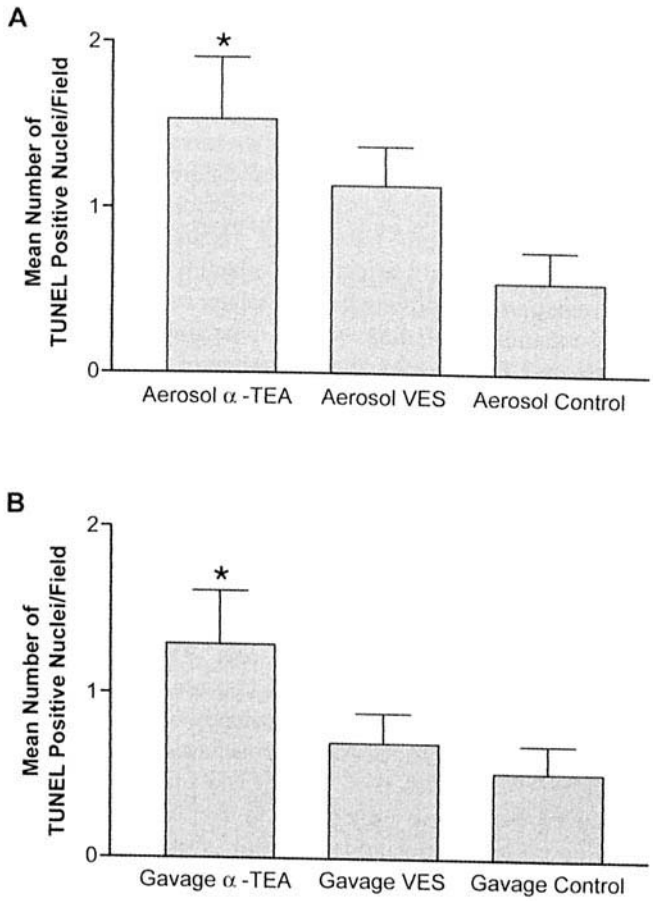

Figure 6. α-TEA, but not VES, significantly induced apoptosis. Comparisons of deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL)-positive nuclei in tumor sections (5 μm) obtained from mice treated with liposomal formulated α-TEA, VES, or liposome control delivered by aerosol (A) or delivered by gavage (B) are given. Number of tumors/treatment group examined were aerosol α-TEA (n = 8), aerosol VES (n = 8), aerosol control (n = 9), gavage α-TEA (n = 8), gavage VES (n = 8), and gavage control (n = 9). The number of TUNEL-positive nuclei were scored in 16 microscopic fields (×400)/tumor. The data are depicted as the mean ± SE of number of TUNEL-positive nuclei/ field. * Designates a significant increase in apoptotic cells in comparison to control.

control mice had a mean ± SE of 0.55 ± 0.19 and 0.54 ± 0.17 apoptotic cells/field, respectively ($P < 0.03$ and $P < 0.05$; Fig. 6A and B). Tumors from mice treated with VES delivered by aerosol or gavage exhibited increased numbers of apoptotic cells but showed no significant increase in apoptotic cells in comparison with control animals, that is, mean ± SE of 1.14 ± 0.23 and 0.71 ± 0.18 apoptotic cells/field ($P < 0.09$ and $P < 0.70$, respectively; Fig. 6A and B).

Discussion

The goal of the studies reported here was to compare the antitumor properties of two vitamin E derivatives *in vitro* and *in vivo* when both compounds were formulated into liposomes and delivered either by aerosol or orally by gavage. Cell culture studies reported here showed that both vitamin E derivatives were effective antitumor agents, capable of inducing dose-dependent DNA synthesis arrest and cell death by apoptosis as well as inhibiting colony formation of murine mammary cancer cells.

In vivo, both α-TEA and VES significantly decreased primary tumor burden when delivered by aerosol. The effectiveness of VES delivered by aerosol is a novel finding because previous studies of antitumor effects of VES in animal xenograft and allograft models administered VES intraperitoneally (12, 22–26). Although both vitamin E derivatives when delivered by aerosol were effective in reducing metastasis, α-TEA decreased lung and lymph node metastasis to a greater degree than VES.

α-TEA was effective when delivered orally; however, oral delivery of VES was ineffective. The ineffectiveness of oral VES delivery was expected based on *in vitro* analyses demonstrating a need for the intact compound for antitumor activities (19, 27–29), and potential for de-esterification by intestinal esterases (30, 31).

A comparison of the tumor efficacy of α-TEA and VES liposomal formulations delivered by aerosol versus gavage showed α-TEA to be effective when delivered by either route, whereas VES was effective only when delivered by

aerosol. As mentioned above, the factor most likely contributing to this difference is that esterases in the intestinal tract most likely cleaved the ester-linked succinic acid moiety of VES, producing free succinic acid and RRR-α-tocopherol, neither of which exhibits anticancer properties (12). Other possible reasons for the difference in efficacy between aerosol and oral delivery include differences in particle size (2.0 μm for aerosol versus 4–10 μm for gavage), which might affect bioavailability; differences in total amounts administered; and differences in the role a liver vitamin E–binding protein, α-tocopherol transfer protein (α-TTP) that selectively incorporates natural vitamin E (RRR-α-tocopherol) from the diet, might play in bioavailability.

There do not appear to be any stability differences between α-TEA and VES liposomal preparations. On the basis of our limited studies with liposomal α-TEA and liposomal VES, lyophilized preparations appear to be stable over several weeks. We did not see any difference in the liposomal-formulated compounds before or after 15 mins of nebulization. Lyophilized α-TEA and VES were resuspended in water, and formation of liposomes and possibly crystals was observed by light microscopy with a polarizing filter to visualize crystals. After aerosolization, the material remaining in the reservoir of the nebulizer was again visualized. No crystals were observed in either the α-TEA or VES liposomal formulations before or after aerosolization. Aerosol concentrations of α-TEA or VES over the 15-min nebulization period gave a similar pattern. High-performance liquid chromatography (HPLC) and UV analyses showed the retention time and area under the peak curve for α-TEA and VES to be similar before and throughout aerosolization. These observations suggest that there were no chemical or physical alterations of α-TEA or VES during the aerosolization process. In summary, lyophilized formulations of α-TEA and VES appear to be stable for several weeks and, once resuspended in water, the liposomal formulations appear to be very stable before and after aerosolization.

Vitamin E compounds are relatively nontoxic and are extremely well tolerated by humans, with reported side effects being of a relatively minor nature, primarily gastrointestinal symptoms, generalized dermatitis, and fatigue in a subset of subjects (32). The Food and Nutrition Board has established the Tolerable Upper Intake Level for vitamin E to be 1000 mg/day for healthy adults aged 19 to 70 (33). Regarding the safety of VES and α-TEA, limited studies in mice suggest that they are also relatively nontoxic because studies have failed to achieve an oral LD50 dose, and daily administration (either orally or via aerosolization) for up to 36 days has not produced any overt signs of toxicity such as weight loss or observable changes in behavior. More studies need to be conducted.

Findings reported here suggest that liposomal formulations of vitamin E derivatives may be beneficial to antitumor efficacy. Regarding oral administration, it is important to note that previous studies reported that α-TEA was not effective in reducing tumor burden when delivered orally in a peanut oil suspension (12). Thus, the positive results achieved here are likely due to liposomal formulation as well as increased dosage achieved by twice-a-day gavaging.

Data showing that α-TEA administered by aerosol inhibited microscopic metastases in lymph nodes is noteworthy because a high percentage of lymph nodes in the α-TEA treatment group did not show any metastases. In comparison, when VES was administered via aerosol, it was effective in significantly reducing total number of microscopic metastases in the lungs, but it it was ineffective in significantly reducing lymph node metastasis. This observation may reflect tissue uptake differences between VES and α-TEA or perhaps tissue-specific inactivation of VES by esterase cleavage. Future studies will be required to understand this difference.

Analyses of cell proliferation and apoptosis by staining tumor sections for the nuclear Ki-67 antigen that is a biomarker for proliferating cells, and staining for TUNEL that is a biomarker for apoptosis, suggested that both α-TEA (aerosol and oral) and VES (aerosol only) reduced tumor burden by decreasing cell proliferation and increasing apoptosis. This correlates directly with mechanisms documented in cell culture studies. Although the ability to decrease cell proliferation and increase apoptosis *in vivo* in comparison with controls was statistically significant for α-TEA only, it is important to point out that no significant differences were detected between the ability of α-TEA (aerosol) and VES (aerosol) to decrease cell proliferation.

In summary, data reported here are promising in that they show that a novel vitamin E analog, α-TEA, when formulated into liposomes and administered either by aerosol or orally, has the ability to decrease primary tumor burden and reduce lung and lymph node metastasis in a syngeneic tumor model. This positive antitumor activity occurred without any overt toxic effects. In comparison, the vitamin E derivative, VES, although effective at decreasing primary tumor burden when formulated into liposomes and delivered via aerosol, was less effective at inhibiting metastasis and had no antitumor properties when delivered orally. These studies support the further development of α-TEA as a potential chemotherapeutic agent.

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