Repeat dose study of the novel proapoptotic chemotherapeutic agent alpha-tocopheryloxy acetic acid in mice

Tobias Hahn and Emmanuel T. Akporiaye

Alpha-tocopheryloxy acetic acid (α-TEA) is an ether derivative of vitamin E and has been shown to suppress tumor growth in various murine and human xenograft tumor models, including melanoma, breast, lung, prostate, and ovarian cancers. The purpose of this study was to assess its safety and pharmacokinetics after repeat dosing in a preclinical murine model. Male and female mice received α-TEA doses of 100, 300, or 1500 mg/kg/day by daily oral gavage for 28 days. α-TEA serum levels were determined weekly by high-performance liquid chromatography with mass spectrometric detection. After 28 days of dosing, complete blood counts were taken, blood chemistry was analyzed, and histology was performed. Pharmacokinetic parameters were determined after single dosing. There was no mortality, and we found no clinical signs of toxicity in any of the α-TEA doses tested. Histopathological evaluation of major organs (heart, lung, kidney, liver, spleen, jejunum, ileum, and cecum) revealed no significant α-TEA treatment-related lesions. Blood counts revealed low-grade anemia but no other significant differences between treatment and control groups. Blood chemistry revealed moderate liver toxicity that was dose dependent and was absent in the lowest dose group. There were no significant sex-specific differences in the toxicity profile. The half-life of orally administered α-TEA was determined to be 52 h. This is the first report comprehensively evaluating the toxicity profile of this novel anticancer drug and will facilitate the design of clinical trials to evaluate the safety and antitumor efficacy of α-TEA in patients with cancer. *Anti-Cancer Drugs* 23:455–464 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: alpha-tocopheryloxy acetic acid, cancer, pharmacokinetics, preclinical safety toxicity study, vitamin E analog

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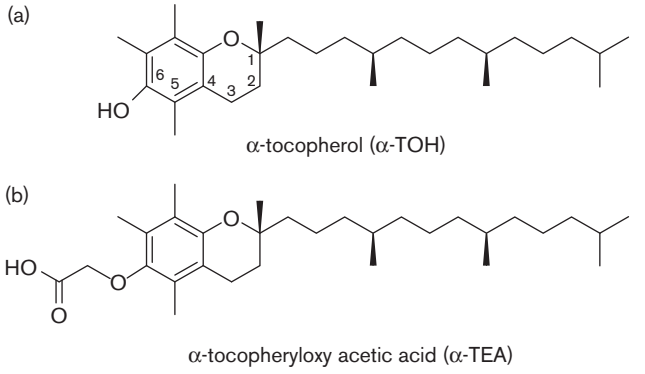
Introduction

Alpha-tocopheryloxy acetic acid (α-TEA) is a novel semisynthetic ether derivative of naturally occurring vitamin E (α-tocopherol). Unlike vitamin E, which lacks in-vivo antitumor activity and fails to prevent cancer in humans [1,2], α-TEA induces tumor cell death at doses that are not harmful to normal cells [3–7]. α-TEA structurally shares the phytyl tail and the chroman head with vitamin E (Fig. 1a); however, α-TEA differs from vitamin E in that the hydroxyl group at the number 6 carbon of the phenolic ring of the chroman head has been replaced by an acetic acid residue by means of an ether bond (Fig. 1b). Because this acetate group is linked by a nonhydrolyzable ether bond [3], α-TEA is resistant to esterase hydrolysis, permitting delivery through the oral route. Oral gavage and aerosol delivery of α-TEA have been successfully used to suppress primary tumor growth and reduce the incidence of metastasis in several murine tumor models [3,5]. We recently reported that oral administration of α-TEA in the diet significantly inhibited the growth of a transplanted, highly metastatic mammary cancer and markedly reduced the incidence of lung metastases [8]. α-TEA in the diet was also able to delay the onset and suppress the growth of spontaneous breast cancer in transgenic mouse mammary tumor virus-PyMT mice [9].

The major mechanism of α-TEA-mediated anticancer activity has been shown to be the induction of tumor cell apoptosis *in vitro* and *in vivo* [6,9–11]. Recent research has

Fig. 1



Structure of (a) α-tocopherol and (b) alpha-tocopheryloxy acetic acid (α-TEA).

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shown that α-TEA may cause tumor cell death by targeting mitochondria and modulation of apoptosis and survival pathways [6,7,12]. Both α-TEA and α-tocopheryl succinate (α-TOS; a second vitamin E analog (VEA) closely related to α-TEA in structure and activity that is esterase sensitive) [4] have been shown to induce the accumulation of reactive oxygen species resulting in mitochondrial depolarization that is considered one of the initiating events that ultimately lead to apoptotic cell death [6,10,11]. Although mitochondria are the major intracellular targets for VEAs, other signaling pathways operating through other organelles such as lysosomes and endoplasmic reticulum may also be involved in VEA-induced tumor cell death [7,11,13]. α-TEA has been shown to inhibit the activation of the survival protein AKT in prostate and ovarian cancer models, thereby inhibiting the expression of c-FLIP and survivin, two major inhibitors of apoptosis [14–16]. In addition to inhibiting survival pathways, α-TEA has also been shown to activate prodeath signaling through the Fas/FasL and JNK/c-Jun signaling pathways in prostate and mammary cancer models [14,17,18]. Furthermore, we recently reported that α-TEA-mediated antitumor activity was partially dependent on a T cell-mediated immune response and that α-TEA therapy modulated the anti-tumor immune response in the tumor microenvironment [19].

Although α-TEA has been used in preclinical animal models to successfully treat cancer, little is known about the toxicity profile of prolonged α-TEA administration. This is the first comprehensive study to evaluate the toxicity profile of this novel anticancer drug and will facilitate the design of first-in-human clinical trials to evaluate the safety and antitumor efficacy of α-TEA in patients with cancer.

Materials and methods

Alpha-tocopheryloxyacetic acid

α-TEA [(2,5,7,8-tetramethyl-(2*R*-(4*R*,8*R*,12-trimethyltridecyl) chroman-6-yloxy) acetic acid)] was synthesized by Marker Gene Technologies Inc. (Eugene, Oregon, USA) using a modification of previously described methods [3,19,20]. A single lot of non-good manufacturing practice α-TEA was synthesized, and purity and identity were confirmed by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance analysis. To make α-TEA soluble in aqueous solution for oral administration, α-TEA was dried under nitrogen in a round-bottom glass flask, and the resulting thin film was vesiculated by sonication in the presence of PBS yielding vesiculated α-TEA as described previously [8].

Animal studies

Eleven-week-old male and female BALB/c mice were purchased from Harlan Laboratories Inc. (Indianapolis, Indiana, USA). Mice were housed in microisolator cages at the Arizona Cancer Center Experimental Mouse Shared Services at The University of Arizona (Tucson, Arizona, USA) in accordance with the Principles of Animal Care (NIH publication No. 85-23). All studies were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Arizona. For the single-dose pharmacokinetic study, female BALB/c mice received one gavage treatment of 4 mg vesiculated α-TEA (hereafter referred to as α-TEA) per mouse, corresponding to approximately 200 mg/kg body weight. Blood was obtained by retro-orbital bleeding at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h, 48 h, 56 h, 96 h, and 102 h after α-TEA administration from each of four individual mice per time point. For the repeat dose study, male and female BALB/c mice ($n = 6$ per sex and treatment group) received 30, 6, or 2 mg α-TEA per mouse per day for 28 days by gavage, corresponding to respective approximate doses of 1500, 300, or 100 mg/kg/day. Control mice ($n = 6$ per sex) received 200 µl of vehicle (PBS). To determine plasma α-TEA levels, blood was obtained from three mice per group by retro-orbital bleeding on days 8, 15, and 22 of dosing and by terminal heart bleed on day 29 (24 h after the last α-TEA administration). To determine the acute toxicity parameters, 24 h after the last α-TEA dose (day 29) blood was collected by terminal heart bleeding, complete differential blood counts (CBC) were taken, and blood chemistry parameters were determined by the University Animal Care Pathology Services at The University of Arizona.

Sample preparation and HPLC analysis

Systemic levels of α-TEA were determined by HPLC with mass spectrometric detection at the Arizona Cancer Center Analytical Chemistry Shared Services at The University of Arizona (Tucson, AZ). For plasma level analysis, blood samples were collected into heparinized tubes from which plasma was obtained and stored at –80°C until analysis. Plasma samples were extracted by adding 200 µl ethanol containing 1% butylated hydroxytoluene (w/v), 100 µl saline (0.9% NaCl, w/v), and 1000 µl 95 : 5 hexane : dichloromethane. The samples were vortexed on a platform shaker for 5 min and centrifuged at 4°C for 10 min at 16 000*g*. The organic phase (900 µl) was then transferred to a fresh tube and dried. The residue was reconstituted with 100 µl 70 : 30 acetonitrile : methanol and transferred to auto sampler vials for injection (5 µl injection volume). Separation was achieved on a Phenomenex Luna C-18 (Phenomenex, Torrance, California, USA) 5µm 2.1 × 50 column using a mobile phase of 70 : 30: 0.25 acetonitrile : methanol : acetic acid and a 500 µl/min flow rate. Mass spectrometric analysis was performed on a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Finnigan, West Palm Beach, Florida, USA) operating in negative polarity electrospray ionization mode. α-TEA was detected by selective reaction monitoring with the transition from parent *m/z* 487 to fragment *m/z* 163 monitored

for α-TEA and the transition from parent *m/z* 429 to fragment *m/z* 163 monitored for the internal standard α-TOS (Sigma, St Louis, Missouri, USA). The transition for the internal standard is that of tocopherol due to the loss of the succinate during ionization. For quantification, an α-TEA standard curve was constructed in mouse plasma, and α-TOS (Sigma) was used as an internal standard to adjust for extraction efficiency and loading control.

Histology

Tissues of major organs (heart, lung, kidney, liver, spleen, jejunum, ileum, and cecum) were dissected and fixed in 10% buffered formalin for 24 h, and paraffin embedded. Thereafter, 3 μm sections were stained with hematoxylin and eosin and evaluated by a veterinary pathologist. Images were captured at ×25 magnification using a BX50 microscope (Olympus, Center Valley, Pennsylvania, USA) and a Paxcam 3 camera with PAX-it Digital Imaging Management and Analysis Software (Midwest Information Systems, Villa Park, Illinois, USA).

Statistical analysis

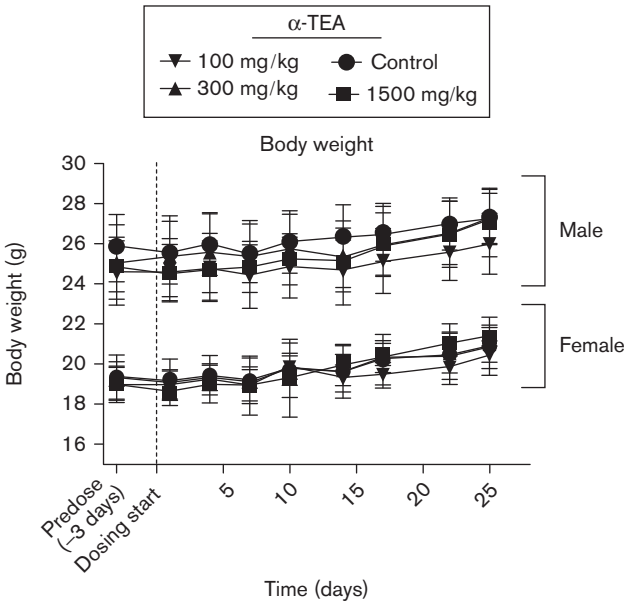
Statistical significance of differences in blood counts and serum chemistry between treated and control groups was assessed by analysis of variances (ANOVA) with Dunnett's Multiple Comparison Test. Treatment association of histopathological lesions was evaluated by Fisher's exact test. Analyses were performed using Prism software (GraphPad, San Diego, California, USA). Probability values (*P*) of less than 0.05 were considered to be significant.

Results

Repeat daily dosing of alpha-tocopheryloxy acetic acid caused no gross toxicity

The purpose of this study was to assess the safety of prolonged oral α-TEA treatment. Male and female BALB/c mice received α-TEA doses of 100, 300, or 1500 mg/kg/day by daily oral gavage for 28 days. The 100 and 300 mg/kg doses were chosen because previous reports have shown that oral administration of these doses was efficacious in murine mammary cancer models [8,9,19]. To determine the maximal tolerated dose, a 1500 mg/kg α-TEA dose group was included in this study. The 1500 mg/kg dose is the maximum feasible oral dose in mice because of the limited solubility of α-TEA in aqueous solvents and the institutional animal use committee-mandated volume limitation of 200 μl for gavage administration in mice. Throughout the treatment period, body weights were determined, and mice were monitored for clinical signs of toxicity. There was no mortality and we found no clinical signs of toxicity (lethargy, abdominal distension, fur loss, hunching) at any of the α-TEA doses tested. Consistent with the common sex-specific weight difference, the female mice were, on average, 4 g lighter than male mice. We found no differences in body weight between the treatment and control groups, and all α-TEA-treated mice displayed weight gains similar to that of the control mice (Fig. 2).

Fig. 2



Body weights of mice during 28-day dosing of alpha-tocopheryloxy acetic acid (α-TEA). Male and female BALB/c mice ($n=6$ per group and sex) received indicated doses of alpha-tocopheryloxy acetic acid by daily gavage. Control mice received vehicle (PBS). Body weights were determined 3 days before the start of the treatment (-3 days) and then in 3–4-day intervals until day 25. Data points represent mean body weight ± SEM.

Hematologic analysis

On day 29, (i.e. 24 h after the last α-TEA administration), differential blood counts were determined from three mice per treatment group and sex (Tables 1 and 2). There were no biologically significant changes in leukocyte counts between any of the dose groups and control animals. Male mice that received 1500 or 300 mg/kg α-TEA experienced a respective erythrocyte count decrease of 1.61 M/μl or 1.44 M/μl from an erythrocyte count of 10.19 ± 0.59 M/μl in control mice. The changes in erythrocyte counts were associated with respective hemoglobin decreases of 2 and 1.3 g/dl and hematocrit decreases of 7.66 and 5.96 percentage points. The male mice that received 100 mg/kg α-TEA dose had no significant changes in erythrocytes, hemoglobin, or hematocrit measurements compared with male control mice (Table 1). Female mice that received 1500, 300, or 100 mg/kg also had respective α-TEA dose-dependent decreased erythrocyte counts of 1.84, 1.22, or 0.79 M/μl compared with a count of 9.82 ± 0.21 M/μl in control mice. Hemoglobin levels in the female mice that received 1500 and 300 mg/kg were decreased by 2.3 and 1.1 g/dl, respectively (Table 2). However, hemoglobin was unaffected in the

Table 1 Hematologic analysis of male mice

	Vehicle			1500 mg/kg				300 mg/kg				100 mg/kg				
Male	Mean	SD	*N*	Mean	SD	*N*	*P*	Mean	SD	*N*	*P*	Mean	SD	*N*	*P*	Unit
Leukocytes																
White blood cell count	6.67	1.04	3	9.07	3.41	3	NS	7.61	2.25	3	NS	8.35	0.57	3	NS	K/μl
Neutrophils	1.37	0.15	3	2.41	0.99	3	NS	2.13	0.48	3	NS	1.91	0.18	3	NS	K/μl
Lymphocytes	4.88	0.97	3	6.14	2.31	3	NS	5.16	1.70	3	NS	6.16	0.37	3	NS	K/μl
Monocytes	0.39	0.02	3	0.46	0.14	3	NS	0.29	0.11	3	NS	0.26	0.07	3	NS	K/μl
Eosinophils	0.02	0.01	3	0.05	0.07	3	NS	0.02	0.02	3	NS	0.02	0.01	3	NS	K/μl
Basophils	0.00	0.01	3	0.01	0.01	3	NS	0.00	0.01	3	NS	0.00	0.00	3	NS	K/μl
Neutrophil %	20.68	2.47	3	26.28	2.27	3	<0.05	28.33	2.71	3	<0.05	22.91	1.16	3	NS	%
Lymphocyte %	72.93	3.26	3	67.89	3.11	3	NS	67.50	2.14	3	NS	73.78	0.89	3	NS	%
Monocyte %	5.94	1.08	3	5.23	0.70	3	NS	3.89	1.03	3	NS	3.12	0.66	3	<0.05	%
Eosinophil %	0.34	0.21	3	0.53	0.62	3	NS	0.23	0.22	3	NS	0.18	0.04	3	NS	%
Basophil %	0.10	0.14	3	0.07	0.11	3	NS	0.04	0.03	3	NS	0.02	0.02	3	NS	%
Erythrocytes																
Red blood cell count	10.19	0.59	3	8.58	0.23	3	<0.05	8.75	0.01	3	<0.05	9.41	0.52	3	NS	M/μl
Hemoglobin	14.50	0.44	3	12.50	0.36	3	<0.05	13.17	0.06	3	<0.05	13.83	0.15	3	NS	g/dl
Hematocrit	56.13	1.62	3	48.47	0.23	3	<0.05	50.17	0.40	3	<0.05	54.03	1.96	3	NS	%
Mean corpuscular volume	55.17	1.67	3	56.50	1.30	3	NS	57.30	0.46	3	NS	57.43	1.17	3	NS	fl
Mean corpuscular hemoglobin	14.23	0.61	3	14.57	0.49	3	NS	15.07	0.06	3	NS	14.77	0.65	3	NS	pg
Mean corp. hemoglobin concentration	35.50	16.37	3	25.77	0.70	3	NS	26.23	0.12	3	NS	25.60	0.62	3	NS	g/dl
RBC distribution width %	19.30	2.52	3	19.77	0.21	3	NS	20.60	0.82	3	NS	20.03	0.21	3	NS	%
Reticulocyte	0.33	0.06	3	0.44	0.11	3	NS	0.29	0.10	3	NS	0.28	0.10	3	NS	M/μl
Reticulocyte %	3.30	0.75	3	5.07	1.19	3	NS	3.37	1.19	3	NS	3.07	1.01	3	NS	%
Thrombocytes																
Platelet	712.00	28.83	3	893.00	85.50	3	<0.05	798.67	66.21	3	NS	784.67	41.31	3	NS	K/μl
Mean platelet volume	4.87	0.38	3	4.93	0.23	3	NS	4.73	0.21	3	NS	4.53	0.15	3	NS	fl

Male BALB/c mice received indicated doses of α-TEA by daily gavage for 28 days. On day 29, 24 h after the last α-TEA dose, whole blood was collected from three mice per treatment group and analyzed.
K, thousand; M, million; *P*, *P*-value in comparison with control group.

Table 2 Hematologic analysis of female mice

	Vehicle			1500 mg/kg				300 mg/kg				100 mg/kg				
Female	Mean	SD	*N*	Mean	SD	*N*	*P*	Mean	SD	*N*	*P*	Mean	SD	*N*	*P*	Unit
Leukocytes																
White blood cell count	8.05	2.63	3	9.55	4.18	3	NS	9.627	1.500	3	NS	10.13	2.43	3	NS	K/μl
Neutrophils	1.61	0.48	3	2.27	1.12	3	NS	1.867	0.510	3	NS	2.22	0.54	3	NS	K/μl
Lymphocytes	6.11	2.06	3	6.53	3.19	3	NS	7.157	0.939	3	NS	7.47	1.73	3	NS	K/μl
Monocytes	0.29	0.08	3	0.68	0.34	3	NS	0.530	0.096	3	NS	0.38	0.12	3	NS	K/μl
Eosinophils	0.04	0.04	3	0.06	0.05	3	NS	0.063	0.067	3	NS	0.07	0.05	3	NS	K/μl
Basophils	0.01	0.02	3	0.01	0.01	3	NS	0.010	0.017	3	NS	0.01	0.02	3	NS	K/μl
Neutrophil %	20.04	0.73	3	23.63	8.47	3	NS	19.150	2.952	3	NS	21.91	1.90	3	NS	%
Lymphocyte %	75.72	1.61	3	68.69	11.02	3	NS	74.590	3.393	3	NS	73.77	1.76	3	NS	%
Monocyte %	3.67	0.29	3	7.05	2.55	3	<0.05	5.490	0.322	3	NS	3.65	0.30	3	NS	%
Eosinophil %	0.43	0.49	3	0.53	0.29	3	NS	0.633	0.708	3	NS	0.59	0.35	3	NS	%
Basophil %	0.12	0.19	3	0.09	0.06	3	NS	0.137	0.150	3	NS	0.09	0.15	3	NS	%
Erythrocytes																
Red blood cell count	9.82	0.21	3	7.98	0.47	3	<0.05	8.597	0.110	3	<0.05	9.03	0.16	3	<0.05	M/μl
Hemoglobin	14.10	0.10	3	11.77	0.86	3	<0.05	13.033	0.513	3	NS	13.73	0.42	3	NS	g/dl
Hematocrit	55.83	1.56	3	47.73	4.12	3	<0.05	52.467	1.069	3	NS	53.53	1.05	3	NS	%
Mean corpuscular volume	56.83	0.95	3	59.73	1.68	3	NS	61.033	0.961	3	<0.05	59.27	1.40	3	NS	fl
Mean corpuscular hemoglobin	14.37	0.31	3	14.77	0.31	3	NS	15.200	0.794	3	NS	15.20	0.36	3	NS	pg
Mean corpuscular hemoglobin concentration	25.27	0.60	3	24.67	0.60	3	NS	24.867	1.250	3	NS	25.63	1.18	3	NS	g/dl
RBC distribution width %	19.63	0.21	3	19.43	0.45	3	NS	19.933	0.709	3	NS	19.73	0.42	3	NS	%
Reticulocyte	0.21	0.02	3	0.74	0.17	3	<0.05	0.370	0.193	3	NS	0.33	0.01	3	NS	M/μl
Reticulocyte %	2.13	0.25	3	8.43	0.59	3	<0.05	4.400	2.402	3	NS	3.63	0.15	3	NS	%
Thrombocytes																
Platelet	678.33	38.81	3	786.00	249.93	3	NS	752.667	92.511	3	NS	702.00	111.23	3	NS	K/μl
Mean platelet volume	4.60	0.10	3	4.93	0.15	3	<0.05	4.767	0.058	3	NS	4.63	0.06	3	NS	fl

Female BALB/c mice received indicated doses of α-TEA by daily gavage for 28 days. On day 29, 24 h after the last α-TEA dose, whole blood was collected from three mice per treatment group and analyzed.
K, thousand; M, million; *P*, *P*-value in comparison with control group.

female 100 mg/kg dose group. In comparison with control mice, the hematocrit level in the female 1500 mg/kg dose group was decreased by 8 percentage points, but the hematocrit decrease was less than 4 percentage points in the 300 and 100 mg/kg dose groups. Female mice that received 1500 mg/kg α-TEA experienced an increase in

reticulocyte counts of 0.53 M/μl that resulted in an increase in reticulocyte frequency of 6.3 percentage points (Table 2). Similar changes were not observed in the male mice (Table 1).

Evaluation of blood chemistry

One day after the last α-TEA administration (day 29), we determined blood chemistry parameters to evaluate liver and kidney toxicity (Tables 3 and 4). The data show that aspartate transaminase (AST) levels were not significantly different in male and female mice in all dose groups. There was a 1.9-fold and 1.6-fold increase in alanine transaminase (ALT) in male mice that received 1500 and 300 mg/kg α-TEA, respectively; however, the ALT levels were unaffected in the 100 mg/kg dose group (Table 3). Although there was a significant ~15-fold increase in ALT levels in the female 1500 mg/kg dose group, ALT levels were normal in the female 300 and 100 mg/kg dose groups compared with control mice (Table 4). Alkaline phosphatase (ALP) levels were also increased by up to 1.6-fold in both male and female mice that received 1500 or 300 mg/kg α-TEA compared with control animals. The ALP levels were normal in the male and female mice that received 100 mg/kg α-TEA. In comparsion with control mice, creatinine kinase (CK) levels were elevated by 1.9-fold to three-fold in female mice at all dose levels (Table 4). In contrast, there was no difference in CK levels between male α-TEA-treated and control mice (Table 3). We also observed a significant decrease in cholesterol levels in both male and female α-TEA-treated mice (Fig. 3). Daily administration of 1500 mg/kg α-TEA to male and female mice resulted in respective three-fold and 2.5-fold decreases in cholesterol levels compared with control mice. These cholesterol

Table 3 Blood chemistry of male mice

	Vehicle			1500 mg/kg				300 mg/kg				100 mg/kg				
Parameter	Mean	SD	*N*	Mean	SD	*N*	*P*	Mean	SD	*N*	*P*	Mean	SD	*N*	*P*	Unit
Aspartate transaminase (AST)	124.7	45.0	3	67.7	26.3	3	NS	90.7	20.0	3	NS	64.3	14.5	3	NS	U/l
Alanine transaminase (ALT)	25.0	6.9	3	48.4	6.8	3	<0.05	40.3	9.5	3	NS	24.2	10.3	4	NS	U/l
Alkaline phosphatase (ALP)	169.7	43.5	3	280.7	21.0	3	<0.05	267.8	22.6	3	<0.05	222.5	16.0	4	NS	U/l
Creatine kinase (CK)	273.3	198.3	3	186.0	52.9	3	NS	211.0	10.5	3	NS	271.0	103.2	4	NS	U/l
Glucose	143.8	19.7	3	150.0	16.6	3	NS	175.0	32.7	3	NS	161.7	36.0	4	NS	mg/dl
Total bilirubin	0.2	0.1	3	0.6	0.2	3	<0.05	0.2	0.1	3	NS	0.2	0.0	4	NS	mg/dl
Blood urea nitrogen (BUN)	17.9	0.9	3	16.0	2.1	3	NS	15.8	0.8	3	NS	15.4	0.9	4	NS	mg/dl
Creatinine	0.4	0.0	3	0.3	0.1	3	NS	0.4	0.1	3	NS	0.4	0.1	4	NS	mg/dl
Total protein	7.0	0.6	3	6.6	0.3	3	NS	6.7	0.5	3	NS	6.8	0.6	4	NS	g/dl
Albumin	3.7	0.4	3	4.0	0.2	3	NS	4.0	0.2	3	NS	3.9	0.2	4	NS	g/dl
Globulin	3.3	0.7	3	2.5	0.3	3	NS	2.7	0.4	3	NS	2.9	0.5	4	NS	g/dl
Cholesterol	145.3	17.2	3	49.7	4.5	3	<0.05	87.6	3.1	3	<0.05	111.8	10.5	4	<0.05	mg/dl
Triglyceride	172.7	2.5	3	528.3	158.1	3	<0.05	200.3	38.7	3	NS	229.7	25.8	3	NS	mg/dl
Amylase	682.8	58.6	3	757.0	51.9	3	NS	685.4	30.3	3	NS	694.6	68.3	4	NS	U/l
Calcium	10.6	0.3	3	11.0	0.3	3	NS	10.9	0.1	3	NS	10.9	0.3	4	NS	mg/dl
Phosphorus	8.2	0.6	3	10.0	1.1	3	NS	7.9	1.5	3	NS	7.7	0.5	4	NS	mg/dl

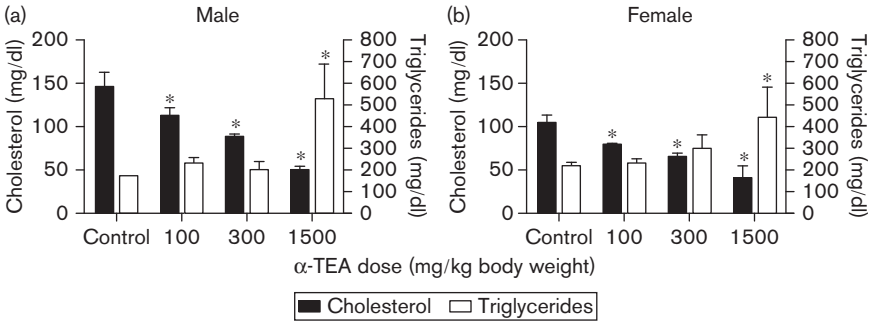
Male BALB/c mice received indicated doses of α-TEA by daily gavage for 28 days. On day 29, 24 h after the last α-TEA dose, blood was collected from three to four mice per treatment group and analyzed.
P, *P*-value in comparison with control group.

Table 4 Blood chemistry of female mice

	Vehicle			1500 mg/kg				300 mg/kg				100 mg/kg				
Parameter	Mean	SD	*N*	Mean	SD	*N*	*P*	Mean	SD	*N*	*P*	Mean	SD	*N*	*P*	Unit
Aspartate transaminase (AST)	85.0	39.5	3	112.3	47.5	3	NS	74.3	36.1	3	NS	93.0	37.7	3	NS	U/l
Alanine transaminase (ALT)	22.1	5.1	3	332.7	15.6	3	<0.05	27.8	1.6	3	NS	20.0	6.4	3	NS	U/l
Alkaline phosphatase (ALP)	241.7	41.1	3	372.7	57.1	3	<0.05	323.4	16.9	3	NS	244.5	14.1	3	NS	U/l
Creatine kinase (CK)	88.0	21.0	3	170.7	51.0	3	NS	273.0	97.6	3	<0.05	165.7	64.9	3	NS	U/l
Glucose	149.4	40.5	3	152.2	6.8	3	NS	173.6	8.2	3	NS	139.0	33.0	3	NS	mg/dl
Total bilirubin	0.1	0.1	3	0.5	0.0	3	<0.05	0.3	0.1	3	NS	0.2	0.1	3	NS	mg/dl
Blood urea nitrogen (BUN)	13.4	1.3	3	15.2	0.4	3	NS	17.3	1.9	3	<0.05	15.3	1.2	3	NS	mg/dl
Creatinine	0.4	0.1	3	0.3	0.1	3	NS	0.3	0.1	3	NS	0.4	0.1	3	NS	mg/dl
Total protein	6.5	0.4	3	6.6	0.3	3	NS	6.4	0.1	3	NS	6.4	0.3	3	NS	g/dl
Albumin	4.4	0.2	3	4.3	0.1	3	NS	4.6	0.2	3	NS	4.5	0.2	3	NS	g/dl
Globulin	2.1	0.3	3	2.3	0.4	3	NS	1.8	0.1	3	NS	1.9	0.3	3	NS	g/dl
Cholesterol	104.0	9.2	3	40.6	14.4	3	<0.05	64.9	3.9	3	<0.05	79.0	1.2	3	<0.05	mg/dl
Triglyceride	221.0	15.4	3	442.7	139.7	3	<0.05	299.7	61.9	3	NS	229.7	21.0	3	NS	mg/dl
Amylase	600.7	56.4	3	759.6	64.1	3	<0.05	570.2	12.1	3	NS	564.2	22.7	3	NS	U/l
Calcium	11.1	0.2	3	10.9	0.2	3	NS	11.3	0.9	3	NS	11.1	0.3	3	NS	mg/dl
Phosphorus	7.5	0.5	3	8.4	0.2	3	NS	6.7	0.2	3	NS	7.3	1.7	3	NS	mg/dl

Female BALB/c mice received indicated doses of α-TEA by daily gavage for 28 days. On day 29, 24 h after the last α-TEA dose, blood was collected from three mice per treatment group and analyzed.
P, *P*-value in comparison with control group.

Fig. 3



Effect of alpha-tocopheryloxy acetic acid (α-TEA) on serum cholesterol and triglyceride levels. Male (a) and female (b) BALB/c mice received indicated doses of α-TEA by daily gavage for 28 days. Control mice received vehicle (PBS). On day 29, 24 h after the last α-TEA dose, blood was collected from three to four mice per group and analyzed for cholesterol and triglyceride levels. *P less than 0.005 compared with control mice.

Table 5 Distribution of lesions per treatment group

	Vehicle		1500 mg/kg		300 mg/kg		100 mg/kg	
Lesion	M (n=3)	F (n=3)	M (n=3)	F (n=3)	M (n=3)	F (n=3)	M (n=3)	F (n=3)
Multifocal epicardial mineralization	1	1	0	0	1	2	0	1
Microgranuloma (liver)	0	0	1	1	0	2	0	0
Extramedullary hematopoiesis (spleen)	1	0	0	2	2	0	2	1

Male and female BALB/c mice received indicated doses of α-TEA by daily gavage for 28 days. On day 29, 24 h after the last α-TEA dose, heart, lung, kidney, liver, spleen, and gastrointestinal tract (jejunum, ileum, and cecum) were dissected and evaluated for gross pathology. Subsequently, tissue sections were stained with hematoxylin and eosin.
F, female; M, male.

decreases were clearly dose dependent, as cholesterol levels gradually increased in mice that received the lower doses (Fig. 3). The decreased cholesterol levels in the 1500 mg/kg dose group were in conjunction with three-fold and two-fold increased triglyceride levels in male and female mice, respectively, compared with control mice. Triglyceride levels in the lower dose groups were not significantly changed compared with control mice (Fig. 3).

Histology of major organs

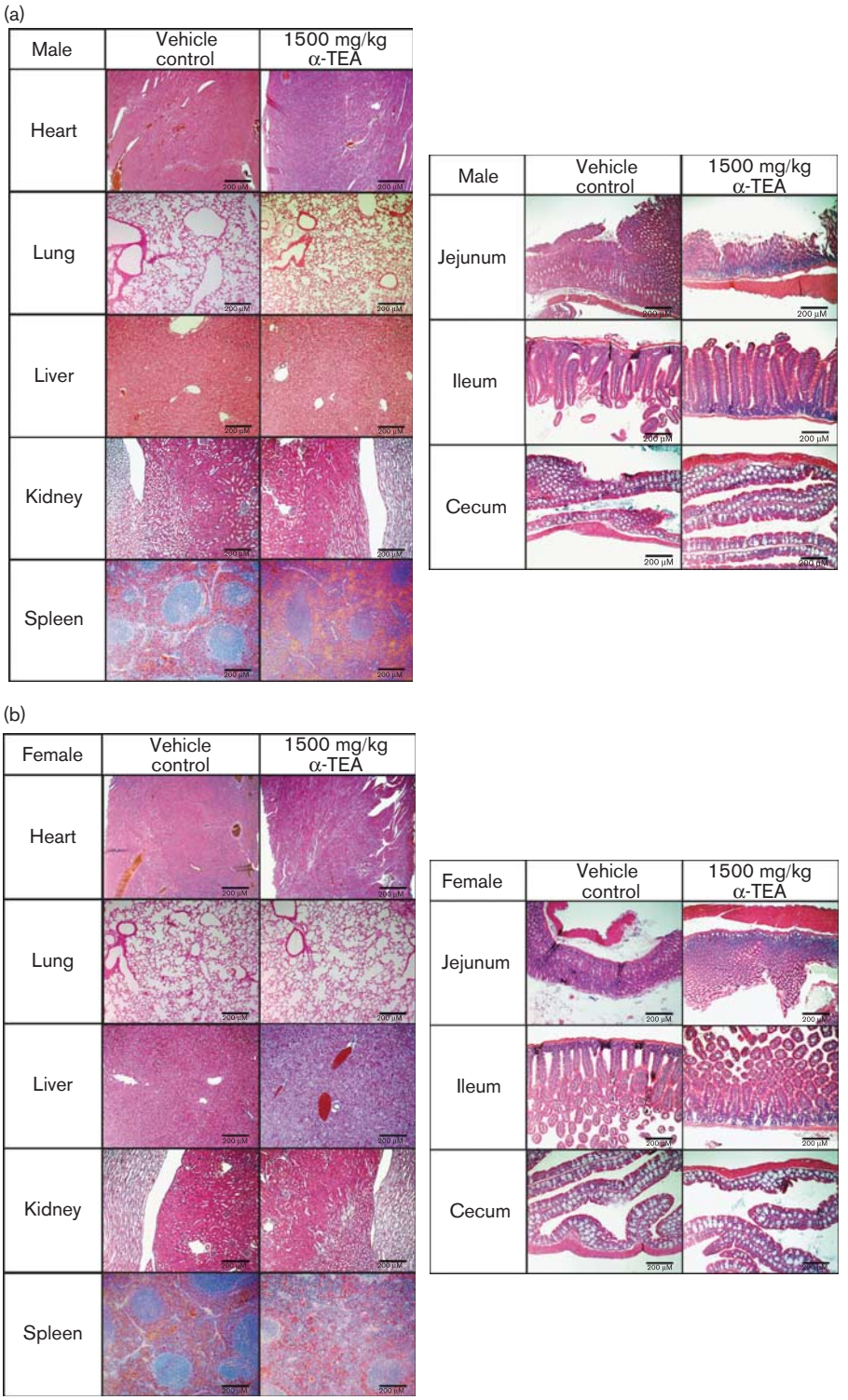
One day after the 28-day treatment period, major organs (heart, lung, kidney, liver, spleen, jejunum, ileum, and cecum) from three mice per treatment group (vehicle control, 1500, 300, and 100 mg/kg) and sex were histopathologically evaluated by a veterinary pathologist. Epicardial mineralization was observed in six mice (Table 5), which is a strain-related lesion common in BALB/c mice. Four mice had few microgranuloma in their livers, and extramedullary hematopoiesis was observed in the spleens of eight mice (Table 5). There was no treatment group association of these lesions, and they are considered to be incidental. Figure 4 shows representative microphotographs of hematoxylin and eosin-stained sections of the evaluated organs for the vehicle (PBS) control mice and mice that received the highest α-TEA dose (1500 mg/kg per day), demonstrating the absence of histological lesions.

Pharmacokinetics of alpha-tocopheryloxy acetic acid

To determine the half-life of orally administered α-TEA, female mice received a single dose of 200 mg/kg α-TEA by oral gavage. Plasma samples were obtained from 15 min to 102 h after α-TEA administration from four mice per time point and analyzed for α-TEA levels by HPLC/MSD. α-TEA plasma levels peaked ~8 h after administration at 23.1 ± 3.4 μg/ml and declined with a half-life of 52.7 h and an area under the plasma concentration–time curve of 1732 (h·μg/ml) (Fig. 5).

We also determined the plasma α-TEA levels after daily repeat dosing of 1500, 300, or 100 mg/kg α-TEA by oral gavage for 28 days (Fig. 6). Plasma was obtained from three mice per treatment group weekly during the dosing period and 24 h after the last α-TEA administration. Administration of a daily dose of 1500, 300, or 100 mg/kg to male mice for 1 week resulted in α-TEA plasma levels of 66.9 ± 6.3, 37.7 ± 3.5, or 41.9 ± 4.1 μg/ml, respectively (Fig. 6a). During the next 3 weeks of dosing, the α-TEA level did not increase further in the 1500 mg/kg dose group and dropped to 43.9 ± 10.7 μg/ml 24 h after the last α-TEA administration. α-TEA levels in the male mice that received 300 and 100 mg/kg/day increased slowly over 3 weeks, reaching 61.1 ± 5.1 and 61.2 ± 10.2 μg/ml at the end of the study. In the female mice, administration of a daily dose of 1500, 300, or 100 mg/kg for 1 week resulted in α-TEA plasma levels of 72.9 ± 2.5, 44.4 ± 5.3,

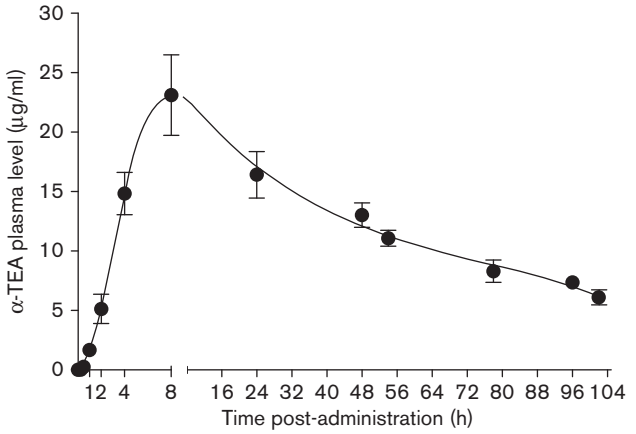
Fig. 4



Histologic evaluation of major organs. Male and female BALB/c mice received 1500 mg/kg alpha-tocopheryloxy acetic acid (α-TEA) by daily gavage for 28 days. Control mice received vehicle (PBS). On day 29, 24 h after the last alpha-tocopheryloxy acetic acid dose, heart, lung, kidney, liver, spleen, and gastrointestinal tract (jejunum, ileum, and cecum) were dissected and sections were stained with hematoxylin and eosin. Microphotographs (× 25 magnification) of (a) male mice and (b) female mice.

or 32.9 ± 1.2 μg/ml, respectively (Fig. 6b). After the initial α-TEA plasma level increases during the first 8 days of treatment, the plasma levels in the female mice that received 1500, 300, and 100 mg/kg α-TEA did not increase further significantly, reaching 58.2 ± 9.2, 39.6 ± 4.4, and 51.7 ± 6.3 μg/ml at the end of the study, respectively (Fig. 6b). α-TEA was not detected in control mice that received vehicle (PBS) alone (data not shown).

Fig. 5



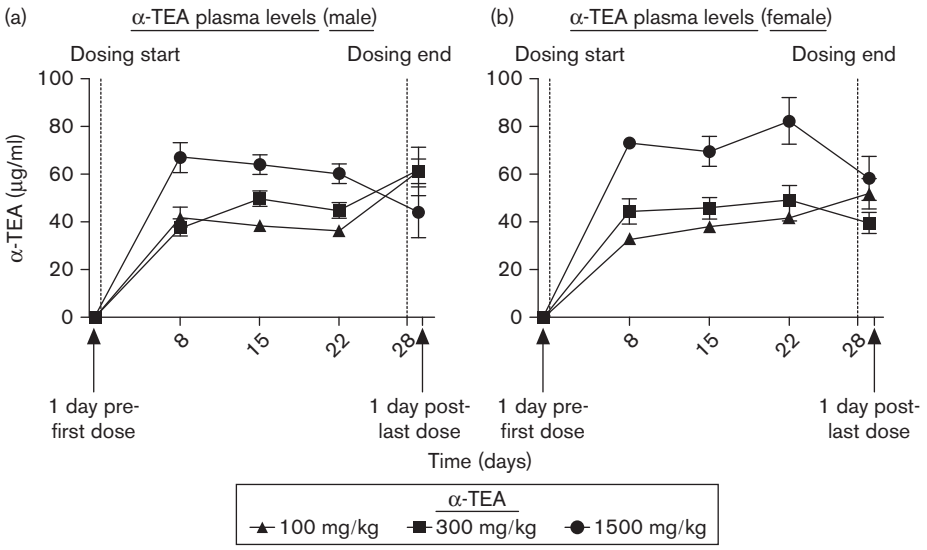
Single-dose pharmacokinetics of alpha-tocopheryloxy acetic acid (α-TEA). Female BALB/c mice received a single dose of 200 mg/kg α-TEA by oral gavage. Plasma α-TEA levels were determined by HPLC with mass spectrometric detection from $n=4$ mice per time point. Values represent mean plasma levels ± SEM.

Discussion

In this report, we evaluated the safety of prolonged (28 day) α-TEA treatment in mice and describe the pharmacokinetic parameters for orally delivered α-TEA. There were no overt signs of toxicity in mice that received daily doses of up to 1500 mg/kg as there was no mortality and all mice displayed normal body weight gains throughout the treatment period. Our findings corroborate previous but limited toxicity studies reporting no signs of gross toxicity after mice received 200 [8] or 250 mg/kg α-TEA by gavage for up to 25 days [21] or 300 mg/kg α-TEA in the diet for up to 20 days [9,19]. However, our study is the first to comprehensively evaluate daily oral α-TEA doses of up to 1500 mg/kg.

To evaluate possible blood and liver toxicity, we performed CBC and serum chemistry analysis after completion of the 28-day treatment course. The CBC revealed that treatment with 1500 mg/kg α-TEA resulted in mild anemia with respective decreases of erythrocyte counts, hemoglobin levels, and hematocrit of up to 1.8 M/μl, 2.3 g/dl, and 8 percentage points. This low-grade anemia was diminished in the male and female 300 mg/kg dose groups and was nonexistent in the 100 mg/kg dose group. The unaffected mean corpuscular volume and red blood cell distribution widths contradict chronic anemia, and acute blood loss due to a mechanism such as gastrointestinal ulcers is unlikely as no ulcerative lesions were apparent. Both male and female mice that received 1500 mg/kg α-TEA had small increases in bilirubin levels, which may indicate hemolytic loss of erythrocytes. Notwithstanding, the mice that received the highest

Fig. 6



Plasma alpha-tocopheryloxy acetic acid (α-TEA) levels during 28-day repeat dosing. Male (a) and female (b) BALB/c mice received 100, 300, or 1500 mg/kg/day α-TEA by daily gavage for 28 days. α-TEA levels were determined by HPLC with mass spectrometric detection at weekly intervals and 24 h after the last dose. Values represent mean plasma levels ± SEM of $n=3$ mice per time point.

α-TEA dose also had small increases in reticulocyte counts and percentages, suggesting that erythropoiesis was still effective. No other significant aberrations in blood cell counts were found after 28 days of up to 1500 mg/kg α-TEA treatment. Serum chemistry analysis revealed moderately elevated ALT levels in male mice that received 1500 or 300 mg/kg α-TEA. In comparison with control mice, 1500 mg/kg α-TEA resulted in 15-fold elevated ALT levels in female mice. However, ALT aberrations were absent in the female 300 mg/kg dose group and the male and female 100 mg/kg dose groups. The elevated ALT levels may indicate hepatocellular injury in the highest dose group, and elevated ALT levels in conjunction with increased ALP levels may indicate bile duct obstruction. In both male and female mice that received 1500 or 300 mg/kg α-TEA, ALP levels were ~1.6-fold higher than those in control mice. ALP levels in the 100 mg/kg group were either ~1.3-fold higher (male mice) or unaffected (female mice). Furthermore, compared with control mice, AST levels were only moderately elevated in the female 1500 mg/kg dose group, with all other animals having unchanged or lower AST levels. Although the increased ALT and ALP levels could be indicators of liver damage, the largely normal AST levels in the absence of α-TEA-related histopathologic changes in the liver suggest that liver damage was likely to be minor even in the highest dose group. Whether α-TEA-mediated increases in ALT and ALP are transient or permanent may be answered by evaluation of blood chemistry parameters after longer discontinuation of the α-TEA treatment; however, this study was designed to only evaluate acute α-TEA-associated toxicity. Although, compared with control mice, there were no abnormal CK levels in any of the treated male mice, α-TEA treatment of female mice resulted in approximately two-fold to three-fold higher CK levels. Although this could indicate α-TEA-mediated muscle damage, the elevated CK levels could have resulted from blood collection by terminal heart puncture.

Histologic analysis revealed no α-TEA treatment-related lesions in the heart, lung, kidneys, spleen, or gastrointestinal tract (ileum, jejunum, and cecum) in any of the dose groups compared with untreated control mice. Taken together, these data suggest that prolonged (28 day) daily dosing with 300 or 100 mg/kg α-TEA has a low toxicity profile and that the maximal feasible oral dose of 1500 mg/kg was relatively safe with no acute, severe adverse effects. Large animal toxicity studies, which are required for an investigational new drug application, will provide more information on how critical endpoints such as maximum tolerated dose and pharmacokinetics scale between species and will enable appropriate dose selection for a future dose-escalation phase I clinical trial.

An interesting finding from our study was the α-TEA-mediated lowering of cholesterol levels that was accompanied by increased triglyceride levels. As this was an unexpected finding, a detailed lipid profile was not determined in this study. The exact mechanism to account for these changes is unknown, and further studies examining α-TEA transport and metabolism are warranted. Given these results, cholesterol and triglyceride levels with a detailed lipid profile will be carefully determined in planned large animal safety and toxicity studies and in a future first-in-human clinical trial.

Pharmacokinetic analysis showed that orally delivered α-TEA was detectable in the blood stream within 1 h and peaked after 8 h. The half-life of α-TEA was determined to be about 2 days after a single dose of 200 mg/kg. This relatively long half-life indicates that α-TEA is stable in circulation and is cleared slowly. Determination of circulating α-TEA levels during daily dosing showed that α-TEA levels in the male and female 1500 mg/kg dose groups increased within a week to ~70 μg/ml and stabilized thereafter. This was also evident in the 100 and 300 mg/kg dose groups, in which the plasma level versus time curves increased with steep slopes during the first week but leveled during the remaining 3 weeks of α-TEA treatment. This leveling of the plasma concentration versus time curves suggests that steady-state plasma α-TEA levels had been achieved after approximately 8 days of daily dosing. Given that the time to reach steady state is commonly three to five half-lives, with an α-TEA half-life of ~52 h, one would expect steady state to be reached between 6 and 10 days of daily dosing, which is consistent with our data. The data show that after steady state was reached, daily dosing with 100 or 300 mg/kg α-TEA achieved plasma levels in the 40–60 μg/ml range. Using murine mammary tumor models, we have shown in a previous study that plasma levels in the 30–60 μg/ml range were effective in reducing primary tumor growth and in markedly reducing metastatic tumor spread [9]. Therefore, the mice that received daily doses of 100 or 300 mg/kg α-TEA achieved plasma levels in the anticancer therapeutic range.

This is the first comprehensive report that examined the safety and toxicity profile of long-term oral administration of the novel antitumor active vitamin E analog α-TEA. Our findings indicate that α-TEA treatment, at doses that have been shown to be efficacious against murine cancer models (100–300 mg/kg), was safe and had few adverse effects. Taken together, our findings highlight the translational potential for α-TEA as a novel treatment for cancer patients and facilitate the design of further investigational new drug-enabling studies and clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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