Research Article

In vitro and *in vivo* evaluation of anticancer actions of natural and synthetic vitamin E forms

Weiping Yu[1,2], Li Jia[2], Pei Wang[2], Karla A. Lawson[3], Marla Simmons-Menchaca[2], Sook-Kyung Park[2], LuZhe Sun[4], Bob G. Sanders[2] and Kimberly Kline[1]

[1] Division of Nutrition/A2703, University of Texas at Austin, Austin, TX, USA
[2] School of Biological Sciences, University of Texas at Austin, Austin, TX, USA
[3] National Cancer Institute, Cancer Prevention Fellowship Program, Bethesda, MD, USA
[4] Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

The goal of these studies was to investigate the potential anticancer properties of two naturally occurring plant sources and two manufactured synthetic forms of vitamin E, *i.e.*, RRR-α-tocopherol (αT), RRR-γ-tocopherol (γT), *all-rac*-α-tocopherol (*all-rac*-αT), and *all-rac*-α-tocopheryl acetate (*all-rac*-αTAc) in breast cancer models. Vitamin E compounds were evaluated *in vitro* for inhibition of colony formation and induction of apoptosis in human MDA-MB-435 and MCF-7 breast cancer cells and murine 66cl-4 mammary cancer cells and *in vivo* for ability to reduce tumor growth and lung and lymph node metastases using the transplantable syngeneic BALB/c mouse 66cl-4-GFP mammary cancer model. γT inhibited colony formation and induced apoptosis in all three cancer cell lines. αT and *all-rac*-αT were less effective and *all-rac*-αTAc was ineffective. γT-induced apoptosis was correlated with activation of caspases-8 and -9 and down-regulation of protein expression of c-FLIP and survivin. *In vivo* study 1 analyses showed that *all-rac*-αT and *all-rac*-αTAc significantly inhibited tumor growth and inhibited both visible and microscopic size lung metastases. *In vivo* study 2 analyses showed that αT and γT reduced tumor growth, but only γT reduced tumor growth significantly in comparison to control. In conclusion, synthetic, but not natural, vitamin E exhibits promising anticancer properties *in vivo*.

Keywords: Anticancer actions / Apoptosis / Human breast cancer / Natural vitamin E / Synthetic vitamin E

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1 Introduction

Vitamin E is a general term used to refer to a group of eight chemically different compounds produced by plants known as tocopherols (α, β, γ and δ) and tocotrienols (α, β, γ and δ), as well as synthetic vitamin E [*all-rac*-α-tocopherol (*all-rac*-αT)], which is prepared commercially and which consists of a mixture of eight stereoisomeric forms of α-tocopherol, only one of which is equivalent to"natural" RRR-α-tocopherol (αT) [1]. Both αT and *all-rac*-αT can be purchased as commercially produced acetate or succinate derivatives. The acetate derivative of *all-rac*-αT (*all-rac*-αTAc) and *all-rac*-αT are common components of dietary supplements. Significant sources of αT and γT are polyunsaturated plant oils and products made from them such as margarines and salad dressings.

Despite many research efforts, the biological functions of natural and synthetic vitamin E compounds remain to be better defined [2 – 7]. Although pre-clinical cell culture and animal model studies suggest promise of certain naturally occurring vitamin E forms (notably γT) and synthesized analogs of vitamin E as anticancer agents, double-blind, placebo-controlled clinical trials fail to provide science-based support for either αT or *all-rac*-αTAc as anticancer agents, with the possible exception of *all-rac*-αTAc as a chemopreventive agent for prostate cancer in heavy smokers [6].

Breast cancer is the most commonly diagnosed cancer in U.S. women, exceeding 214 000 cases annually, with approx-

Correspondence: Dr. Kimberly Kline, Division of Nutrition/A2703 University of Texas at Austin, Austin, TX 78712-1097, USA
E-mail: k.kline@mail.utexas.edu
Fax: +1-512-232-7040

Abbreviations: ***all-rac*-αT**, *all-rac*-α-tocopherol; ***all-rac*-αTAc**, *all-rac*-α-tocopheryl acetate; **αT**, RRR-α-tocopherol; **α-TEA**, RRR-α-tocopherol ether-linked acetic acid analog ; **DAPI**, 4′,6-diamidino-2-phenyindole; **DLPC**, 1,2-dilauroyl-sn-glycerol-3-phosphocholine; **GAPDH**, glyceraldehyde-3-phosphate dehydrogenase; **γT**, RRR-γ-tocopherol; **PARP**, poly (ADP-ribose) polymerase; **PI**, propidium iodide

imately 88% of women surviving 5 years [8]. Thus, there is a need to better understand how vitamin E compounds, found naturally in the diet or consumed as food additives or supplements, may influence breast cancer development and recurrence. Two extensive reviews concluded that vitamin E from dietary sources may provide women with modest protection from breast cancer; however, there was no evidence that vitamin E supplements (αT or *all – rac*-αTAc) conferred any protection against breast cancer [9, 10]. Increasing evidence in other cancer types, suggest that γT, the most abundant form of vitamin E in the American diet, may possess anticancer properties [9, 11 – 16].

Thus, the goal of this study was to evaluate two "natural" forms of vitamin E (αT and γT) and two synthetic forms of vitamin E (*all-rac*-αT and *all-rac*-αTAc) for their anticancer actions *in vitro* and *in vivo*.

2 Materials and methods

2.1 Vitamin E compounds

all-rac-αT and *all-rac*-αTAc were purchased from Sigma Chemical Co (St. Louis, MO, USA). αT (RRR-α-tocopherol) and γT (RRR-γ-tocopherol) were purchased from TAMA Biochemical Company (Tokyo, Japan). α-TEA (RRR-α-tocopherol ether-linked acetic acid analog), an analog of RRR-α-tocopherol, that exhibits potent anticancer properties both *in vitro* and *in vivo* [6, 17] was used as a positive control and prepared as previously described [18]. α-TEA was chosen as the positive control because it shows anti-cancer efficacy *in vivo* when delivered orally [17, 18].

2.2 Cell lines

Original sources and cell culture conditions for maintenance of the human breast cancer cell lines MDA-MB-435 (estrogen non-responsive) and MCF-7 (estrogen responsive) and the mouse mammary cancer cells used in this study have described previously [18, 19]. It is important to note that there are questions about the origin of MDA-MB-435 cells. Some investigators have data indicating that the MDA-MB-435 cells are derived from the M14 melanoma cell line [20], while other investigators have data supporting breast origin [21, 22]. For experiments, FBS was reduced to 2% to better mimic the *in vivo* low serum exposure of these type cells. The cells were plated at 3×10^5 cells/100-mm dish or 6×10^5/150-mm dish for apoptosis and Western blot analyses, respectively.

2.3 Colony formation assay

Effects of vitamin E compounds on colony formation of human breast cancer cells and 66cl-4-GFP cells were determined as described previously with the following modifications [23]. Vitamin E compounds were dissolved in DMSO at 200 mM, then further diluted in ethanol to achieve 40 mM stock solutions. Equivalent levels of DMSO/ethanol (1:4) were used as vehicle controls (VEH). Cells were seeded at 200 or 400 cells/well in 12-well plates and incubated overnight. Cells were treated with vitamin E forms or VEH at indicated concentrations for 12 days. Cells were washed with PBS, fixed with methanol and stained with 0.1% methylene blue in PBS. Colony forming cells are expressed as cell survival (%), which was calculated as number of colonies in treatment/number of colonies in control × 100%. IC_{50} values for inhibition of colony formation were calculated using CalcuSyn software (Biosoft, Cambridge, UK).

2.4 Evaluation of apoptosis

Apoptosis was evaluated by morphological analyses of 4′,6-diamidino-2-phenyindole (DAPI)-stained cells as described in detail previously [24] and quantified using the Annexin V-FITC/propidium iodide (PI) assay following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The latter assay measures amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to membranes of apoptotic cells) and the amount of PI, a dye that does not cross the plasma membrane of viable cells but readily enters dead cells and binds DNA. Fluorescence was measured using FACSCalibur flow cytometry and data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA). Cells displaying phosphatidylserine on their surface (positive for annexin-V fluorescence) were considered to be apoptotic. IC_{50} values for apoptosis were calculated using CalcuSyn software

2.5 Western blot analyses

Western blot analyses to assess protein levels in whole cell extracts were performed as described previously [24]. Antibodies to poly (ADP-ribose) polymerase (PARP), c-FLIP and survivin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to caspase-8 and -9 were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to GAPDH were produced in house. Following transfer, blots were reacted with primary antibody in 0.1% BSA/TBST overnight at 4°C, washed three times with TBST and reacted with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse (Jackson Immunoresearch, Rockford, IL, USA) secondary antibodies. Protein bands were imaged and quantified after correcting for GAPDH loading control using Scion Image Software (Scion Corporation, Frederick, MD, USA).

2.6 Animals and treatment protocols

All animal experiments were conducted according to ‚Guidelines for the Humane Treatment of Animals' as des-

ignated by the University of Texas Institutional Animal Care and Use Committee. Female BALB/c mice at 6 weeks of age (approximately 20 g in weight) were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

Two animal studies were conducted. All mice were injected subcutaneously with 2×10^5 BALB/c 66cl-4-GFP mammary cancer cells/100 μL in the inguinal area at a point equal distant from the fourth and fifth nipples on the right side. In study 1, the mice were randomly assigned to four groups (10 mice/group): control (liposome only), *all-rac*-αT, *all-rac*-αTAc and α-TEA when the tumors reached an average volume of 3.1 mm^3 (control, 3.2 mm^3; *all-rac*-αT, 2.8 mm^3; *all-rac*-αTAc, 3.4 mm^3; and α-TEA, 2.9 mm^3). Treatments were initiated on the day 10 after tumor cell inoculation. In study 2, the mice were randomly assigned to four groups (10 mice/group): control (liposome only), α-TEA, αT and γT, with average tumor volume/group of 1.5, 1.4, 1.5 and 1.4 mm^3, respectively. Treatments were initiated on day 7 after tumor inoculation. For both studies, tumors were measured using calipers every other day, and the volumes were calculated using the formula: volume (mm^3) = (width × width × length/2). Body weights were determined weekly.

Vitamin E compounds were formulated in liposomes in an effort to enhance vitamin E uptake and retention and delivered by gavage. Vitamin E/liposome treatments was prepared as previously described [18]. Mice in both studies were gavaged with 100 μL of vitamin E treatments twice each day (morning at 8 – 9 am and evening at 4 – 5 pm) so that mice in treatment groups received 6 mg vitamin E/day in study 1 and 5 mg vitamin E/day in study 2.

2.7 Determination of lung and lymph node metastases

For both studies, macroscopic metastases in all five lung lobes were counted visually at euthanasia. The left lung lobe as well as brachial and axillary lymph nodes were harvested and frozen for analyses of micrometastatic foci. Fluorescent micrometastatic foci were determined as described previously [18].

2.8 Statistical analyses

Tumor growth was evaluated by transforming volumes using a logarithmic transform (base 10) and analyzed using a nested two-factor analysis of variance (ANOVA) with SPSS (SPSS Inc, Chicago, IL, USA). Differences in number of fluorescent microscopic metastases/group and visible metastatic foci were determined using the two-tailed Mann-Whitney rank test with Prism software version 4.0 (Graphpad, San Diego, CA, USA). Differences in incidence of lung macroscopic foci were determined using Chi-Square test with Prism software version 4.0. A level of $p < 0.05$ was regarded as statistically significant.

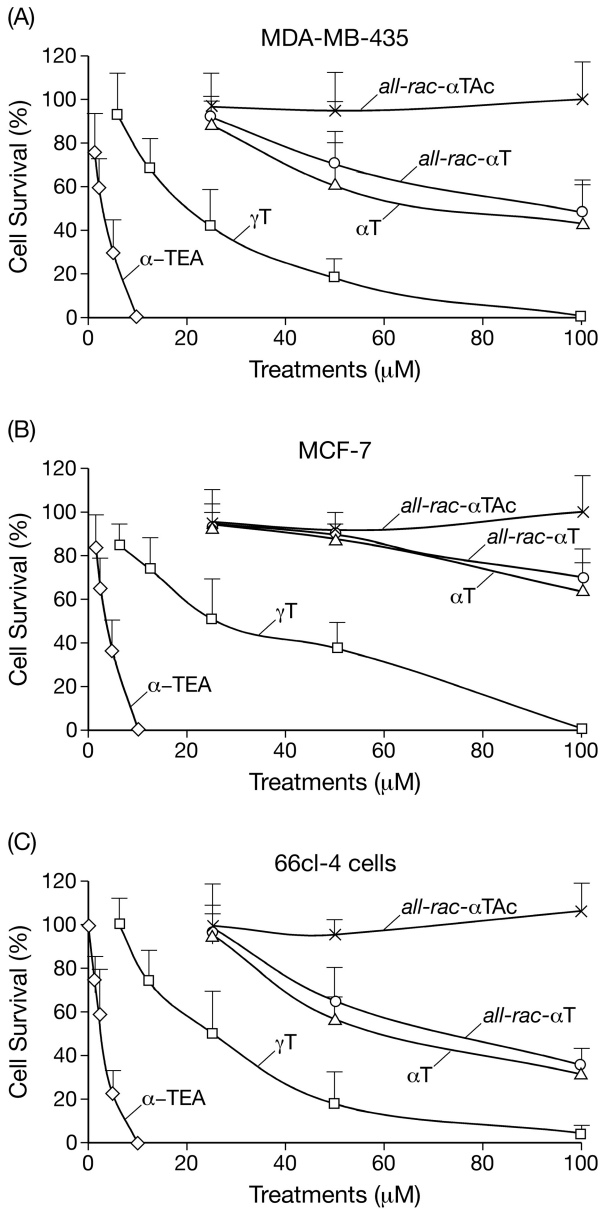

Figure 1. Colony formation assays. Cells were treated with a range of concentrations of the five vitamin E compounds for 12 days, and colonies counted (A – C). Colonies are expressed as cell survival (%), which was calculated as number of colonies in treatment/number of colonies in control × 100%. Data are mean ± SD of at least three separate experiments.

3 Results

3.1 Effect of vitamin E compounds on cancer cell colony formation

Vitamin E treatments for 12 days in concentrations ranging from 1.25 to 100 μM were evaluated for ability to reduce colony formation by MDA-MB-435, MCF-7, and 66cl-4-GFP cells. Cell survival (%) curves for the three cell lines are given in Figs. 1A – C. A summary of the IC_{50} values for

Table 1. Comparison of IC_{50} values of vitamin E compounds for inhibition of colony formation and induction of apoptosis[a)]

	MDA-MB-435		MCF-7		66cl-4	
	CF	APO	CF	APO	CF	APO
α-TEA	2.4 ± 0.5	8.8 ± 1.8	2.9 ± 0.8	9.4 ± 0.7	3 ± 0.83	9.4 ± 2.0
γT	19.2 ± 3.6	70.3 ± 12.6	22 ± 6.6	77.3 ± 22	25 ± 6.6	79.3 ± 14.8
αT	91 ± 44	>200	>100	>200	67 ± 18	>200
all-rac-αT	95 ± 39	>200	>100	>200	72 ± 17	>200
all-rac-αTAC	NE[b)]	>200	NE	>200	NE	>200

a) IC_{50} = level of vitamin E compound resulting in 50% inhibition for colony formation (CF) or inducing apoptosis (APO). IC_{50} values for inhibition of colony formation were calculated from Fig. 1 data, and IC_{50} values for apoptosis were calculated from dose-response experiments generated by DAPI staining (data not shown; 2.5–200 μM concentrations tested). Data are depicted as mean ± SD of three experiments.

b) NE, not established; no inhibition of colony formation was observed at any concentration tested (1.25–100 μM).

the different vitamin E forms is presented in Table 1. Based on IC_{50} values, the rank order from most to least effective was: α-TEA > γT > αT > *all-rac*-αT. The IC_{50} value for *all-rac*-αTAc could not be determined since it did not inhibit colony formation in any of the three cells lines at any of the concentrations tested.

3.2 Evaluation of pro-apoptotic properties of vitamin E compounds

DAPI staining was used to identify apoptotic nuclear morphological changes and quantify apoptosis. FACS analyses of phosphatidylserine translocation to the cell surface using FITC-tagged Annexin V were used as an alternative method to identify and quantify apoptosis. γT- and α-TEA-treated cancer cells exhibited condensed and fragmented nuclei indicative of apoptosis following 3 days of treatment (Fig. 2A). When αT, *all-rac*-αT and *all-rac*-αTAC were tested at 40 μM (*i.e.*, same concentration as used to demonstrate pro-apoptotic activity of γT) none of these compounds exhibited apoptotic morphology (data not shown). Both γT and α-TEA-induced apoptosis in a time-dependent manner (Fig. 2B). Based on IC_{50} values calculated from dose-response experiments (2.5–200 μM; data not shown) analyzing DAPI-stained cells, the rank order of effectiveness for induction of apoptosis was: α-TEA > γT (Table 1). The IC_{50} values for apoptosis induction for αT, *all-rac*-αT, and *all-rac*-αTAc could not be determined at any of the concentrations tested (Table 1). Annexin V assays further confirmed the presence or absence of pro-apoptotic properties of the vitamin E compounds, showing that both α-TEA and γT induced increased binding of annexin V in all three cancer cell lines (Fig. 2C). However, αT and the two synthetic vitamin E forms tested at 200 μM (*i.e.*, fivefold higher concentration than γT) caused only a modest percentage of annexin V-positive cells, *i.e.*, about 10–20% (Fig. 2C), confirming that the IC_{50} values of these compounds are more than 200 μM.

3.3 Mechanisms of γT-induced apoptosis

γT and α-TEA induced apoptosis in a similar manner in MDA-MB-435 cells; *i.e.*, *via* activation of caspases-8 and -9 and cleavage of PARP (Fig. 3A). Also both compounds down-regulated two pro-survival factors, c-FLIP and survivin, which should sensitize the cells to apoptosis (Figs. 3B and C). In contrast, αT did not activate caspases or cause the cleavage of PARP, and enhanced rather than decreased survivin protein expression (Figs. 3A–C). These studies suggest that γT induces apoptosis in a manner similar to α-TEA, which has been studied more extensively [6, 17].

3.4 Evaluation of synthetic *all-rac*-αT and *all-rac*-α-TAc for ability to reduce tumor burden and metastases

In vivo study 1 compared the ability of *all-rac*-αT and *all-rac*-α-TAc to reduce 66cl-4-GFP tumor burden and inhibit metastases. The mean tumor volume-time curves for treatment and control groups are shown in Fig. 4A. Data show a significant reduction of tumor volume in α-TEA, *all-rac*-αT and *all-rac*-αTAc treatment groups in comparison to control group ($p < 0.001$). The average tumor volumes (mean ± SE) at 19 days treatment were: 752 ± 225, 561 ± 182, 316 ± 85, and 220 ± 47 mm^3 for control, *all-rac*-αTAc, *all-rac*-αT and α-TEA treatments, respectively. There were no significant differences in ability to inhibit tumor growth among the vitamin E treatment groups. Although the two synthetic vitamin E forms were ineffective as anticancer agents in cell culture, both were effective in reducing tumor volume *in vivo*.

The number of mice/group with macroscopic lung metastases was reduced in all three vitamin E-treated groups, but only significantly reduced in the *all-rac*-αT treatment groups in comparison to control ($p < 0.004$; Table 2). The average number of macroscopic lung tumor foci was significantly reduced in both *all-rac*-αT and α-TEA treatments in

comparison to control ($p < 0.05$) but not with *all-rac*-αTAc treatment ($p = 0.12$; Table 2).

The average number of total lung micrometastatic foci for *all-rac*-αT (18.2 ± 3.7), *all-rac*-αTAc (24.6 ± 3.9), and α-TEA (22.8 ± 5.3) were significantly decreased in comparison to control (87 ± 12.6) ($p = 0.0003$, 0.0002 and 0.0004, respectively; Fig. 4B). The average number of lymph node micrometastatic foci in *all-rac*-αTAc (2.3 ± 0.5) and in α-TEA (1.8 ± 0.5) treatment groups in comparison to control group (6.1 ± 1.3) were significantly reduced ($p = 0.023$ and $p = 0.012$, respectively; Fig. 4C). Although, the average number of total lymph node micrometastatic foci in the *all-rac*-αT treatment group (3.4 ± 1.5) was reduced in comparison to control, it was not statistically significant ($p = 0.17$; Fig. 4C).

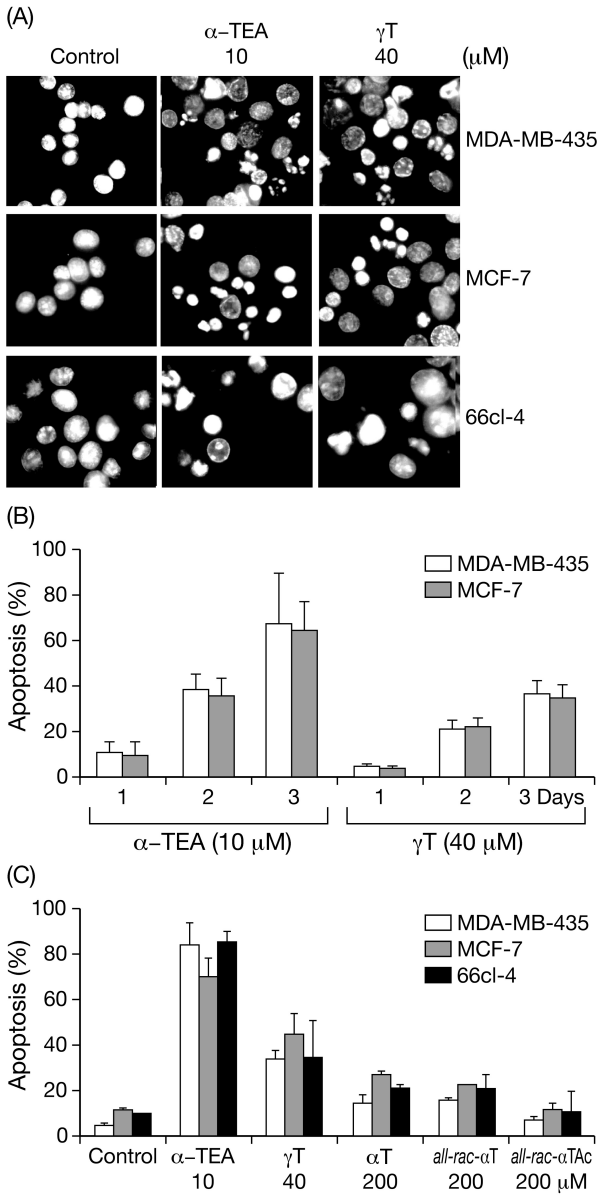

Figure 2. Evaluation of pro-apoptotic properties. Cells were treated with vitamin E compounds at the indicated concentration for 3 days. (A) Photomicrographs of DAPI-stained cells showing condensed or fragmented chromatin in nuclei from apoptotic cells in comparison to non-apoptotic nuclei exhibiting an overall dull staining pattern. (B) Time-dependent apoptosis of α-TEA (10 μM) and γT (40 μM) for 1, 2, and 3 days was analyzed by DAPI staining. (C) Quantification of percent apoptotic cells by FACS analyses of annexin V-positive cells. Data in (A) are representative of multiple experiments and data in (B) and (C) are mean ± SD of at least two independent experiments.

Table 2. Ability of synthetic vitamin E forms to prevent visible lung metastases (study 1)

Treatments	No. of animals/group with macroscopic lung metastases[a)]	Average no. macroscopic lung tumor foci[b)]
Control	7/10	1.9 ± 0.7
all-rac-αT	0/8[c)] *	0*
all-rac-αTAc	3/10	0.4 ± 0.2
α-TEA	2/9[c)]	0.3 ± 0.2*

a) Macroscopic foci in all lung lobes for each animal in control and treatment groups were counted visually at the time of sacrifice. Numbers of mice with macroscopic lesions/total number of mice in each group are given.
b) Data are expressed as the average number of macroscopic lung tumor foci per mouse ± SE.
c) One mouse in the α-TEA group and two mice in the *all-rac*-αT group were accidentally killed during gavage.
* Significantly different from control $p < 0.05$.

3.5 Evaluation of αT and γT for ability to reduce tumor burden and metastases

In vivo study 2 compared the ability of αT and γT to reduce 66cl-4-GFP tumor burden and inhibit metastases. Data showed that γT, but not αT, significantly reduced tumor burden in comparison to control (Fig. 5A; $p < 0.001$). As expected, α-TEA significantly reduced tumor burden in comparison to control, and it also significantly reduced tumor burden in comparison to γT (Fig. 5A).

The number of mice/group with macroscopic lung metastases were reduced in all three vitamin E-treated groups in comparison to control, but only significantly reduced in the α-TEA-treated group ($p < 0.01$; Table 3); whereas, the average number of macroscopic lung tumor foci were significantly reduced in α-TEA and γT treatment groups ($p = 0.0052$ and $p = 0.04$), but not in the αT treatment group ($p = 0.14$), when compared to the control group (Table 3).

Neither γT nor αT significantly decreased the number of either lung or lymph node micrometastatic foci in comparison to control, while α-TEA significantly decreased both the average number of micrometastatic lung foci ($p = 0.009$; Fig. 5B) and lymph node micrometastatic foci ($p = 0.004$; Fig. 5C) in comparison to control. As positive control, α-TEA exhibited significant antitumor activities in both study 1 and study 2; however, anticancer efficacy was

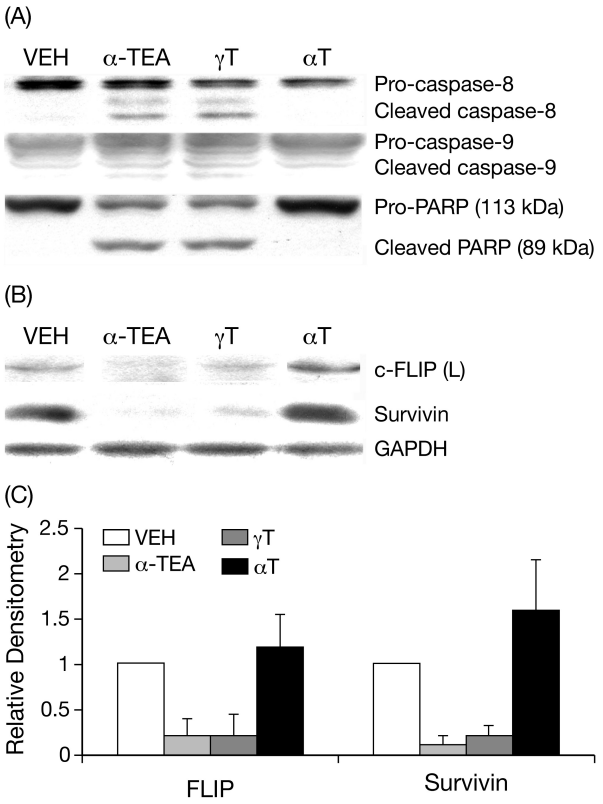

Figure 3. Assessment of biomarkers of apoptosis. Activation of caspase-8 and 9 and PARP cleavage, as well as expression of pro-survival proteins c-FLIP and survivin were detected in MDA-MB-435 cells treated with α-TEA (10 μM), γT (40 μM) or αT (40 μM) for 3 days. (A, B) Protein levels of biomarkers were determined by Western immunoblot analyses. (C) Relative protein levels of c-FLIP and survivin in comparison to control were determined by densitometric analyses. Data are presented as mean ± SD of three independent experiments.

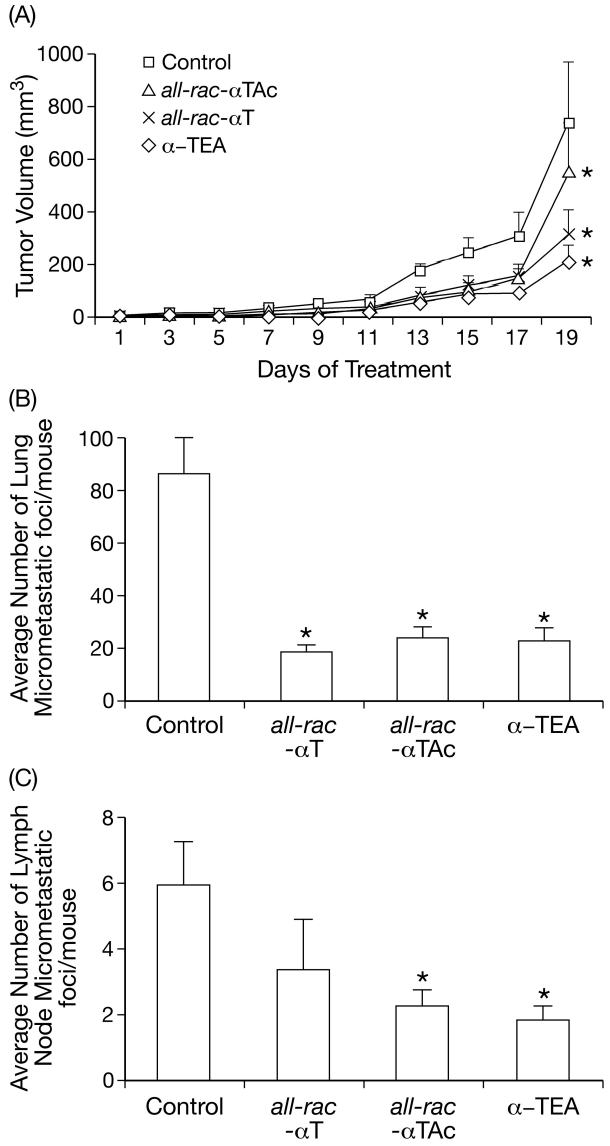

Figure 4. Animal study 1. Treatments were started 10 days after tumor injection. (A) Tumor volumes (mm^3) were determined at 2-day intervals and are depicted as mean ± SE. (B) Average number of lung microscopic metastatic foci for control and treatment groups depicted as mean ± SE. (C) Average number of lymph node microscopic metastatic foci for control and treatment groups depicted as mean ± SE. *Significant difference from control group ($p < 0.05$).

better in study 1. The difference may be attributed to biological variation in the two studies or to the lower dose of α-TEA used in study 2.

Mice in control and treatment groups in both animal studies gained weight throughout the studies and there were no significant differences in body weights among animals in control and treatment groups for studies 1 and 2 (data not shown), suggesting no overt toxicities generated by any of the treatments.

4 Discussion

Data presented in this study show different anticancer efficacy outcomes for different forms of vitamin E when tested *in vitro* and *in vivo*: (i) γT but not αT, *all-rac*-αT or *all-rac*-αTAc, markedly reduced cancer cell growth as determined by inhibition of colony formation *in vitro*. Furthermore, γT but not the other vitamin E forms was demonstrated to be an effective inducer of apoptosis *in vitro*, capable of activating caspases-8 and -9, causing the cleavage of PARP and down-regulating the expression of two survival factors, c-FLIP and survivin; (ii) although synthetic forms of vitamin E (*all-rac*-αT and *all-rac*-αTAc) were either modestly effective or completely ineffective, respectively, *in vitro*, they both significantly reduced tumor volume and metastases in the transplantable syngeneic mouse mammary cancer model; (iii) αT, the form of vitamin E with the highest bioavailability in humans was only modestly effective *in vitro*

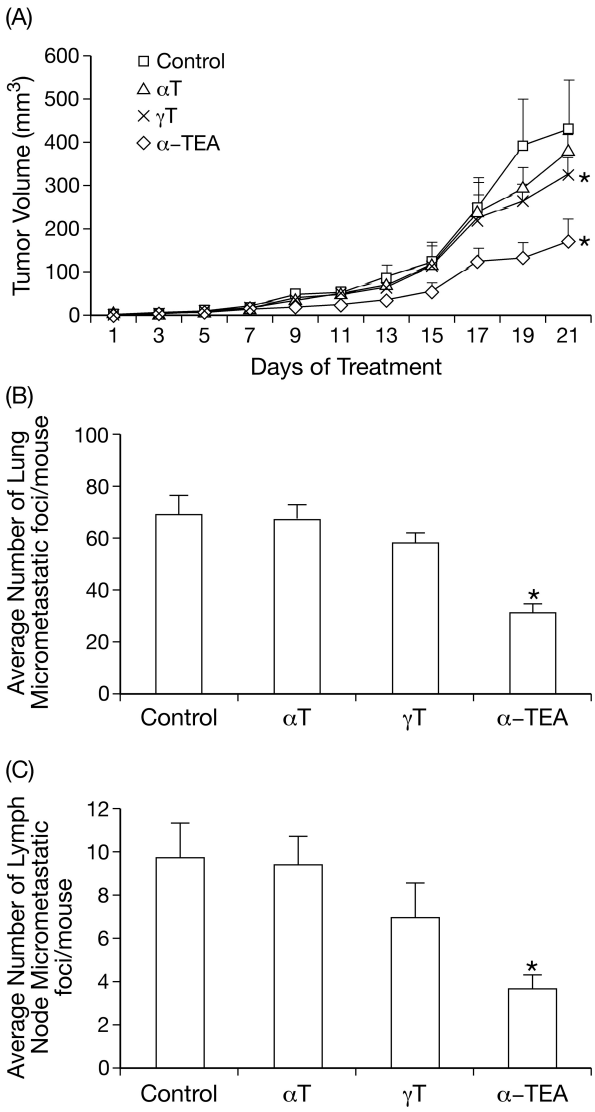

Figure 5. Animal study 2. This study was conducted in an identical manner to study 1 except treatments were started 7 days after tumor injection and mice received 5 mg vitamin E/day *versus* 6 mg vitamin E/day in study 1. (A) Tumor volumes (mm^3) were determined at 2-day intervals and depicted as mean ± SE. (B) Average number of lung microscopic metastatic foci for control and treatment groups depicted as mean ± SE. (C) Average number of lymph node microscopic metastatic foci for control and treatment groups depicted as mean ± SE. *Significant reduction in tumor burden in comparison with control. **Significant difference from all other treatments.

and not significantly effective *in vivo*; (iv) γT was effective *in vitro* and based on statistical analyses significantly reduced BALB/c 66cl-4 tumor volume *in vivo*, but it was not effective against either lung or lymph node micrometastases; and (v) α-TEA (used in these studies as a positive control), was an effective anticancer agent in all experiments conducted. Thus, these studies illustrate the complexities of assessing different vitamin E compounds for

Table 3. Ability of natural vitamin E forms to prevent visible lung metastases (study 2)

Treatments	No. of animals/group with macroscopic lung metastases[a)]	Average no. macroscopic lung tumor foci[b)]
Control	8/10	1.9 ± 0.6
αT	5/10	0.9 ± 0.4
γT	5/10	0.6 ± 0.2*
α-TEA	2/10*	0.2 ± 0.1*

a) Macroscopic foci in all lung lobes for each animal in control and treatment groups were counted visually at the time of sacrifice. Numbers of mice with macroscopic lesions/total number of mice in each group are given.
b) Data are expressed as the average number of macroscopic lung tumor foci per mouse ± SE.
* Significantly different from control $p < 0.05$.

anticancer activities and demonstrate that cell culture studies may or may not be a reliable predictor for efficacy in pre-clinical animal models. Of special note, *in vitro* assessment was not a reliable predictor for *in vivo* anticancer effectiveness of the synthetic forms of vitamin E. These studies support the hypothesis that certain forms of vitamin E have unique anticancer properties that are not shared by other vitamin E forms.

Pre-clinical data generated by animal model studies supporting anticancer activities of various vitamin E forms for established breast cancer rather than chemoprevention of breast cancer development are limited. Such studies have been conducted using either human breast cancer cells transplanted (inoculated) into immune-compromised mice (*i.e.*, xenograft studies) or murine mammary cancer cells transplanted into a syngeneic host, and the forms of vitamin E that have been used are *all-rac*-αT, the succinate derivative of αT (RRR-α-tocopheryl succinate) or the vitamin E analog α-TEA [6, 17, 25–27]. Regarding animal studies assessing any of the four vitamin E forms tested here for efficacy against advanced breast cancer, there is one report by Cameron and co-investigators [26] showing that *all-rac*-αT formulated in the diet reduced tumor burden of human MDA-MB-231 breast cancer cells in a xenograft model. These investigators speculated that one or more of the steroisomers in *all-rac*-αT may have played a role in the tumor burden reduction. Our data extend this observation to another pre-clinical animal model, a mouse metastatic mammary cancer model, confirming the anticancer efficacy of *all-rac*-αT, and show for the first time that *all-rac*-αTAc can also significantly reduce tumor burden. Furthermore, the studies reported here show that the synthetic vitamin Es (*all-rac*-αT and *all-rac*-αTAc) can significantly reduce lung and lymph node metastases in the mouse model of metastatic breast cancer.

Our studies demonstrated only limited anti-proliferative and low pro-apoptotic activity for *all-rac*-αT and no activity for *all-rac*-αTAc *in vitro* in comparison to α-TEA and γT.

Our cell culture studies are in general agreement with previously published studies on the synthetic vitamin Es [28, 29]. Therefore, it was surprising that both synthetic forms of vitamin E showed similar abilities to α-TEA to reduce tumor volume and metastasis *in vivo*. In speculating about what might account for this *in vitro* compared to *in vivo* difference in anticancer activity by the synthetic forms, possibilities include: (i) the synthetic vitamin Es must be metabolized to more active components that only occurs *in vivo*; and (ii) the synthetic vitamin Es do not act directly on the tumor cells but rather produce indirect effects; such as, activation of antitumor responses by the immune system, anti-angiogenic effects on endothelial cells or perhaps targets cells supportive of tumor cell proliferation for elimination. Absorption efficiencies among the vitamin E forms most likely do not account for differences in anticancer efficacy since all the various vitamin E forms, including α- and γ-tocopherols and the 2R- and 2S-α-tocopherols in the synthetic vitamin Es have similar efficiencies of intestinal absorption and secretion in chylomicrons in humans and mice [30]. Other possibilities include differences in bioavailability. We do not know the concentrations of the tested vitamin E compounds in cell culture or in plasma or tumor tissue in our studies. However, data from other labs showed that the half-life of RRR-α-tocopherol and the 2R-α-tocopherol forms in synthetic vitamin E is threefold greater than other forms due to preferential secretion of RRR-α-tocopherol and the 2R-α-tocopherol isoforms of synthetic vitamin E from liver into the plasma [30].

Data regarding the antitumor activities of αT on breast cancer *in vitro* and *in vivo* are limited. Inability of αT to induce apoptosis in human breast cancer cells and murine mammary cancer cells in culture has been reported previously [19, 31, 32]. The study reported here did not show marked pro-apoptotic actions by αT in either human breast cancer or murine mammary cancer cells (IC_{50} for apoptosis > 200 μM). However, inhibitory effects of αT on colony formation in 66cl-4 cells was observed (IC_{50} = 67 μM), which is at an achievable concentration in humans, whereas the IC_{50} levels for MDA-MB-435 and MCF-7 cells are above physiological concentrations [4, 13]. In agreement with the *in vitro* data, αT exhibited modest but not statistically significant inhibitory effects on tumor burden and visible lung metastasis *in vivo*. Perhaps αT in our study did not reach an effective concentration in serum and tumor tissue.

In contrast to our earlier report on the ability of different forms of vitamin E to induce apoptosis in human MDA-MB-435 and MCF-7 breast cancer cells [19] and in contrast to studies by Sylvester and co-workers [31, 32] investigating the effects of tocopherols and tocotrienols on preneoplastic, neoplastic and highly malignant mouse mammary epithelial cell culture, as well as studies by Neuzil and co-workers [33] reporting that γT has no apoptotic effect on MCF-7 cells, our data here shows γT to be an effective pro-apoptotic agent against both human breast and murine mammary cancer cells in cell culture. Differences among these studies may be attributed to several factors; including: (i) differences in bioavailability due to different techniques used to dissolve the highly hydrophobic γT; and (ii) differences in cell densities and cell culture conditions.

Although γT was a less potent apoptotic inducer than α-TEA, both vitamin E forms induced apoptosis that involved caspase-8 and caspase-9 activation (cleavage), cleavage of PARP, and reductions in pro-survival c-FLIP and survivin protein expression. While Azzi and co-workers [34] reported on the ability of γT to block cell proliferation in human prostate and colon cancer cells, they concluded it did not cause cell death. In contrast, investigations of anticancer mechanisms of γT in human colon cancer cell lines showed γT treatment to result in apoptosis with evidence for activation of caspases-3, -7 and -8 but not -9 and cleavage of PARP [34, 35]. Likewise, investigations of pro-apoptotic mechanisms of γT in human LNCaP prostate cancer cells showed γT treatment to result in induction of cytochrome c release, activation of caspases-9 and -3, cleavage of PARP, and involvement of caspase-independent pathways [36]. Thus, our study supports involvement of mitochondria-dependent pathways in γT-induced apoptosis in human breast cancer cells in culture. Also, it is of interest that γT appears to show selectivity for cancer cells and not normal cells in culture. γT has been reported to induce both prostate and colon cancer cells but not normal prostate or colon cells to undergo apoptosis [35, 36], and we observed (data not shown) that γT had no pro-apoptotic effect on normal human mammary epithelial cells.

Although γT exhibited anticancer activity in cell culture, it exhibited only modest anticancer activity *in vivo*. This difference may be accounted for by bioavailability issues. It is well established that plasma levels of γT are much lower than αT due to selective metabolism of γT by liver P450 enzymes [4, 13, 37]. Thus, the IC_{50} values of γT for inhibition of colony formation and induction of apoptosis *in vitro* most likely were not reached *in vivo.* However, the ratio of γT to αT concentrations in some tissues is higher than in plasma due to greater uptake of γT [13]. Strategies to increase plasma concentrations of γT as a potential way to improve its antitumor activity *in vivo* are being sought. It has been reported that γT supplementation produces increased plasma γT levels [38] and sesamin, a lignan of sesame, enhances γT concentrations in serum and tissues due to inhibition of P450 enzymes [39, 40]. In contrast to sesamin, αT reduces γT concentrations in plasma and tissues [41]. Further studies are needed to optimize γT as an anticancer agent.

In summary, further studies are needed to understand whether or not synthetic or natural vitamin E forms may be of any benefit in helping eliminate advanced breast tumor cell burden and metastases.

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