Orally active α-tocopheryloxyacetic acid suppresses tumor growth and multiplicity of spontaneous murine breast cancer

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Abstract

We recently demonstrated the antitumor efficacy of orally administered α-tocopheryloxyacetic acid (α-TEA), a redox silent and nonhydrolyzable derivative of naturally occurring vitamin E. In order to move α-TEA closer to the clinic to benefit patients with breast cancer, the present study had two goals. First, to determine the minimal effective treatment dose; and second, to test the efficacy of dietary administration of α-TEA in the clinically relevant MMTV-PyMT mouse model of spontaneous breast cancer that more closely resembles human disease. The minimal effective dose of α-TEA was evaluated in the transplantable 4T1 tumor model and we show a dose-dependent decrease of primary tumor growth and reduction of metastatic spread to the lung. Six-week-old MMTV-PyMT mice were treated with oral α-TEA for 9 weeks, with no apparent signs of drug toxicity. The α-TEA treatment delayed tumor development and significantly slowed tumor progression, resulting in a 6-fold reduction of the average cumulative tumor size. In addition, oral α-TEA caused an 80% reduction in spontaneous metastases. In situ analysis of tumor tissue identified apoptosis as an important mechanism of α-TEA-mediated tumor suppression in addition to inhibition of tumor cell proliferation. This study shows, for the first time, the ability of orally administered α-TEA to delay tumor onset and to inhibit the progression and metastatic spread of a clinically relevant model of spontaneous breast cancer. Our finding of the high efficacy in this tumor model highlights the translational potential of oral α-TEA therapy. [Mol Cancer Ther 2009;8(6):1570–8]

Introduction

Recently, we and others have demonstrated the antitumor efficacy of a novel class of chemotherapeutic drugs, the redox silent, semisynthetic derivatives of naturally occurring vitamin E (1–3), which have been shown to exhibit selective toxicity toward tumor cells (1, 4–7). This novel drug class is epimerized by vitamin E succinate (α-tocopheryl succinate; α-TOS) and α-tocopheryloxyacetic acid (α-TEA). Both derivatives structurally share the phytanyl tail and the chroman head with vitamin E. However, the hydroxyl group at the number 6 carbon of the phenolic ring of the chroman head is replaced by an acid residue (8) that confers antitumor activities (1, 2, 5, 7, 9–11). α-TEA is of particular interest to us because, unlike α-TOS, α-TEA is nonhydrolyzable and therefore can be administered through the more clinically relevant oral route. In order to move α-TEA closer to the clinic to be used as a new treatment option benefiting patients with breast cancer, the present study had two main goals. The first goal was to determine the minimal effective dose of α-TEA. For this purpose, α-TEA was incorporated into mouse chow at different concentrations and was fed to mice bearing established transplantable 4T1 tumors. We show a dose-dependent decrease of primary tumor growth and reduction of metastatic spread to the lung.

The second goal was to test the efficacy of orally administered α-TEA in a more clinically relevant murine model of spontaneously arising breast cancer. Although, transplantable tumor models are valuable tools in cancer research, they do not fully recapitulate human cancer etiology as they lack the physiologically relevant host and tumor microenvironments that influence spontaneous tumor progression. For this reason, we employed the transgenic mouse mammary tumor virus (MMTV)-PyMT mouse model of spontaneous breast cancer to test the efficacy of α-TEA in the endogenously appropriate setting. The MMTV-PyMT mice carry the polyoma middle T oncogene driven by the mouse mammary tumor virus (MMTV) promoter (12). These mice rapidly develop multifocal mammary adenocarcinoma involving the entire mammary pad with secondary metastasis to the lung (12). MMTV-PyMT mice received a low dose of 2 mg of α-TEA per day starting at 6 weeks of age until 15 weeks of age when the mice on the control diet were sacrificed due to large tumor burden. Our results show that α-TEA treatment delayed the development of palpable tumors by more than 2 weeks,
and significantly \( P < 0.0001 \) slowed tumor progression, resulting in 85% reduction of the average cumulative tumor area, and inhibiting lung metastases by 80%. In addition, the orally administered \( \alpha\)-TEA resulted in a 50% reduction of the average number of tumors per mouse. We also determined that apoptosis is an important mechanism of \( \alpha\)-TEA-mediated tumor suppression in vivo.

Together these results show, for the first time, the efficacy and ease of delivery of orally administered \( \alpha\)-TEA against spontaneous murine breast cancer that more closely resembles human breast cancer. The dramatic antitumor effect of the lower \( \alpha\)-TEA dose in this model may lay the foundation for clinical testing of \( \alpha\)-TEA in humans.

**Materials and Methods**

**Preparation of \( \alpha\)-TEA**

\( \alpha\)-TEA \([2,5,7,8\text{- tetramethyl-}[2R-(4R,8R,12\text{-trimethyltridecy1 chroman-6-yl] acetic acid}]\) was synthesized in-house using a combination of previously described methods (1, 13), and purity was confirmed by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance analysis.

**Tumor Cells and Cell Culture**

The 4T1 tumor cell line is a variant of 410.4, a tumor subline that was isolated from a spontaneous mammary tumor in a BALB/cfC3H mouse. The 4T1 tumor is poorly immunogenic and highly metastatic and spontaneously metastasizes to the liver, lungs, bone marrow, and brain (14–16), a characteristic that is shared with human breast cancers. The tumor cells were maintained in Iscove's modified Dulbecco's medium (JRH Biosciences), containing 100 units/mL of penicillin, 100 \( \mu \)g/mL of streptomycin (Invitrogen), 0.75 \( \mu \)g/mL of Fungizone, and 10% fetal bovine serum (both from Gemini Bio-Products).

**Animal Studies**

Six- to eight-week-old female BALB/c mice were purchased from the National Cancer Institute Frederick Facility (Frederick, MD) and the MMTV-PyMT transgenic mice [FVB/N-Tg(MMTV-PyVT)634Mul/J; ref. 17] were purchased from the University of Arizona Animal Facilities in accordance with the Principles of Animal Care (NIH publication no. 85–23) and all studies were approved by the University of Arizona Institutional Animal Use Committee. For the transplantable tumor model studies, \( 5 \times 10^4 \) viable 4T1 breast cancer cells were injected i.c. into the right mammary fat pad of BALB/c mice. The mice received control diet until tumor establishment (day 10 post–tumor implantation, \( \sim 17 \text{ mm}^2 \)) and were then switched to mouse chow containing different amounts of \( \alpha\)-TEA. For the spontaneous tumor model, MMTV-PyMT transgenic mice received control diet until 6 weeks of age (day 42), and were then fed \( \alpha\)-TEA chow. Tumor growth was monitored by measuring the tumor length \((L)\) and width \((W)\) using calipers and calculating the tumor area as, \( A = (L \times W) \). Visible metastatic lung nodules were enumerated by staining with India ink and Fekete's solution.

\( \alpha\)-TEA was incorporated into the AIN93G diet by Harlan Teklad at a concentration of 3 g \( \alpha\)-TEA per kilogram of chow (0.3%), 1 g/kg chow (0.1%), 0.5 g/kg chow (0.05%), and 0.25 g/kg chow (0.025%).

**Histopathology of Mammary Glands from MMTV-PyMT Mice**

MMTV-PyMT mice received 0.1% \( \alpha\)-TEA diet (~2 mg/d) starting at 6 weeks of age for 4 weeks (until 10 weeks of age, when mice were sacrificed). Mammary glands were dissected and whole mounts prepared. Briefly, the tissues were dried flat on a microscope slide, fixed and stained with carmine red (18), and examined under a microscope (Nikon TE-2000S) at \( \times 20 \) magnification. At the same time, tissues were fixed in 10% buffered formalin and paraffin embedded, and 5-\( \mu \)m sections were stained with H&E and evaluated by a veterinary pathologist based on the system described by Lin et al. (19) and guidelines for mouse models of mammary cancer described by Cardiff et al. (20). Apoptosis and cell proliferation were determined on paraaffinized 5-\( \mu \)m sections by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and using an antibody specific for nuclear antigen Ki-67, respectively. The TUNEL assay was done using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International) according to the protocols of the manufacturer. Detection of the anti–Ki-67 primary antibody was done on a Discovery XT Automated Immunostainer (Ventana Medical Systems, Inc.). Antibodies were diluted in Discovery XT diluent and all staining was done using VMSI validated reagents, including streptavidin-HRP and diaminobenzocate. Because the malignant tissues in the \( \alpha\)-TEA–treated mice, particularly at the early time point (day 70), were much smaller and less contiguous in comparison to the tissues from untreated mice, microscopic fields were only scored from solid cell sheets within the sections to remove bias toward lower numbers in the \( \alpha\)-TEA–treated groups. Fluorescein TUNEL-stained nuclei were scored as positive for apoptosis in 15 microscopic fields (\( \times 200 \) magnification, Nikon TE-2000S) per section. Brown-stained Ki-67–positive cells were counted in 15 microscopic fields (\( \times 400 \) magnification, Nikon TE-2000S) per section.

**Determination of \( \alpha\)-TEA Levels**

Sera and tissues were analyzed for \( \alpha\)-TEA content by HPLC with mass spectrometric detection (HPLC/MSD). Blood was collected by terminal heart puncture and serum was isolated using microtainer serum separator tubes (BD). Sera and tissues were analyzed for \( \alpha\)-TEA content by HPLC with mass spectrometric detection (HPLC/MSD). Blood was collected by terminal heart puncture and serum was isolated using microtainer serum separator tubes (BD) and stored at \(-80^\circ \text{C}\). Tissue samples were snap-frozen and stored in liquid nitrogen until analysis. Tissues (10 mg) were minced and digested in 15 mg/mL of collagenase (Worthington Biochemical Corporation) and 6 mg/mL of Pronase E (Sigma-Aldrich) at 37°C for 1 h in 300 \( \mu \)L of PBS. Subsequently, 300 \( \mu \)L of 1% SDS was added and the tissue samples were homogenized by repeated pulling through a 21-gauge needle.

For HPLC/MSD detection of \( \alpha\)-TEA, a 50-\( \mu \)L aliquot of serum or tissue digest was added to a capped 15 mL polypropylene centrifuge tube containing 200 \( \mu \)L of ice-cold ethanol. The tubes were vortex-mixed briefly and allowed to
stand for 5 min. The volume of each sample was brought up to 1.0 mL with saline and extracted with hexane/dichloromethane, 95:5 (v/v), by vortex mixing. The samples were then centrifuged to facilitate the separation of the two phases. The lower aqueous layer was frozen in an acetone/dry ice bath, and the upper organic layer was carefully decanted into a 15 mL clean-capped polypropylene centrifuge tube. The organic extract was dried at room temperature under a gentle stream of nitrogen and then reconstituted with 100 μL of the mobile phase used to analyze the samples. Spiked drug-free serum samples were prepared in the same manner and were used to prepare a calibration curve. An injection of 2.0 to 5.0 μL was used for each analysis. The chromatographic system was an Agilent LC/MSD 1100 using atmospheric pressure electrospray ionization in negative ion mode. A C18 column with a mobile phase of acetonitrile/methanol/glacial acetic acid, 70:30:0.25 (v/v/v), at a flow rate of 0.5 mL/min was used for the chromatographic separation. Calibration curves, from 1.0 to 50 μg/mL, were generated by least-squares quadratic curvilinear regression and had correlation coefficients of \( r^2 \geq 0.997. \)

### Statistical Analysis

Statistical significance of differences among data sets of treatment groups was assessed either by Student's \( t \) test, where applicable, or by one-way ANOVA, including Tukey-Kramer post-tests for multiple comparisons. To compare tumor growth rates, growth curves were transformed to linearity, and linear regression analysis was used to determine slopes that were then compared by \( t \) test. To compare average cumulative tumor growths in the MMTV-PyMT model, growth curves were determined by nonlinear regression analysis and statistically evaluated for difference by \( f \) test. Differences of the mean number of lung metastases were evaluated by Mann-Whitney test. All analyses were done using Prism software (GraphPad). \( P \leq 0.05 \) was considered indicative of significant differences between data sets.

### Results

Orally Administered \( \alpha \)-TEA Inhibits the Growth and Spread of Established Transplantable 4T1 Mammary Tumors in a Dose-Dependent Manner

Prior to testing the efficacy of \( \alpha \)-TEA in the more clinically relevant MMTV-PyMT mouse model of spontaneous breast cancer, we wanted to assess the minimal effective dose of \( \alpha \)-TEA against transplantable, poorly immunogenic, and highly metastatic 4T1 murine mammary cancer. Because we had already shown that the relatively high dose of 6 mg \( \alpha \)-TEA per day reduced primary tumor burden and metastatic spread in this tumor model (2), 6 mg \( \alpha \)-TEA per day was chosen as the highest dose (0.3% \( \alpha \)-TEA diet). Mice were injected with 4T1 tumor cells and treated daily with \( \alpha \)-TEA diet from day 1 to day 18 post-tumor cell injection. Untreated mice received control diet throughout the study.

#### Figure 1.

Effect of dietary delivery of \( \alpha \)-TEA on primary tumor growth. BALB/c mice were injected with 4T1 mammary tumor cells in the right mammary fat pad (day 0). When tumors became palpable (arrow; 17 mm², day 10 post-tumor cell injection), mice received \( \alpha \)-TEA in the diet for 18 d. Untreated mice received control diet throughout the study. **A.** Points, mean tumor areas of nine mice per group; bars, SE. To compare tumor growth rates, growth curves were transformed to linearity and linear regression analysis was used to determine slopes that were then compared by \( t \) test. **B.** The values represent tumor areas of individual mice on day 28 post-tumor injection. Boxed numbers are mean tumor areas ± SE. Differences of the mean tumor areas were determined by ANOVA including Tukey-Kramer post-tests for multiple comparisons. **C.** Effect of dietary delivery of \( \alpha \)-TEA on tumors spread. BALB/c mice with implanted 4T1 mammary tumors from the above study were sacrificed on day 28 post-tumor cell injection. To determine the number of pulmonary metastases, lungs were inflated with India ink and removed, and the surface lung metastases were counted. Boxed numbers are mean number of lung metastases ± SE. Differences of the mean number of lung metastases were determined by ANOVA including Tukey-Kramer post-tests for multiple comparisons.
and received control diet until day 10 when tumors became palpable (~17 mm²). The mice were then transferred to mouse chow containing different doses of α-TEA (0.3%, 0.1%, 0.05%, and 0.025%) for 18 days. Figure 1A shows that all doses of dietary α-TEA, except for the lowest dose (0.025%), resulted in significant and dose-dependent reductions in primary tumor growth rate and tumor size. Our results also show that whereas the highest α-TEA dose tested (0.3%) achieved a 64% reduction in final tumor size, the 0.1% diet still caused a 50% reduction in tumor size (Fig. 1B). Even the 0.05% diet caused a moderate, but statistically significant, 38% reduction in tumor size. Not only did the oral α-TEA treatment slow down tumor growth, the 0.3%, 0.1%, and 0.05% α-TEA diet also significantly reduced metastatic spread of the primary tumor to the lung by 85%, 92%, and 71%, respectively (Fig. 1C). Similar to the effect on primary tumors, the lowest dose tested (0.025% α-TEA diet) failed to significantly decrease lung metastases. Interestingly, in comparison to the highest dose tested (0.3%), the 0.1% dose was as efficacious in suppressing lung metastases.

**Determination of α-TEA Levels In vivo**

In order to correlate the clinical outcome with the actual α-TEA dose the mice received, food intake was monitored every 2 to 3 days throughout the treatment period and α-TEA serum levels were determined at the end of the study. The average food consumption among the different groups was comparable and ranged from 2.0 g chow per mouse per day (0.3% diet group) to 2.5 g chow per mouse per day (0.05% diet group), with the mice on the control diet eating an average of 2.2 g chow per mouse per day, suggesting that there is no aversion to α-TEA in the food. According to this food consumption profile, the mice on the 0.3%, 0.1%, 0.05%, and 0.025% diets received an average daily α-TEA dose of 5.9, 2.4, 1.2, and 0.6 mg, respectively, that is equivalent to 295, 122, 62, and 28 mg α-TEA per kilogram of bodyweight. To determine the actual level of circulating α-TEA, serum was collected at the end of the study (day 28 post–tumor injection) and analyzed by HPLC/MSD. Our results show a dose-dependent decrease in serum α-TEA levels ranging from 73.5 ± 25.4 μg/mL in the mice at the highest dose (0.3% diet) to 19.3 ± 4.9 μg/mL at the lowest dose (0.025% diet; Fig. 2A). Meanwhile, mice on the control diet had no detectable α-TEA levels (data not shown).

It is self-evident that an anticancer therapeutic needs to reach its target in order to be successful in eradicating a tumor. Therefore, we were interested in how much α-TEA was present in tumor tissues during α-TEA therapy. Because we had determined that the 0.3% and 0.1% diets resulted in the most primary tumor growth inhibition, we measured the α-TEA levels that were achieved after mice received the 0.3% or the 0.1% diet for 8 days. Similar to the serum levels, α-TEA levels in the tumors were dose-dependent and ranged from 0.53 ± 0.01 μg/mg tumor tissue (0.3% diet) to 0.37 ± 0.02 μg/mg tumor tissue (0.1% diet). Mice on the control diet had undetectable α-TEA levels (data not shown).

**Low-Dose Oral α-TEA Treatment Does Not Cause Gross Toxicity**

Although vitamin E analogues, including α-TEA, have been shown to be preferentially toxic to malignant cells (1, 4–7), and we have shown in an earlier report (2) that mice on oral α-TEA therapy lacked signs of toxicity, we wanted to determine if the prolonged α-TEA treatment resulted in toxic effects. For this purpose, the body weights of mice were monitored every 5 to 7 days throughout the study. On average, all groups, including the control group, had no significant weight change during the study except for the lowest dose tested (0.025% diet group), with the mice on the control diet eating an average of 2.2 g chow per mouse per day, suggesting that there is no aversion to α-TEA in the food. According to this food consumption profile, the mice on the 0.3%, 0.1%, 0.05%, and 0.025% diets received an average daily α-TEA dose of 5.9, 2.4, 1.2, and 0.6 mg, respectively, that is equivalent to 295, 122, 62, and 28 mg α-TEA per kilogram of bodyweight. To determine the actual level of circulating α-TEA, serum was collected at the end of the study (day 28 post–tumor injection) and analyzed by HPLC/MSD. Our results show a dose-dependent decrease in serum α-TEA levels ranging from 73.5 ± 25.4 μg/mL in the mice at the highest dose (0.3% diet) to 19.3 ± 4.9 μg/mL at the lowest dose (0.025% diet; Fig. 2A). Meanwhile, mice on the control diet had no detectable α-TEA levels (data not shown).

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**Oral Administration of α-TEA Inhibits Growth and Spread of Spontaneous Mammary Tumors**

After we established that the lower 0.1% α-TEA dose was efficacious in treating established primary tumors in the transplantable 4T1 mammary cancer model, we wanted to investigate the efficacy of this dose in treating breast disease in the more clinically relevant transgenic MMTV-PyMT model of spontaneous breast cancer. For this purpose,
MMTV-PyMT mice were treated with the 0.1\% α-TEA diet starting at 6 weeks of age (42 days). At this age, MMTV-PyMT mice display alveolar hyperplasia and moderate mammary intraepithelial neoplasia in comparison to wild-type mice. The mice received the α-TEA diet *ad libitum* for 9 weeks until day 105 of age, when the mice on the control diet were sacrificed due to large tumor burden. Both groups of mice consumed similar amounts of food, with the control mice eating 2.7 g/d and the mice in the 0.1\% diet group eating 2.4 g/d. Therefore, the mice on the 0.1\% diet received an average daily α-TEA dose of 2.4 mg/d. Serum analysis showed that the mice had an average circulating α-TEA level of 27.2 ± 4.8 μg/mL, which was somewhat lower than the α-TEA serum level of BALB/c mice on the 0.1\% diet (38.2 ± 4.9 μg/mL). The α-TEA treatment delayed the development of palpable tumors by more than 2 weeks (17 days) and significantly (*P* < 0.0001) slowed tumor progression over the duration of the study, resulting in an 83\% reduction of the average cumulative tumor area (Fig. 3A). In addition, the α-TEA treatment reduced tumor multiplicity by 50\% (Fig. 3B). The average number of tumors in the α-TEA-treated group was 4.8 ± 1.0 in comparison to an average number of tumors of 9.9 ± 0.1 in the control diet group (Fig. 3B). At the study end point (day 105), we also determined the metastatic spread of the primary tumors to the lung. Over the course of the study, the α-TEA diet significantly reduced (*P* = 0.0041) the average number of lung metastases almost 5-fold from 58.3 ± 23.0 to 12.3 ± 8.8 (Fig. 3C) with 40\% (4 of 10) of the mice lacking visible metastatic lung nodules.

We also tested the efficacy of the 0.3\% and 0.05\% α-TEA diets to treat tumors in the MMTV-PyMT mice. Although we had shown in our previous studies that the 0.3\% diet did not cause gross toxicity in BALB/c mice (2), several of the MMTV-PyMT mice on the 0.3\% α-TEA diet died after ∼10 to 19 days of α-TEA treatment, suggesting that these transgenic mice may be more sensitive to this higher α-TEA dose. In addition, the 0.05\% α-TEA dose inhibited tumor development in the MMTV-PyMT mice in a dose-dependent manner similar to the 4T1 tumor model (data not shown).

**Effect of Oral α-TEA on Tumorigenesis in MMTV-PyMT Mice**

In order to determine the effect of α-TEA on the early development of spontaneous tumors, whole mounts of mammary glands from control and α-TEA diet–fed mice were prepared after 4 weeks of α-TEA treatment. In comparison to younger mice (6 weeks of age), untreated mice at 10 weeks of age displayed a marked increase in irregularly formed ductal side branches, enlarged terminal buds, and multilobular tumor masses (Fig. 4, compare whole mount panels 2 and 4). This is in contrast to mammary glands from mice that received short-term (4 weeks) α-TEA treatment (Fig. 4, whole mount panel 3), which displayed only moderately irregular side branches and few enlarged terminal buds. Examination of H&E-stained tissue sections revealed that 10-week-old untreated mice had early mammary carcinoma signified by coalescing acini with solid sheets of cells filling their lumen, nuclear atypia, and loss of intact basement membranes with neoplastic epithelial cells invading the stroma. In addition, inflammation was observed (Fig. 4, H&E panel 4). The mice that received 4 weeks of α-TEA treatment displayed only adenomas indicated by expansile masses composed of relatively uniform cells within

![Figure 3](image-url)
acini, the basement membranes were intact and only mild inflammation was observed (Fig. 4, H&E panel 3).

Orally Active α-TEA Increases Apoptosis and Inhibits Cell Proliferation in Spontaneously Arising Breast Tumors

After we showed the ability of orally administered α-TEA to reduce primary tumor burden and metastatic spread, we wanted to determine if the delay of tumor development and tumor progression was due to the proapoptotic or antiproliferative properties of α-TEA. For this purpose, we evaluated apoptosis and cell proliferation in tumor sections after 4 weeks (day 70 of age) and 9 weeks (day 105 of age) of α-TEA treatment by TUNEL and Ki-67 staining, respectively. Our results show (Fig. 5A and B) that at both time points, the average number of TUNEL-positive cells per field in the α-TEA-treated group was significantly higher than in the control group (P < 0.0001). There was no difference in the number of Ki-67-positive cells after short-term α-TEA treatment (4 weeks, day 70) compared with mice on the control diet (Fig. 5C). However, the number of Ki-67-positive cells was significantly lower after 9 weeks of α-TEA treatment (day 105) compared with control treatment (Fig. 5D). Together, these results suggest that tumor cell killing by apoptosis, at least in the earlier stages of the disease, seems to play a bigger role in the α-TEA-mediated inhibition of tumor progression.

Discussion

The antitumor activities of vitamin E compounds, exemplified by α-TOS and the closely related α-TEA, have been extensively characterized using in vitro systems and using transplantable xenograft and syngeneic tumor models of various origin. In recent years, it has been established that α-TOS and α-TEA have the ability to inhibit tumor progression in vivo (1, 5, 7, 9, 10, 22–28). In addition, we have recently shown, using the transplantable, highly aggressive and poorly immunogenic syngeneic model of murine breast cancer (4T1), that the esterase-stable α-TEA can be administered orally with high antitumor efficacy (2).

In order to move α-TEA closer to the clinic to be used as a new treatment option for breast cancer, the present study had two main goals: first, to determine the minimal effective dose needed to treat breast cancer; and second, to test the efficacy of orally administered α-TEA in a more clinically relevant murine model of spontaneously arising breast cancer.

To examine the minimally effective α-TEA dose, BALB/c mice with established 4T1 mammary tumors (day 10 post–tumor implantation) were fed mouse chow containing decreasing amounts of α-TEA. The relatively high α-TEA dose (0.3% diet) of ~6 mg α-TEA per day (equivalent to 300 mg/kg body weight) caused a 64% reduction of primary tumor size and 85% decrease in lung metastases (Fig. 1), confirming our earlier studies in this tumor model (2). The next dose tested was a 3-fold lower formulation of
the α-TEA diet designed to deliver 2 mg of α-TEA per day (equivalent to 100 mg/kg body weight) that resulted in an antitumor effect similar to the 0.3% diet, achieving a 50% reduction in tumor size (Fig. 1). Our findings indicate that increasing the α-TEA dose does not translate into a directly proportional antitumor benefit. This seems to be particularly true for the ability of α-TEA to inhibit metastasis formation, as the 0.1% diet inhibited metastatic tumor spread (92% reduction) with the same efficacy as the 0.3% α-TEA diet (85% reduction; Fig. 1C). This is also underscored by the result that the even lower 0.05% α-TEA diet dose (∼1 mg/d), although it had only a moderate effect on the primary tumor (38% reduction in tumor size), was almost as effective in preventing metastasis formation as the 0.1% or the 0.3% diet. This finding suggests that the low ∼1 mg/d α-TEA intake (equivalent to 50 mg/kg body weight) is below a necessary α-TEA threshold level needed to significantly suppress the primary tumor; however, it is still high enough to inhibit metastatic tumor spread. Studies using a model of residual disease, in which primary tumors are surgically removed after metastasis formation (29), are currently under way to examine this possibility. If these results are confirmed, it may open the possibility to use a low α-TEA dose as a safe long-term therapy option to prevent or treat recurrent metastatic disease after debulking of the primary tumor. Another possible explanation of the findings that the 0.1% and 0.05% α-TEA diets significantly reduced lung metastasis, although there was only moderate or no significant effect on the primary tumor, may be that α-TEA is able to suppress tumor spread via different mechanisms at lower doses. In a recent report, we showed that low α-TEA and α-TOS doses (ranging from ∼2.5 to ∼12.5 μg/mL) significantly decreased wound healing and tube formation of endothelial-like EAhy926 cells in vitro and that α-TOS also inhibited in vivo angiogenesis by induction of apoptosis of proliferating endothelial cells (30). A similar mechanism may be responsible for the anti-metastatic activity of α-TEA.

Because our results suggested that the dose-dependent suppression of tumor growth and metastatic spread may not be a linear function of the apparent α-TEA intake, we determined the circulating α-TEA serum levels. Although the 0.3% diet contained three times as much α-TEA as the 0.1% diet, the α-TEA serum level in the mice on the 0.3% diet was approximately twice as high as the α-TEA serum level in the mice on the 0.1% α-TEA diet (Fig. 2). However, according to the average food intake, the daily dose of the mice on the 0.3% diet (5.9 mg/d) was indeed close to twice the average calculated dose of the mice on the 0.1% diet (2.4 mg/d). This may explain the observed similarities in antitumor efficacy of the 0.3% and 0.1% diet. Surprisingly, the serum level of the mice on the 0.05% diet was close to the α-TEA serum level of the mice that received the 2-fold higher α-TEA dose (0.1% diet), which may help to explain the interesting result that the low 0.05% α-TEA diet dose still had a significant suppressive effect on lung metastasis.

Figure 5. α-TEA increases apoptosis and inhibits cell proliferation in MMTV-PyMT tumors. After 4 wk (day 70) or 9 wk (day 105) of α-TEA treatment (0.1% diet), tumor sections were examined for cell proliferation by Ki-67 staining and apoptosis by TUNEL assay. Data are depicted as average number of Ki-67 or TUNEL-positive cells in 15 microscopic fields per tumor sample (Ki-67, magnification, ×400; apoptosis, magnification, ×200). Differences were evaluated by t test.

1576 α-TEA Inhibits Spontaneous Murine Breast Cancer

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The transplantable 4T1 tumor model enabled us to quickly determine the minimal effective α-TEA dose, establishing that the 0.1% diet is an effective α-TEA dose against murine mammary tumors. However, the second goal of this study was to evaluate the efficacy of α-TEA therapy in a more clinically relevant murine mammary tumor model. Although transplantable tumor models are valuable tools in preclinical cancer research, and many mimic human disease reasonably well, they do not fully resemble human cancer etiology as they lack the physiologically intact tumor bed and spontaneously arising tumor microenvironment (31). For this reason, we used the transgenic MMTV-PyMT mouse model of spontaneous breast cancer to test the efficacy of α-TEA therapy in a setting more closely resembling human disease. The MMTV-PyMT mice (12) develop multifocal mammary adenocarcinoma involving the entire mammary pad with secondary metastasis to the lung, making it a valuable model of human breast cancer (19). The MMTV-PyMT mice were started on the 0.1% TEA diet at 6 weeks of age, when these mice displayed alveolar hyperplasia and moderate mammary intraepithelial neoplasia and therefore represent an early disease model of breast cancer (Fig. 3). This dose of ∼2 mg α-TEA per day delayed the development of palpable tumors by 17 days and reduced tumor size by 85% in comparison to untreated mice (Fig. 3A and B). In addition, a promising result was that α-TEA was able to prevent lung metastases in 40% of the treated animals and reduced the average number of spontaneous lung metastases by 80% (Fig. 3C). These results corroborate our earlier findings using the transplantable 4T1 tumor model that α-TEA is effective in inhibiting tumor progression and spread and suggests that α-TEA therapy may be particularly valuable to treat metastases formation.

Although the lower 0.1% diet dose was very effective in delaying tumor development and suppressing primary tumor growth and metastasis, we also evaluated the 0.3% α-TEA diet, but treated MMTV-PyMT mice died after ∼3 weeks of drug exposure. This is in contrast to our finding that this same dose had no adverse effects in BALB/c mice as reported here and previously (2). Although we have no compelling explanation for this different outcome, it seems to suggest that the transgenic MMTV-PyMT mice are more sensitive to higher doses of α-TEA. However, more detailed toxicologic studies are needed to determine the differential effect of α-TEA on mice of different genetic backgrounds.

To elucidate the mechanism of α-TEA–mediated tumor suppression, we evaluated what roles the proapoptotic and antiproliferative properties of α-TEA play *in vivo*. Using *in vitro* experiments, we (2, 29) and others (3, 7, 32) have shown that vitamin E analogues such as α-TOS and α-TEA induce apoptosis and inhibit the proliferation of breast cancer and other tumor cell lines. However, this is the first report examining the mechanism of how α-TEA inhibits tumor progression in an *in vivo* model of spontaneous breast cancer. After 4 weeks of α-TEA treatment and at the end of the study (after 9 weeks of treatment), we examined tumor tissues for apoptosis and cell proliferation *in situ*. Our results show that after short-term treatment (4 weeks), there was no difference in cell proliferation in the tumor tissue (Fig. 5C). Meanwhile, at the study end point, tumor cell proliferation was affected by α-TEA therapy. However, at both time points, there were significantly more apoptotic cells in the tumor tissue from α-TEA–treated animals in comparison to control animals. This suggests that the well-documented proapoptotic activities of α-TEA play a significant role in the α-TEA–mediated inhibition of tumor progression in the MMTV-PyMT tumor model.

This study shows, for the first time, the ability of orally administered α-TEA to delay tumor onset and to inhibit the progression and metastatic spread of a clinically relevant model of spontaneous breast cancer. Our finding of the high efficacy in this tumor model highlights the translational potential of oral α-TEA therapy and may lay the foundation for clinical testing of α-TEA in patients with metastatic breast cancer.

**Disclosure of Potential Conflicts of Interest**

Laurence H. Hurley is a patent holder for the "Preparation of tocophorins, tocotrienols, other chroman and side chain derivatives for use as antitumor agents and for inducing cell apoptosis." No commercial applications have been realized or are under development.

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