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Research Article

Inhibition of lung cancer growth in mice by dietary mixed tocopherols

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Tocopherols are lipophilic antioxidants found in vegetable oils. Here, we examined the growth inhibitory effect of a γ-tocopherol-enriched tocopherol mixture (γTmT) against CL13 murine lung cancer cells grown in culture and as subcutaneous tumors in A/J mice. We found γTmT had no effect after 2 days and weakly inhibited the growth of CL13 in culture after 5 days (28% growth inhibition at 80 μM). Dietary treatment with 0.1 and 0.3% γTmT for 50 days inhibited the growth of CL13 tumors in A/J mice by 53.9 and 80.5%, respectively. Histopathological analysis revealed an increase in tumor necrosis compared to control tumors (80 and 240% increase by 0.1 and 0.3% γTmT, respectively). Dietary treatment with γTmT dose-dependently increased γ- (10.0 – 37.6-fold) and δ-tocopherol (8.9 – 26.7-fold) in the tumors of treated mice compared to controls. Dietary treatment with γTmT also increased plasma γ- (5.4 – 6.7-fold) and δ-tocopherol (5.5 – 7-fold). Whereas others have demonstrated the cancer preventive activity of γTmT against mammary and colon cancer, this is the first report of growth inhibitory activity against lung cancer. Further studies are needed to determine the underlying mechanisms for this anticancer activity, and to determine if such activity occurs in other models of cancer.

Keywords: Lung cancer / 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone / Tocopherols / Tumor necrosis / Syngeneic tumor model

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1 Introduction

Tocopherols are plant-derived polyphenolic compounds characterized by the presence of chromanol-ring attached to a phytl tail (Fig. 1). These compounds are found in high amounts in vegetable oils commonly found in the human diet: the most abundant tocopherols being α-tocopherol (α-T), β-tocopherol (β-T), γ-tocopherol (γ-T), and δ-tocopherol (δ-T) [1, 2]. These naturally occurring tocopherols have an *R*, *R*, *R* stereochemistry, but differ in terms of the number and location of methyl substituents on the chromanol ring. These methyl substituents affect the relative antioxidant activity of the various tocopherols by hindering access to the phenolic hydroxyl group. Although α-T is considered the "Classical Vitamin E" based on studies of fertility maintenance, various studies have shown that γ-T and δ-T have stronger antioxidant and anti-inflammatory activity [3]. This suggests that γ-T and δ-T may have greater cancer preventive activity.

Human intervention and epidemiological studies are mixed in regard to the potential cancer preventive activities of tocopherols. The NHANES I cohort study found no significant correlation between lung cancer risk and α-T [4]. In contrast, a nested case-control study by Mahabir *et al*. [5], found a significant inverse correlation between increase in intake of α-T, β-T, γ-T, or total tocopherols and lung cancer risk. Although an association between higher blood levels of γ-T and reduced risk of prostate cancer has been found in two nested case-control studies [6], no such association was observed in the Physician's Health Study [7]. In the Women's Health Study, supplementation with 600 IU α-T every other day had no significant effect on incidence of invasive cancer or cancer death [8].

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Abbreviations: α-T, α-tocopherol; **AOM,** azoxymethane; **H&E,** hematoxylin and eosin; **β-T,** β-tocopherol; **γ-T,** γ-tocopherol; **γTmT,** mixed tocopherols; **δ-T,** δ-tocopherol

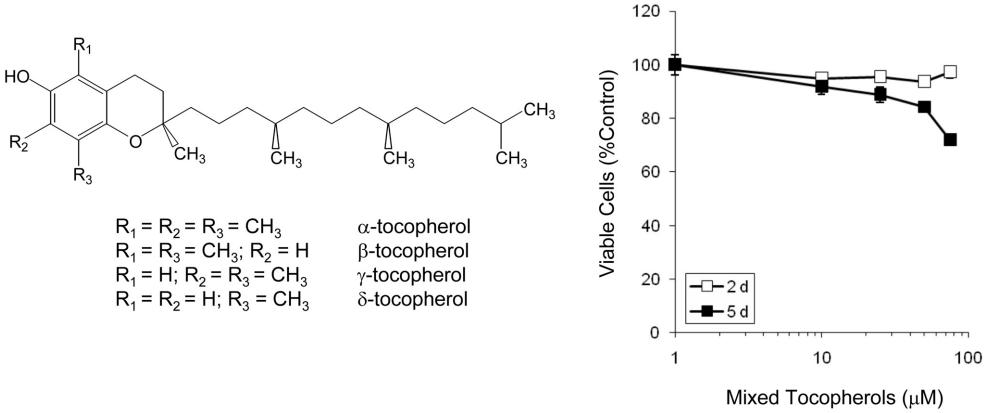

Figure 1. Structures of the tocopherols and the effect of mixed tocopherols (γTmT) on the growth of CL13 cells in culture. Effects on cell growth and viability were assessed using the MTT assay. Cells were treated for 2 or 5 days with γTmT in serum-complete cell culture medium. Each point represents the mean of $n = 18$. Error bars represent the SEM. For points were no error bar is visible, it indicates that the error is smaller than the size of the point.

Recent studies have shown that dietary supplementation with a mixture of tocopherols can prevent tumorigenesis in animal models of mammary and colon cancer [9–11]. In a recent study in our laboratory, we found that dietary treatment of azoxymethane (AOM)/dextran sulfate sodium-treated mice with a 0.3% w/w γ-T-rich tocopherol mixture (γTmT) reduced the multiplicity of total colon tumors, adenomas, and adenocarcinomas by 87.5, 83.3, and 83.3%, respectively, compared to control-treated mice [11]. Short-term studies in the same model showed that treatment with γTmT decreased markers of oxidative stress and inflammation in the colon and plasma including: prostaglandin E2, leukotriene B4, and nitrotyrosine [11]. Similar preventive effects were also reported in the *N*-methyl-*N*-nitrosourea-induced mammary tumor model in rats and AOM-induced aberrant crypt foci (ACF) model [9, 10]. Treatment with 0.1% γTmT reduced mammary tumor burden and multiplicity by 43.5 and 26.5%, respectively in the former model, whereas induction of ACF by AOM was reduced by 55% compared to control treated rats in the latter model.

Lung cancer is the second most commonly diagnosed cancer and is the leading cause of cancer death in the United States [12]. Although cessation of smoking is the most effective means of preventing the development of lung cancer, there is still a significantly elevated risk of developing lung cancer in former smokers, and lung cancer incidence due to occupational exposure and other factors not related to smoking are significant [13, 14]. Although advances have been made in the treatment of various types of cancer, the 5-year survival rate for lung cancer has only slightly improved (13% in 1975 *vs.* 16% in 2006) [15]. Clearly, new strategies are needed to prevent the development of new lung cancer cases and treat cases once they have developed. In the present study, we determined the efficacy of γTmT against CL13 murine lung cancer cells grown in culture and as subcutaneous syngeneic tumors in A/J mice. This cell line was derived from lung adenocarcinomas generated in female A/J mice using the classical 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorgenesis model. The subcutaneous syngeneic tumor model represents an economical method to rapidly establish the efficacy of test compounds and to establish a dose-range prior to undertaking long-term mouse lung carcinogenesis studies. This study represents the first report on the effect of γTmT in an animal model of lung cancer.

2 Materials and methods

2.1 Chemicals and diets

γTmT (57% γ-T, 24% δ-T, 13% α-T, and ~0.5% β-T) was obtained from Cognis Corporation (Kankakee, IL). γTmT was formulated into AIN93M basal diet at 0.1 or 0.3% w/w by Research Diets, Inc. (New Brunswick, NJ) and stored at 4°C in sealed bags. The tocopherol content of all diets is listed in Table 1. All other chemicals were of the highest purity available and purchased from VWR International (West Chester, PA).

2.2 Cell culture and cell viability assay

CL13 mouse lung adenocarcinoma cells were a gift from Dr. Steven A. Belinsky (Lovelace Respiratory Research Institute, Albuquerque, NM). Cells were maintained in log-phase growth in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cells were maintained at 37°C in a 5% CO_2 atmosphere with 95% relative humidity.

To determine the growth inhibitory activity of γTmT, CL13 cells were plated in 96-well plates (5×10^3 cells *per* well) and allowed to attach for 24 h. The medium was replaced with fresh, serum-complete medium containing

Table 1. Tocopherol content of the experimental diets

	mg/kg diet			
Diet	α-T	β-T	γ-T	γ-T
AIN93M[a)]	79.8	0.4	24.4	7.6
AIN93M + 0.1% γTmT w/w	199.8	15.4	604.4	217.6
AIN93M + 0.3% γTmT w/w	439.8	45.4	1764.4	637.6

a) Tocopherol content in AIN93M diet is due to α-T in the vitamin mix (V10037) which is added to the diet at 1% and soybean oil which is added as 4%. The amounts of tocopherols present in soybean oil are based on previously reported values [24].

0 – 80 μM γTmT. Cells were incubated for 2 or 5 days at 37°C. The medium was removed, the cells were washed once with medium containing no γTmT, and the number of viable cells were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [16]. Treatment effect determined by comparison to DMSO (vehicle)-treated controls. Final DMSO concentration was less than 0.1% in all treatments.

2.3 Subcutaneous tumor studies

Animal experiments were conducted according to a protocol (91-024) approved by the Institutional Animal Care and Use Committee at Rutgers University (Piscataway, NJ). Female A/J mice (7 wk old) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed ten *per* cage in air-conditioned quarters with room temperature of 20 ± 2°C, relative humidity of 50 ± 10%, and alternating 12 h light/dark cycle. After a one-week acclimation period, mice were randomized based on weight into the following treatment groups: control (AIN93M diet), 0.1% γTmT (w/w in AIN93M diet), or 0.3% γTmT (w/w in AIN93M) diet. After one week of feeding with experimental diet, each mouse was inoculated subcutaneously with 1×10^6 CL13 cells (in 100 μL PBS) on both rear flanks. Mice had access to food and water *ad libitum*. Body weight and food consumption were determined weekly. Tumors were measured weekly and volume was calculated using the following formula:

$$V = \left(\frac{LW^2}{2}\right)$$

where V is tumor volume, L is the longest, and W is the shortest tumor dimension. We elected to begin treatment with γTmT prior to tumor cell inoculation in order to better model cancer prevention rather than cancer therapy. In cancer prevention (or secondary prevention), treatment is begun when only a small population of cancerous or precancerous cells is present, rather than an established tumor mass. All mice were sacrificed by CO_2 asphyxiation when the mean tumor volume of the control group reached 400 mm^3. Blood was collected by cardiac puncture. Plasma was prepared by centrifugation and frozen at −80°C for later analysis. Tumors were excised and halved. Half of the tumor was snap frozen on dry ice for HPLC analysis of tocopherols levels and the other half was fixed in 10% buffered formalin. Formalin-fixed tumors were dehydrated, embedded in paraffin, and cut into 5 μm sections. Sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) for histopathological analysis. Necrotic areas in tumors were defined as areas composed of either large amounts of dead cell debris or the empty space in the center of tumors formed after cell death. The total tumor area and the necrotic area of each tumor were measured using the Image-pro Plus program and quantified as a percentage.

2.4 HPLC analysis of tocopherols

The procedure for the determination of tocopherol levels in plasma was described previously [11]. In brief, 10 μL plasma was combined with 140 μL deionized water and 150 μL ethanol. Fat-soluble materials, including tocopherols, were extracted with hexane. For tumors, 10 mg samples were homogenized with a mechanical dounce homogenizer in 140 μL deionized water and 150 μL ethanol. After homogenization, the supernatant was extracted with hexane. The hexane-extractable material was dried under vacuum and redissolved in 100 μL ethanol for HPLC analysis.

HPLC separation was achieved using a Supelcosil C18 RP column (150 × 4.6 mm^2; 5 μm particle size; Bellefonte, PA). An isocratic mobile phase was used consisting of 82% aqueous ethanol containing 20 mM ammonium acetate (pH = 4.4). The flow rate was 1.2 mL/min. The eluant was monitored with an ESA 5600A Coulochem electrode array system (ESA Inc. Bedford, MA) with potentials set at 200, 300, 500, and 700 mV. Quantification was accomplished by comparing the peak area of the sample tocopherol peaks with the peak area in reference plasma. The reference plasma was prepared as previously described [11].

2.5 Statistical analysis

Tumor growth inhibition and body weight were analyzed by repeated measured ANOVA with Bonferroni post-test. Tumor necrosis, and plasma and tumor levels of tocopherols were analyzed by one-way ANOVA with Tukey's post-test. Significance was achieved at $p < 0.05$.

3 Results and discussion

3.1 Inhibition of lung cancer cell growth *in vitro* and *in vivo*

The effect of γTmT on the growth and viability of CL13 cells in culture was assessed using the MTT assay. Treatment for 2 days with 0 – 80 μM γTmT had no significant

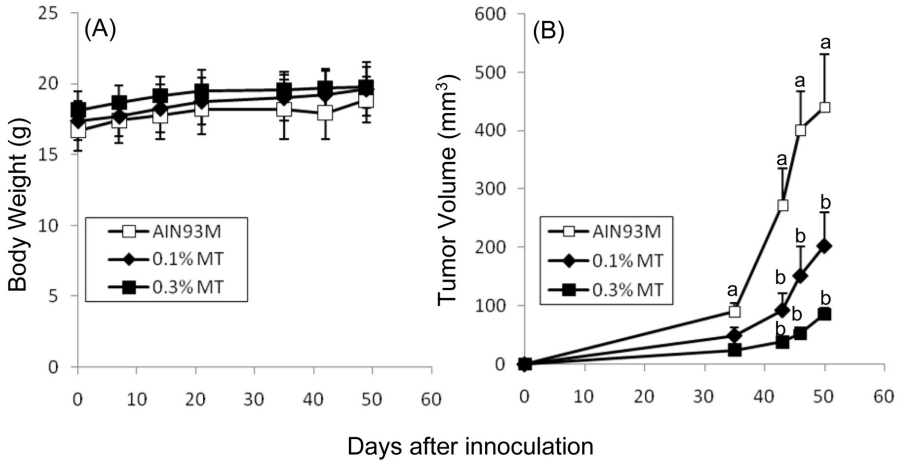

Figure 2. Effect of γTmT treatment on the growth of subcutaneous CL13 tumors in A/J mice. A/J mice were treated with AIN93M diet supplemented with 0, 0.1 or 0.3% γTmT for one week prior to injection with CL13 cells. Treatment γTmT-supplemented diet continued until sacrifice. Body weight (A) and tumor volume (B) were determined throughout the course of the experiment. Each point represents the mean of $n = 14-15$ mice (for body weight) and 25–27 tumors (for tumor volume). Error bars represent the SEM. Data points with different superscripts are significantly different ($p < 0.05$) by repeated measures analysis of variance with Bonferroni post-test.

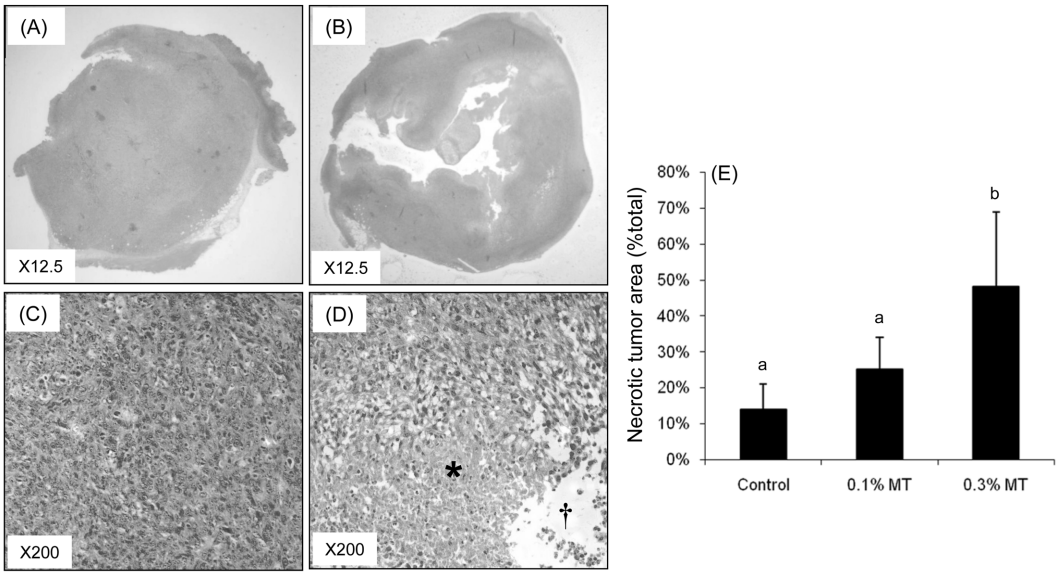

Figure 3. Histopathological analysis of CL13 tumors in A/J mice treated with 0.3% γTmT. Representative tumors from the AIN93M-treated mice (A,C) and 0.3% γTmT -treated mice (B,D) were stained with H&E and the necrotic area of the tumor was quantified by microscopy and normalized to total tumor area in each section (E). Each bar represents the mean of $n = 10$ tumors. Error bars represent the SD. Bars with different superscript are significantly different ($p < 0.05$) by one-way ANOVA with Tukey's post-test. *, † indicate areas defined as tumor necrosis.

effect on the number of viable cells relative to control (Fig. 1). However, treatment for 5 days dose-dependently decreased the number of viable cells, with inhibition of 28% at 80 μM (Fig. 1).

To determine the growth inhibitory effects of γTmT *in vivo*, we randomized female A/J mice and treated them for 1 wk with 0.1 or 0.3% γTmT in the diet. Mice were then implanted subcutaneously with CL13 cells and dietary treatment with γTmT was continued throughout the duration of the experiment. Treatment with γTmT had no significant effect on the body weight of treated mice compared to mice fed AIN93M diet (Fig. 2A). γTmT dose-dependently inhibited the growth of subcutaneous CL13 tumors in A/J mice (Fig. 2B). γTmT at 0.1 and 0.3% in the diet reduced final tumor volume by 53.9 and 80.5%, respectively, compared to the control.

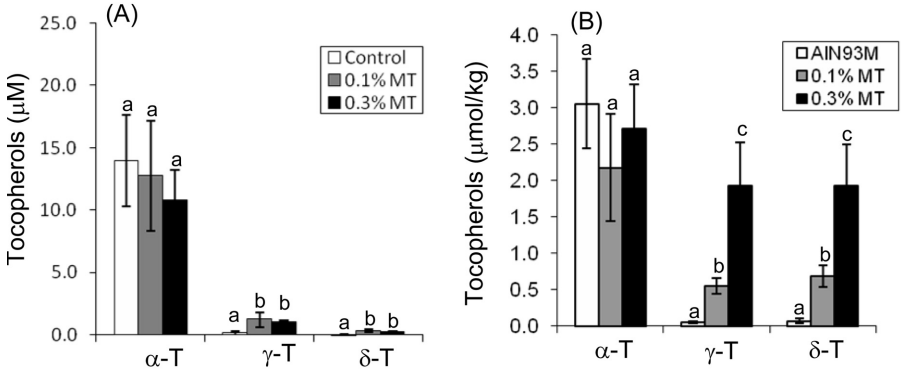

Figure 4. Plasma (A) and tumor (B) levels of tocopherols in A/J mice treated with 0.1 or 0.3% γTmT in the diet. The levels of α-T, γ-T, and δ-T were determined by HPLC with electrochemical detection. Each bar represents the mean of $n = 7$. Error bars represent the SEM. Bars with different superscripts are significantly different ($p < 0.05$) by one-way ANOVA with Tukey's post-test.

Tumors from control and γTmT-treated animals were sectioned and stained with H&E (Fig. 3). Histopathological analysis revealed that tumors from control-treated mice were uniformly composed of living cells with a small population of dead cells (Fig. 3A and C). In contrast, tumors from γTmT-treated mice had large amounts of tumor cell necrosis (Fig. 3B and D). The necrotic areas were defined as areas composed of either large amount of dead cell debris (labeled as * in Fig. 3D) or the empty space in the center of tumors formed after cell death (labeled as † in Fig. 3D). Quantification of the necrotic tumor area as a percentage of the total tumor showed that treatment with 0.3% γTmT increased tumor necrosis by 2.4-fold compared to the control (Fig. 3E). The underlying mechanisms for this increase in tumor cell necrosis remains unclear. It is possible that γTmT can directly induce cell death, but the results of our *in vitro* studies with CL13 cells do not support this notion. Rather, our data suggest that γTmT may work by an indirect mechanism such as disruption of tumor cell – stromal cell interactions as has been reported in other studies [17–22]. This possibility remains to be tested in the current model.

3.2 Plasma and tumor levels of tocopherols

The plasma and tumor levels of individual tocopherols (α-T, γ-T, and δ-T) were determined by HPLC following treatment of tumor-bearing mice for 50 days. Although the diet was supplemented with higher levels of γ-T (57%) and δ-T (24%) than α-T (13%), the plasma and tumor levels of α-T were significantly higher than γ-T and δ-T in this study (Fig. 4). Dietary treatment with γTmT had no significant effect on the levels of α-T in either the plasma (Fig. 4A) or the tumors (Fig. 4B) compared to control. In contrast, treatment with γTmT increased plasma levels of γ-T and δ-T by 5.4–6.7-fold and 5.5–7.0-fold, respectively, compared to control (Fig. 4A). Dietary γTmT also dose-dependently increased tumor levels of γ-T (10–37.6-fold increase) and δ-T (8.8–26.7-fold increase) (Fig. 4B).

The apparent contradiction between dietary supplementation with lower levels of α-T compared to γ- or δ-T, but much higher levels of α-T resulting in plasma and tissues has been observed in other studies [10, 11]. It has been reported that hepatic α-T transfer protein (α-TTP), which maintains plasma α-T levels, preferentially binds to α-T over γ-T [23]. The result is a 3-fold greater half-life for α-T compared to γ-T. There is also evidence to suggest that nonα-T tocopherols are metabolized more readily that α-T by cytochromes P450 [23]. The lack of change in α-T after dietary supplementation, again, is probably due to the tight regulation by α-TTP. These data indicate that dietary administration is effective at increasing tumor levels of tocopherols. It is unclear why the increase in tumor levels of γ-T and δ-T is higher than the increase in plasma levels of these compounds. It is likely that the tocopherol's partition into the tumor because of the more nonpolar environment of the tissue relative to the plasma.

4 Concluding remarks

In the present study, we observed that dietary administration of γTmT inhibited the growth of CL13 mouse lung adenocarcinoma subcutaneous tumors in A/J mice, even though these compounds were only weakly active against CL13 cells in culture. Given the apparent antitumor activity of γTmT in this short-term study, long-term studies in the NNK-induced carcinogenesis model in A/J mice are warranted. Furthermore efficacy studies should be conducted in other animal models of other tumor types to determine the spectrum of anticancer activity of γTmT.

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The authors have declared no conflict of interest.

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