Novel Vitamin E Analogue Decreases Syngeneic Mouse Mammary Tumor Burden and Reduces Lung Metastasis[1]

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Abstract

A nonhydrolyzable ether analogue of RRR-α-tocopherol, 2,5,7,8-tetramethyl-2R-(4R, 8R, 12-trimethyltridecyl)chroman-6-yloxyacetic acid, called RRR-α-tocopheryloxyacetic acid or RRR-α-tocopherol ether-linked acetic acid analogue (α-TEA), exhibits antitumor activity *in vitro* and *in vivo* using a syngeneic BALB/c mouse mammary tumor model (line 66 clone 4 stably transfected with green fluorescent protein). Treatment of cells with 5, 10, and 20 μg/ml α-TEA for 3 days produced 6, 34, and 50% apoptosis, respectively, and treatment of cells with 10 μg/ml for 2, 3, 4, and 5 days produced 20, 35, 47, and 58% apoptosis, respectively. A liposomal formulation of α-TEA administered by aerosol reduced s.c. tumor growth and lung metastasis. α-TEA-treated animals showed a significant decrease in tumor volumes over 17 days of aerosol treatment ($P < 0.001$). Forty percent of aerosol as well as untreated control mice had visible, macroscopic lung metastases *versus* none (0%) of the α-TEA-treated mice. On the basis of fluorescence microscopic examination of the surface (top and bottom) of flattened whole left lung lobes, an average of 60 ± 15 and 102 ± 17 *versus* 11 ± 4 fluorescent microscopic metastases was observed in aerosol control and untreated control *versus* α-TEA-treated animals, respectively. α-TEA formulated in ethanol + peanut oil (5 mg/mouse/day) delivered by gavage did not reduce s.c. primary tumor burden; however, fluorescent microscopic lung metastases were significantly reduced ($P < 0.0021$). In summary, α-TEA formulated in liposomes and delivered by aerosol is a potent antitumor agent and reduces lung metastasis.

Introduction

Several studies have described the potent antitumor activity of RRR-α-tocopheryl succinate (VES),[3] a hydrolyzable ester derivative of RRR-α-tocopherol (natural vitamin E). Prasad and Edwards-Prasad (1) were the first to describe the capacity of VES but not other forms of vitamin E to induce morphological alterations and growth inhibition of mouse melanoma B-16 cells and to suggest that VES might be a useful therapeutic agent for tumors. Additional studies have demonstrated that VES is a potent growth inhibitor of a wide variety of epithelial cancer cell types, including breast, prostate, lung, and colon, as well as hematopoietic-lymphoid leukemia and lymphoma cells, *in vitro* (2–7).

Recent studies have demonstrated VES to have antitumor and antimetastatic activity in animal xenograft and allograft models when administered i.p. (8–12), suggesting a possible therapeutic potential. VES administered i.p. or p.o. has also been shown to have inhibitory effects on carcinogen [benzo(*a*)pyrene]-induced forestomach carcinogenesis in mice, suggesting potential as an anticarcinogenic agent (13). Investigations have demonstrated that VES induces concentration- and time-dependent inhibition of cancer cell growth via DNA synthesis blockage, induction of cellular differentiation, and induction of apoptosis (5, 6, 10, 14–16).

VES is noteworthy not only for its induction of growth inhibitory effects on tumor cells but also for its lack of toxicity toward normal cells and tissues (2–7, 11). The use of a nonhydrolyzable VES derivative has shown that it is the intact compound and not either of its cleavage products (namely, RRR-α-tocopherol or succinic acid) that are responsible for the antiproliferative effects (4). Thus, the antiproliferative actions of this vitamin E derivative are not related to antioxidant properties.

In an effort to develop a clinically useful vitamin E-based chemotherapeutic agent and to administer it in a clinically

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[3] The abbreviations used are: VES, vitamin E succinate; α-TEA, 2,5,7,8-tetramethyl-2R-(4R, 8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid; 66cl-4-GFP, Balb/c mouse mammary tumor line 66 clone 4 stably transfected with GFP; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; PARP, poly(ADP-ribose) polymerase; THF, tetrahydrofuran; VEH, vehicle.

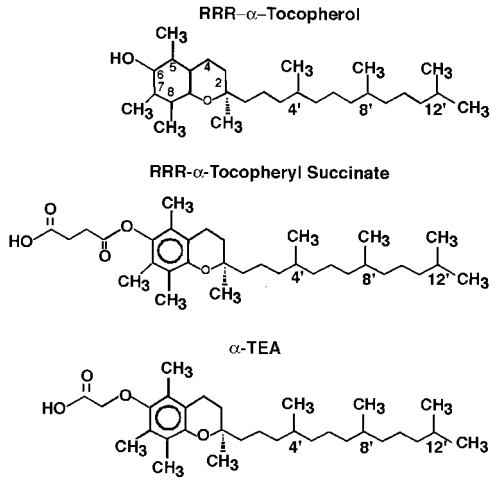

Fig. 1. Structures for RRR-α-tocopherol, VES, and α-TEA. Common names for RRR-α-tocopherol are D-α-tocopherol or natural vitamin E. Chemical name is 2,5,7,8-tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol. M_r = 430.69, empirical formula = $C_{29}H_{50}O_2$. Common names for VES are D-α-tocopheryl succinate, D-α-tocopherol acid succinate, and RRR-α-tocopheryl succinate. M_r = 530.76, empirical formula = $C_{33}H_{54}O_5$. Common name for α-TEA is ethyl 6–0-carboxymethyl-α-tocopherol. Chemical name is 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid, referred to as RRR-α-tocopheryloxyacetic acid or RRR-α-tocopherol ether-linked acetic acid analogue. M_r = 488, empirical formula = $C_{31}H_{52}O_4$.

relevant manner, a nonhydrolyzable ether analogue of RRR-α-tocopherol, namely, α-TEA (Fig. 1) has been produced. As shown in Fig. 1, α-TEA differs from RRR-α-tocopherol by an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head by an ether linkage. VES differs from α-TEA in that a succinic acid moiety is linked by an ester linkage to the phenol at carbon 6 of the chroman head (Fig. 1). α-TEA, like VES, is capable of inducing human breast (MCF-7, MDA-MB-231, MDA-MB-435), ovarian (CP-70), cervical (ME-180), endometrial (RL-952), prostate (LnCaP, PC-3, DU-145), colon (HT-29, DLD-1), lung (A-549), and lymphoid (Raji, Ramos, Jurkat) cells to undergo apoptosis. Also like VES, α-TEA does not induce apoptosis in normal human mammary epithelial cells or normal PrEC human prostate epithelial cells.

Because α-TEA is a lipid that is insoluble in water, aerosol delivery of liposomal preparations was chosen as a potentially effective, clinically relevant method of delivery. Aerosol delivery of lipophilic chemotherapeutic agents to mice has been shown to increase drug concentrations in the lungs and other organs compared with i.m. injection, as well as to increase drug effectiveness against breast, lung, and colon xenograft growth in nude mice when compared with either i.m. injection or p.o. administration (17, 18). In addition, this method of delivery appears to be highly effective against pulmonary metastasis of melanoma and osteosarcoma in mice (19). Of major importance, aerosol delivery of drugs shows increased efficacy and is well tolerated by humans (20). α-TEA was tested in this study using either oral (peanut oil VEH) or aerosol (liposome VEH) delivery.

In this article, we report that α-TEA, a novel RRR-α-tocopherol analogue, is a potent concentration- and time-dependent inducer of apoptosis of murine mammary tumor cells *in vitro* and can effectively inhibit s.c. murine mammary tumor burden and lung metastasis when formulated in liposomes and delivered by aerosolization.

Materials and Methods

α-TEA Production in Sufficient Quantity for Animal Studies. For scaled-up production, α-TEA was prepared as follows. NaH (5.0 g, 124.9 mmol) was suspended in dry THF (300 ml) and stirred under argon at 0°C for 10 min before the addition via cabbula of RRR-α-tocopherol (ICN Biomedicals; 41.3 g, 96.1 mmol) that was dissolved in 100 ml of dry THF. This mixture was stirred at 0°C for 15 min while under argon pressure, then ethyl bromoacetate (19.26 g, 115.3 mmol) was added via syringe. The reaction was monitored by thin layer chromatography (hexane:ethyl acetate = 10:1, R_f = 0.65) and was completed in 3.5 h. The reaction mixture was diluted with 150 ml of CH_2Cl_2, washed with saturated NaCl solution (150 ml × 3) until the organic phase was clear, dried over anhydrous Na_2SO_4, and the solvent removed under a reduced pressure. The crude product still contained a small amount of free α-tocopherol, which could be removed by column chromatography on silica gel using hexane:ethyl acetate (30:1 to 20:1) to yield pure product α-TEA ethyl ester (41.6 g, 84%).

The α-TEA ethyl ester (21.0 g, 40.7 mmol) was dissolved in 250 ml of THF, then 75 ml of 10% KOH (122.1 mmol) was added and the mixture stirred at room temperature for 6 h. The reaction was monitored by thin layer chromatography ($CHCl_3$:methanol:CH_3COOH = 97:2.5:0.5, R_f = 0.18) and was quenched with 100 ml of water. The solution was adjusted to pH 3 using 1 N HCl and the product extracted with CH_2Cl_2 (100 ml × 4), washed with saturated NaCl solution, dried over Na_2SO_4, and the solvent was removed under a reduced pressure, providing the final product α-TEA as a white waxy solid (18.5 g, 93%) with a melting point of 54–55°C.

Murine Mammary Tumor Cell Line. 66cl-4-GFP cells are a mouse mammary tumor cell line originally derived from a spontaneous mammary tumor in a Balb/cfC3H mouse and later isolated as a 6-thioguanine-resistant clone (21, 22). Subsequently, these cells were stably transfected with the enhanced GFP by Dr. L-Z. Sun (one of the authors). 66cl-4-GFP cells are highly metastatic with ~40% of animals developing visible macroscopic metastases and 100% of animals developing florescent microscopic metastases in the lungs 26 days after s.c. injection of 2×10^5 tumor cells into the inguinal area. Before use in these studies, cells were sent to the University of Missouri Research Animal Diagnostic and Investigative Laboratory (Columbia, MO) where they were certified to be pathogen free. 66cl-4-GFP cells were maintained as monolayer cultures in growth media: McCoy's media (Invitrogen Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA); 100 μg/ml streptomycin; 100 IU/ml penicillin; 1 × (vol/vol) nonessential amino acids; 1× (vol/vol) MEM vitamins; 1.5 mM sodium pyruvate; and 50 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO). Treatments were given using this same McCoy's supplemented media, except fetal

bovine serum content was reduced to 5%. Cultures were routinely examined to verify absence of *Mycoplasma* contamination.

Determination of Apoptosis by Morphological Evaluation of DAPI-stained Nuclei. Apoptosis was determined using previously published procedures (23). Briefly, 1×10^5 cells/well in 12-well plates were cultured overnight to permit attachment. Next, the cells were treated with α-TEA, VES (Sigma Chemical Co.), or ethanol control (0.1% ethanol F.C. vol/vol) in experimental media at various concentrations of α-TEA and VES for various time intervals. After treatment, floating cells plus scraped-released adherent cells were pelleted by centrifugation for 5 min at 350 $\times$ *g*, washed one time with PBS [137 mM NaCl, 2.7 mM KCL, 10.4 mM Na_2HPO_4, 10.5 mM KH_2PO_4 (pH 7.2)], and stained with 2 μg/ml DAPI (Boehringer Mannheim, Indianapolis, IN) in 100% methanol for 15 min at 37°C. Cells were viewed at $\times$400 magnification with a Zeiss ICM 405 fluorescent microscope using a 487701 filter. Cells in which the nucleus contained condensed chromatin or cells exhibiting fragmented nuclei were scored as apoptotic. Data are reported as percentage of apoptotic cells/cell population (*i.e.,* number apoptotic cells/total number of cells counted). Three different microscopic fields were examined, and 200 cells counted at each location for a minimum of 600 cells counted/slide. Apoptotic data are presented as mean $\pm$ SD for three independently conducted experiments.

Western Immunoblot Detection of PARP Cleavage Fragment. PARP cleavage was analyzed as an alternate method for detecting apoptosis. 66cl-4-GFP cells were treated as described above for the DAPI assay. After the PBS wash, cells were suspended in lysis buffer [1$\times$ PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM DTT, 2 mM sodium orthovanadate, 10 μg/ml phenylmethylsulfonyl fluoride] for 30 min at 4°C, vortexed, and supernatants collected by centrifugation at 15,000 $\times$ *g* for 10 min. Protein concentrations were determined using the Bio-Rad (Bradford) protein assay (Bio-Rad Laboratories, Hercules, CA), and samples (100 μg/lane) were resolved on 7.5% SDS-polyacrylamide gels electrophoresed under reducing conditions. Proteins were electroblotted onto a nitrocellulose membrane (0.2-μM pore Optitran BA-S-supported nitrocellulose; Schleicher and Schuell, Keene, NH). After transfer, membranes were blocked with blocking buffer [25 mM Tris-HCl (pH 8.0), 125 mM NaCl, 0.5% Tween 20, and 5% nonfat dry milk] for 45 min at room temperature. Immunoblotting was performed using 1 μg of primary rabbit antihuman PARP antibody [PARP (H-250), Santa Cruz Biotechnology, Santa Cruz, CA], and horseradish peroxidase-conjugated goat antirabbit immunoglobulin was used as the secondary antibody (Jackson Immunoresearch Laboratory, West Grove, PA) at a 1:3000 dilution. Horseradish peroxidase-labeled bands from washed blots were detected by enhanced chemiluminescence (Pierce, Rockford, IL) and autoradiography (Kodak BioMax film; Rochester, NY).

BALB/c Mice. Female Balb/cJ mice at 6 weeks of age (~25 g in weight) were purchased from Jackson Labs (Bar Harbor, ME) and were allowed to acclimate at least 1 week. Animals were housed at the Animal Resource Center at the University of Texas at Austin at 74 $\pm$ 2°F with 30–70% humidity and a 12-h alternating light-dark cycle. Animals were housed 5/cage and given water and standard lab chow (Harlan Teklad no. 2018 Global 18% Protein Rodent Diet; Madison, WI) *ad libitum*. 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, centrifuged, and resuspended in McCoy's media, containing no supplements, at a density of 2×10^5/100 μl. Mice were injected in the inguinal area at a point equal distance between the fourth and fifth nipples on the right side using a 23-gauge needle.

Fifty mice were assigned (10/group) into five groups [(group 1: untreated control; group 2: liposome/aerosol control; group 3: liposomal α-TEA aerosol treatment; group 4: peanut oil + ethanol/gavage control; group 5: α-TEA in ethanol + peanut oil/gavage treatment) such that the mean tumor volume of each group was closely matched. Each group had an average tumor volume/group = 6.35 mm^3 at the start of treatments, which were begun 9 days after tumor cell inoculation. Ten additional mice, not injected with tumor cells, were treated with aerosol or oral α-TEA (5 each) for 17 days, removed from treatment, and observed for an additional 11 months to evaluate long-term safety. Tumors were measured using calipers every other day, and volumes were calculated using the formula: volume (mm^3) = [width (mm^2) $\times$ length (mm)]/2 (24). Body weights were determined weekly.

Preparation of α-TEA Solubilized in Peanut Oil for Delivery by Gavage. α-TEA was dissolved in 100% ethanol (400 mg/ml) and then mixed with peanut oil (100% peanut oil; nSpired Natural Foods, San Leandro, CA) at a ratio of 1:8 (v/v). Control treatment consisted of equivalent amounts of ethanol and peanut oil as contained in the α-TEA treatment. The mixtures were vortexed vigorously, stored at 4°C, and brought back to room temperature and revortexed vigorously immediately before administration.

Preparation of α-TEA Liposomes for Delivery by Aerosol. An α-TEA/liposome ratio of 1:3 (w/w) was determined empirically to be optimal by methods described previously (18). To prepare the α-TEA/lipid combination, the components were first brought to room temperature. The lipid (1,2-dilauroyl-sn-glycero-3-phosphocholine; Avanti Polar-Lipids, Inc., Alabaster, AL) at a concentration of 120 mg/ml was dissolved in tertiary-butanol (Fisher Scientific, Houston, TX) then sonicated to obtain a clear solution. α-TEA at 40 mg/ml was also dissolved in tertiary-butanol and vortexed until all solids were dissolved. The two solutions were then combined in equal amounts (v:v) to achieve the desired ratio of 1:3 α-TEA/liposome, mixed by vortexing, frozen at $-80°$ C for 1–2 h, and lyophilized overnight to a dry powder before storing at -20°C until needed. Each treatment vial contained 75 mg of α-TEA.

Aerosol Delivery. Aerosol was administered to mice as described previously (18). Briefly, an air compressor (Easy Air 15 Air Compressor; Precision Medical, Northampton, PA) producing a 10 liter/min airflow was used with an AeroTech

II nebulizer (CIS-US, Inc., Bedford, MA) to generate aerosol. The particle size of α-TEA liposome aerosol discharged from the AeroTech II nebulizer was determined using an Anderson Cascade Impactor to have a mass median aerodynamic diameter of 2.01 μm, with a geometric SD of 2.04. About 30% of such particles when inhaled will deposit in the respiratory tract of the mouse and the remaining 70% will be exhaled (18). Aerosolized liposomal drug delivery also includes oral ingestion because of swallowing of nasal and lung mucus secretions, and in mice, there is the added oral ingestion factor created by their inborn grooming behavior, which rapidly facilitates the translocation of any drug deposited onto the fur into the digestive tract (18).

Before nebulization, the α-TEA/lipid powder was brought to room temperature and reconstituted by adding 3.75 ml of distilled water to achieve the final desired concentration of 20 mg/ml α-TEA. The mixture was allowed to swell at room temperature for 30 min with periodic inversion and vortexing and then added to the nebulizer. 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. Animals were exposed to aerosol until all α-TEA/liposome was aerosolized (~15 min).

Oral Delivery. α-TEA/peanut oil mixture was brought to room temperature and revortexed vigorously immediately before administration by gavage 100 μl/mouse/day (final concentration 5 mg α-TEA/mouse/day).

Lung Metastasis. Macroscopic metastases in all five lung lobes were counted visually at time of sacrifice. Fluorescent microscopic metastases were counted using a Nikon fluorescence microscope (TE-200; ×200 magnification). For analyses, lung tissue (left lung lobes) was flattened, and the entire surface (top and bottom) scored for fluorescent green microscopic metastases. Fluorescent microscopic metastases were scored by size into three size grouping: <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.

Terminal Deoxynucleotidyl Transferase-mediated Nick End Labeling 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/tumor. Data are presented as the mean ± SE number of apoptotic cells counted in three separate tumors from each group.

Statistical Analyses. Animal numbers for experiments were determined by power calculations derived from data generated by preliminary pilot studies. Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor ANOVA using SPSS (SPSS, Inc., Chicago, IL). Difference in number of fluorescent microscopic metastases/group were determined using the 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 Induce Apoptosis in 66cl-4-GFP Cells, *in Vitro*. Previous studies indicate that VES is a potent apoptotic inducer in many human cancer cell lines, including breast cancer. For comparative purposes, we included VES in the *in vitro* analyses of α-TEA induced apoptosis. BALB/c mammary cancer 66cl-4-GFP cells were treated with VES or α-TEA, and apoptosis was assessed by morphological analyses of DAPI-stained cells for condensed nuclei and fragmented DNA.

Nuclei from 66cl-4-GFP cells treated with 10 μg/ml α-TEA or VES for 3 days exhibited condensed and fragmented DNA, characteristics of apoptosis, whereas nuclei from untreated cells did not exhibit these morphological characteristics (Fig. 2*A*). The level of apoptosis of 66cl-4-GFP cells treated for 3 days with 2.5, 5, 10, and 20 μg/ml α-TEA or VES in comparison to controls was 2.5-, 3-, 17- and 24-fold higher for α-TEA and 1.5-, 2.5-, 8-, and 17-fold higher for VES (Fig. 2*B*). Untreated, VEH, and ethanol controls exhibited background levels of apoptosis: 2, 2, and 3%, respectively (Fig. 2*B*).

α-TEA was shown to induce apoptosis in a time-dependent manner. 66cl-4-GFP cells treated with 10 μg/ml α-TEA for 2–5 days exhibited 10-, 18–24-, and 28-fold increases in apoptosis over baseline (2%), respectively (Fig. 2*C*). Induction of apoptosis was confirmed by the presence of PARP cleavage after treatment of 66cl-4-GFP cells with 5, 10, and 20 μg/ml α-TEA for 48 h (Fig. 2*D*). The M_r 84,000 cleavage fragment of PARP was evident at both 10 and 20 μg/ml α-TEA treatment, whereas only intact PARP protein was detected in cells treated with 5 μg/ml α-TEA or in the untreated control cells (Fig. 2*D*).

Aerosol Characteristics of α-TEA Incorporated into Liposomes. 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 (18): delivered drug dose = drug concentration (μg/liter) × volume of air intake/min/unit of body weight (1 ml/min/g body weight) × 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 were deposited in the respiratory tract of each mouse each day. Thus, for the 17-day treatment period, we estimate that each mouse received 612 μg of α-TEA from liposomal aerosol delivery. Although mice received 5 mg/day for 13 days of treatment by gavage for a total of 65 mg, we do not know the bioavailability of α-TEA delivered by this method.

Liposomal α-TEA/Aerosol Treatment Suppressed 66cl-4-GFP Tumor Growth in BALB/c Mice and Reduced Lung Macroscopic and Microscopic Metastases. Mean tumor volumes of the liposomal α-TEA/aerosol treatment group, in comparison to aerosol control, was significantly lower over

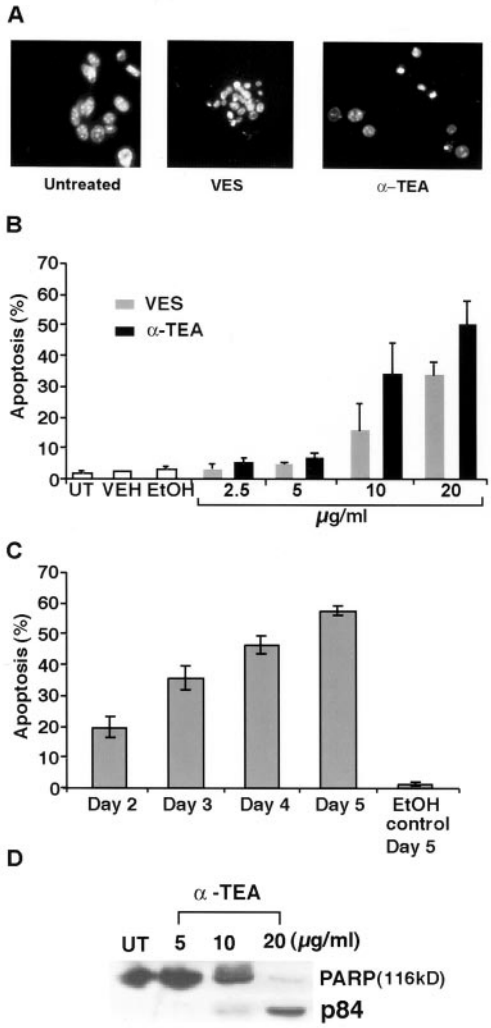

Fig. 2. Documentation of α-TEA-induced apoptosis. *A*, 66cl-4-GFP murine mammary cells were treated with 10 μg/ml α-TEA or VES (positive control) or untreated and cultured for 3 days. Cells were harvested, nuclei were labeled with the fluorescent DNA binding dye DAPI, and cells were examined using a Zeiss ICM 405 fluorescent microscope (×400), using a 487701 filter. Nuclei of cells with condensed chromatin or fragmented nuclei were scored as apoptotic. Data are representative of numerous experiments. *B* and *C*, analyses of nuclei of DAPI-stained cells show α-TEA to induce apoptosis in a concentration- and time-dependent manner. Data are depicted as mean ± SD of three separate experiments. *D*, additional evidence of α-TEA induction of apoptosis by PARP cleavage. 66cl-4-GFP cells were treated with 5, 10, or 20 μg/ml α-TEA for 48 h, and cellular lysates were analyzed for PARP cleavage by western immunoblot analyses. Data are representative of three separate experiments.

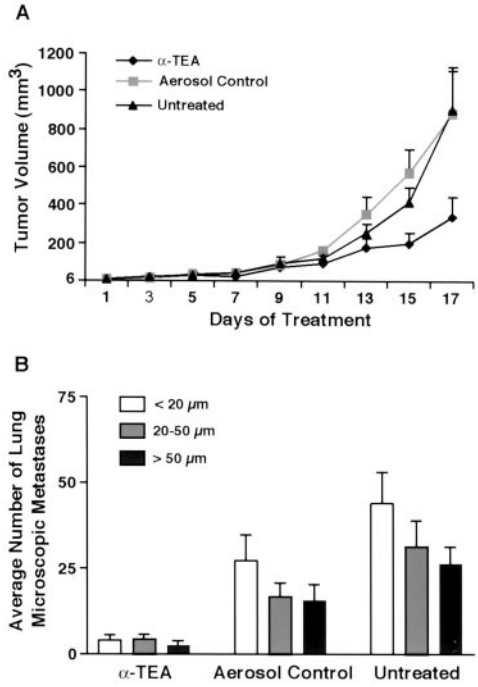

Fig. 3. Liposomal α-TEA delivery by aerosol inhibits tumor burden and microscopic metastases in the lung. *A*, 66cl-4-GFP cells at 2×10^5/mouse were injected into the inguinal area at a point equal distance between the fourth and fifth nipples. Nine days after tumor injection, mice were assigned to control and α-TEA treatment groups such that the mean tumor volume of each group was closely matched (average tumor volume/group = 6.35 mm^3). Mice (10/group) were not treated or treated daily with liposomal α-TEA/aerosol or aerosol only for 17 days. Tumor volume/mouse was determined at 2-day intervals. Tumor volumes (mm^3) are depicted as mean ± SE. *B*, with the aid of a Nikon fluorescent microscope, the number of fluorescent microscopic metastases on the surface (top and bottom) of flattened left lung lobes from liposomal α-TEA/aerosol (8 mice), aerosol only (10 mice), and untreated mice (10 mice) were determined. Data are depicted as mean ± SE.

Table 1 66cl-4-GFP mammary cancer cell lung metastasis in Balb/c mice receiving liposomal α-TEA or liposome control by aerosol or no treatment

Treatments	No. of animals/group with visible lung macroscopic metastases[a]	No. of visible lung macroscopic metastases/animal[b]
No Treatment	4/10	3.25 ± 1.7
Aerosol/liposome control	4/10	4.25 ± 0.5
Aerosol/liposomal α-TEA	0/10	0

[a] Macroscopic metastases 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 mean ± SD of visible lung macroscopic metastases observed in the four lung macroscopic metastases bearing animals in the two control groups.

17 days of treatment ($P < 0.001$; Fig. 3*A*). At sacrifice, all five lung lobes from each animal were examined visually for macroscopic metastases. No visible macroscopic metastases were seen in the α-TEA treatment group, whereas 40% each of untreated and aerosol control animals exhibited macroscopic metastases with an average of 3.25 ± 1.7 and 4.25 ± 0.5 visible tumors/animal, respectively (Table 1). Use of a Nikon fluorescence microscope permitted measurement of green fluorescing microscopic metastases into three size groupings (<20 μm, 20–50 μm, and >50 μm). 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 metastases of all three size groupings in the α-TEA treatment group in comparison to either the aerosol or untreated controls (Fig. 3*B*). The mean number of microscopic metastases in the α-TEA treatment group (11.4 ± 3.5; $n = 8$), in com-

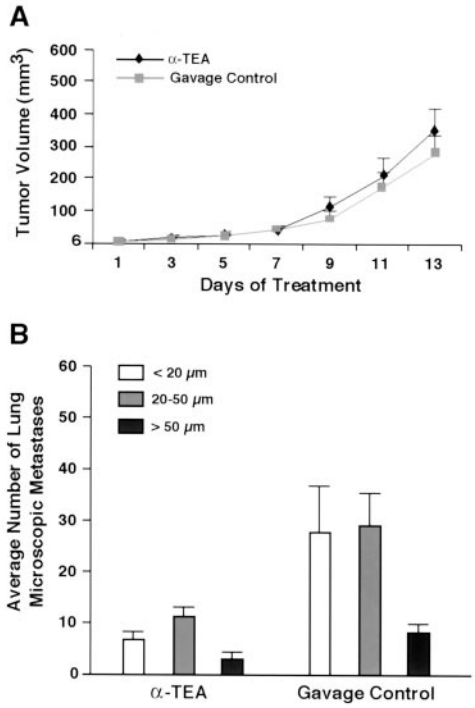

Fig. 4. *A* and *B*, α-TEA administered by gavage does not inhibit primary tumor burden but does reduce lung microscopic metastases. These studies were conducted in parallel with the studies described in Fig. 3 and differ only in that the mice were treated daily by gavage with 5 mg of α-TEA dissolved in ethanol and peanut oil or ethanol + peanut oil VEH control and were treated for only 13 days. Tumor volume and lung microscopic metastases data were determined as described in the legend to Fig. 3. Data are depicted as mean ± SE.

parison to aerosol control (60.0 ± 15; $n = 10$), was significantly reduced ($P < 0.002$). Although the mean number of microscopic metastases in the aerosol control group *versus* the untreated control ($n = 10$) was reduced (60 ± 15.2 *versus* 101.7 ± 17.0), the difference was not significant ($P < 0.063$; Fig. 3*B*).

Although this exact experiment has not been repeated, we have conducted several studies evaluating the antitumor properties of α-TEA using the 66cl-4-GFP syngeneic mammary cancer model. Data from experiments comparing the antitumor properties of aerosolized α-TEA formulated in liposomes with different aerosolized vitamin E compounds, and data from an experiment comparing α-TEA alone and in combination with 9-nitrocamptothecin, consistently and repeatedly show that tumor volume, visible macroscopic lung metastases, and fluorescent microscopic lung metastases are significantly reduced in comparison to controls (unpublished data).

Delivery of α-TEA by Gavage Did Not Reduce Tumor Burden at the s.c. Inoculation Site but Did Reduce the Number of Lung Microscopic Metastases. In contrast to liposomal α-TEA/aerosol treatment, mean tumor volumes from mice receiving 5 mg/day/mouse α-TEA formulated in peanut oil by gavage did not differ from the mean tumor volume of the gavage control (Fig. 4*A*). However, administration of α-TEA by gavage significantly reduced the number of lung microscopic metastases (21.2 ± 3.5 *versus* 65.5 ± 15.3; $P < 0.0021$; $n = 10$ for both groups). The average numbers of microscopic metastases, based on three size groupings (<20 μm, 20–50 μm, >50 μm), were 6.8 ± 1.5, 11.3 ± 1.8, and 3.1 ± 1.2 for mice administered α-TEA by gavage, whereas average numbers of microscopic metastases in gavage control mice were 27.9 ± 9.0, 29.2 ± 6.3, and 8.4 ± 1.5, respectively (Fig. 4*B*).

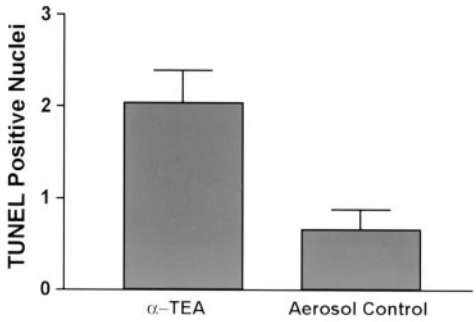

Fig. 5. α-TEA induces 66cl-4-GFP cells to undergo apoptosis *in vivo*. α-TEA induction of apoptosis was determined using 5-μm tumor sections derived from liposomal α-TEA/aerosol treatment and liposome aerosol control group animals ($n = 3$). Apoptotic cells were determined using ApopTag *in Situ* Apoptosis Detection kit (Intergen, Purchase, NY).

No differences in mean body weights among any of the treatment or control groups were observed (data not shown). Nontumor-bearing mice that were treated with either aerosol/α-TEA or gavage/α-TEA for 17 days and then kept for 11 months to assess long-term effects did not show any adverse effects of the α-TEA treatments.

Histological Evaluation of Tumors. Tumors from aerosol control and α-TEA-treated animals were examined after H&E staining. No evidence for α-TEA-induced differentiation was seen because tumors from both control and α-TEA-treated animals were judged to be poorly differentiated spindle cell carcinomas with high mitotic index.

Induction of Apoptosis by α-TEA *in Vivo*. In view of the *in vitro* data showing that α-TEA inhibits 66cl-4-GFP tumor cell growth via induction of apoptosis, three tumors from each of the liposomal α-TEA/aerosol treatment and aerosol control groups were examined for apoptosis using terminal deoxynucleotidyl transferase-mediated nick end labeling staining of 5-μm tumor sections. Tumors from mice treated with α-TEA had a mean ± SE of 2.04 ± 0.23 apoptotic cells/field, whereas tumors from aerosol control mice had a mean ± SE of 0.67 ± 0.15 apoptotic cells/field (Fig. 5).

Discussion

Analyses of natural occurring vitamin E compounds and vitamin E analogues for antitumor activity for breast cancer has been reviewed recently (25). Our goal in developing α-TEA is to produce and characterize a tocopherol-based antitumor agent with favorable characteristics suitable for use in humans. Studies reported here demonstrate that the novel vitamin E derivative referred to as α-TEA is an effective antitumor agent, inducing 66cl-4-GFP cells to undergo ap-

optosis both *in vitro* and *in vivo*. Furthermore, α-TEA reduces lung metastasis and does not exhibit toxicity to normal cells and tissues *in vivo*.

Structurally, vitamin E (RRR-α-tocopherol) consists of a chroman head with two rings: one phenolic and one heterocyclic and a saturated phytyl tail (Fig. 1; Ref. 26). α-TEA is a synthetic derivative of vitamin E (RRR-α-tocopherol) with a nonhydrolyzable acetic acid moiety attached to the no. 6 carbon of the phenolic ring of the chroman head by an ether linkage (Fig. 1). In contrast, VES has an ester-linked succinic acid moiety attached to the no. 6 carbon of the phenolic ring of the chroman head (Fig. 1). Thus, α-TEA should be resistant to hydrolysis by cellular esterases, possibly providing a superior apoptotic inducing agent for *in vivo* use in comparison to VES.

This is the first article of the antitumor properties of α-TEA, both *in vitro* and *in vivo*. There are numerous studies showing that natural vitamin E (RRR-α-tocopherol) does not possess antitumor properties for epithelial cells and that VES can induce tumor cells of epithelial origin to undergo apoptosis (1–13, 16). VES has been shown to exhibit antitumor properties when administered i.p. but not by gavage, with the exception of experiments involving stomach cancer (8–13). In this study, we choose to administer α-TEA either via liposomal/aerosol or gavage, two established delivery methods for treatment of cancers both in clinical and home environments.

Administration of α-TEA by aerosol was superior to administration by gavage in these studies in that α-TEA administered by gavage did not reduce tumor burden at the site of s.c. tumor injection in comparison to tumor burden of control mice. Nevertheless, it is of interest that the number of lung microscopic metastases were reduced in comparison to control when α-TEA was administered by gavage, suggesting that α-TEA might be effective via this route of administration. Recent preliminary experiments used gavage delivery of a liposomal α-TEA formulation rather than the ethanol-peanut oil α-TEA formulation used in these studies, and treatments were administered twice a day to achieve a higher daily dose (6 mg/day/mouse) rather than the once a day treatment schedule used in these studies to achieve a total daily dose of 5 mg/day/mouse. Liposomal α-TEA delivered by gavage significantly reduced tumor burden ($P < 0.001$) and resulted in zero animals exhibiting lung macroscopic metastases and significantly reduced lung microscopic metastases in comparison to gavage control (21.5 ± 4.9 *versus* 52.7 ± 4.2; $P < 0.0005$) after 21 days of treatment (unpublished data).

Regarding lung metastasis, it is important to point out some differences in the experimental protocol used in these studies in comparison with studies describing the metastatic nature of the parental 66cl-4 cells (22). In the studies by Miller *et al.* (22), the parental 66cl-4 cells were injected s.c., permitted to grow to 12 × 12-mm (4–7 weeks after tumor cell injection), primary tumors were surgically removed, and animals were sacrificed 3 weeks later. Using this experimental protocol, 89% of the animals exhibited macroscopic metastases. In contrast, in our studies, the GFP-transfected subline of 66cl-4 cells were injected s.c., the primary tumors were permitted to grow for 26 days at which time both primary tumors and lung metastases, both visible macroscopic and fluorescent microscopic metastases, were assessed. Using this experimental protocol, 40% of control animals (both untreated control and aerosol control) had visible macroscopic metastases in the lungs, whereas none (0%) of the α-TEA-treated animals had visible macroscopic metastases in the lungs (Table 1), whereas 100% of control and α-TEA-treated animals exhibited fluorescent microscopic metastases.

Regarding the <20-μm grouping of microscopic metastases, based on recent studies by Chambers *et al.* (27, 28), solitary tumor cells such as these may be potential contributors to dormancy, and if such cells remain viable in sufficiently large numbers, they could contribute to metastatic recurrence after a period of clinical dormancy. Whether the fluorescent microscopic metastases seen in the studies reported here represent viable cells, namely cells that could be removed and demonstrated to grow both in cell culture and after injection into the mammary fat pad, remains to be determined. Nevertheless, it is interesting to note that α-TEA was very effective in markedly decreasing the number of these small fluorescent microscopic metastases, as well as the larger microscopic metastases after both aerosol or oral treatment.

In an effort to try to address the question of whether or not α-TEA is preventing tumor cells from trafficking from the primary s.c. tumor to the lungs via the lymphatic system, we have counted fluorescent tumor cell foci in the axillary and brachial lymph nodes. In these experiments we observed that aerosolized, liposomal formulated α-TEA treatments significantly reduce the number of fluorescent tumor cell foci in the lymph nodes in comparison to aerosol treated controls (0.38 ± 0.1 *versus* 7.0 ± 1.6; $P < 0.0001$). This suggests that α-TEA may be having an effect on the process of metastasis, but more studies are needed (unpublished data).

In summary, data reported here are promising in that they show that a novel vitamin E analogue exhibits the ability to decrease primary tumor burden and reduce lung metastasis in a rather rapid and aggressive syngeneic tumor model without any overt toxic effects when administered by a clinically relevant route, namely, aerosol delivery. Increased rates of tumor cell apoptosis imply that the antitumor effect is due, at least in part, to analogue triggering of tumor cell death. The mechanism of how α-TEA reduces lung metastasis in this model system is unknown and warrants additional investigation.

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