α-TEA Plus Cisplatin Reduces Human Cisplatin-Resistant Ovarian Cancer Cell Tumor Burden and Metastasis

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A novel nonhydrolyzable ether-linked acetic acid analog of vitamin E, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)-chroman-6-yloxyacetic acid (α-TEA) in combination with cisplatin, reduces tumor burden of A2780/cp70 (cp70) cisplatin-resistant human ovarian cancer cells xenografted into immune compromised nude mice. Two xenograft studies were conducted using cp70 cells stably expressing green fluorescent protein (cp70-GFP) subcutaneously transplanted into NU/NU mice. For studies 1 and 2, α-TEA was formulated in liposomes and delivered by aerosol such that approximately 36 μg and 72 μg of α-TEA were deposited in the respiratory tract of each mouse each day, respectively. Cisplatin at 5 mg/kg was administered by intraperitoneal injections once weekly for the first 3 weeks in Study 1 and on the third and 10th days following treatment initiation in Study 2. The combination α-TEA + cisplatin treatment reduced tumor burden and metastasis of cp70-GFP cells in comparison to control mice or mice treated with α-TEA or cisplatin singly. A significant reduction ($P < 0.001$) in growth of subcutaneous transplanted tumors was obtained with α-TEA + cisplatin for both studies. Visible metastases were observed in the lungs of animals from control and cisplatin-treated groups but not in animals from the α-TEA- or α-TEA + cisplatin–treated groups. The α-TEA + cisplatin significantly reduced the total number of lung and axillary lymph node micrometastasis ($P < 0.03$ and $P < 0.0001$, respectively). Analyses of tumor sections showed the α-TEA + cisplatin treatment group, in comparison to control, to have a significantly lower level of cell proliferation (Ki-67 staining; $P < 0.0001$) and a significantly higher level of apoptosis (terminal deoxynucleotidyl transferase–mediated nick end labeling [TUNEL]; $P < 0.0001$). In summary, combinations of α-TEA + cisplatin significantly reduced tumor burden and metastases in a xenograft model of cisplatin-resistant human ovarian cancer cells. These data show promise for combination α-TEA + cisplatin chemotherapy for ovarian cancer. Exp Biol Med 229:1169–1176, 2004

Key words: vitamin E analog (α-TEA); cisplatin; metastasis; antitumor agents; xenograft ovarian cancer model

Ovarian cancer is the fourth leading cause of cancer-related deaths among women, and the overall cure rate, which has remained relatively constant for the past 20 years, is approximately 45% (1, 2). The localized invasive nature of ovarian cancer makes early detection difficult; thus, treatment often begins when the cancer is at an advanced stage. Although most ovarian tumors respond well to chemotherapeutic drugs and may completely regress, there is a high prevalence of recurrence in advanced stage disease (2). Recurrent tumor growth is a poor prognostic indicator, and second- and additional-line chemotherapy often fail (2, 3), most likely because of acquired resistance to drug therapy. The development of cross-resistance to unrelated chemotherapeutic agents is a serious problem in ovarian cancer patients (4).

DNA-damaging agents constitute one important class of chemotherapeutic drugs. These drugs may be employed against many different types of malignancies with great efficacy. By binding to DNA, alkylating agents form cross-links, adducts, and strand breaks, which disrupt DNA replication (3). One such drug is *cis*-dichlorodiammineplatinum(II), cisplatin. This platinum-based compound is well known for its effectiveness when used for the treatment of ovarian, testicular, bladder, head and neck, and some lung

This work was supported by Public Health Service Grant CA59739 (to K.K., B.G.S.), CA75253 (to L.-Z.S.), the Foundation for Research (to K.K., B.G.S.), the National Institute of Environmental Health Sciences Center Grant ES07784 (K.K., B.G.S. are members), and Toxicology Training Grant T32 ES07247 (predoctoral support for K.A.).

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Received July 2, 2004.
Accepted August 31, 2004.

1535-3702/04/22911-1169$15.00

cancers (5). Cisplatin is classified as a pro-drug, and once activated, it interacts with nucleophilic sites in DNA, creating predominantly purine-purine intrastrand cross-links. This genotoxic stress activates signal transduction pathways, which in most cells leads to DNA synthesis arrest and programmed cell death (6). Unfortunately, the initial success of cisplatin chemotherapy is often short-lived, as recurrence of ovarian cancer occurs in a majority of cases (3). The potential of the platinum-based chemotherapeutics is limited by several factors, including dangerous side effects, limited solubility, and intrinsic or acquired resistance. Thus, new therapies are needed for relapsed, cisplatin-resistant patients.

Recently our laboratory has been focusing on establishing the anticancer efficacy and understanding the mechanisms underlying the potent pro-apoptotic properties of a novel vitamin E analog, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid, α-TEA. The parent compound for making α-TEA is natural vitamin E (RRR-α-tocopherol). α-TEA has an acetic acid moiety linked to the phenolic oxygen at carbon 6 of the chroman head by an ether linkage yielding a stable, nonhydrolyzable compound (α-tocopherol ether-linked acetic acid analog, α-TEA). Cell culture studies using a variety of human tumor cells, including breast, prostate, colon, lung, ovarian, cervical, and endometrial cells, as well as normal human mammary epithelial cells and normal human prostate epithelial cells, have demonstrated that α-TEA is a potent pro-apoptotic agent for cancer cells but not normal cells (7, 8). Cell culture studies have also shown that α-TEA induces human ovarian and breast cancer cells to undergo DNA synthesis arrest and apoptosis in a dose- and time-dependent fashion (7, 8) and that α-TEA-induced apoptosis involves restoration of TGF-β and Fas/Fas ligand signaling pathways that signal apoptosis *via* c-Jun NH2-terminal kinase (7). Testing of α-TEA in a preclinical human breast cancer xenograft model (9) as well as a syngeneic mouse mammary cancer model (10, 11) have shown it to significantly reduce tumor burden and lung and lymph node metastasis when administered alone as well as in combination with celecoxib or 9-nitro-camptothecin without any observable toxicities.

Since α-TEA is a lipid, we are investigating formulating it into liposomes to make it more soluble. Also, since 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 of vitamin E compounds other than RRR-α-tocopherol when delivered orally, we are investigating if aerosol is a more effective means of delivering α-TEA.

Based on the ability of α-TEA to induce apoptosis in a number of ovarian cancer cells with EC50 values of 5–20 μg/ml (10.2–41 μ*M*; Ref. 8), we were interested in determining its *in vivo* efficacy. In this paper we investigated the ability of α-TEA singly and in combination with cisplatin to reduce tumor burden and metastasis of cisplatin-resistant A2780/cp70-GFP ovarian cancer cells transplanted into immune compromised mice.

Materials and Methods

Chemicals. The α-TEA (F.W. = 488.8) was prepared as described previously (10). Cisplatin (CDDP; *cis*-dichlorodiammine platinum[II]; F.W. = 300.1] was purchased from Sigma (St. Louis, MO).

Human Ovarian Cancer Cells. The human ovarian cisplatin-resistant A2780/cp70 cells were obtained from Dr. Michael J. Birrer (National Institutes of Health, Rockville, MD). The cp70 subclone was created through intermittent exposure of A2780 cells to increasing levels of cisplatin up to 70 μ*M in vitro* (12). Cells were maintained as monolayers on plastic as previously described (8).

Expression of EGFP in cp70 Cells. The cp70 cells were induced to express green fluorescent protein by viral infection. Briefly, cp70 cells were stably transfected with an expression vector of the enhanced green fluorescence protein (EGF; pEGFP-N1 from Clontech Laboratories, Inc., Palo Alto, CA). The cp70 cells expressing EGFP were then sorted with FACS twice to obtain a population of cp70-GFP cells expressing high levels of green fluorescent protein. cp70-GFP cell populations are routinely monitored for expression of a high level of GFP. Prior to conducting *in vivo* studies, the cp70-GFP cells were sent to the University of Missouri Research Animal Diagnostic and Investigative Laboratory (Columbia, MO), where they were certified to be pathogen free.

Immune Compromised Nude Mice. Female outbred NU/NU immunodeficient mice 4–6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA) and housed in a sterile environment in the transgenic facilities at the Animal Resource Center at the University of Texas at Austin at 74 ± 2°F with 30%–70% humidity and a 12-hr alternating light:dark cycle. Animals were given water and standard lab chow *ad libitum*. Guidelines for the humane treatment of animals were followed as approved by the University of Texas Institutional Animal Care and Use Committee.

Preparation of α-TEA Liposomes for Aerosol Delivery. An α-TEA/lipid ratio of 1:3 (w/w) was previously determined to be optimal for concentration of drug in lipid carrier (10). Briefly, DLCP (1,2-dilauroyl-sn-glycero-3-phosphocholine; Avanti Polar-Lipids Inc., Alabaster, AL) at 120 mg/ml was dissolved in tertiary-butanol (Fisher Scientific, Houston, TX) and then sonicated to obtain a clear solution. The α-TEA at 40 mg/ml was dissolved in tertiary-butanol and vortexed until all solids were dissolved. Equal amounts (v/v) of DLPC and α-TEA were combined to yield a 1:3 ratio of α-TEA/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 Delivery. Liposome control and liposomal formulated α-TEA were administered by aerosol, as previously described (10). Briefly, aerosol was generated using an Easy Air 15 Compressor (Precision Medical, Northampton, PA) producing a 10-L/min airflow with an

AeroTech II nebulizer (CIS-US, Inc. Bedford, MA). This produces liposome particles of a mass median aerodynamic diameter of approximately 2 μm (10). Prior to nebulization, the α-TEA/lipid powder was reconstituted in 3.75 ml of sterile-filtered distilled water to achieve the final desired concentration of 20 mg/ml α-TEA. The mixture was vortexed for approximately 1 min until dissolved and then added to the nebulizer. Six to 10 mice at a time were placed in plastic cages (7 × 11 × 5 in.) with a sealed top in a sterile 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 or liposome only (control) was aerosolized (approximately 15 mins). As previously determined, we estimate that approximately 36 μg or 72 μg of α-TEA were deposited in the respiratory tract of each mouse each day for Study 1 and Study 2, respectively (10). Thus, for Study 1 and Study 2, we estimate that each mouse received a total of 1.4 mg (36 μg/day × 39 days) and 1.08 mg (72 μg/day × 15 days) of α-TEA, respectively.

Treatments. Two animal studies were conducted with 40 and 24 mice, respectively, being initially randomized to have approximately equal tumor burden prior to beginning the treatments (10 mice/group for Study 1 and 6 mice/group for Study 2). Treatments were as follows: control (liposomes by aerosol and isotonic saline ip injections), α-TEA (formulated into liposomes and delivered by aerosol), cisplatin (dissolved in isotonic saline and administered ip one time/week for the first 3 weeks of Study 1 and on the third and 10th days following treatment initiation in Study 2) or combination treatment α-TEA + cisplatin. For Study 1, mice received 1×10^6 cells/100 μl cp70-GFP cells injected subcutaneously into the inguinal area using a 23-gauge needle. For Study 2, mice received 2.5×10^6/100 μl cp70-GFP cells injected subcutaneously into the flank areas of both hind legs using a 23-gauge needle.

Study 1 treatments were initiated 4 days after tumor cell inoculation when tumor volumes were small. Tumor-bearing mice were randomly divided into the four groups with the following mean tumor volume/group of 10 mice: α-TEA = 3.4 mm^3, cisplatin = 4.6 mm^3, combination treatment = 3.9 mm^3, and control = 4.2 mm^3. Study 2 treatments were initiated 21 days after tumor cell inoculation when tumor volumes were larger. Tumor-bearing mice were randomly divided into the four groups with the following mean tumor volumes (two tumors/mouse; six mice/group): α-TEA = 33.4 mm^3, cisplatin = 49.8 mm^3, combination treatment = 34.5 mm^3, and control = 36.6 mm^3). In Study 1, α-TEA was administered at 75 mg α-TEA/cage, and cisplatin was dissolved in 0.9% NaCl at a concentration of 1 mg/ml and administered 5mg/kg once weekly for the first 3 weeks. (Note: These animals received only three ip injections to avoid toxicity.) In Study 2, the level of α-TEA was doubled (150 mg α-TEA/cage), but cisplatin concentration and administration remained the same as for Study 1. For each study, control and α-TEA–treated mice were weighed once weekly, and cisplatin-treated mice were weighed daily to evaluate toxicity. Weight loss of less than 10% of body weight was regarded as safe for treatment continuation. Tumor volume was measured every second day using calipers, and data were recorded. Tumor volume was calculated using the formula $V = (L \times W^2)/2$, where V is the volume of each tumor, L is the length, and W is the width (13).

Determination of Lung and Lymph Node Metastasis. At sacrifice, visual macroscopic lung metastases were counted, and axillary lymph nodes and the left lung lobe were taken for analyses of fluorescent green microscopic metastases. (Note: Since the lungs of mice are not symmetrical, the single lobe of the left lung was chosen for comparative purposes instead of the multilobed right lung.) Lung lobes and lymph nodes were flattened, and the entire surface (both sides for lung and one side only for lymph nodes) of each of the tissues was scored for fluorescent green microscopic metastases using a Nikon fluorescence microscope (TE-200; ×200 magnification) equipped with an ocular grid for use for size determination of metastases. Fluorescent microscopic metastases were placed into three size groupings, <20 μm, 20–50 μm, and >50 μm, which, based on the diameter of cp70-GFP cells, are thought to represent solitary cells, 2–5 cells, and >5 cell groupings.

Ki-67 Staining for Detection of Proliferation *In Vivo*. Deparaffinized 5-μm tumor sections from mice were stained using antibody to the Ki-67 nuclear antigen, which is expressed only in actively proliferating cells, as previously described (10). 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 Corp., Carpinteria, CA), washed, then incubated with biotinylated rabbit–anti-rat IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 30 mins at room temperature. 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. Five fields per sample were scored for Ki-67 positive stained cells. Data are presented as mean ± standard error (SE) of total number of tumors in each group.

Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling [TUNEL] Assay for Detection of Apoptosis *In Vivo*. Deparaffinized 5-μm tumor sections were assessed for apoptosis using reagents supplied in the ApoTag *In Situ* Apoptosis Detection Kit (Intergen, Purchase, NY) according to the manufacturer's instructions. Brown-stained nuclei were scored as positive for apoptosis, and those stained blue were scored as negative. Sixteen microscopic fields (×400) were scored/tumor. Data are

presented as the mean ± SE number of apoptotic nuclei counted in all tumors from each group of treated animals.

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 analysis of variance with the Tukey posttest using SPSS (SPSS Inc., Chicago, IL). Differences 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

α-TEA + Cisplatin Reduced Cp70-GFP Tumor Burden in Nude Mice. In Study 1, mean tumor volume in the α-TEA + cisplatin-combination treatment group was significantly lower over 39 days of treatment than control, α-TEA–, or cisplatin-treated groups ($P < 0.001$; Fig. 1A). One animal was eliminated from the α-TEA treatment group because of an extremely high tumor volume not observed in any other mice. The mean tumor volume at sacrifice for control, α-TEA, cisplatin, and α-TEA + cisplatin groups were 34.6 ± 4.4 mm^3, 41.7 ± 7 mm^3, 35 ± 2.3 mm^3, and 7.1 ± 1.3 mm^3, respectively.

For Study 2, where a greater number of tumor cells were injected subcutaneously into both right and left flank regions and tumors were larger at treatment initiation, mean tumor volumes of the combination treatment group were significantly lower over 15 days of treatment than either control or cisplatin alone-treated groups ($P < 0.001$ and $P < 0.003$, respectively; Fig. 1B). These studies had to be terminated after 15 days because of the large size of tumors in the control and cisplatin-treated animals. The mean ± SE tumor volume (mm^3) at sacrifice for the combination treatment, cisplatin, and control groups was 717.1 ± 359.5 mm^3, 2750 ± 1126.7 mm^3, and 2754 ± 1054.4 mm^3, respectively. The mean tumor volume for the α-TEA group was also significantly lower than control ($P < 0.02$; mean ± SE tumor volume for α-TEA was 2313 ± 1150.4 mm^3; Fig. 1B).

No differences in mean body weight among any of the treatment or control groups for Study 1 and Study 2 were observed. The mean beginning and ending body weights (in grams) for each group were Study 1: control 20.8 ± 0.57 g and 22.7 ± 0.74 g; α-TEA 21.6 ± 0.78 g and 21.8 ± 0.34 g; cisplatin 21.1 ± 0.41 g and 22.4 ± 0.57 g; α-TEA + cisplatin 21.8 ± 0.46 g and 21.9 ± 0.44 g; Study 2: control 20.98 ± 0.67 g and 24.7 ± 0.68 g; α-TEA 23.7 ± 0.50 g and 23.6 ± 0.50 g; cisplatin 22.6 ± 0.73 g and 21.8 ± 0.68 g; α-TEA + cisplatin 24.5 ± 0.63 g and 23.9 ± 0.65 g.

α-TEA + Cisplatin Reduced Visible Lung Metastases. For both Study 1 and Study 2, at the completion of the experiments the five lung lobes from each mouse were examined for visible metastasis. For Study 1, no visible lung metastases were seen in control or treatment groups. For Study 2, visible metastases were observed in four of six control mice with a total of seven visible metastases being detected (two, two, one, and two per mouse). Three visible metastases were observed in one of six cisplatin-treated mice. The α-TEA alone and α-TEA + cisplatin treatment groups had no visual lung lesions (Table 1).

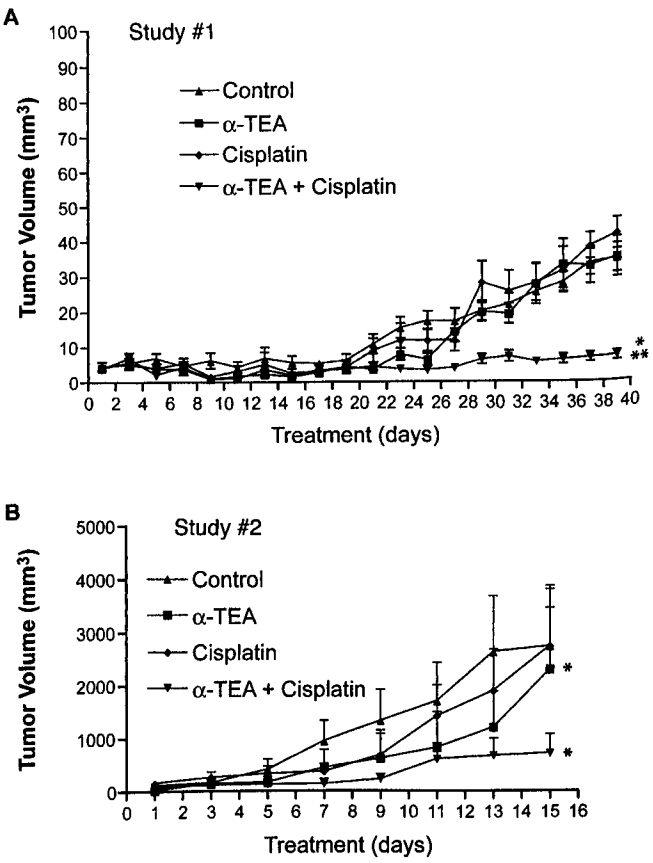

Figure 1. Combined 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA) + cisplatin treatments significantly reduce tumor burden. (A) Study 1. Female NU/NU mice were injected with 1 × 10^6 cp70-GFP–expressing human ovarian cancer cells in the inguinal area. After 4 days, mice were divided into groups of 10 and treated with either 75 mg liposomal formulated α-TEA/treatment chamber delivered *via* aerosol daily, cisplatin (5mg/kg) injected ip once weekly for the first 3 weeks combination of α-TEA + cisplatin, or control (liposome only + ip saline). Mice were treated for 39 days. (B) Study 2. Female nude mice were injected with 2.5 × 10^6 cp70-GFP–expressing human ovarian cancer cells in both lower flank areas. After 21 days, mice were divided into groups of six and treated with either 150 mg liposomal formulated α-TEA/treatment chamber delivered *via* aerosol daily, cisplatin (5 mg/kg) injected ip once weekly (total of two injections), combination of α-TEA + cisplatin, or control (liposome only + ip saline). Mice were treated for 15 days. For both A and B, tumor volumes (mm^3) are depicted as mean ± standard error (SE). *, significantly different from control; **, significantly different from all other treatment groups.

α-TEA + Cisplatin Reduced Microscopic Lung Metastasis. Green fluorescing microscopic lung metastases were classified into three size groupings: <20 μm, 20–50 μm, and >50 μm (Fig. 2). In Study 1, the total number of lung micrometastatic lesions (all sizes) in the α-TEA +

Table 1. Study 2 cp70-GFP Human Ovarian Cancer Cell Macroscopic Lung Metastasis in NU/NU Mice Receiving α-TEA and Cisplatin Alone and in Combination[a]

Delivery/treatments	No. of animals/ group with macroscopic lung metastases[b]	Total no. of macroscopic lung tumor foci[c]
Aerosol/liposomal control	4/6	7
Intraperitoneal/cisplatin	1/6	3
Aerosol/liposomal α-TEA	0/6	0
Aerosol/liposomal α-TEA + intraperitoneal/cisplatin	0/6	0

[a] α-TEA, 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid.
[b] Macroscopic lesions in all five lung lobes from each animal ($N = 6$) in all treatment groups were counted visually.
[c] Data are expressed as the number of animals/group with visible lung metastases and as total number of macroscopic lung tumor foci observed in each group.

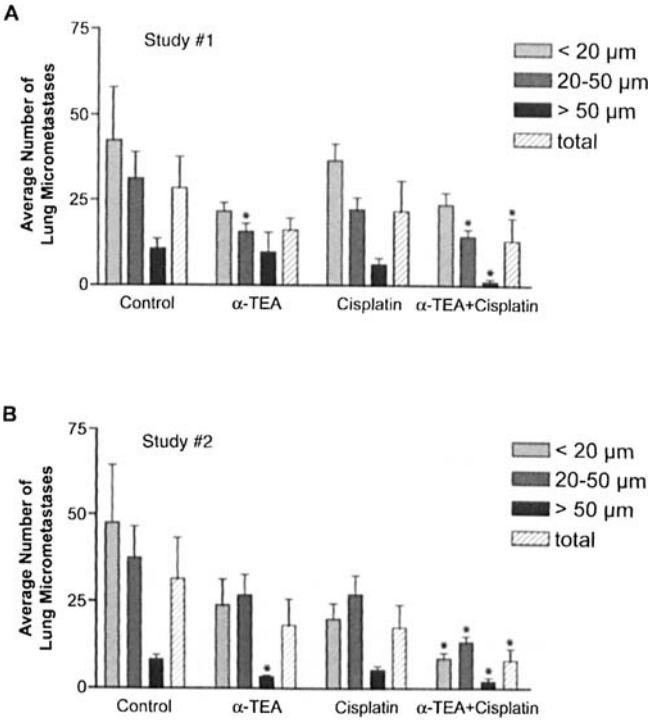

Figure 2. Combined 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA) + cisplatin treatments significantly reduce total number of lung micrometastatic lesions. For Study 1 (A) and Study 2 (B), the left lung lobe from control and treatment groups was flattened, and both sides were examined for fluorescent microscopic lesions. Lesions were grouped into three size groupings (<20 μm, 20–50 μm, or >50 μm). Average number of total lesions was calculated. Data are depicted as mean ± standard error (SE). *, significantly different from control.

cisplatin group was significantly lower than the number of lesions in the control group ($P < 0.03$; Fig. 2A). Analysis within the three size groupings showed a significant reduction in large micrometastatic lesions (>50 μm) in the α-TEA + cisplatin-combination treatment group compared to the control ($P < 0.008$). The mean number of large (>50 μm) lesions in the combination treatment group ($N = 9$), in comparison to the control group ($N = 10$), was reduced by 88%. The mean number of medium lesions (20–50 μm) in the α-TEA and combination treatment groups were significantly reduced, $P < 0.05$ and $P < 0.008$, respectively (Fig. 2A).

In Study 2, lungs from mice treated with α-TEA + cisplatin also had significantly less total micrometastatic lesions than the control group ($P < 0.03$; Fig. 2B). The mean number of large lesions (>50 μm) in the α-TEA and α-TEA + cisplatin treatment groups was significantly different from control, $P < 0.008$ and $P < 0.008$, respectively. The mean number of medium (20–50 μm) and small (<20 μm) lesions in the α-TEA + cisplatin treatment group were significantly different from control, $P < 0.008$ and $P < 0.008$, respectively (Fig. 2B).

α-TEA + Cisplatin Reduced Microscopic Lymph Node Metastasis. Green fluorescent microscopic lymph node metastatic lesions were counted and expressed as number of metastatic lesions/lymph node/group. Actual means ± SE for total number. of metastatic lesions/lymph node/group for the four groups (control, α-TEA, cisplatin, and α-TEA + cisplatin, respectively) in Study 1 were 47.3 ± 5.6, 41.9 ± 8.6, 40.2 ± 8.8, and 24.7 ± 3.5 and in Study 2 were 41.1 ± 6.5, 29.7 ± 4.4, 24.5 ± 2.8, and 17.5 ± 2.7. Microscopic lymph node metastatic lesions in α-TEA and α-TEA + cisplatin–treated mice in Study 1 were significantly lower than control ($P < 0.04$ and $P < 0.0001$; Fig. 3A). For Study 2, the number of metastases/lymph node from α-TEA + cisplatin–treated mice were significantly different from control ($P < 0.005$; Fig. 3B).

α-TEA + Cisplatin Reduced Tumor Cell Proliferation *In Vivo*. The mean number of proliferating cells determined by Ki-67 staining was assessed in tumor sections in Study 2. Tumors from mice receiving the combination α-TEA + cisplatin treatment had a mean ± SE of 97.6 ± 5.9 Ki-67 positive cells/field, whereas tumors from aerosol control mice had a mean ± SE of 243.5 ± 14.9 Ki-67 positive cells/field ($P < 0.0001$; Fig. 4A). The Ki-67 staining of tumor sections from mice treated with α-TEA alone had a mean ± SE of 195.9 ± 21.9 Ki-67 positive cells/field but were not significantly different from control ($P < 0.09$), whereas tumor sections from mice treated with cisplatin alone had a mean ± SE of 153.8 ± 20 Ki-67 stained cells, showing a significant decrease in proliferation in comparison to control ($P < 0.0008$; Fig. 4A).

α-TEA + Cisplatin Increased Tumor Cell Apoptosis *In Vivo*. The TUNEL staining of tumor sections (5 μm) from mice from Study 2 was used to assess the induction of apoptosis by the treatments *in vivo* (Fig. 4B). The mean ± SE number of TUNEL positive cells/field for α-TEA + cisplatin, α-TEA, and cisplatin treatment groups were 5.1 ± 0.36, 2.3 ± 0.3, and 1.5 ± 0.29, whereas

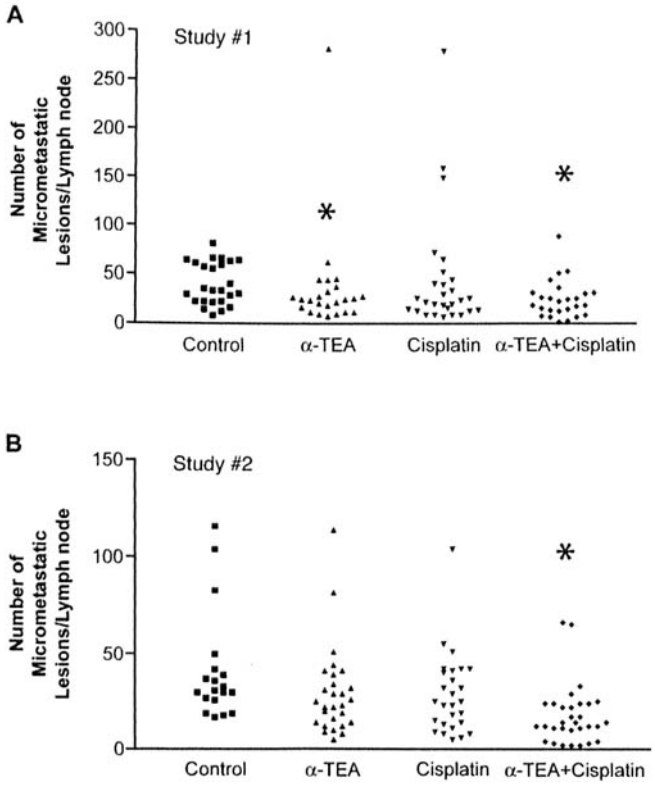

Figure 3. Combined 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA) + cisplatin treatments significantly reduce total number of lymph node micrometastatic lesions. For Study 1 (A) and Study 2 (B), on sacrifice axillary lymph nodes were removed (at least two per animal) and evaluated using a fluorescence microscope for presence of micrometastatic lesions, which were placed into three size groupings (<20 μm, 20–50 μm, or >50 μm). Average number of total lesions was calculated. Data are depicted as mean ± standard error (SE). *, significantly different from control.

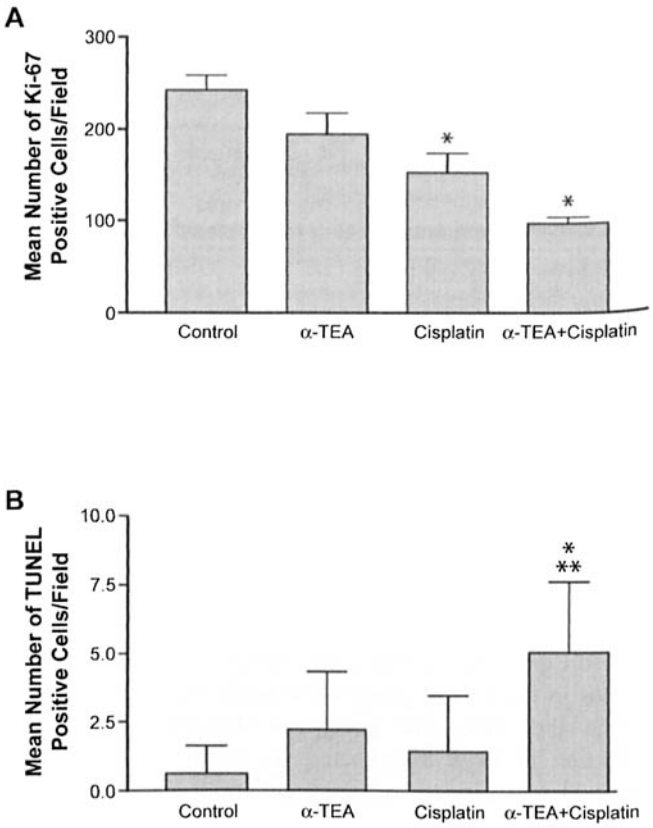

Figure 4. Combined 2,5,7,8-tetramethyl-2R-(4R,8R,12-trimethyltridecyl)chroman-6-yloxyacetic acid (α-TEA) + cisplatin treatments significantly reduce proliferation (Ki-67 staining) and significantly increased apoptosis (terminal deoxynucleotidyl transferase–mediated nick end labeling [TUNEL]). Five-micron tumor tissue sections from control and treatment groups in Study 2 ($N = 10$ tumors/group) were examined by immunohistology for levels of cell proliferation by Ki-67 (A) and for apoptosis by TUNEL (B). Data are depicted as mean ± standard error (SE) of Ki-67 positive-stained cells/field or TUNEL positive-stained cells/field. *, significantly different from control.

tumors from the aerosol control group had a mean ± SE of 0.6 ± 0.14 number of TUNEL positive cells/field. The combination treatment group had significantly more apoptotic cells/tumor than all other groups (combination treatment vs. α-TEA, $P < 0.0001$; combination treatment vs. cisplatin, $P < 0.0007$; and combination treatment versus control $P < 0.0001$, respectively; Fig. 4B).

Discussion

In these studies we compared the ability of a liposome formulation of the vitamin E analog α-TEA administered by aerosol alone and in combination with cisplatin to inhibit tumor growth in a human cisplatin-resistant ovarian cancer xenograft model. Data show that when low-dose α-TEA treatments were initiated when tumor volumes were small, the combination α-TEA + cisplatin treatment was the only treatment that significantly decreased tumor volume in comparison to control. In a second study in which treatments were initiated when tumor volumes were larger and the level of α-TEA was doubled, the combination α-TEA + cisplatin treatment significantly decreased tumor volume better than either α-TEA or cisplatin single treatments, but the α-TEA single treatments also showed a significant decrease in tumor volume in comparison to control.

In addition to reduction in tumor volume, these studies showed that only mice receiving α-TEA + cisplatin had a statistically significant reduction in micrometastatic lesions in both lungs and lymph nodes in comparison to control.

Furthermore, combination treatment with α-TEA + cisplatin significantly decreased the number of proliferating cells in tumor tissue and increased the number of apoptotic cells. Thus, data from these studies show that the combination α-TEA + cisplatin treatment is an effective *in vivo* anticancer drug combination for cisplatin-resistant A2780/cp70 human ovarian cancer cells and that both growth arrest and enhanced apoptosis are involved in mediating the antitumor effects.

These findings are potentially significant in that platinum-based chemotherapy alone and with other chemotherapeutic agents are used in treatment of human ovarian cancer with the majority of patients becoming refractive (14–16). Thus, a compound such as α-TEA, with no known

adverse side effects, that can convert cisplatin resistant cells to some type of responsiveness to cisplatin in a combined therapy is noteworthy. In addition to showing promise for human ovarian cancer, α-TEA alone has been shown to be effective in inducing human breast and mouse mammary cancer cells to undergo apoptosis *in vitro* and to reduce tumor burden and metastasis to lungs and lymph nodes in a syngeneic murine mammary cancer model (10) and in a xenograft model (9). Furthermore, α-TEA + 9-nitro-camptothecin or α-TEA + celecoxib combination treatments have been shown to significantly reduce tumor burden and metastasis in animal models (9, 11). Taken together, these studies support the need for further development of α-TEA as a chemotherapeutic agent.

Although data reported here showed that when α-TEA was administered singly, it was inconsistent in significantly reducing tumor burden or metastasis, this is in contrast to all other studies performed to date, which demonstrate that α-TEA alone is a very effective anticancer agent (9–11). It is hoped that current studies focused on improving the bioavailability of α-TEA will increase its potential as a single agent against ovarian cancer.

How α-TEA + cisplatin is able to restore cisplatin responsiveness in cp70 cells is not known. One possibility is restoration of pro-apoptotic signaling, more specifically the Fas death receptor signaling pathway. Mansouri and co-workers (17) showed that the JNK > c-Jun > FasL > Fas pathway is important in mediating cisplatin-induced apoptosis in ovarian cancer cells and that the duration of JNK activation is critical in determining whether the cells survived or underwent apoptosis. These investigators showed that cisplatin induced a prolonged activation (8–12 hrs) of JNK and p38 in sensitive cells and only a transient activation (1–3 hrs) in resistant cells. Since α-TEA has been shown to induce prolonged activation (6–24 hrs) of JNK and activate Fas signaling (7), perhaps the α-TEA + cisplatin combination reverses a dysregulated JNK > c-Jun > FasL > Fas pathway leading to apoptosis. As further support for this possibility, a recent study by Abedine *et al.* (18) supports the important role Fas signaling plays in cisplatin chemoresistance. These investigators showed that Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (FLIP), a cell survival factor, plays a role in cisplatin chemoresistance in human ovarian cancer cells. They showed that cisplatin significantly decreased FLIP protein level and induced cleavage of caspase-8 and caspase-3 and apoptosis in cisplatin-sensitive but not -resistant cells. Furthermore, they showed that ectopic overexpression of FLIP attenuated cisplatin apoptosis in sensitive cells and that downregulation of FLIP in resistant cells led to increased apoptosis induced by cisplatin. The ability of α-TEA to modulate FLIP is under study.

In summary, data presented here show that treatment of mice bearing cisplatin-resistant cp70 human ovarian cancer cell tumors with a novel vitamin E–based compound, α-TEA, in combination with cisplatin significantly reduces tumor burden and inhibits metastasis. Since the use of cisplatin in ovarian cancer chemotherapy is limited by the development of resistance, we propose that α-TEA + cisplatin holds promise as a chemotherapeutic approach for treatment of advanced cisplatin resistant ovarian cancer in patients.

We thank the Histology Core Facility for preparation of H&E and immunohistochemically stained tissues and the director of the Biostatistics and Data Processing Core, Dr. Howard Thames, for help with statistical analyses (National Institute of Environmental Health Sciences Center Grant ES07784; University of Texas M. D. Anderson). We thank Dr. Richard Willis (Division of Nutrition, University of Texas at Austin) for statistical advice and Dr. Vernon Knight's and Dr. Brian Gilbert's laboratories at Baylor College of Medicine, Houston, Texas, for the methods of liposome preparation and aerosol analyses.

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