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ORIGINAL ARTICLE

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Novel vitamin E analogue and 9-nitro-camptothecin administered as liposome aerosols decrease syngeneic mouse mammary tumor burden and inhibit metastasis

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Abstract *Purpose*: To test the anticancer properties of a nonhydrolyzable ether-linked acetic acid analogue of vitamin E, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA), and a derivative of camptothecin, 9-nitrocamptothecin (9-NC) singly and in combination against mouse mammary tumor cells (line 66 clone 4 stably transfected with green fluorescent protein; 66c1-4-GFP) cultured in vitro or transplanted subcutaneously into the inguinal region of female BALB/c mice to form established tumors. *Methods*: Following in vitro treatment of 66cl-4-GFP cells with α-TEA and suboptimal concentrations of 9-NC, singly or in combination, apoptosis was measured by morphological evaluation of nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI), and DNA synthesis arrest was measured by tritiated thymidine uptake. For in vivo analyses α-TEA and 9-NC, both water-insoluble compounds, were formulated into liposomes using dilauroylphosphatidylcholine and administered by aerosol to deliver doses calculated to be 36 and 0.4 μg/mouse per day, respectively, (singly or each separately for combined treatments) 7 days per week. *Results*: Treatment of 66cl-4-GFP cells in culture for 3 days with a combination of α-TEA (10 μg/ml; singly produces 38% apoptosis), and suboptimal concentrations of 9-NC (15.6, 31.3, 62.5, or 125 ng/ml; singly produce 2–7% apoptosis), produced 47%, 58%, 64%, and 69% apoptosis. Likewise, combinations of α-TEA + 9-NC inhibited DNA synthesis more than either agent administered singly. A significant reduction ($P < 0.001$) in growth of subcutaneous transplanted tumors was observed with liposome-formulated and aerosolized delivery of α-TEA + 9-NC to BALB/c mice. The incidence of macroscopic lung metastasis was 83% in control vs 8% in α-TEA-, 9-NC-, or combination-treated mice. Fluorescence microscopic examination of lungs and axillary and brachial lymph nodes showed a statistically significant decrease in metastasis observed in α-TEA-, 9-NC-, and combination- vs control-treated animals. Analyses of primary tumor tissue for proliferation and apoptosis showed treatment groups to have lower Ki-67 and higher terminal deoxynucleotidyl transferase-mediated nick end labeling, respectively. Treatments showed no measurable effects on two angiogenesis parameters, namely intratumoral blood volume as assessed by

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hemoglobin content and intratumoral blood vessel density as assessed with CD31 staining. *Conclusions*: Combination treatments enhanced antiproliferative and proapoptotic activities in cell culture, and when formulated in liposomes and delivered via aerosolization to treat an aggressive and metastatic syngeneic murine mammary tumor, the combination treatment showed a significant reduction in tumor volume in comparison to either treatment alone. Mechanistically, it appears that neither enhanced apoptosis, reduced cell proliferation, nor reduced blood vessel density can fully account for the enhanced effects of the combination treatment.

Keywords Vitamin E analogue α-TEA · 9-nitrocamptothecin · Metastasis · Antitumor · Syngeneic mouse mammary cancer model

Abbreviations α-TEA: 2,5,7,8-Tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid · 9-NC: 9-Nitro-camptothecin · 66cl-4-GFP: BALB/c mouse mammary tumor line 66 clone 4 stably transfected with GFP · DAPI: 4′,6-Diamidino-2-phenylindole · DMSO: Dimethylsulfoxide · GFP: Green fluorescent protein · PECAM-1: Platelet-endothelial cell adhesion molecule · THF: Tetrahydrofuran · TUNEL: Terminal deoxynucleotidyl transferase-mediated nick end labeling

Introduction

Camptothecin, a naturally occurring anticancer agent, was first extracted from the Chinese tree *Camptotheca acuminata* in the early 1960s [19]. Since this time, several less-toxic, clinically active, derivatives have been identified including CPT (irinotecan or CPT-11), 9-amino-camptothecin (9-AC) and 9-nitrocamptothecin (9-NC, or rubitecan) [19]. Camptothecin derivatives are used clinically for the treatment of several forms of cancer including breast, ovarian, lung, and colorectal [7].

Liposomal formulations of 9-NC delivered by aerosol are being investigated as a more effective, less-toxic formulation and delivery method [8, 10, 13, 21]. Dosages used in these studies were chosen based on previous studies in which a range of doses were investigated for both α-TEA [14] and 9-NC [10]. More specifically, efforts to limit 9-NC toxicity by administering it in liposome aerosol at concentrations lower than those traditionally used in mice treated with 9-NC by other routes, have demonstrated this approach to be very effective against human breast, colon and lung cancer xenografts in nude mice [10] and experimental pulmonary metastasis in mice [13]. Furthermore, liposomal formulations of 9-NC delivered by aerosol have been used successful in toxicology studies in dogs [8] and a dose escalation and toxicologic evaluation of cancer patients [21]. A therapeutic trial in cancer patients is in progress (V. Knight, B. Gilbert, and coworkers).

Alpha-tocopherol ether analogue (α-TEA), is a stable, nonhydrolyzable acetic acid derivative of vitamin E (RRR-α-tocopherol) and has been shown recently to be a potent dose-dependent antitumor agent in vitro and in vivo [14]. Because α-TEA is a lipid which complicates i.v. administration and because there is a specific RRR-α-tocopherol transfer protein in the liver that selectively mediates the transfer of RRR-α-tocopherol into lipoproteins thus limiting bioavailability following oral administration of vitamin E compounds other than RRR-α-tocopherol, we are investigating the formulation of α-TEA into liposomes and aerosol delivery as a potential clinically-relevant cancer treatment strategy. In preclinical syngeneic transplantable mouse mammary cancer studies, α-TEA formulated into liposomes and delivered by aerosol has been shown to: (1) significantly decrease tumor growth rate ($P < 0.001$) over 17 days of treatment; (2) completely inhibit visual macroscopic metastasis, reducing incidence from 40% in control animals to 0% in animals treated with α-TEA; and (3) significantly reduce microscopic metastasis ($P < 0.002$) [14]. In all in vivo studies conducted to date, mice receiving α-TEA have exhibited no significant difference in mean body weight or any visible indication of toxicity such as lethargy or loss of hair [14]. Thus, based on its anticancer effectiveness in vivo and lack of overt toxicity, α-TEA is a promising agent for investigation separately and in combination with other anticancer drugs.

Although the antitumor mechanisms of action of 9-NC and α-TEA are not completely understood, 9-NC has been shown to inhibit cell growth by inhibition of DNA synthesis and induction of cell death by apoptosis [2, 4, 17] and α-TEA has been shown to be a potent proapoptotic agent [14]. Of potential interest to these studies are recent investigations suggesting that the death receptor CD95 (APO-1/Fas) signaling pathway is important for 9-NC- and α-TEA-induced apoptosis [4, 21]. Chatterjee et al. [4] have shown that 9-NC induces de novo synthesis of both CD95 ligand and CD95 as well as downregulation of the antiapoptotic protein c-FLIP (Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein) in human prostate carcinoma DU145 cells.

Taken together with the findings of Shun et al. [18] showing that functional knockdown of the Fas signaling pathway by treatment of human MDA-MB-431 breast cancer cells with neutralizing antibodies to CD95 partially abrogates α-TEA-induced apoptosis, we speculated that a combination of α-TEA and 9-NC might exhibit additive or synergistic proapoptotic activity. Of additional interest in considering potential cooperative activity between α-TEA and 9-NC is experimental evidence demonstrating that both of these agents exhibit antimetastatic potential in animal models although mechanistic insights into this aspect of their antitumor activities are currently lacking [13, 14]. α-TEA has been shown in our laboratory to be effective in reducing tumor burden and inhibiting metastasis, but it does not totally eliminate tumor growth or metastasis [14].

The rationale for the animal studies reported here were based on promising cell culture data presented in this paper showing that combinations of α-TEA + 9-NC enhanced apoptosis more than either agent administered separately. Thus, studies were initiated to investigate the hypothesis that α-TEA in combination with 9-NC would enhance the in vivo therapeutic effects over either drug administered separately. The primary outcomes from these studies were the ability of α-TEA and 9-NC in combination to reduce tumor burden and inhibit metastasis to a greater degree than either compound administered separately.

We report here that the combination of α-TEA + 9-NC produced a higher degree of apoptosis and DNA synthesis arrest in culture than either agent alone, significantly reduced subcutaneous syngeneic mouse mammary tumor growth ($P < 0.001$) to a greater degree than either agent alone, reduced the total incidence of visible macroscopic metastasis by 90%, reduced the total incidence of microscopic lung metastasis by 71%, and axillary and brachial lymph node metastasis by 88%, reduced the number of proliferating tumor cells in vivo by 68%, and increased tumor cells undergoing apoptosis by 90%. Investigations of parameters of tumor angiogenesis revealed that neither reductions in primary tumor blood volume nor number of CD31 positive-staining blood vessels in tumors appear to be involved in the antitumor effects of α-TEA and 9-NC administered separately or in combination.

Materials and methods

α-TEA and 9-NC and in vitro treatments

α-TEA was synthesized, its structure confirmed, and production scaled up to provide sufficient amounts for preclinical animal studies by one of the authors (J.A.) as previously described [14]. 9-NC was purchased from ChemWerth (Woodbridge, Ct.). For in vitro treatments, stock (40 m *M* and 318 μM) solutions of α-TEA and 9-NC were made in ethanol (EtOH) and dimethyl sulfoxide (DMSO), respectively. Final concentrations of chemicals were achieved by dilution in medium. Final concentrations (v/v) of EtOH and DMSO in the cell treatments were 0.1% and 0.1%, respectively.

66cl-4-GFP murine mammary tumor cell line

The 66cl-4 cell line is 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 [6, 15]. The 66cl-4 cells were stably transfected with the enhanced green fluorescent protein and selected for a high degree of fluorescence by L.-Z.S. (one of the authors). 66cl-4-GFP cells are highly metastatic with approximately 40% of animals developing visible macroscopic metastases and 100% of animals developing fluorescent microscopic metastases in the lungs 26 days after subcutaneous injection of 2×10^5 tumor cells into the inguinal area [14]. 66cl-4-GFP cells were maintained as monolayer cultures in McCoy's medium (Invitrogen Life Technologies, Carlsbad, Calif.) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, Calif.), 100 μg/ml streptomycin, 100 IU/ml penicillin, 1× (v/v) nonessential amino acids, 1× (v/v) MEM vitamins, 1.5 m *M* sodium pyruvate, and 50 μg/ml gentamicin (Invitrogen Life Technologies). Treatments were given using this same McCoy's supplemented medium except the fetal bovine serum content was reduced to 5%. Cultures were routinely examined to verify absence of *Mycoplasma* contamination.

Determination of DNA synthesis by incorporation of [^{3}H]thymidine

The effects of α-TEA and 9-NC, separately and in combination, on inhibition of DNA synthesis of 66cl-4-GFP cells were determined by [^{3}H]thymidine incorporation as described previously [3]. Briefly, 66cl-4-GFP cells at 2×10^4 cells/well in 96-well plates were cultured separately and in combination with 10 μg/ml of α-TEA and 8, 15, 31, or 62 ng/ml of 9-NC for 24 h, adding 0.5 μCi [^{3}H]thymidine 6 h prior to harvesting the cells. [^{3}H]Thymidine incorporation was measured using a Beckman LS5000TD liquid scintillation counter.

Determination of apoptosis by morphological evaluation of DAPI-stained nuclei

Apoptosis was determined using previously published procedures [14, 18]. 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 per slide. Apoptotic data are presented as the mean ± SD for three independently conducted experiments.

BALB/c mice

Female BALB/c mice at 6 weeks of age (20–25 g in weight) were purchased from Jackson Laboratories (Bar Harbor, Me.). Mice, five per cage, were housed at the Animal Resource Center at the University of Texas at Austin at 74 ± 2°F with 30–70% humidity and a 12-h alternating light/dark cycle. Mice were given water and standard laboratory chow (Harlan Teklad #2018 Global 18% Protein Rodent Diet; Madison, Wis.) 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 and tumor growth measurement

Mice were injected (100 μl) subcutaneously with 2×10^5 cells in the inguinal area at a point equidistant from the fourth and fifth nipples on the right side. A total of 48 mice (12 mice/group) were assigned to α-TEA, 9-NC, α-TEA + 9-NC, or liposome-only (control group) such that the mean tumor volume of each group was closely matched. The groups had average tumor volumes per group of 1.55, 1.34, 1.26, and 1.60 mm^3, respectively, at the start of the treatments, which were begun 9 days after tumor cell inoculation. Tumors were measured using calipers every other day, and volumes were calculated using the formula: volume (mm^3) = [width (mm^2)×length (mm)]/2 [5]. Body weights were determined weekly.

Preparation of α-TEA and 9-NC liposomes

α-TEA/liposome and 9-NC/liposome were prepared as previously described [10, 14]. Briefly, the lipid (1,2-dilauroyl-*sn*-glycerol-3-phosphocholine (DLPC); Avanti Polar-Lipids, Alabaster, Ala.) at a concentration of 120 mg/ml was dissolved in *t*-butanol (Fisher Scientific, Houston, Tx.) then sonicated to obtain a clear solution. α-TEA at 40 mg/ml was dissolved in *t*-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 −80°C until needed. Each treatment vial contained 75 mg of α-TEA. 9-NC treatments were made as described previously [10]. Briefly, 9-NC (100 mg/ml) was dissolved in DMSO and warmed to 37°C. DLPC (100 mg/ml) was dissolved in *t*-butanol. Drug and phospholipid solutions were added at a 1:50 (w/w) ratio, and mixed by vortexing. Samples were frozen at −80°C for 1–2 h before lyophilizing overnight. Each treatment vial contained 1 mg of 9-NC.

Aerosol delivery

All treatments were administered by aerosol as described previously [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, Bedford, Mass.) to generate aerosol. Particle sizes of liposomal formulated α-TEA and 9-NC aerosol discharged from the AeroTech II nebulizer were determined using an Anderson Cascade Impactor to have a mass median aerodynamic diameter (MMAD) of 2.01 μm ± geometric SD of 2.04 and 1.6 μm ± geometric SD of 2.04, respectively [10, 14].

Before nebulization, the α-TEA/lipid powder (75 mg/vial) and 9-NC/lipid powder (1 mg/vial) were brought to room temperature then reconstituted by adding 3.75 and 5 ml distilled water, respectively, to achieve the final desired concentration of 20 mg/ml α-TEA and 200 μg/ml 9-NC. The mixtures were allowed to swell at room temperature for 30 min with periodic inversion and vortexing, and then all 3.75 ml of α-TEA or 5 ml of the 9-NC were added to the nebulizer. 9-NC was administered 8 h after administration of α-TEA. Treatments were administered 7 days per week. Animals were exposed to aerosol until all α-TEA or 9-NC was aerosolized (approximately 15 min). Based on previous calculations of amount of aerosolized α-TEA or 9-NC delivered to mice under these treatment conditions, it was estimated that approximately 36 μg α-TEA and 0.4 μg 9-NC per mouse per day were deposited in the lungs, respectively. Calculations were based on a 25-g mouse with an inhalation volume of 1 l/min/kg body weight and an average aerosol retention factor of 30% [12].

Lung and lymph node metastasis

Macroscopic metastases in all five lung lobes were counted visually at the time the mice were killed. Fluorescent microscopic metastases were counted using a Nikon fluorescence microscope (TE-200; ×200 magnification) as described previously [14]. For analyses, the left lung lobe was flattened and the top and bottom surfaces were scored for fluorescent green microscopic metastases. Fluorescent microscopic metastases were scored by size into three size groupings: <20, 20–50, 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 was thought to represent solitary cells, the 20–50 μm grouping two to five cells, and the >50 μm grouping microscopic metastases of more than two to five cells.

Ki-67 staining for detection of proliferation in tumor tissue

Deparaffinized sections (5 μm) of tumor tissue were used to assess proliferation using antibody to the Ki-67 antigen, a nuclear antigen expressed in cells undergoing active cell division. Briefly, endogenous peroxidase activity was blocked using a 3% H_2O_2 solution for 10 min followed by washing with PBS. Rabbit serum (10%) in PBS was applied to 5 μm tumor tissue sections to block nonspecific antibody binding, before incubation with primary antibody (rat-anti-mouse Ki-67 antibody,1:200 dilution; DAKO Corporation, Carpinteria, Calif.) overnight at 4°C. After primary antibody incubation, slides were washed then incubated with biotinylated rabbit-anti-rat IgG (Vector Laboratories, Burlingame, Calif.) at a 1:200 dilution for 30 min at room temperature. Sections were then incubated with avidin–biotin complex (ABC-HRP; Vector Laborato-

ries) for 30 min at room temperature. Immunoreactivity was visualized via incubation with diaminobenzidine dihydrochloride. Slides were lightly counterstained with hematoxylin. Ki-67-positive stained cells were counted in five separate fields per sample. The data are presented as the means ± SE of all tumors in each group ($n = 11$ for α-TEA, 9-NC, and combination groups; $n = 12$ for control group).

TUNEL assay for detection of apoptosis in tumor tissue

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, N.Y.) 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 separate random fields at high power (×400 magnification) were scored per tumor. The data are presented as the mean ± SE numbers of apoptotic cells counted in seven or more individual tumors from each treatment group.

CD31 staining for determination of blood vessel formation in tumor tissue

Immunohistochemistry was used to assess the presence of the endothelial antigen CD31 (also referred to as PECAM-1, platelet-endothelial cell adhesion molecule) as an indicator of small capillaries in primary tumor tissue. Formalin-fixed paraffin-embedded tissues were sectioned (5 μm) and tissue sections adhered onto charged slides (Fisher Scientific, Chicago, Ill.). Tissue sections were deparaffinized, hydrated through a series of graded ethanol solutions to water and endogenous peroxidase activity blocked by incubation in 3% H_2O_2 for 10 min. Tissue sections were then pretreated with 0.06% Protease Type XXIV (Sigma) for 10 min at room temperature before incubation with antibody to CD31 (PECAM-1; PharMingen, San Diego, Calif.) at a 1:400 dilution overnight at 4°C. Detection utilized the Tyramide Signal Amplification Biotin System-Peroxidase (PerkinElmer Life Sciences, Boston, Mass.) with diaminobenzidine dihydrochloride development. For contrast, the sections were lightly counterstained with hematoxylin. Entire tumor sections ($n = 3$ for α-TEA and 9-NC groups; $n = 4$ for combination and control groups) were scored for CD31-stained vessels and then were adjusted for tumor size, by dividing number of CD31 vessels by length×width of tumor tissue mounted on the slide.

Hemoglobin assay

Frozen tumor samples ($n = 4$ for each treatment group) were analyzed to determine hemoglobin levels in an effort to corroborate the blood vessel density data obtained by CD31 staining. Tumors were carefully cleaned to remove any surface blood vessels and then hemoglobin was extracted as described previously [1]. Briefly, frozen samples were weighed, frozen in liquid nitrogen and pulverized using mortar and pestle. Extraction was performed with ice-cold buffer containing 10 m *M* Tris (pH 7.5), 0.1 *M* NaCl, 0.5% Triton-X-100, 1.0 m *M* ethylene diaminetetraacetic acid, and 0.1 m *M* phenylmethylsulfonyl fluoride. Six milliliters of extraction buffer was used for each gram of tissue. Extracts were centrifuged at 10,000 *g* for 15 min, and hemoglobin was measured via a hemoglobin extraction kit (Sigma) following the manufacturer's instructions. The hemoglobin content in systemic blood obtained at the time the animals were killed was also measured to obtain the hemoglobin level per unit of blood so that the blood volume of each tumor could be calculated.

Statistical analyses

Differences among the treatment groups for the cell culture data were evaluated using the unpaired *t*-test with unequal variances. The degree of enhancement of apoptosis among cells cotreated with α-TEA + 9-NC was evaluated using two-way analysis of variance. Animal numbers for experiments were determined by power calculations derived from data generated in previous studies. Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor analysis of variance with Tukey post-test using SPSS (SPSS, Chicago, Ill.). The significance of the differences in number of fluorescent microscopic and macroscopic metastases per group, Ki-67-stained cells per group, and TUNEL-positive nuclei per group were determined using the two-tailed Mann-Whitney rank test using Prism software version 3.0 (GraphPad, San Diego, Calif.). A level of $P < 0.05$ was regarded as statistically significant.

Results

Structure of 9-NC and α-TEA

The structures of 9-NC and α-TEA are depicted in Fig. 1. 9-NC is a less toxic, clinically active derivative of camptothecin, a pentacyclic alkaloid isolated from the Chinese tree *C. acuminata* [22]. α-TEA is a nonhydrolyzable ether analogue of natural vitamin E, RRR-α-tocopherol. 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 [14].

α-TEA, 9-NC, and combination treatments induce apoptosis in 66cl-4-GFP cells in cell culture

66cl-4-GFP cells treated with α-TEA (5 μg/ml) for 3 days exhibited 8% apoptosis (Fig. 2a). Cells treated

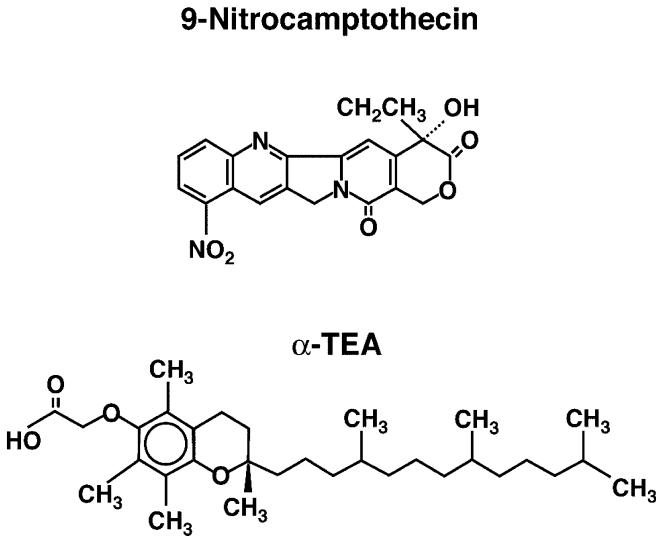

Fig. 1 Structure of 9-NC and α-TEA

with 9-NC (15, 31, 62, or 125 ng/ml) exhibited 2%, 4%, 6% and 7% apoptosis, whereas cells treated with combinations of 5 μg/ml α-TEA plus various concentrations of 9-NC exhibited elevated levels of apoptosis (14%, 24%, 34% and 61%). A significant increase in apoptosis was observed for two of the cotreatments (5 μg/ml α-TEA plus 62 or 125 ng/ml 9-NC) in comparison to α-TEA and 9-NC alone ($P < 0.045$, 0.00072; and $P < 0.04$, 0.0028, respectively; Fig. 2a).

66cl-4-GFP cells treated with 10 μg/ml α-TEA for 3 days exhibited 38% apoptosis, and treatment with 9-NC at the four different doses induced 2%, 4%, 6% and 7% apoptosis, whereas cells treated with combinations of 10 μg/ml α-TEA plus various concentrations of 9-NC exhibited elevated levels of apoptosis (47%, 58%, 64% and 68%; Fig. 2b). A significant increase in apoptosis was observed for three of the cotreatments (10 μg/ml α-TEA plus 31, 62, and 125 ng/ml 9-NC) in comparison to

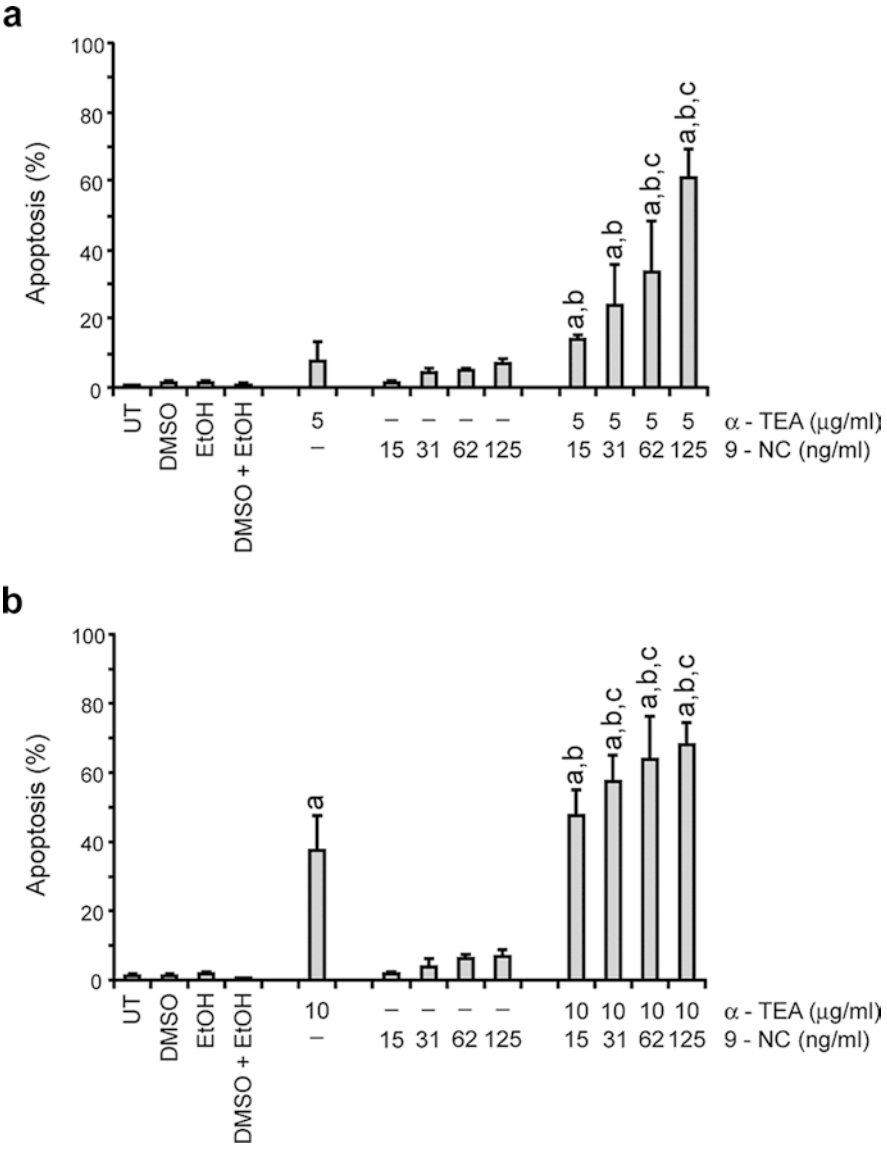

Fig. 2a, b α-TEA and 9-NC singly and in combination induced 66cl-4-GFP murine mammary cells to undergo apoptosis. Cells were treated with α-TEA at (**a**) 5 μl/ml or (**b**) 10 μg/ml and with various levels of 9-NC for 3 days. Nuclei were labeled with the fluorescent DNA-binding dye DAPI. Nuclei exhibiting condensed chromatin or fragmented DNA were scored as apoptotic. Data are means ± SD of three separate experiments ([a]significantly different from control, [b]significantly different from 9-NC, [c]significantly different from α-TEA; $P < 0.05$)

Table 1 Inhibition of DNA synthesis in 66cl-4-GFP mammary cancer cells following treatment with α-TEA, 9-NC, and their combinations. 66cl-4-GFP cells cultured at 2.0×10^4 cells/well in 96 well-plates were treated with α-TEA (10 μg/ml), 9-NC (8, 15, 31, or 62 ng/ml), combinations of α-TEA (10 μg/ml) + 9-NC (8, 15, 31, or 62 ng/ml) or DMSO + EtOH (control), or untreated, for 1 day. DNA synthesis was monitored by [^{3}H]thymidine uptake. DNA synthesis arrest was calculated by comparing [^{3}H]thymidine uptake by cells receiving treatments with [^{3}H]thymidine uptake by cells receiving no treatment. Values are means ± SD of three separate experiments

Treatment	DNA synthesis arrest (%)	Fold increase in inhibition in comparison to cells treated with	
		α-TEA alone	9-NC alone
α-TEA (10 μg/ml)	35 ± 5.0		
9-NC (8 ng/ml)	29 ± 5.5		
9-NC (15 ng/ml)	46 ± 4.9		
9-NC (31 ng/ml)	77 ± 4.4		
9-NC (62 ng/ml)	90 ± 1.5		
α-TEA (10 μg/ml) + 9-NC (8 ng/ml)	53 ± 2.5	1.5	1.8
α-TEA (10 μg/ml) + 9-NC (15 ng/ml)	65 ± 2.0	1.9	1.4
α-TEA (10 μg/ml) + 9-NC (31 ng/ml)	84 ± 2.5	2.4	1.1
α-TEA (10 μg/ml) + 9-NC (62 ng/ml)	92 ± 1.0	2.6	1.0
DMSO + EtOH	6 ± 3.6		

α-TEA and 9-NC alone ($P < 0.050$, 0.034 and 0.019; and $P < 0.0008$, 0.001 and 0.0007, respectively; Fig. 2b).

Untreated, EtOH, DMSO, and EtOH + DMSO controls exhibited background levels of apoptosis of 1%, 1%, 2%, and < 1% apoptosis, respectively (Fig. 2).

α-TEA, 9-NC, and combination treatments inhibit DNA synthesis in 66cl-4-GFP cells in cell culture

66cl-4-GFP cells were treated to determine the effect of each agent separately and in combination on DNA synthesis (Table 1). Cells treated with 10 μg/ml α-TEA exhibited 35% reduction in DNA synthesis when compared to untreated controls. 9-NC at 8, 15, 31, or 62 ng/ml inhibited DNA synthesis by 29%, 46%, 77%, and 90%, respectively, in comparison to untreated controls. Cells treated with combinations of 10 μg/ml α-TEA plus the various concentrations of 9-NC exhibited 53%, 65%, 84%, and 92% DNA synthesis inhibition in comparison to untreated controls. EtOH + DMSO vehicle-treated cells exhibited 6% DNA synthesis arrest in comparison to untreated control cells (Table 1).

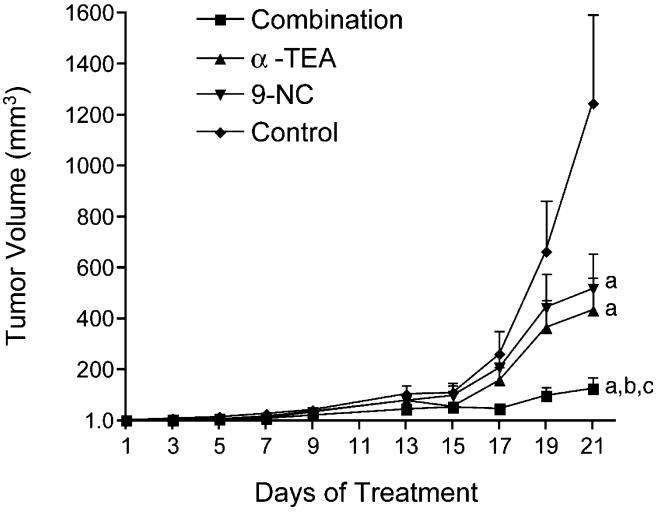

Fig. 3 Aerosolized α-TEA, 9-NC, and combination treatments reduced tumor volume. Treatments were started 9 days after subcutaneous injection of 2×10^5 66cl-4-GFP cells/mouse and continued 7 days per week for 3 weeks ([a]significantly different from control, [b]significantly different from 9-NC, [c]significantly different from α-TEA; $P < 0.05$)

Liposomal formulated α-TEA, 9-NC, and combination treatments delivered by aerosolization decreased 66cl-4-GFP tumor burden in BALB/c mice

Mean primary tumor volumes of aerosol control-treated animals over 21 days of treatment were significantly higher than all other groups ($P < 0.001$; Fig. 3; mean ± SE 1245 ± 344 mm^3). Both 9-NC- and α-TEA-treated groups had mean tumor volumes significantly lower than control animals ($P < 0.001$; means ± SE 518 ± 134 mm^3 and 434 ± 124 mm^3, respectively); and the size of the tumors in the α-TEA and 9-NC single treatment groups were not significantly different from one another ($P < 0.2$; means ± SE 518 ± 134 mm^3 and 434 ± 124 mm^3, respectively). Animals receiving α-TEA + 9-NC had mean tumor volumes over 21 days of treatment that were significantly lower than those of control animals and those of both α-TEA- and 9-NC-treated animals ($P < 0.001$; Fig. 3; mean ± SE 126 ± 42 mm^3). There were no significant differences in mean body weights among any of the treatment or control groups (data not shown).

Liposomal formulated α-TEA, 9-NC, and combination treatments delivered in aerosol suppressed 66cl-4-GFP lung and lymph node metastasis in BALB/c mice

At the time the animals were killed, all five lung lobes and axillary and brachial lymph nodes were taken,

Table 2 66cl-4-GFP mammary cancer cell lung metastasis in BALB/c mice receiving liposomal formulated α-TEA, 9-NC, or α-TEA + 9-NC, or liposome control by aerosol

Treatments delivered by aerosol	No. of animals per group with visible macroscopic lung metastases[a]	Total no. of visible lung macroscopic metastatic foci[b]
Control	10/12	36
9-NC	1/12	2
α-TEA	1/12	2
α-TEA + 9-NC	1/12	2

[a]Macroscopic metastatic lesions in all five lung lobes for each animal in all treatment groups were counted visually at the time the animals were killed.
[b]The values presented the total number of visible lung macroscopic metastases observed in the 12 mice in each group.

examined visually for macroscopic metastatic lesions and frozen for subsequent analyses of microscopic metastases by fluorescent microscopy. Visible macroscopic lung metastases were observed in 1 of 12 animals (8%) in each of the α-TEA, 9-NC, and α-TEA + 9-NC treatment groups ($P < 0.002$), in comparison to 10 of 12 mice (83%) in the aerosol control group (Table 2). Total numbers of macroscopic lung lesions observed differed greatly between the control and treatment groups, with a total of 36 lesions observed in the aerosol control group in comparison to only 2 lesions observed in each of the treatment groups (Table 2). No macroscopic metastases were observed in any of the lymph nodes.

Use of a Nikon fluorescence microscope permitted measurement and classification of green fluorescing microscopic metastases in lung tissue into three size groupings (< 20, 20–50, and > 50 μm; Fig. 4a). Since the tumor cells are approximately 10–20 μm in diameter, the < 20 μm group most likely represents single cells. This analysis showed a decrease in the number of microscopic lung metastases in all three size groups and all three treatment groups in comparison to the control group. The mean numbers of microscopic metastases in the α-TEA treatment group (28.4 ± 3.3, $n = 11$), the 9-NC treatment group (43.5 ± 8.7, $n = 11$), and the combination treatment group (27.6 ± 5.2, $n = 12$) were significantly reduced in comparison to the aerosol control group (96.7 ± 12.2, $n = 12$; $P < 0.0001$, < 0.002, and < 0.0001, respectively). However, the mean numbers of microscopic metastases in the α-TEA + 9-NC combination treatment group and the α-TEA and 9-NC single treatment groups were not significantly different.

The number of microscopic metastases in the axillary and brachial lymph nodes from the different treatment groups were significantly reduced in comparison to the control group (α-TEA, $P < 0.0001$; 9-NC, $P < 0.05$; α-TEA + 9-NC, $P < 0.0004$; Fig. 4b). It is important to note that 71%, 33%, and 52% of lymph nodes from mice treated with α-TEA, 9-NC, or α-TEA + 9-NC, respectively, had no microscopic metastases, whereas

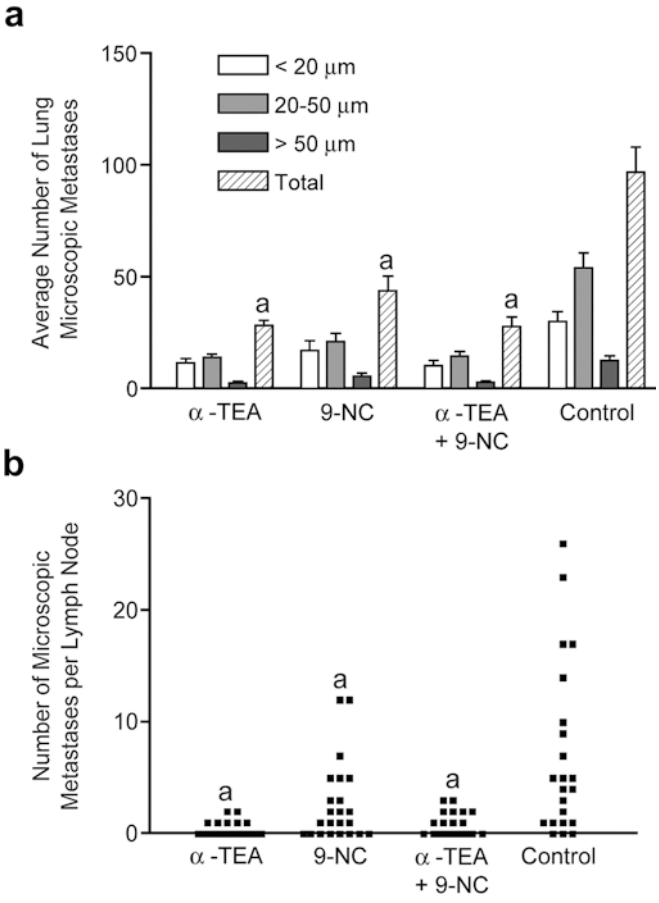

Fig. 4a, b Aerosolized α-TEA, 9-NC, and combination treatments inhibited lung and lymph node metastasis. The number of fluorescent microscopic metastases (**a**) on the surface of the left lung lobe or (**b**) on the surface of individual lymph nodes were determined ([a]significantly different from control; $P < 0.05$)

only 14% of lymph nodes from the control mice were free of microscopic metastases.

Inhibition of cell proliferation by α-TEA, 9-NC, and combination treatments in vivo

Tumor sections from each of the treatment groups as well as the aerosol control group were examined by immunohistochemistry for proliferation status using the nuclear Ki-67 antigen expressed in proliferating cells as a biomarker. The number Ki-67-positive cells in tumors from mice treated with α-TEA was 106 ± 41.4 cells/field (mean ± SE) compared with 241 ± 35.7 cells/field in tumors from aerosol control mice ($P < 0.008$; Fig. 5a). The number Ki-67-positive cells in tumors from mice treated with 9-NC (218 ± 45.6 cells/field, mean ± SE) showed no significant decrease compared with the number in tumors from aerosol control mice (Fig. 5a). The number Ki-67-positive in tumors from mice treated with α-TEA + 9-NC (77 ± 20.2 cells/field, mean ± SE) was also significantly lower than the number in tumors from aerosol

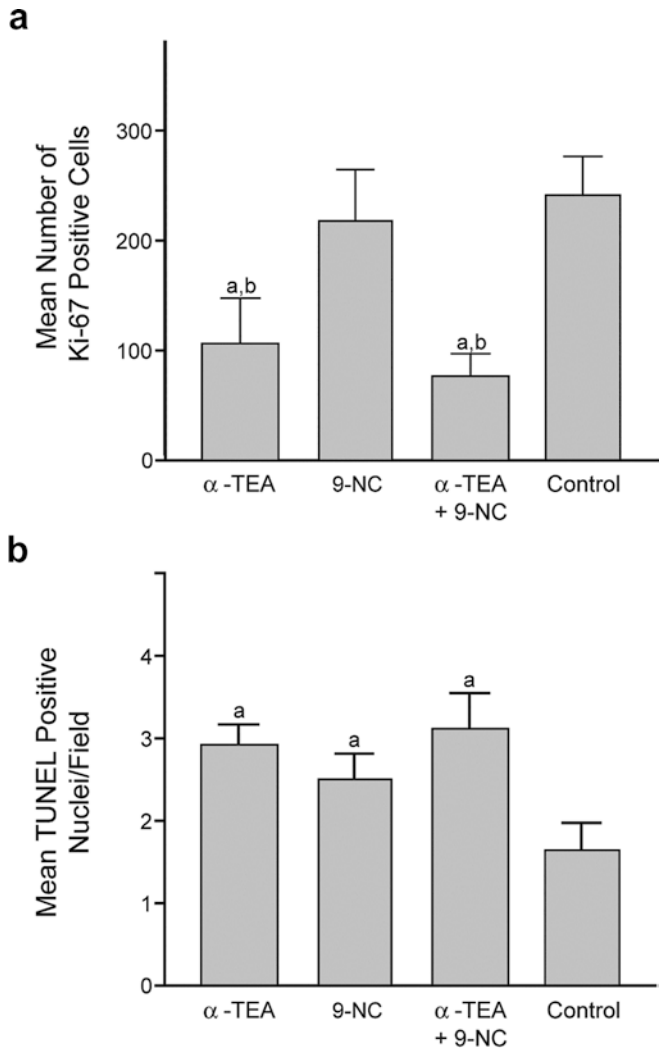

Fig. 5a, b Aerosolized α-TEA, 9-NC, and combination treatments inhibited 66cl-4-GFP cell proliferation and increased apoptosis in vivo. Cell proliferation (**a**) and apoptosis (**b**) were determined using immunohistochemical analyses of 5-μm tumor sections ([a]significantly different from control, [b]significantly different from 9-NC; $P < 0.05$)

control mice ($P < 0.001$; Fig. 5a). The mean numbers of Ki-67-positive cells in tumors from mice treated with α-TEA, and α-TEA + 9-NC were significantly lower than in tumors from mice treated with 9-NC only ($P < 0.02$, $P < 0.004$, respectively). However, there was no significant difference in the mean number of Ki-67 positive cells between the α-TEA treated group and the α-TEA + 9-NC combination treatment group (Fig. 5a).

Induction of apoptosis by α-TEA, 9-NC, and combination treatments in vivo

In view of the in vitro data showing that combinations of α-TEA + 9-NC inhibited 66cl-4-GFP tumor cell growth via induction of apoptosis to a greater extent than either agent administered singly, tumors from each of the treatment groups were examined for apoptosis using TUNEL staining of 5-μm tumor sections. Tumors from mice treated with α-TEA, 9-NC, and α-TEA + 9-NC had a mean ± SE of 2.93 ± 0.24, 2.51 ± 0.31, and 3.12 ± 0.42 apoptotic cells/field, respectively, whereas tumors from aerosol control mice had a mean ± SE of 1.64 ± 0.32 apoptotic cells/field ($P < 0.009$, $P < 0.05$, and $P < 0.014$, respectively; Fig. 5b).

α-TEA, 9-NC, and combination treatments did not have a significant effect on tumor angiogenesis as determined by CD31 staining and hemoglobin content

CD31 expression and hemoglobin levels in tumors were examined in an effort to determine if the antimetastatic effects of α-TEA, 9-NC, and α-TEA + 9-NC treatments might be due to reduced blood vessel formation. Tumors were examined for the presence of blood vessels using staining for CD31 (PECAM-1), an endothelial cell marker (Fig. 6a). There were no significant differences in the number of blood vessels per tumor section among the treatment and control groups when standardized for tumor size (Fig. 6a). As an alternate method for obtaining information on intratumoral blood vessel density, tumor blood volume was determined using a hemoglobin content assay (Fig. 6b). Consistent with the CD31 staining results, tumors tested from treatment groups did not show a reduction in blood volume when compared to aerosol-treated controls.

Discussion

The studies reported here showed that α-TEA, a new vitamin E analogue, and 9-NC significantly increased tumor cell death by apoptosis and blocked cell proliferation in cell culture, and significantly reduced tumor burden in a mouse mammary tumor model.

9-NC is a less-toxic derivative of camptothecin. Preclinical animal data show 9-NC to inhibit human breast cancer xenograft growth in athymic nude mice by as much as 100% [9, 10]. 9-NC, which is currently in clinical trials for treatment of ovarian and pancreatic cancer, is being considered for treatment of breast cancer [11, 16, 20]. Although 9-NC possesses impressive anticancer activity, it also exhibits significant toxic effects, thus the need for seeking treatment strategies that might circumvent this problem by combining 9-NC at a lower dosage with another anticancer drug. In the cell culture studies reported here, combinations of α-TEA (10 μg/ml) and suboptimal doses of 9-NC (31, 62, or 125 ng/ml) gave significantly greater levels of apoptosis than either drug administered as a single agent. Furthermore, combinations of α-TEA + suboptimal doses of 9-NC resulted in higher levels of DNA synthesis arrest than either drug alone. Based on these encouraging in vitro results, investigations of combination treatment on tumor growth and metastasis in a syngeneic transplantable mouse mammary cancer model were undertaken. No

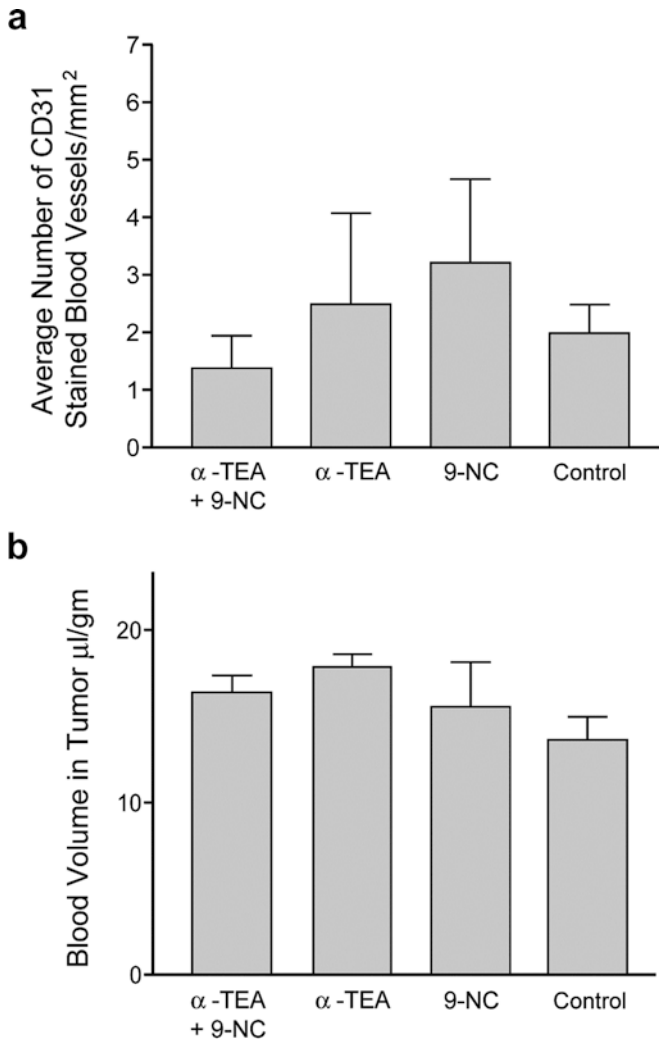

Fig. 6a, b Aerosolized α-TEA, 9-NC, and combination treatments had no effect on the number of blood vessels or blood volume in 66cl-4-GFP tumors. **a** CD31-positive blood vessels per entire 5 μm tumor section were counted then adjusted for tumor size by dividing by length × width of tumor tissue. **b** Levels of hemoglobin in tumor extracts and systemic blood from each mouse were determined using a hemoglobin assay kit, and the corresponding blood volume of each tumor was calculated. Data presented are means ± SE

differences in mean body weights among control or treatment groups were observed (data not shown).

We do not understand the mechanisms whereby a significant increase in apoptosis as measured by TUNEL, a significant decrease in cell proliferation as measured by Ki-67, or a significant decrease in blood vessel density as measured by CD31 or hemoglobin levels was not seen in the combination treatment of 9-NC + α-TEA in comparison to the single treatments since the combination treatment was capable of reducing tumor burden significantly better than the single treatments alone.

Likewise, the mechanisms whereby α-TEA singly and in combination with 9-NC reduced both visible and microscopic metastases are not known. Based on data showing no significant differences between treatment and control groups in CD31 expression and hemoglobin levels in tumors, it appears that neither the single agent treatments nor the combination treatment were effective in reducing tumor volume or preventing metastases by reduction of number of blood vessels or blood volume in tumors.

Data showing that α-TEA, 9-NC, and α-TEA + 9-NC inhibit microscopic metastases in lymph nodes are of interest in that metastasis was reduced in a site that is not directly targeted by the aerosol treatment. It is possible that these drugs are having a direct killing effect on cells at secondary sites. Alternatively, since a high percentage of lymph nodes in the treatment groups did not show any metastasis, it is possible that the treatments are preventing tumor cells from trafficking from the primary subcutaneous tumor to lungs and lymph nodes via the lymphatic system.

In summary, these results suggest that combination treatments using α-TEA, a novel vitamin E analogue, and lower doses of 9-NC should be investigated further as a chemotherapeutic strategy for breast cancer.

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