Vitamin E analog, alpha-tocopherol ether-linked acetic acid analog, alone and in combination with celecoxib, reduces multiplicity of ultraviolet-induced skin cancers in mice

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The goals of this study were to determine whether alpha-tocopherol ether-linked acetic acid analog (α-TEA), a novel vitamin E analog, and celecoxib, alone or in combination, when administered as a late intervention can reduce the ultraviolet-induced nonmelanoma skin-tumor burden of established tumors, prevent additional tumors from developing, and prevent tumor recurrence once treatments are stopped. Hairless SKH-1 female mice were ultraviolet-irradiated for 24 weeks, divided into treatment groups so that each group had approximately 5.8 tumors/mouse, and then treated with 72 μg of liposome-formulated α-TEA by aerosol inhalation, 500 p.p.m. celecoxib in AIN-76A diet, or a combination of α-TEA and celecoxib for 4 weeks. At the end of 4 weeks of treatment, each treatment group was subdivided, with one subgroup continuing to receive treatment and with treatment being stopped in the other. Skin-tumor development was monitored visually throughout the study and by histologic evaluation at the end. After 4 weeks of treatment, all treatments showed statistically significant reductions in tumor number when compared with controls. After termination of treatment, only α-TEA prevented a significant increase in tumor recurrence; however, continuous combination treatment resulted in the lowest total number of tumors. In conclusion α-TEA is an effective late-stage chemopreventive agent for nonmelanoma skin cancer that exhibits lasting benefits. *Anti-Cancer Drugs* 19:175–181 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The incidence of nonmelanoma skin cancer has increased significantly in the past two decades, particularly in the United States, Canada, Australia, the United Kingdom, and the Scandinavian countries [1]. Nonmelanoma skin cancer is the most frequently diagnosed malignancy in the United States, and more than 1 million cases of basal-cell and squamous-cell skin cancer are expected to be newly diagnosed in 2007 [2]. If left untreated, nonmelanoma skin cancers can become invasive and require surgery, which can result in disfigurement. Formerly this was a concern for people above 50 years; a recent study indicates a rise in incidence in individuals less than 40 years of age [3]. Therefore, effective treatments are needed.

Excessive exposure to ultraviolet (UV) irradiation in sunlight, which induces DNA damage, is considered to be the major etiologic factor in the development of nonmelanoma skin cancer in humans [4,5]. Among the many events leading to tumor initiation and progression, one that is relevant to the studies reported here is that continuous UV irradiation leads to the constitutive expression of the cyclooxygenase-2 enzyme and increased prostaglandin production [6,7]. Prostaglandin E2 contributes to the tumorigenic process by enhancing the proliferation of UV-damaged cells, resulting in skin tumors [8,9]. Celecoxib, a selective cyclooxygenase-2 inhibitor, has been studied as a preventive and a therapeutic agent for the skin [6,7,9–11]. Celecoxib has been shown to be a potent chemopreventive agent producing a dose-dependent reduction in tumor yield (60 and 89%) when 150 or 500 p.p.m. of celecoxib was fed through an AIN-76A diet during UV irradiation [6]. Furthermore, celecoxib is capable of serving as a chemotherapeutic agent, producing a 25% reduction in tumor number when fed (500 p.p.m.) through the diet following papilloma/carcinoma development [11]. Of significance to this study, the antitumor effects of celecoxib have been shown to be transient, with tumors recurring to the same degree as in control mice within 1 month of the cessation of celecoxib treatment [11].

The absence of lasting effects of celecoxib highlights the need for an effective combination treatment. Combinations of agents with dissimilar targets have the potential for being more effective than single agents alone. In an effort to investigate a compound that has the potential to

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enhance antitumor efficacy when used in combination with celecoxib, we tested the alpha-tocopherol ether-linked acetic acid analog (α-TEA), a nonhydrolyzable ether analog of natural vitamin E (RRR-α-tocopherol), which has been shown previously to significantly reduce tumor volumes and numbers of lung metastases when used in combination with celecoxib, in a xenograft model of human breast cancer [12]. This xenograft study showed that, when α-TEA was administered by aerosol (inhalation) and celecoxib was fed through the diet at 1250 mg/kg either separately or in combination, apoptosis was significantly enhanced and cell proliferation was significantly decreased in the tumor tissue [12]. Furthermore, this study showed that the combination treatment of α-TEA and celecoxib significantly reduced blood-vessel density, as determined by CD-31 staining [12]. An additional rationale for using α-TEA emerges from the fact that α-TEA has been shown to restore Fas/Fas-ligand signaling [13,14]. This signaling, when taken in combination with results from studies showing that UV-damaged keratinocytes are eliminated by the Fas/Fas-ligand interaction – but also that this pathway becomes dysregulated during UV skin carcinogenesis [15,16] – suggests a potential relevant target for α-TEA in UV-induced skin cancer.

The goals of this study were thus to determine whether α-TEA and celecoxib, if administered as a late intervention, either alone or in combination, could prevent additional UV-induced nonmelanoma skin tumors from developing, reduce tumor burden of established tumors, and/or prevent tumor recurrence once treatments were stopped.

Materials and methods

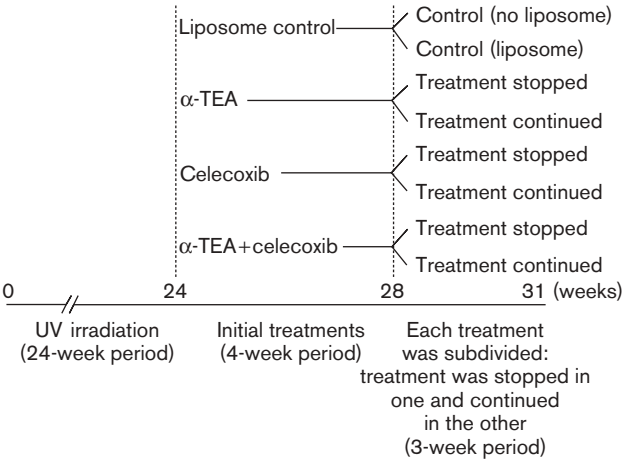
Animals

Hairless SKH-1 female mice at 3 weeks of age were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA) and were used at 5 weeks of age. Animals were housed at five per cage. The weight of each animal was monitored and recorded weekly for the duration of the study. Average weight for each group was compared, and no significant overall differences were observed using the Mann–Whitney rank test (data not shown).

Irradiation and celecoxib and alpha-tocopherol ether-linked acetic acid analog treatments

One hundred and twenty mice were placed on control AIN-76A diets (Dyets Inc., Bethlehem, Pennsylvania, USA) 1 week before beginning the UV-irradiation protocol. UV irradiation was carried out as previously described [11]. Briefly, mice were initially exposed to 90 mJ/cm^2 UV irradiation three times per week, and the dose was increased by 10% each week until a final dosage of 175 mJ/cm^2 was reached [11]. UV irradiation was stopped after 24 weeks (Fig. 1), and 30 mice were assigned randomly to one of four groups (control, α-TEA, celecoxib, and α-TEA + celecoxib), such that each group had an average of 5.8 visible (>1 mm^2) tumors per mouse. In this manner, all the treatment groups started with approximately the same numbers and sizes of tumors. Treatments were administered for 4 weeks. At the end of this 4-week period, each treatment group was subdivided, with one subgroup continuing to receive treatment and with treatment being stopped in the other subgroup. After an additional 3 weeks, the experiment was terminated.

Fig. 1



Schema of treatment protocol. During the first 24 weeks, ultraviolet (UV)-induced skin tumors were being generated. At the end of week 24, the mice were divided into four groups and were treated as indicated for the next 4 weeks. At the end of week 28, half of the mice in each of the treatment groups continued on treatment, whereas the rest were removed from treatment (treatment stopped).

Celecoxib was purchased from LKT Laboratories (St Paul, Minnesota, USA). AIN-76A diet with celecoxib supplementation (500 mg celecoxib/kg diet) was prepared by Dyets Inc. Celecoxib (500 p.p.m.) in AIN-76A diet has previously been shown to be effective in UV-induced skin-carcinogenesis studies [6]. The diet was stored at 4°C, and fresh diet was supplied three times per week.

α-TEA was synthesized and purified in-house and formulated into liposomes and delivered by aerosol (inhalation), as previously described [17]. This method of α-TEA formulation and delivery was chosen because it has been demonstrated earlier to significantly inhibit both tumor burden and metastasis in a syngeneic mouse mammary-cancer model and in xenograft models of human ovarian and breast cancers [12,17–20]. On the basis of previous studies, it is estimated that 72 μg of α-TEA was deposited in the respiratory tract of each mouse daily [17].

Characterization of skin-tumor development and termination of study

Skin-tumor development was monitored weekly throughout the study. Mice were visually inspected for tumors. Skin-tumor data were expressed as the mean number of tumors per mouse (multiplicity) and percent of mice with tumors (incidence).

At the completion of the study (31 weeks), mice were euthanized by carbon dioxide asphyxiation, and tumors were removed. Tumors were fixed in formalin, and were processed for histologic evaluation of the tumor type (papilloma or squamous-cell or spindle-cell carcinoma).

Statistical analysis

Data were analyzed using analysis of variance with the Tukey honestly significant difference mean-rank tests and Poisson regression methods. Weekly weights were analyzed using the Mann–Whitney rank test. A level of P less than 0.05 was regarded as statistically significant.

Results

Tumor generation (ultraviolet irradiation for a 24-week period)

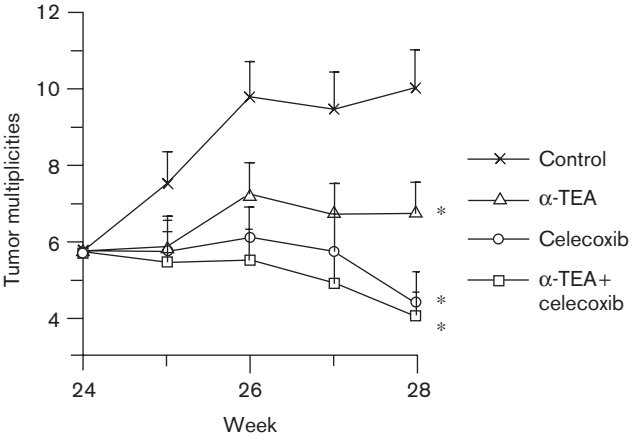
Tumors were generated by UV irradiation three times per week for 24 weeks (Fig. 1). At this point, UV treatments were stopped and the mice were randomly divided into four groups, such that each group had an average of 5.8 tumors per mouse and an incidence of 100% (control) or 97% (all other groups).

Prevention of new tumor formation and tumor regression (response to the initial 4-week period of treatments)

For the next phase of the experiment, mice were administered the designated drug (Fig. 1), and tumor multiplicities were determined weekly. In the control group, there were approximately 10 visible exophytic tumors per mouse by week 28 (Fig. 2). This reflects a 72% increase in tumor multiplicity over this 4-week period. At 28 weeks, tumor multiplicities in the different treatment groups were as follows. The α-TEA group had significantly fewer tumors than the control group ($P = 0.0181$). The α-TEA group had an average of 6.8 tumors per mouse, reflecting a gain of one tumor per mouse, and a tumor multiplicity 32% less than that of the control group.

Tumor multiplicity for the celecoxib group was also significantly less than that of the control group ($P = 0.0001$), averaging 4.4 tumors per mouse. This represents a loss of over one tumor per mouse during this initial 4-week treatment period, and an overall 56% reduction in tumor multiplicity in comparison with the control group. Similarly, mice in the α-TEA + celecoxib group showed a significant reduction in the number of tumors ($P < 0.00005$), averaging 4.1 tumors per mouse and a 59% reduction in tumor multiplicity in comparison with controls. In cases of tumor regression, some seemed to have regressed completely, with the skin appearing normal. In contrast, the location of the former tumor in the other cases was only evident as a red-colored spot that could not be palpated.

Fig. 2



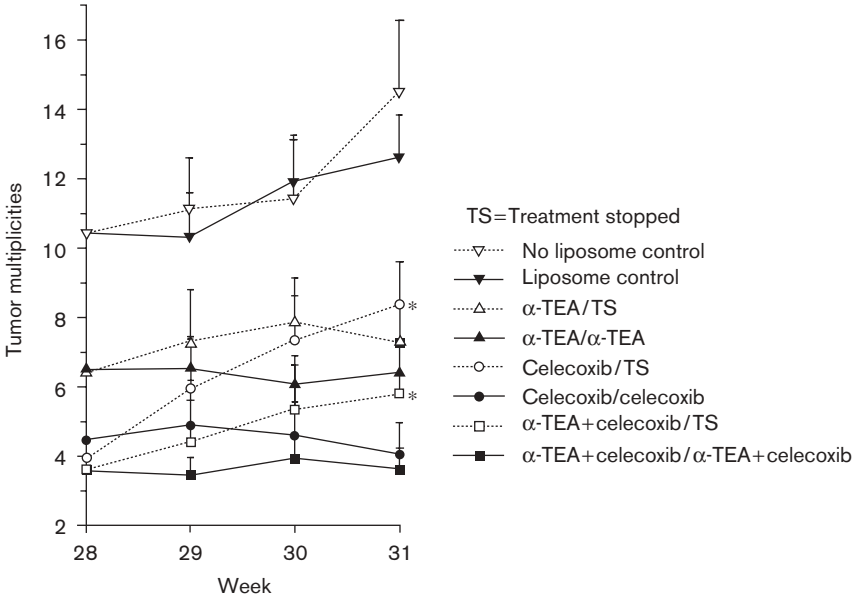
Tumor development during first 4 weeks of drug treatment. After 24 weeks of ultraviolet exposure, female SKH-1 hairless mice were placed in treatment groups, in such a way that there was an average of 5.8 tumors per mouse. Daily (7 days/week) treatments consisted of the deposition of liposome-formulated α-TEA (approximately 72 μg/respiratory tract/mouse), consumption of AIN-76A diet containing 500 p.p.m. celecoxib in it, and of both treatments or neither for 4 weeks. Tumor multiplicities were calculated as the average number of tumors per mouse ± SE. *$P < 0.05$ treated vs. control. α-TEA, alpha-tocopherol ether-linked acetic acid analog.

Tumor regrowth (comparison of tumor multiplicity in animals continuing to receive treatment vs. animals removed from treatment)

To determine whether the antitumor effects were permanent or required continuous treatment, each treatment group was divided into two subgroups. One subgroup continued to receive treatment, whereas treatment was stopped in the other subgroup (Fig. 1). The average number of tumors in all the treatment groups was significantly reduced, compared with the two controls (Fig. 3). The subgroup of mice in which α-TEA had been removed (treatment stopped = TS; α-TEA/TS) gained less than one tumor per mouse over the 3-week period (Fig. 3). The subgroup of mice that continued to receive α-TEA (α-TEA/α-TEA) showed no net change in tumor multiplicity over the 3-week period. The difference in tumor multiplicity between these two groups was thus not significant ($P = 0.3652$). This suggests that the effects of α-TEA are lasting, as the group in which α-TEA treatment had been stopped did not regain a significant number of tumors.

The group in which celecoxib had been discontinued (celecoxib/TS) showed a significant ($P = 0.0000345$) increase in tumor multiplicity, compared with the group

Fig. 3



Effects of cessation or continuation of treatment on tumor multiplicity. At the end of week 28 of the experiment, each treatment group was divided in half with drug treatment stopped (TS) in one half (open symbols and dashed lines) whereas treatment was continued in the other half (solid symbols and solid lines). Tumor multiplicities were calculated as the average number of tumors per mouse ± SE. Average numbers of tumors in all treatment groups were significantly reduced in comparison with either control group. *$P<0.05$ continuously treated vs. treatment stopped per type of treatment. α-TEA, alpha-tocopherol ether-linked acetic acid analog.

that continued to receive celecoxib. As reported previously [11], this indicates that the tumor regression is not permanent, even though, visually or by palpation, the tumors were not present at week 28.

The group in which the combined α-TEA + celecoxib treatment was discontinued (α-TEA + celecoxib/TS) regained 2.1 tumors per mouse. The α-TEA + celecoxib group that continued to receive treatment evidenced no net change in tumor number during this 3-week period; hence, the mice averaged 3.7 tumors at the start and finish (Fig. 3). The difference in tumor multiplicity between these two groups was significant ($P = 0.0184$). [Note: The reason for the slight change in tumor number from the end of the initial 4 weeks of treatment (4.1 tumors) to the start of treatment subdivision (3.7 tumors) for this group is that one mouse had to be killed: when the group was split and the tumors counted again, the average number of tumors dropped. This was accounted for in the statistical analyses.] Please see Fig. 4 for a summary of data on tumors/groups in both the treatment phases.

Comparison of the effect of treatment vs. stoppage of treatment on tumor size was analyzed (Table 1), and no differences in tumor sizes were observed between controls and treatment groups. Over 90% of all the tumors were less than 5 mm in diameter. In fact, none of the treatments influenced the size of the tumors over the course of the study (all $P > 0.05$).

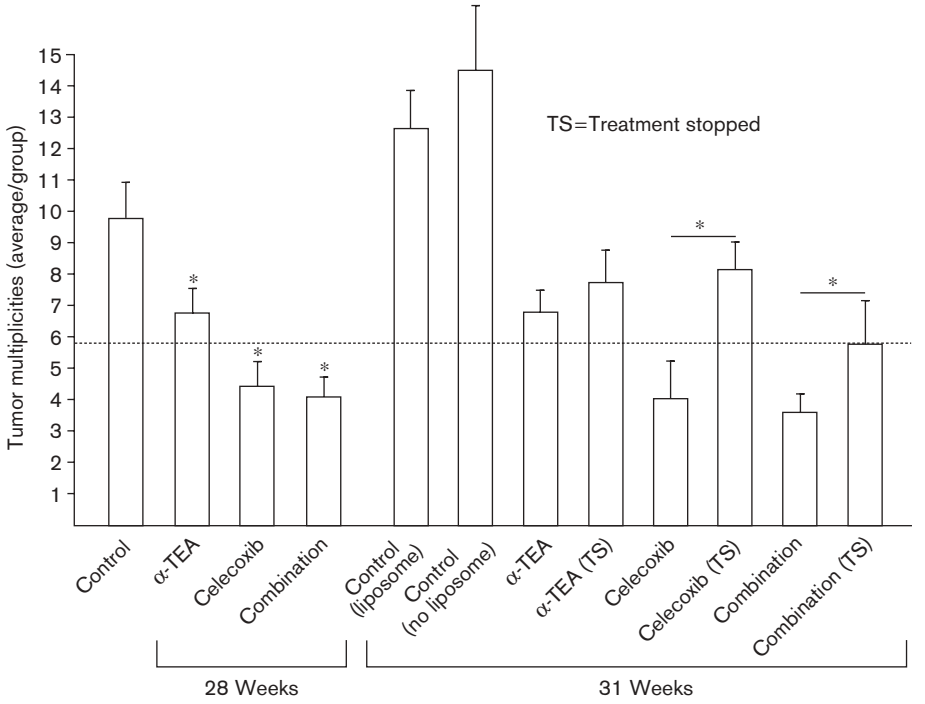
At the conclusion of the experiment, all the mice were euthanized, and the tumors were removed and histologically classified (Table 2). In all treatment groups, the total number of squamous-cell carcinomas and spindle-cell carcinomas were not significantly different from those in either of the controls.

Discussion

As the incidence of human nonmelanoma skin cancer is increasing at a rapid rate, there is an urgent need to identify agents that are effective when administered late in the tumorigenic process, to prevent the appearance of new cancers and to treat the existing ones. With this goal in mind, we hypothesized that a combination of α-TEA (a vitamin E analog) and celecoxib (an effective chemopreventive/chemotherapeutic agent) would be more effective than either agent alone in (i) preventing the formation of new tumors following UV termination, (ii) reducing the tumor burden of established tumors, and (iii) preventing the recurrence of tumors once treatment ceased, in the SKH-1 hairless-mouse animal model.

The studies reported here showed that after 4 weeks of treatment, α-TEA, celecoxib, and combination treatments produced significant ($P < 0.05$) reductions in

Fig. 4



Summary of tumor development. Average number of tumors ± SE per mouse per treatment group are presented as bar graphs. The dotted line indicates the average number of tumors per mouse (i.e. 5.8) after 24 weeks of ultraviolet (UV) treatment prior to initiation of α-TEA, celecoxib or combination (α-TEA + celecoxib) drug treatments. *$P < 0.05$ for treated vs. control for 28-week data and continuously treated vs. treatment stopped (TS) for 31-week data. α-TEA, alpha-tocopherol ether-linked acetic acid analog.

Table 1 Comparison of effect of treatment vs. stoppage of treatment on tumor size[a]

Groups/treatment	Tumor sizes			Total number of tumors
	<5 mm (%)	5–10 mm (%)	>10 mm (%)	
Control (no liposome)				
Begin ($n=14$)	133 (92)	12 (8)	0 (–)	145
End ($n=13$)	174 (93)	14 (7)	0 (–)	188
Control (liposome)				
Begin ($n=14$)	136 (93)	10 (7)	0 (–)	146
End ($n=13$)	153 (93)	10 (6)	1 (1)	164
α-TEA/TS				
Begin ($n=12$)	75 (97)	2 (3)	0 (–)	77
End ($n=12$)	86 (98)	2 (2)	0 (–)	88
α-TEA/α-TEA				
Begin ($n=13$)	80 (96)	3 (4)	0 (–)	83
End ($n=13$)	81 (98)	2 (2)	0 (–)	83
Celecoxib/TS				
Begin ($n=12$)	46 (96)	2 (4)	0 (–)	48
End ($n=12$)	91 (92)	8 (8)	0 (–)	99
Celecoxib/celecoxib				
Begin ($n=13$)	55 (96)	2 (4)	0 (–)	57
End ($n=13$)	49 (92)	4 (8)	0 (–)	53
α-TEA + celecoxib/TS				
Begin ($n=12$)	43 (98)	1 (2)	0 (–)	44
End ($n=12$)	66 (96)	3 (4)	0 (–)	69
α-TEA + celecoxib/α-TEA + celecoxib				
Begin ($n=13$)	46 (96)	1 (2)	1 (2)	48
End ($n=13$)	47 (98)	1 (2)	0 (–)	48

[a]For each tumor size category, data are expressed as the total number of tumors/group and as a percentage of the total tumors (in parentheses).
α-TEA, alpha-tocopherol ether-linked acetic acid analog; *n*, number; TS, treatment stopped.

tumor number, compared with control: in the latter, the average number of tumors increased from approximately 5.8 to 10 per mouse. The data on celecoxib as a single agent are in agreement with those of an earlier study that showed that celecoxib is effective in preventing the formation of new tumors when administered as a late intervention [11]. The data reported here are the first reports that show that α-TEA is effective as a chemopreventive agent for skin cancer.

Regarding the ability of the treatments to reduce the tumor burden of established tumors (5.8/mouse), after 7 weeks of continuous treatment, both celecoxib and combination treatments produced reductions (29 and 36%, respectively); whereas, no reduction was seen in the group of animals receiving continuous α-TEA treatment (10% increase).

The third question posed in these studies was whether the treatment benefits lasted after the cessation of treatment. In this regard, statistically significant increases in tumor burden were observed in both celecoxib and combination treatment groups in which the treatments had been stopped for the preceding 3 weeks, compared with their companion groups that continued to receive treatment. This demonstrates that the initial tumor

Table 2 Distribution of tumors by type at the completion of the study (31 weeks)[a]

Groups/treatment	Total number of all types of tumors[b]	Average[c]	Total number of SCC tumors	Average[d]	Total number of SpCC tumors	Average[d]
Control (no of liposomes), $n=13$	188	14.5 ± 2.0	8	0.6 ± 0.1	1	0.1
Control (liposome), $n=13$	164	12.6 ± 1.2	8	0.6 ± 0.2	0	–
α-TEA/TS, $n=12$	88	7.4 ± 1.1	14	1.2 ± 0.3	1	0.1
α-TEA/α-TEA, $n=13$	83	6.4 ± 0.7	9	0.7 ± 0.2	0	–
Celecoxib/TS, $n=12$	99	8.3 ± 1.2	11	0.9 ± 0.3	1	0.1
Celecoxib/celecoxib, $n=13$	53	4.1 ± 0.9	8	0.6 ± 0.2	1	0.1
α-TEA + celecoxib/TS, $n=12$	69	5.8 ± 1.4	16	1.3 ± 0.4	0	–
α-TEA + celecoxib/α-TEA + celecoxib, $n=13$	48	3.7 ± 0.6	9	0.7 ± 0.2	0	–

[a]At the termination of the experiment, mice were euthanized; tumors were removed, fixed in formalin and processed for histologic evaluation of tumor type: papilloma, squamous-cell carcinoma (SCC), or spindle-cell carcinoma (SpCC).
[b]Total number of all types of tumors represents the sum of all papillomas and carcinomas (SCCs and SpCCs).
[c]Average number of all types of tumors/mouse/group ± SE.
[d]Average number of either SCCs ± SE or SpCCs/mouse/group rounded to the nearest 10th.
n, number; α-TEA, alpha-tocopherol ether-linked acetic acid analog; TS, treatment stopped.

regressions in these treatment groups were not permanent. Loss of celecoxib benefit following the cessation of treatment has been reported earlier [11]. The difference in tumor recurrence between celecoxib/treatment stopped and α-TEA + celecoxib/treatment stopped might reflect the possibility that α-TEA and celecoxib are effective at preventing different subsets of tumors. As a result, tumors susceptible to celecoxib reappeared when the treatment stopped, whereas tumors responsive to α-TEA did not. Alternatively, α-TEA might be working in combination with celecoxib to produce a lasting effect on a subset of regressed tumors. In contrast to celecoxib alone and to the combination treatment, α-TEA seems to produce lasting benefits: no significant difference in tumor multiplicity was seen between the animals for whom treatment had been stopped vs. animals continuing to receive α-TEA-treatment.

At the completion of the study, reductions in total numbers of tumors were observed in all the treatment groups in comparison with controls; also, in comparison with the controls, the number of squamous cell carcinomas and spindle-cell carcinomas did not increase in any of the groups in which treatments were being continued. The number of squamous cell carcinomas and spindle-cell carcinomas did increase in all groups in which treatments had been stopped; however, in all treatment groups, the total number of squamous cell carcinomas and spindle-cell carcinomas were not significantly different from those in either control group.

In summary, α-TEA administered as a single, late intervention proved to be effective in the prevention of new UV-induced skin tumors; however, it was not as effective as celecoxib as a single, late intervention: the combination treatment of α-TEA + celecoxib offered no statistically significant benefit over celecoxib treatment alone. Although not as effective as celecoxib in the overall reduction of numbers of new tumors, it is of interest that the antitumor benefits of α-TEA, unlike those of celecoxib, were not lost with the cessation of treatment. The mechanisms involved in the ability of α-TEA to prevent UV-induced tumor formation, when administered as a late intervention, and its lasting impact are not known and are likely to be complex. Investigations of the mechanisms of action of α-TEA in these later stages of skin-tumor progression, as well as the determination of its potential as an early intervention chemopreventive agent against skin cancer merit future study.

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