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Research Article

The Safety of Oral Telomerase Activator in UV-Induced Skin Cancer with A Review of Telomerase in Aging and Skin Carcinogenesis

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Abstract

The supplement telomerase activator TA-65 (purified from *Astragalus membranaceus*) has been shown to retard cellular senescence, boost the aging immune system, and retard age-related symptoms. Lengthened telomeres retard aging, but because cancers often maintain longevity by lengthening telomeres, dietary telomerase activator might possibly increase tumorigenesis. This study investigated whether oral TA-65 effects the timing of onset and/or the incidence of skin cancers induced by UVB-irradiation and whether that possible effect is different if the oral supplementation is begun only after tumors are first detected clinically or if supplementation is begun before initiation of tumors as well as during and after the inciting UVB exposure. Three groups of ten Skh:1 hairless, nonpigmented mice exposed to UVB for twenty weeks were given (1) no supplementation, (2) TA-65 supplementation starting when the first UV-induced skin cancers were clinically observed, after which the UV exposure was

terminated, and (3) TA-65 supplementation before, during, and after UV exposure (as more tumors subsequently appeared). Except for two time points when Group 3 had borderline or statistically more tumors ≥ 2mm per mouse, overall, there was no statistically significant difference in the time of onset, the incidence, or the tumor load of skin cancers with TA-65 with either timing, confirming the safety of this anti-aging supplement in this model of the most frequent human malignancy.

Keywords
Telomerase and skin cancer; telomerase and aging; telomerase activator; telomerase reverse transcriptase promoter (TERTp) mutations (TPMs) and skin cancer; UV-induced skin cancer

1. Introduction

Telomeres are the protective DNA protein complexes at the ends of eukaryotic chromosomes. Their function is to prevent chromosome fusion that would result in chromosomal breaks, leading to abnormal segregation of genetic information in progeny cells [1]. Telomerase is a cellular ribonucleoprotein reverse transcriptase enzyme [TERT] with a telomerase RNA template component (TERC) that adds de novo TTAGGG repeats to the G-rich 3' overhang of telomeres in late S-phase in cells proficient for the enzyme [2]. Telomere maintenance can also occur in cells without telomerase activity by alternative mechanisms [3].

Telomerase is activated during periods of dramatic cell expansion, especially during fetal development [4, 5]. Telomerase is then repressed before birth in most tissues, although tissues requiring continual cell turnover or periods of rapid proliferation can and do up-regulate telomerase as needed [6, 7]. Some human somatic cells are proficient in telomerase activity, such as B-cells, T-cells, and endothelial cells. Also, adult stem cells are capable of activating telomerase during tissue regeneration [7]. However, human cells lose telomeric DNA in different tissues at different rates, depending upon natural proliferation rate, age, and metabolic and oxidative stress. Postmitotic cells and tissues do not lose telomere sequences since they do not divide. Overall, the loss of telomeric DNA is relatively slow; in cross-sectional studies, humans lose telomere DNA at a rate of about 15-60 base pairs per year [8]. This modest rate of base pair loss is an indication (1) that in proliferative tissues, there are only a small number of stem cells that are actually actively dividing compared to the total stem cell reserve, and (2) that other (primarily nonproliferative) tissues have mostly quiescent cells. This base pair loss reflects or possibly causes cellular aging as manifested by decreased tissue regeneration and reduced cellular function. Telomere shortening has been described as a sort of "molecular clock" that triggers cells to count their divisions so that they divide only a limited number of times *in vitro*, the phenomenon of all replicative senescence, characteristic of each particular cell type or strain, the so-called "Hayflick limit" [9]. However, decreased telomere length is not only an indication of natural aging [8, 10] but is also compounded and modified by stress [11], psychologic and metabolic [12] as well as oxidative [13]: As well as counting cell division, telomere shortening indicates the cumulative number of mutations induced by, for example, oxidative stress [13]. Von Zglinicki [13] suggests "that telomeres act as cellular sentinels for genomic damage [in order to] remove 'dangerous' cells from further proliferation."

Infection accelerates telomere loss causing decreased efficacy of the immune system [14], especially in chronic viral infections such as cytomegalovirus (CMV) and human immunodeficiency virus (HIV). Both of these infections show symptoms of premature aging of the immune system with severe compromise to the functioning of viral cytotoxic T-cells [15-18]. Telomere shortening has been studied *in vitro* in human cells of genetic diseases with mutations in telomerase [19] such as idiopathic pulmonary fibrosis [20], dyskeratosis congenita [21], aplastic anemia [22, 23], and myelodysplasia [21]. All of these studies associate cellular aging and aging-related diseases with telomere shortening. Further epidemiological studies indicate that short telomeres in humans are a risk factor for common diseases such as hypertension [20], atherosclerosis [21], cardiovascular disease [21], stroke [21, 24], diabetes type 2 [24, 25], arthritis [24], osteoporosis [24], cataracts [24], cancer [21, 26], and overall mortality [27]. Razgonova et al. [28] recently reviewed the role of telomere length in the development of aging-related diseases, discussing diverse effects on mitochondrial activity, chronic stress, and circadian rhythm.

In addition to maintaining and extending telomere length, the telomerase subunit TERT has been shown to have important non-telomeric functions. As early as 1999, Elizabeth Blackburn's laboratory [29] showed that a growth crisis (failure to proliferate) in human fibroblasts (with a viral oncogene-extended lifespan *in vitro*) was curtailed by ectopic expression of hTERT, though the telomeres continued to shorten, demonstrating a protective function of human telomerase that allows cell proliferation without lengthening of telomeres. That ectopic expression of TERT could impart a tumorigenic phenotype to an immortal cell line without maintaining telomere length was further demonstrated by Steward, et al [30] *in vitro* and *in vivo*. Also a recent zebra fish model of dyskeratosis congenita showed that TERC acts as a transcription factor by binding to specific DNA sequences of myeloid genes to control their expression by recruiting RNA polymerase II [31]. The full spectrum of telomerase function in post-mitotic cells, in normal cells, and in tumorigenesis is currently being further investigated.

Possibly dietary supplementation with telomerase activator could be advantageous not only in retarding age-related symptoms and diseases, but also in slowing cellular senescence. A small molecule telomerase activator (TA-65) has been purified from a dried root extract of *Astragalus membranaceus*, a plant that has been commonly used for 2000 years in traditional Chinese medicine to protect against myocardial infarction, to boost immunity, and to retard aging. Purified from this *Astragalus* species, TA-65 is a single chemical entity which has been demonstrated to increase telomerase activity and lengthen telomeres in zebra finches, mice, and humans [3, 32, 33], as measured in blood monocytes and lymphocytes (particularly in the CD8+ and CD28+ populations). Safety of TA-65 has been well documented [8, 32, 34, 35], and TA-65 has been granted "generally recognized as safe" (GRAS) status. No product-related toxicity was reported in a study of over five years (encompassing 7000 person-years) which showed that TA-65 improves markers of metabolic, cardiovascular, and bone health [8, 36]. Two later randomized placebo-controlled studies over one-year duration showed no adverse effects [32, 34].

TA-65 has shown promise in delaying clinical and cellular senescence. TA-65 elongated telomers and improved the health status of older female mice without increasing the incidence of cancer [3]. In the mouse model, the slowing of senescence and the concomitant improvement of the healthspan was measured by biomarkers including improved scores in glucose tolerance test and fasting insulin levels, higher levels of red blood cells and hemoglobin count, and higher bone density as well as by clinical and histologic parameters such as enhancement of subcutaneous fat and

epidermal thickness, increased *in vitro* wound healing capacity of keratinocytes, and enhanced hair regrowth *in vivo* after hair plucking [3]. Significantly, with these antiaging improvements in health, the mice treated with TA-65 telomerase activator did not experience any increase in cancer incidence [3].

Further research has shown a significant age-reversal of immune function with telomerase activator supplementation. In a human dietary supplement study with a total baseline population of 114 adults (average age=63yo; 72% men), TA-65 supplementation for 6-12 months decreased the percentage of senescent cytotoxic ($CD8^+/CD28^-$) T-cells and natural killer cells with a marked reduction in the percentage of short telomeres in some patients' leukocytes [8]. A greater decrease in senescent cytotoxic T-cells counts (up to a 20% reversal of senescence) was seen in cytomegalovirus (CMV) seropositive (CMV+) subjects (who initially had a higher percentage of senescent T-cells), resulting in a more "youthful" profile of circulating T-lymphocytes ($CD8^+/CD28^+$), similar to the CMV- subjects. These results were confirmed in a recent larger placebo-controlled study of 500 individuals (average age 60yo; 54% women) who showed a decrease of 13% in senescent cells after nine months of TA-65 supplementation, with greater decreases observed in the CMV+ patients [37]. Correspondingly, in not only aging but also in chronic HIV+ infection, dysfunctional $CD8^+$ cytotoxic T-lymphocytes with short telomeres predominate. Incubation with the telomerase activator TA-65 (previously named TAT2 or cycloastragenol) was found to retard telomere shortening (particularly in the cytotoxic T-cells with the shortest telomeres) and to increase proliferative potential and cytokine production, thus enhancing antiviral function [16]. In *in vitro* CD4 and CD8 T-cells from six healthy donors, TA-65 increased the telomerase activity through regulation of MAPK-specific interactions and increased proliferative activity [38].

Telomerase activity is tightly controlled physiologically in most non-cancer cells, while most upregulation of telomerase activity in cancer cells is believed to occur via human telomerase reverse transcriptase (hTERT) promoter mutations (TPMs). Telomerase may also associate with tumor development via non-telomeric functions, as discussed above. In more than 90% of malignant tumors, telomerase activity is detected [39]. Since telomerase is so frequently up-regulated to high levels in cancer, leading to the unchecked proliferation of cancer cells [6], dietary telomerase activator could potentially be dangerous by increasing the incidence and/or growth and metastatic activity of cancer. Because many individuals take telomerase activators to retard clinical manifestations of senescence, to extend overall healthspan, and to improve immune function, this study was undertaken to determine the safety of telomerase activator TA-65 supplementation in a mouse model of skin cancer induced by UVB irradiation. This research investigated whether oral telomerase activator TA-65 has any effect on the timing of onset and/or the incidence of skin cancers induced by UVB irradiation and whether that possible effect is different if the oral supplementation is begun only *after* tumors are first detected clinically or if supplementation is begun *before* initiation of tumors as well as during UVB exposure and after UVB exposure as more tumors are subsequently detected. (Note that the former timing of supplementation corresponds to that of many individuals who begin healthy lifestyles and supplements only after they acquire a disease such as skin cancer!)

This model of UVB-induced skin cancer is of importance because the most common human malignancy is skin cancer. Indeed, the number of patients with skin cancer is equal to the number of patients with all other cancers combined. When immune suppression is given after organ transplants, many patients develop numerous skin cancers, even if they had none before. If oral

supplementation with telomerase activator were to increase the incidence of cancer, it is likely that this adverse effect would be noted in this skin cancer model.

2. Materials and Methods

2.1 Animals and Treatments

Thirty Skh:1 hairless, nonpigmented, female mice of age 5-6 weeks were purchased from Charles River Laboratories, Wilmington, MA. The mice were pathogen-free by bacteriology and parasitology. The mice were habituated for one week before starting the supplemented diet and two weeks later UV exposure was begun, at which time they were of age 8-9 weeks and initial weight of 22.2 ± 1.0 gm/mouse. The thirty mice were randomly assigned to be housed with 5 mice/cage in the Mount Sinai Medical Center Animal Facility (New York, NY) under standard conditions of 12 hr light/12 hr dark cycle, humidity 50 ± 15%, and temperature 22 ± 2º C. The study protocol and animal care was approved by the Animal Care Committee of Mount Sinai Medical Center in accordance with this institution's ethical guidelines.

A base diet of AIN-76A laboratory chow (supplied as pellets) was purchased from Purina Test Diet (Richmond, IN). In those mice to be given oral telomerase activator, the supplement was incorporated into the pellets to contain 60 mg TA-65/kg chow. The TA-65 (supplied with dicalcium phosphate excipient at a 4:1 excipient/TA-65 ratio) was premixed into the pellets of chow at the beginning of the experiment to deliver an expected dose of about 7.2 mg TA-65/kg mouse weight/day. This dose is three times the dose recommended for human consumers and is comparable to the maximum amount given to human volunteers in the original health maintenance research program described by Harley et al. [8], in which the dose was started at 5 or 10 mg/day with a few subjects given a maximal dose of up to 25 or 50 mg/day in later months. (Only minimal increases in salutary effects were seen with the doses up to 20 mg/day.) These doses did lengthen short telomeres and decrease the percentage of senescent cytotoxic T-cells in CMV+ patients to the levels of youthful CMV- patients, proving efficacy at these concentrations in humans. The commercially available capsules of TA-65 contain 88mg/capsule; the suggested personal daily dose is 1-2 capsules/day, thus delivering about 1.3-2.7mg TA-65/kg body weight/day. The higher doses of 10 or 25 mg TA-65/kg mouse weight/day [40] or 25mg TA-65/kg mouse weight/day [3] have been used previously in mouse research. The unsupplemented and supplemented pellet chow was stored frozen until use. All chow for the entire experiment was from the same batch. In feeding the mice, the chow was replenished weekly and thawed weekly. The mice were exposed to UVB as described below and supplemented in three ways (n=10 in each group), as shown in Figure 1: Group 1 served as a control and was given no TA-65 supplementation (base/base); group 2 was given TA-65 only *after* the UV exposure was terminated, which was when UV-induced skin cancers were first clinically observed (base/tel); group 3 was given TA-65 beginning two weeks *before* and continuing *during* and *after* UV exposure (tel/tel).

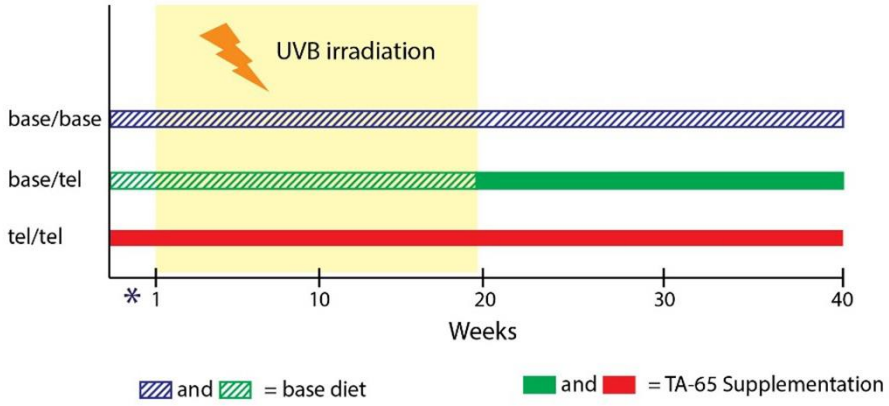

Figure 1 Timing of Oral Telomerase Activator (TA-65) Supplementation and UV Irradiation. * TA-65 supplementation was begun 2 weeks before beginning UV irradiation in the tel/tel group and at week 20 in the base/tel group. UVB irradiation was initiated starting at 15 seconds per session until mid-second week when the maintenance exposure time of 33 seconds per/session was attained. UV irradiation was terminated at week 20 when all three treatment groups had an incidence of 2 mice with at least one tumor ≥ 1mm.

Figure 1 shows the timing of oral telomerase activator (TA-65) supplementation and UV irradiation. After arriving at the Mount Sinai laboratory, the mice were randomly divided into 6 cages with 5 mice/cage and habituated for one week. The TA-65 supplementation was then begun two weeks before beginning UV irradiation for week 1. UV exposure was continued until the twentieth week, when all three treatment groups had an incidence of two mice with at least one tumor ≥ 1mm. (The very first tumor appeared in the control base diet treatment group at week 18).

With the onset of the first tumor in each initial supplementation category, each mouse was identified and distinguished with different numbers of nicks in the ear(s) for each individual mouse within each group. Within each group, the first mouse to get a tumor was mouse #1, the second mouse, #2, etc. Mice with no tumor at the time of tumor onset were designated numbers randomly.

Dietary consumption for each cage of five mice was measured weekly and graphed as grams food intake per gram mouse body weight per day. To monitor the general health of the mice, they were weighed weekly as a group of the mice in each cage. A graph was kept of the average weight per mouse in each of the treatment groups to monitor possible changes and differences. To check further the animals' overall health, each mouse was specifically examined weekly for general appearance (skin, muscle tone, and movement) and observed carefully for any signs of discomfort as judged by their behavior and weight.

At the termination of the experiment, the mice were euthanized with CO_2 followed by cervical dislocation. The weight of each mouse was recorded; the tumors were excised and weighed. The "tumor load" (= tumor weight ÷ total body weight) was calculated. The final data included the one mouse in the tel/tel group that died prematurely at week 33 and the two mice in the base/tel group euthanized at week 39 for calculation of incidence and multiplicity of tumors and the two mice euthanized for calculation of tumor load. Biopsies of the skin tumors were also taken and analyzed histologically.

2.2 UV Irradiation

The light source was 8 FS24T12-UVB-HO lamps (Voltaire Company, Fairfield, CT) with a predominant emitting peak at 280-320 nm. These bulbs provide a homogenous field of irradiation. During UV irradiation, the lights were placed 40 cm from back of mice. The output was monitored weekly with an IL-1700 radiometer (International Light, Newburyport, MA).

For the induction of skin cancer, the irradiation was initiated at 15 seconds per session (which is equal to about 50% of the average minimal erythema dose as measured on 6 mice) and increased by 5 seconds per session until the maintenance exposure time of 33 seconds was reached within the second week, giving 0.3 kJ/m^2/session. This dose is reported to be comparable to sunlight exposure for thirty minutes at noon in New York City's Central Park during the summer [41]. UVB irradiation was continued three times per week until the twentieth week, when all three treatment groups had an incidence of two mice with at least one tumor $\geq$ 1mm. This dose of UV irradiation has been shown to induce skin cancers in this breed of mouse [41].

2.3 Evaluation of Skin Damage Induced by UV Irradiation

During the initial exposure to UVB (until one week after the maintenance dose was attained), all animals were examined three times per week to determine the degree of short-term sun damage. Inflammation was assessed clinically by grading the degree of erythema (skin redness, the clinical manifestation of UV-induced inflammation or "sunburn"), and the number of blisters (indicative of more severe "sunburn" inflammation induced by UV exposure) on each animal was counted.

From the time that the first tumor was observed at week 18, the numbers and sizes of tumors on each animal were noted weekly. One or two raters counted the tumors (unblinded) throughout weeks 1 to 34; for weeks 35-40, two raters counted the tumors (each blinded to the individual mouse identification and its treatment group). Tumors approximately 1mm in size were counted only if they were present for at least one week. Tumors $\geq$ 1mm and < 2mm and those $\geq$ 2mm were counted separately. Tumors $\geq$ 2mm were counted as soon as they were observed. Occasionally small tumors enlarged to co-join becoming one large tumor: In those cases, the tumor count remained two. The diagnosis of tumor was confirmed by biopsy and histological examination of clinically representative tumors from each animal. One or more tumors from each tumor-bearing mouse was biopsied for histological examination.

When mice were euthanized at the end of study after week 40, all tumors from each mouse were excised and weighed together: The "tumor load" (= tumor weight / total body weight) was calculated. Tumor load was also measured on the two mice that were sacrificed prematurely.

2.4 Tissue Analyses

Biopsies of representative tumors were taken from each mouse in each treatment group to confirm the diagnoses of squamous cell carcinomas by histologic staining with hematoxylin and eosin (which stains extracellular matrix blue and cytoplasm pink).

2.5 Statistical Analysis

All statistical tests were conducted with SAS® Software [42] using non-directional alternative hypotheses, which reflect the case that *a priori* arguments could be made that TA-65 could increase or decrease tumor growth. A p-value of 0.05 was used as the cutoff point for stating if the testing was statistically significant or not significant. P-values of 0.04 to 0.05 were interpreted as "borderline significant"; p-values of >0.05 were considered not significant.

Treatment effect on tumor incidence and tumor counts were explored using, (1) Wilcoxon rank sum tests [43], (2) Fisher's exact tests of proportion [43], (3) Kaplan-Meier time-to-event analyses [43], and (4) repeated measure ANOVA [43]. Frequency of tumor occurrence was compared using Wilcoxon rank sum tests for the base/base and base/tel groups, for the base/base and tel/tel groups, and for the base/tel and tel/tel groups. The incidence of tumors $\underline{\geq}$ 1mm and $\underline{\geq}$ 2mm was also analyzed at each week using Fisher's exact test [43]. The number of tumors ≥ 2mm was analyzed by Proc GLM with pairwise contrast estimates among groups [43].

Time to observation of the first tumor $\underline{\geq}$ 1mm and $\underline{\geq}$ 2mm was compared across the three treatment groups (base/base, base/tel, and tel/tel) by two separate Kaplan-Meier survival analyses [43]. In each analysis, the log rank statistic was used to evaluate the omnibus hypothesis of equality in survival function among all three groups, with follow-up pairwise comparisons in the presence of a significant omnibus effect.

Another separate statistical analysis was done to compare results of the tumor counts by two different raters, each blinded with respect to mouse identification in its treatment group. Exploratory analysis on treatment effects for four variables ((i) total tumors for Rater 1, (ii) total tumors for Rater 2, (iii) tumors $\underline{\geq}$ 2mm for Rater 1 and (iv) tumors $\underline{\geq}$ 2mm for Rater 2) were performed using (1) repeated measures of "analyses of variance" (ANOVA) and (2) Wilcoxon rank sum tests [43]. In the repeated measures of ANOVAs, time served as the within-subject (or repeated) factor; group (base/base, base/tel, and tel/tel) served as the between-subject factor. Three effects were analyzed: (a) time, (b) group, and (c) the time x group interaction. In addition, a contrast was performed between the combined base/tel and tel/tel groups and the base/base group.

3. Results

3.1 Mouse Growth Rate and Food Intake

As seen in Figure 2a, the average dietary intake was the same in all three treatment groups (0.119 gm/gm body wt/day). Note that food intake could only be measured per cage; the food intake of each individual mouse could not be determined. Throughout the experiment, the five mice in each cage were weighed together. Only at week 40 were they weighed individually. At week 30 (when the mice were fully mature and not yet possibly debilitated by their skin cancers) the average body weight was 30.5 ± 1.8 gm/mouse. As can be seen by the graph in Figures 2a and, 2b, the dietary consumption and the body weight of the mice was essentially the same in all three treatment groups. Thus, dietary telomerase activator TA-65 did not significantly affect food intake and consequent body weight. There were no symptoms of failure to thrive in any mice in any group: All animals had normal skin in non-UV-exposed areas, normal muscle tone, and normal movement and activity throughout the 40-week experiment. With the exception of only two mice (see below), both

supplemented and non-supplemented mice thrived and did not become debilitated by their tumor load.

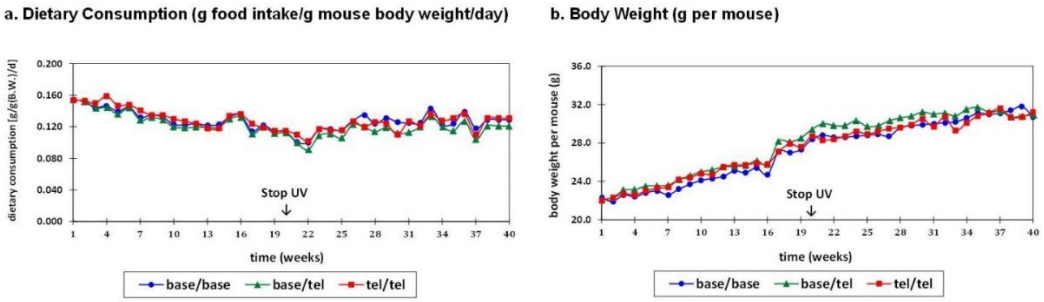

Figure 2 The weekly dietary consumption and body weight and food intake of Skh:1 mice without and with TA-65 supplementation. (a) The food intake was measured per cage; the food intake of each individual mouse could not be determined. (b) Throughout the experiment, the five mice in each cage were weighed together. Only at week 40 were they weighed individually.

3.2 UVB-Induced Carcinogenesis

Although the mice showed very minimal erythema after the second exposure to UVB, there was no difference in degree of erythema in any of the treatment groups, and no mouse had any blistering from the initial or subsequent UV exposure.

During the course of the 40-week experiment, only one mouse died prematurely (tel/tel group, 33rd week). Because the body was destroyed, no data to calculate tumor load on that one mouse was available. Two mice (both from base/tel group) were euthanized at week 39 because of severe body weight loss: The tumor load of one of these mice was quite high (8.7%); the second had a tumor load equal to 2.0% (the average tumor load seen in this study). Because the experiment was planned to be terminated only days later, euthanizing early was not expected to change tumor count or tumor load significantly. All other mice showed no signs of discomfort or change in muscle tone, movement, or activity.

Skin tumors were induced in all animals exposed to UVB irradiation. The Skh:1 mice characteristically developed multiple tumors. Some animals were riddled with tumors (up to 25-30 small tumors $\underline{\geq}$1mm/mouse and 7-9 larger tumors ≥2mm/mouse) and others had fewer large tumors. Clinically and histologically these tumors were fibrosarcomas (spindled-cell squamous cell carcinomas with marked invasion of the dermis and many multinucleated anaplastic spindle-shaped cells), or they were keratoacanthoma-like (with ulceration and marked hyperkeratosis and acanthosis of the epithelium and with invasive endophytic papillary projections). Whorls of cornfield cells and atypical keratinocytes were noted with dense dermal inflammatory infiltrates. All tumors biopsied were squamous cell carcinomas varying from well differentiated to poorly differentiated. No benign papillomas persisted for more than one week.

Figure 3a illustrates the tumor incidence, the number of mice with tumor(s) $\underline{\geq}$ 1mm detected in each of the three irradiated groups of mice. Clearly the time of onset of tumors was similar in all three groups as confirmed by Kaplan-Meier analysis (see Section 3.3). The first tumor appeared in the base diet treatment group at week 18. UV exposure was discontinued after week 20 – after at

least two mice in each group had at least one tumor > 1mm. It is evident that even after UVB radiation was terminated, the mice continued to develop tumors. By week 28, every mouse in the base/tel and in the tel/tel groups had at least one tumor ≥ 1mm. One mouse in the base/base group had no tumor ≥1mm until week 33. These differences were not statistically significant, as discussed below in Section 3.3. Kaplan-Meier analysis showed no statistically significant difference among the three treatment groups in the time of onset of skin tumors ≥ 1mm or in incidence of tumors ≥ 1mm (as discussed below). Although in Figure 3b the time of onset of tumors ≥ 2mm appears earlier in the tel/tel group than in the two other treatment groups, Kaplan-Meier analysis demonstrated that this apparent difference was not statistically significant. As seen in Figure 3b, by week 27 all groups had at least two mice with a tumor ≥ 2mm. At weeks 24 to 27 and weeks 32 to 36, there appears to be a higher incidence of tumors ≥ 2mm in the tel/tel treatment group, but repeated measure ANOVA analysis showed that these differences were not statistically significant because the appearance in the graph reflects only a difference of one or two mice. That these differences were not significant was confirmed by Fischer's Exact test except for only the comparison of base/base vs. tel/tel at week 26 which was borderline significant (p=0.0433) (as shown below in Table 1 and Table 2 and Section 3.3 of statistical analysis results).

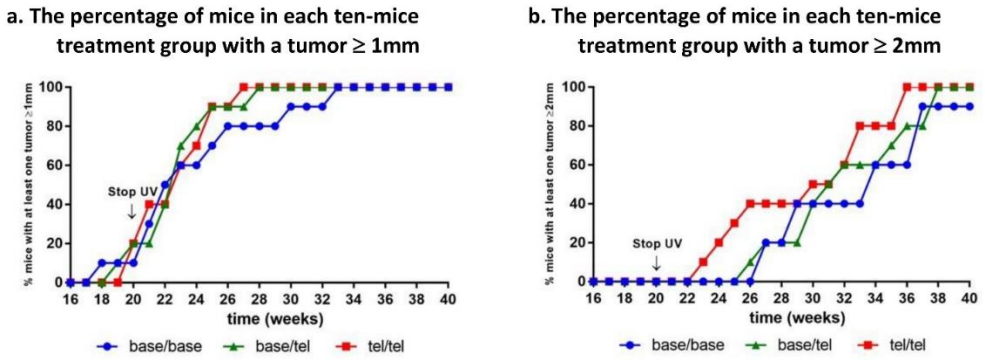

Figure 3 The weekly incidence of UVB-induced skin tumors. UVB irradiation was discontinued after week 20. The number of mice with at least one tumor were noted weekly, and the number of tumors on each mouse was counted. Tumors ≥ 1mm in size were counted only if they were present for at least one week.

Table 1 Analysis by Fisher's Exact Test [43] in incidence of tumors ≥ 2mm.

	*p-value**	
Week	*base/base vs. base/tel*	*base/base vs. tel/tel*
26	Not significant[a]	0.0433[b]
33	Not significant[a]	0.0750[c]
36	0.0867[c]	0.0867[c]

*This table lists only weeks with p-values <0.1.

[a] All p > 0.1053 for all other comparisons

at all other weeks of trial

with no specific p-value listed here

[b] Borderline significant difference

[c] Not significant difference

Table 2 Contrast estimate results [43] for tumor incidence (≥2mm) among pairwise groups.

contrast groups	*p-values*
base/tel vs. base/base	0.6056
tel/tel vs. base/base	0.1903
tel/tel vs. base/tel	0.2305

Figure 4 shows the tumor multiplicity: Figure 4a illustrates the number of tumors ≥ 1mm per mouse observed in each of the three treatment groups of irradiated mice, and Figure 4b shows the multiplicity of larger tumors (the number of tumors ≥ 2mm per mouse) detected in each of the three groups of irradiated mice. From these graphs it can be seen that even after UVB radiation was terminated, the mice continued to develop more tumors. Although in Figures 4a and 4b, there seem to be some differences among the three treatment groups in multiplicity (i.e., number of tumors per mouse) when counting all tumors ≥ 1mm (Figure 4a) or when counting only larger tumors ≥ 2mm (Figure 4b), the only statistically significant differences were at weeks 25 and 26 with more tumors ≥ 2mm in the tel/tel group compared with the base/base group. A discussion of this statistical analysis follows in Section 3.3.

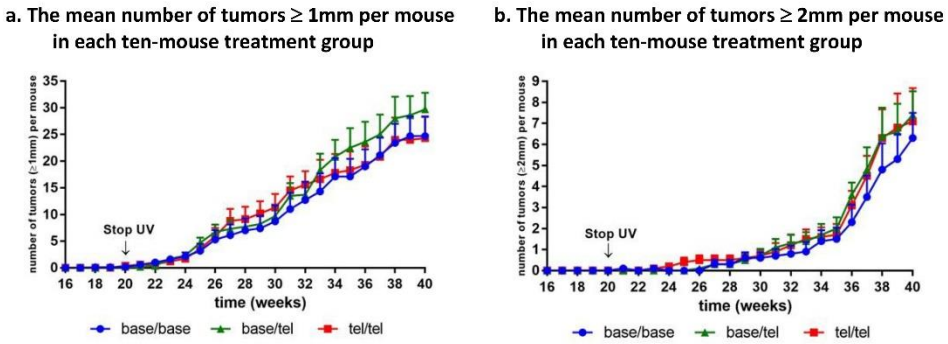

Figure 4 The weekly multiplicity of skin tumors. UVB irradiation was discontinued after week 20. Each week tumors ≥ 1mm and < 2mm and those ≥ 2mm were counted separately. Tumors ≥ 2mm were counted as soon as they were observed. Occasionally small tumors enlarged to co-join, becoming one large tumor: In those cases, the tumor count remained two. The diagnosis of tumor was confirmed by biopsy and histological examination of clinically representative tumors from each animal.

The average tumor load per mouse for each treatment group at the end of study is shown in Figure 5. Although the tumor load of the base/tel group (equal to 1.7%) is slightly less than that of the base/base group (equal to 1.9%) and that of the tel/tel group (2.2%) is slightly higher, these minimal differences are not statistically significant by ANOVA tests (F=0.24, p=0.7864). The standard errors were rather high since each treatment had one (base/tel) or two (base/base and tel/tel) mice with an exceptionally high tumor load.

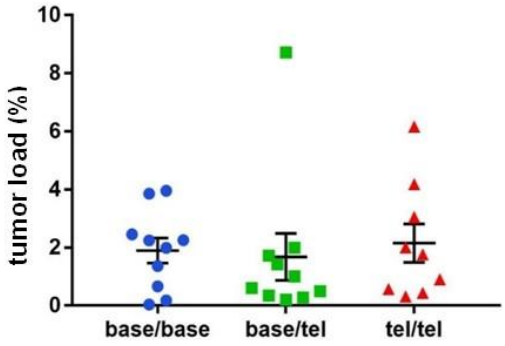

Figure 5 The effect of oral TA-65 on UVB-induced skin tumor load in each 10-mouse treatment group at week 40. The bar represents mean ± SEM (standard error of mean) along with the individual value plotted. Tumor load (%) was calculated using the formula: tumor load (%) = tumor weight (gram) ÷ total body weight (gram) x 100. Note that one mouse from the tel/tel group died prematurely at week 33, so the tumor load for that one mouse could not be determined.

3.3 Statistical Analysis Results

The Kaplan-Meier analysis showed no significant differences among the treatment groups in the time of clinical observation of the first tumors $\geq$ 1mm ($p = 0.3575$) or $\geq$ 2mm ($p = 0.1222$). Therefore there was no significant difference in the time of onset of the first tumors ≥ 1mm or $\geq$2 mm among the treatment groups.

As enumerated in Table 1, the Fisher's exact test results indicated only one borderline significant difference in the incidence of tumors $\geq$ 2mm at week 26 (base/base vs. tel/tel, $p = 0.0433$). Differences at week 33 (base/base vs. tel/tel, $p = 0.0750$) and at week 36 (base/base vs. tel/tel, $p = 0.0867$) were not significant. No other significant differences were observed (all $p > 0.1053$). There were no significant differences among the groups in incidence of tumors $\geq$ 1mm. The repeated measure ANOVA analysis performed for tumors ≥2 mm showed no treatment effect overall among the three treatment groups in tumor incidence by Proc Genmod (Chisq=1.97, p=0.3730) and the p-values for contrast estimate results among groups listed in Table 2 were all > 0.05. (Note that all calculated p-values were > 0.19.) Therefore TA-65 treatment has no statistically significant effect on tumor incidence.

As shown in Table 3, the Wilcoxon results indicated only one borderline and one significant difference in mean number of tumors $\geq$ 2mm between the base/base and tel/tel groups at week 25 ($p = 0.0468$) and week 26 ($p = 0.0239$), respectively. No significant differences were observed in the mean number of tumors $\geq$ 2mm at week 24 (base/base vs. tel/tel, $p = 0.0918$), week 33 (base/base vs. tel/tel, $p = 0.0887$), and week 36 (base/base vs. base/tel, $p = 0.0717$) as well as other weeks of trial. No significant differences were observed among the groups in number of tumors $\geq$ 1mm (all $p > 0.1291$ except at week 25 (base/base vs. base/tel, $p = 0.0993$).

Table 3 Analysis by Wilcoxon Test [43] in mean number of tumors $\geqslant$ 2mm[a].

	*p-value**	
Week	*base/base vs. base/tel*	*base/base vs. tel/tel*
24	Not significant[a]	0.0918[b]
25	0.0993[b, c]	0.0468[d]
26	Not significant[a]	0.0239[e]
33	Not significant[a]	0.0887[b]
36	0.0717[b]	Not significant[a]

*This table lists only weeks with p-values <0.1.

[a] All p > 0.129 for all other comparisons

at other weeks of trial

with no specific p-value listed here

[b] Not significant difference

[c] Comparison for tumors ≥ 1mm

[d] Borderline significant difference

[e] Significant difference

The tumor (≥ 2 mm) multiplicity by repeated measure ANOVA was performed with SAS® Procedure GLM with week as repeated measurement within subjects. The least square means method was used for pairwise comparison at each week to determine whether treatment effect was significant. An almost borderline significant model effect at week 25 (F=3.27, p= 0.0534) and a borderline model effect at week 26 (F=3.50, p=0.0445) were detected, and the pairwise comparison results are shown in Table 4. For example, significant differences were observed at week 25 (p=0.0353) and at week 26 (p=0.0188) between base/base vs tel/tel (Table 4) which are similar to Wilcoxon p-value 0.0468 at week 25 and 0.0239 at week 26 (Table 3). No statistically significant differences were observed in any other weeks. No treatment effect was observed among the three treatment groups in tumor multiplicity in tumors ≥1mm.

Table 4 Analysis by reported measure ANOVA [43] in mean number (tumors/mouse) ≥ 2mm[a].

	*p-value**	
Week	*base/base vs. base/tel*	*base/base vs. tel/tel*
24	Not significant[a]	Not significant[a]
25	Not significant[a]	0.0353[b]
26	Not significant[a]	0.0188[b]

*This table lists only weeks with p-values <0.1.

[a] p > 0.3 for all other comparisons

at all other weeks of trial

[b] Significant difference

Thus overall, the analyses did not demonstrate compelling statistical evidence to support either a significant decrease or increase in tumor incidence or tumor number by oral supplementation with

TA-65 either when given only after tumors were clinically noted or when given before, during, and after the UVB irradiation which induced these skin tumors.

Analysis of tumor multiplicity for both all tumors $\geq$ 1mm and only larger tumors $\geq$ 2mm by two raters (each blinded) showed no significant differences between treatment groups (base/base vs. tel/tel or base/tel vs. tel/tel) based on the t-test results (all $p > 0.18$). Only one comparison of data from Rater 2 was significantly different based on the Wilcoxon non-parametric test for base/base vs. base/tel at week 36 for tumors $\geq$ 2mm: one-sided exact $p = 0.0495$. All other comparisons showed no significant difference among treatment groups (all $p > 0.14$).

In analyzing tumor load at the end of the study (at week 40), there was no significant difference in tumor load between treatment groups and the control group at the end of the study ($F=0.24$; $p=0.7864$). Because one mouse in the base/tel group had an exceptionally large tumor load and two mice in both the base/base and the tel/tel groups similarly had particularly large tumor loads, the standard errors were high.

4. Discussion

Skin cancer is by far the most frequent human malignancy: One in five Americans will develop skin cancer by the age of 70 [44]. About 90% of nonmelanoma skin cancers [45] and about 86% of melanomas [46] are associated with exposure to solar UV. Basal cell carcinoma is the most common and squamous cell carcinoma, the second most common nonmelanoma skin cancer (80% and 20 %, respectively) with 4.3 million and > 1.0 million cases per year [47], resulting in 3000 [48] and 15,000 deaths [49] per year, respectively, in the USA. Although telomerase activity is usually suppressed in adult somatic cells, cancer cells often show reactivation of telomerase which is thought to be responsible for their rapid proliferation. Recent studies indicate that indeed telomerase activation plays a role in the onset and progression of basal and squamous cell carcinomas as well as of melanomas [50].

Mice are frequently used in studies of photocarcinogenesis of the skin because squamous cell carcinomas are reproducibly induced [41, 51-54]. Obviously, hairless mouse breeds are preferred to eliminate the necessity to shave. With their thin stratum corneum [55] and limited capacity to repair UV-induced pyrimidine dimers [56, 57], Skh:1 hairless mice are particularly vulnerable to skin cancer. Furthermore, these mice are not immunologically compromised and can tolerate large tumor loads without failure to thrive [41, 51, 54]. Since skin cancer is by far the most common human malignancy, this mouse model can be used to check the safety of telomerase activator by determining whether UVB-induced skin cancer incidence and growth is increased by telomerase activator supplementation.

Although the concentrations of TA-65 in the skin were not directly measured in this study, prior research has shown that oral supplementation does effectively deliver TA-65 to the skin with evidence of telomerase activation. Preclinical testing of oral TA-65 fed to pigs indicated the presence of TA-65 in the skin by both biopsy and by analysis of the skin collected after sacrifice (Personal Communication, 2020 [58]), demonstrating that oral TA-65 does result in effective concentrations in skin cells. Two independent studies show that TA-65 does activate telomerase in keratinocytes *in vivo* [3, 8]. Mice fed TA-65 – and not the control – exhibited significant alterations in the subcutaneous fat layer and more proliferation of epidermis [3]. Also, female Sprague-Dawley rats increased mRNA levels of TERT and telomerase associated protein (Tep1) in the cerebral prefrontal

cortex and hippocampus after oral supplementation with TA-65 following repetitive traumatic brain injury; unsupplemented controls showed no increase [59]. Another demonstration of tissue distribution of TA-65 after oral supplementation was shown in a transgenic mouse model of Parkinson's disease [40]: Oral TA-65 increased TERT protein in the brain neocortex and hippocampus, resulting in decreased α-synuclein protein levels and improvement of motor functions. Such effects in these four studies would not have been possible without the circulatory distribution of TA-65.

In our study the seeming differences observed in Figure 3b showing tumor incidence at weeks 25 and 26 may imply that TA-65 might have some influence on tumor initiation and progression, statistical analysis comparing base/base vs. tel/tel by Fisher's Exact Test showed only one statistically significant increase in incidence of tumors ≥ 2mm in the tel/tel group. This comparison by Wilcoxon Test for mean number of tumors ≥ 2mm similarly showed only one statistically significant increase at week 26 and a borderline statistically significant increase at week 25. Reported measure ANOVA indicated statistically significant increases in mean number of tumors ≥ 2mm only at weeks 25 and 26. Except for these two times, overall statistical analysis showed that TA-65 has no treatment effect on tumor multiplicity considering sample size is small and there is no remarkable difference at other times in the 40-week study (Tables 3 and 4).

The TA-65 supplementation did not cause any noticeable adverse symptoms in the mice. The non-supplemented and supplemented mice appeared of normal weight and showed normal movement and activity with no sluggishness throughout the 40-week experiment. Both unsupplemented and supplemented mice thrived. All had normal, healthy skin on non-UV-exposed sites, as well as normal muscle tone.

No mouse in any treatment group showed symptoms of sunburn with significant erythema or blistering during the initial or subsequent UV irradiation. This demonstrates that oral TA-65 is not sun-sensitizing (as are many oral medications) – further testimony confirming the safety of TA-65.

These experiments demonstrate that TA-65 supplementation did not affect the incidence or multiplicity of tumors induced by UVB-exposure in this mouse model, whether given only *after* tumors are first detected clinically or if supplementation is begun *before* initiation of tumors as well as *during* and *after* the inciting UVB exposure. Overall there was not a statistically significant difference among the treatment groups in time of onset of skin tumors, incidence of tumors (number of mice with at least one tumor), multiplicity of tumors (number of tumors/mouse), or tumor load. Although Figure 3b seems to show that there is a higher incidence of tumors $\underline{\geq}$ 2mm for the tel/tel treatment group for the few weeks 22 to 29 and 32 to 34, this difference was not statistically significant except at week 26 when there was a borderline statistically significant increase in tumor incidence. In tumor multiplicity, only at weeks 25 and 26 were there borderline or statistically significantly more tumors ≥ 2mm in the tel/tel group compared with the base/base group. Also, one mouse in the tel/tel group died in week 33, so for that one mouse, tumor number could not further increase and tumor load could not be determined. This is a limitation that may mean that there may have been more tumors in the tel/tel group. However, overall apparent differences in tumor load were not statistically significant.

These results are very reassuring, since oral supplementation with telomerase activator might have greatly increased the incidence of UV-induced skin cancer. Cancer cells proliferate rapidly and typically have a high level of telomerase which might override natural protective anti-proliferative or apoptosis signals to enable and/or enhance cancer growth. Thus cancer cells might have a significant growth advantage in the presence of supplemental telomerase activator.

Possible evidence of this growth advantage was observed by Stampfer et al. [60]: Growth-inhibiting, anti-proliferative actions of tumor growth factor-β (TGF-β) were not effective in cultured, telomerase-positive human breast epithelial cells, whereas the control, telomerase-negative breast epithelial cells were sensitive to TGF-β. Ectopic expression of hTERT in the telomerase-negative cells was sufficient to convert these TGF-β responsive cells to a TGF-β-resistant state.

Telomerase reverse transcriptase (TERT), the ribonucleoprotein enzyme that synthesizes telomerase DNA (TTAGGG hexamers), is responsible for maintaining telomere length [61]. TERT promoter (TERTp) mutations (TPMs) create binding sites for transcription factors that result in telomerase expression and increased telomere length and stability, preventing senescence and apoptosis of cancer cells, thereby allowing cancer cells to divide. Recurrent somatic TPMs have been found to be high in many human cancers [62], particularly those of the central nervous system [63, 64], bladder cancers [65], and follicular cell-derived thyroid cancers [66], as well as in cutaneous melanomas [67, 68]. The signature UV- radiation-induced mutations (cytidine-to-thymidine (C→T, CC→TT)) do generate TPMs that are seen in melanomas [69], cutaneous squamous cell and basal cell carcinomas [70-72], periocular basal and squamous cell carcinomas [73], and conjunctival intraepithelial neoplasia [73]. In fact, in analyzing melanomas with contiguous benign nevus precursors, TPMs were identified in areas of the tumor thought to be intermediate between nevus and melanoma [74].

Recently published research has demonstrated that indeed TPMs are significantly more common in both primary and recurrent melanomas compared with recurrent nevi and that the relative number of TPMs can help distinguish recurrent nevi from recurrent melanomas [75]. Also distinguishing between Spitz nevi, atypical Spitz tumors, and Spitzoid melanomas can be histopathologically challenging. Recent analysis indicates that Spitzoid lesions with TPMs exhibit a more clinically aggressive course, so TPMs can serve as an additional predictive marker [76].

Further experiments by De Unamuno et al. [77] analyzed not only TPMs, but also telomerase expression at the protein level. Telomerase protein was found in all melanocytic lesions, but is higher in melanomas than in nevi. A heterogeneous pattern of expression is linked to a more aggressive tumor. Curiously, the mutational state of the TERT gene did not correlate with differences in telomerase expression. Nevertheless, TERTp mutation status may serve as an independent prognostic factor in cutaneous melanoma [78]. A new *in situ* hybridization technique, RNAscope, can detect hTERT mRNA in formalin-fixed paraffin-embedded tissue [79]. In comparing 17 melanomas and 13 benign nevi, hTERT mRNA was indeed expressed more abundantly in melanomas compared to benign nevi with correlation to Breslow thickness and Ki67 proliferation index [79], suggesting prognostic potential. After analysis of 86 primary melanomas, 72 melanocytic nevi, and 40 diagnostically difficult melanocytic lesions, Thomas et al. [39] conclude that TERT positivity as a test for melanoma versus nevis has an accuracy of 87%, a sensitivity of 78%, a specificity of 99%, a positive predictive value of 99%, and a negative predictive value of 79%.

Although there is often a familial association in the tendency to acquire an environmentally-induced melanoma, TPMs were not identified in a study of 228 hereditary cutaneous melanoma families, though 2% of individuals from these families had mutations in POT1, a part of the shelterin complex that binds to telomerase to protect these chromosomal ends [80]. Other studies confirmed that TPMs are rare in familial melanoma [69, 81].

Interestingly in analyzing TERTp mutations in primary and secondary melanomas, the mutation status was discordant between the primary tumor and metastasis [82]. TERTp mutated melanomas

tended to be thicker, have a higher mitotic count, and higher patient age than TERTp wild-type tumors, but there was no significant association with reduced survival. As in the De Unamuno et al. [77] study cited above, TERT protein level did not correlate with mutational status. The telomerase protein level showed discordance between primary and first mutational lesions and was significantly associated with reduced patient survival [82]. Furthermore, TERT mutations vary in gene expression and impact on absolute telomere length: For example, in evaluating 60 melanoma cell lines, mutations at positions -124/125 and -146 were found to be associated with the highest levels of TERT gene expression but had no impact on telomere length while the common mutation at position -245 resulted in long telomere length [83]. As Shaughnessy et al. [83] conclude, TPMs comprise a "complex mutational landscape." The failure of TERT promoter to correlate consistently with TERT expression and telomere length suggests an alternative method whereby tumor cells escape the critical shortening of telomeres [83].

The discrepancy may be because *senescence-mediated aging* is clearly distinct from *epigenetic aging* [84]. By stimulation of telomerase to lengthen telomeres, cells bypass replicative senescence and fortunately rejuvenate. This longer lifespan ironically allows more time for the inherent processes of epigenetic aging to occur [84]. For example, epigenetic aging induced by histone methylation (or acetylation or phosphorylation) alters chromatin accessibility – not only to the expression of TERT, but also to the expression of other transcription factors [84, 85]. Certainly, exposure to environmental pollutants and infectious agents affects the endogenous cascades that cause epigenetic aging.

Nonmelanoma skin cancer (NMSC) is the most common of all cancers, with distinct subtypes basal cell carcinoma (BCC), cutaneous squamous cell carcinoma (cSCC), and Merkel cell carcinoma (MCC) – all of which invade locally into deeper layers of the skin and can metastasize. Actinic keratoses (AK) are precancers (precursors to cSCCs), and Bowen's Disease (BD) is cSCC *in situ*. Each type has distinct causes (exposure to ultraviolet (UV) light and pollution, genetic heritage, infectious agents) and distinct clinical and histopathological presentations and progression. Telomere length varies within each type of NMSC, and even between original tumors and metastases [86].

Interestingly, a recent study of 53 biopsied AK's in 29 patients, TERTp mutations were detected in 21% of the AK's; 83% of these TPM+ AK's also had increased p53 expression [87]. Treatment with daylight-mediated photodynamic therapy decreased the histologic grading of dysplasia as well as the frequency of TPM mutations and p53 expression. This study included 20 tumors, of which 30% showed TPMs and 11 SCCs, of which 45% carried TPMs.

Both long and short telomeres have been noted in cutaneous squamous cell carcinoma. TPMs have been identified in 32-70% of squamous cell carcinomas [88]. (These mutations are most frequently UV-signature mutations, thus confirming the role of UV exposure.) No significant correlation was found between the TPMs and clinical or pathologic characteristics, though these mutations did predict recurrences and metastases [88]. Other recent studies have also demonstrated that TPM+ squamous cell carcinomas have higher risks for local recurrence and lymph node metastasis [89]. TPMs may in the future be included in the prognostic assessment of patients with cutaneous squamous cell carcinomas.

In the study presented here, fortunately oral telomerase TA-65 was not photosensitizing and did not increase UVB-induced skin cancer, thus confirming the safety of TA-65 supplementation. This study is limited because telomere length and the precise proliferation of cancer cells using PCNA or Ki67 were not analyzed. However, the fact that oral telomerase activator TA-65 did not increase the

incidence of UVB-induced skin cancer is of particular significance because of the high prevalence of skin cancer. Skin cancer is by far the most common type of cancer, and its incidence is rising [90-94]. Indeed, each year there are more new cases of skin cancer than the combined incidence of breast, prostate, lung, and colon cancer [90]. Also, the fact that Gonzalez-Suarez and colleagues [95] have shown that *in vivo* overexpression of the mouse telomerase reverse transcriptase mTERT in basal keratinocytes promoted proliferation of these cells (despite the observation that the keratinocyte telomeres, already very long, were not appreciably changed in length upon expression of mTERT) suggests that basal cell and squamous cell skin cancers could be particularly susceptible to adverse effects from telomerase activation. Fortunately, this proliferation of basal cells and keratinocytes known to be induced by UVB-exposure of human skin was not observed in the mouse model studied here.

Oral telomerase activator supplement TA-65 may prove to be very beneficial by reducing the load of very short telomeres, thereby counteracting the telomere shortening, which occurs with natural aging and physiologic stress. Human subjects taking TA-65 for one year attained a net increase of telomere length of 530±180 base pairs per year, rather than an annual loss of about 60 base pairs [32]. Maintenance of telomeres possibly has been proven to slow many of the manifestations of aging, which lead to declining health and an increased risk of disease – particularly in enhancing immune function with aging and with viral infection [8, 17, 36, 37]. In fact, "high-performing" centenarians (>100 years old) were found to have (i) longer telomeres, (ii) greater proliferation of their T-cells with *in vitro* stimulation (markedly better not only when compared to "low-performing" centenarians, but also when compared to 67-83 year-old controls), and (iii) enhancement of telomerase following stimulation with expression of many genes related to telomere length [96].

Thus, the possible advantages of telomerase activator to overall health are potentially extraordinary, but safety must be tested. The observation that oral telomerase activator does not increase the incidence or proliferation of the most prevalent human cancer – UVB-induced skin cancer – certainly further supports the safety of TA-65. The fact that oral telomerase activator TA-65 was found to be safe in this one model (used frequently in cancer investigation) gives evidence for the safety of this supplement.

Further studies are currently underway in human volunteers to measure other parameters of aging that may be slowed by oral TA-65. Indeed, even very low (nanomolar) levels of TA-65 have been shown to be effective in maintaining telomere length [8, 36, 37]. Other new telomerase activators are currently being investigated for therapeutic and antiaging efficacy with monitoring for safety, including a *Centella asiatica* extract formulation, oleanotic acid, mastinic acid, and other proprietary nutrients [97]. More than 200 compounds have been isolated from *Astragulus membranaceus* including 14 polysaccharides, 161 saponins, many flavonoids, as well as other compounds (such as astragalactosides) – many of which have been proven to have not only anti-aging but also even anti-carcinogenic efficacy in animal and *in vitro* human cell studies [98]. Anti-neurodegenerative, anti-vascular disease, immunomodulatory, and anti-oxidative effects have been demonstrated for many of these specific compounds, as excellently reviewed by Liu et al. [98]. With the further evidence of safety demonstrated by this research, the ultimate hope is that taking a telomerase activator supplement will lead to the prevention or inhibition of the degenerative diseases of aging so that we can indeed "add years to our life and life to our years."

5. Conclusion

The rates of tumor formation, the incidence and multiplicity, and the sizes of skin tumors were similar in all three treatment groups of Skh:1 mice (whether placebo or TA-65 supplementation began before and continued during and after the inciting UVB exposure or TA-65 given only after UVB-induced skin cancers appeared), indicating that oral telomerase activator TA- 65 supplementation does not significantly influence UV-induced skin carcinogenesis. This is indeed encouraging because a possible adverse effect of dietary telomerase might have been the enhancement of tumorigenesis. In fact, many researchers (who have themselves demonstrated the efficacy of TA-65 in slowing age-related diseases and cellular senescence and possibly even mortality) realize the importance of ensuring "that administration of a telomerase activator does not increase susceptibility to / or risk for cancer or cancer related diseases" [59]. Because skin cancer is by far the most common human cancer, the absence of any change in the magnitude of skin cancer incidence with oral TA-65 attests to the safety of this supplement.

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Author Contributions

Karen E. Burke, M.D., Ph.D. (KEB), conceptualized, acquired the funding, supervised, and administered this research. Xueyan Zhou, M.D, M.S. (XZ), with KEB performed the laboratory investigation (enhancing the protocol when indicated) and curated the data. Yongyun Wang, Ph.D. (YW) did the statistical analysis. KEB and XZ collected the data and together with the statistician YW analyzed this data. Huachen Wei, M.D., Ph.D. (HW), provided scientific advice about the protocol and analyses as well as laboratory space and animal facilities. All authors reviewed and edited the protocol prior to the investigation, critically reviewed the intellectual content and presentation of research data and analysis, and revised, edited, and approved the final manuscript. As principal author, KEB did design the study and decide to publish with the consent of all co-authors but with no involvement from the supporting source. KEB with coauthors XZ and YW prepared the manuscript which all coauthors read and revised when indicated. All authors ensure the accuracy and integrity of the work.

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Competing Interests

None of the authors have any competing interests or any financial, non-financial, professional, or personal competing interests that would bias this research. Please note that YW was chosen as a consultant to do the statistical analysis of the data because he had previously worked in our laboratory and had collaborated on statistical analysis for other past publications with similar protocols to study the prevention of skin cancer by topical and oral antioxidants. His current commercial affiliation (employment) at Medtronic Diabetes is in no way associated with the research presented in this publication. His employment does not in any way alter adherence to the policies of ***OBM Geriatrics*** on sharing data and/or materials. The sponsor of this research was TA Sciences through Calvin Bruce Harley, Ph.D., who did not participate in the specific study design (except to advise supplement dose) or in the collection, analysis, or interpretation of data. He has no conflict of interest regarding this manuscript.

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