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Topical AC-11 abates actinic keratoses and early squamous cell cancers in hairless mice exposed to Ultraviolet A (UVA) radiation

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Abstract:

AC-11 is an aqueous extract of the botanical, *Uncaria tomentosa*, which has a variety of effects that enhance DNA repair and down regulate inflammation. AC-11 is essentially free of oxindole alkaloids (< 0.05%, w/w) but contains more than 8% carboxy alkyl esters (CAEs) as their active ingredients. Three groups of 10 outbred SK-1 hairless or SK-II hairless strains of mice each were treated with AC-11 at 0.5%, 1.5%, and 3.0% in a non-irritating, dye-free, perfume-free, and fragrance-free vanishing cream vehicle. Ten mice used vehicle only and 10 were untreated. Each concentration of AC-11 and was applied daily to the backs of the mice prior to exposure to a 1,600-watt solar simulator used in this work (Solar Light Co. Philadelphia, PA) emitting (mainly Ultraviolet A (UVA) and B (UVB) radiation) duration of the experimental period with UVB wavelengths was filtered out with a 1.0 cm Schott WG 345 filter. AC-11 with a peak absorption at 200nm does act as a sun block. We tested for and focused on clinical appearance of mice and histological appearance of tumors in mice rather than metrics of radiation generated inflammation. Tumor progression scores were assigned as follows: 4+ = extensive tumor development; 3+ = early malignancies (raised palpable plaques)(early squamous cell cancers) 2+ = firm scaling, palpable keratosis (actinic keratoses); 1+ = light scaling with erythema. Following a total cumulative dose of 738 J/cm², 85.7% all of the irradiated control animals, which did not receive AC-11 had precancerous actinic keratosis (AK)-type lesions (2+) (64.3% versus 42.9%) or early squamous cell carcinoma (SCC) (3+) (21.4% vs. 4.8%), in comparison with 47.7 % of AC-11-treated animals. There were no significant differences between the AC-11 groups. Three months after cessation of exposure to UVA radiation, the lesions in all but three of the 14 animals which were treated with AC-11 that were still evaluable irradiated with UVA radiation progressed to papillomas and frank squamous



cell carcinomas (+4 responses). AC-11 retarded, but did not stop, carcinogenesis progression. It is possible that if AC-11 was continuously applied tumors would not have in mice treated with AC-11 for a limited period. While we do not know how AC-11 exerts its DNA repair and anti-inflammatory effects, AC-11 is therapeutic for the treatment at the time of development of actinic keratoses and squamous cell carcinomas in mice and by extension humans. Without the constant presence of AC-11 these protective effects do not occur.

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Letter

Topical AC-11 abates actinic keratoses and early squamous cell cancers in hairless mice exposed to Ultraviolet A (UVA) radiation

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Abstract

AC-11 is an aqueous extract of the botanical, *Uncaria tomentosa*, which has a variety of effects that enhance DNA repair and down regulate inflammation. AC-11 is essentially free of oxindole alkaloids (< 0.05%, w/w) but contains more than 8% carboxy alkyl esters (CAEs) as their active ingredients. Three groups of 10 outbred SK-1 hairless or SK-II hairless strains of mice each were treated with AC-11 at 0.5%, 1.5%, and 3.0% in a non-irritating, dye-free, perfume-free, and fragrance-free vanishing cream vehicle. Ten mice used vehicle only and 10 were untreated. Each concentration of AC-11 and was applied daily to the backs of the mice prior to exposure to a 1,600-watt solar simulator used in this work (Solar Light Co. Philadelphia, PA) emitting (mainly Ultraviolet A (UVA) and B (UVB) radiation) duration of the experimental period with UVB wavelengths was filtered out with a 1.0 cm Schott WG 345 filter. AC-11 with a peak absorption at 200nm does act as a sun block. We tested for and focused on clinical appearance of mice and histological appearance of tumors in mice rather than metrics of radiation generated inflammation. Tumor progression scores were assigned as follows: 4+ = extensive tumor development; 3+ = early malignancies (raised palpable plaques)(early squamous cell cancers) 2+ = firm scaling, palpable keratosis (actinic keratoses); 1+ = light scaling with erythema. Following a total cumulative dose of 738 J/cm², 85.7% all of the irradiated control animals, which did not receive AC-11 had precancerous actinic keratosis (AK)-type lesions (2+) (64.3% versus 42.9%) or early squamous cell carcinoma (SCC) (3+) (21.4% vs. 4.8%), in comparison with 47.7 % of AC-11-treated animals. There were no significant differences between the AC-11 groups. Three months after cessation of exposure to UVA radiation, the lesions in all but three of the 14 animals which were treated with AC-11 that were still evaluable irradiated with UVA radiation progressed to papillomas and frank squamous cell carcinomas (+4 responses). AC-11 retarded, but did not stop, carcinogenesis progression. It is possible that if AC-11 was continuously applied tumors would not have in mice treated with AC-11 for a limited period. While we do not know how AC-11 exerts its DNA repair and anti-inflammatory effects, AC-11 is therapeutic for the treatment at the time of development of actinic keratoses and squamous cell carcinomas in mice and by extension humans. Without the constant presence of AC-11 these protective effects do not occur.

Introduction

Exposure to sunlight results in both oxidative damage and photochemical (cyclobutyl pyrimidine dimers, 6-4 photoproducts) DNA damage [1-6] and often mutated p53 genes AC-11 is an aqueous extract of the botanical, *Uncaria tomentosa*, which has been shown to both enhance the repair of cyclobutyl pyrimidine dimers in human living skin equivalents and to decrease inflammation and DNA damage in humans and animals by enhancing DNA repair [7-17]. Both these processes involve regulating the nuclear transcription factor kappa beta (NF-kB). How AC-11 down regulates NF-kB is not known. NF-kB is well known to control both the nuclear events that salvage cells from apoptotic cell death as well as pro-inflammatory cytokine production. Thus, in aqueous extract of the botanical, *Uncaria tomentosa*, has a variety of effects that enhance DNA repair and decrease inflammation. In cells AC-11 is essentially free of oxindole alkaloids (< 0.05%, w/w) but contains more than 8% carboxy alkyl esters (CAEs) as their active ingredients [16]. Thus, use of AC-11 can prevent the development of actinic keratoses and squamous cell cancers through its ability to enhance DNA repair and its down regulation of NF-kB.

Animals

Albino hairless mice which had been inbred in our laboratories (over 30 generations) from the outbred SK-1 hairless or SK-II hairless strains obtained from Drs. F. Urbach and R. Davies (Philadelphia) and from Dr. J. Epstein (San Francisco) were used (the latter strain contained some pigmentation on the ears). Each experiment involved mice, which were initially 6 weeks old. Previous experiments have established that their biological responses to light are essentially the same within $\pm 5\%$ to obtain the minimal erythema reaction, and that spontaneous tumor development is extremely low (< 2% after one (1) year of age). The animals were kept in a room with dim incandescent lighting, of constant temperature and humidity. They received standard laboratory food and drinking water.

Using mice for research purposes requires appropriate Institutional Review Board (IRB) approval and all necessary approvals were obtained for using the mice in the experiments reported in this article.

Methods

Fifty Skh-1 hairless mice were repeatedly exposed to a 1,600-watt xenon arc solar-simulator radiation (290 nm – 400 nm) as previously described by Willis et al¹. The spectral output of this source is shown in Figure 1, solid curve.

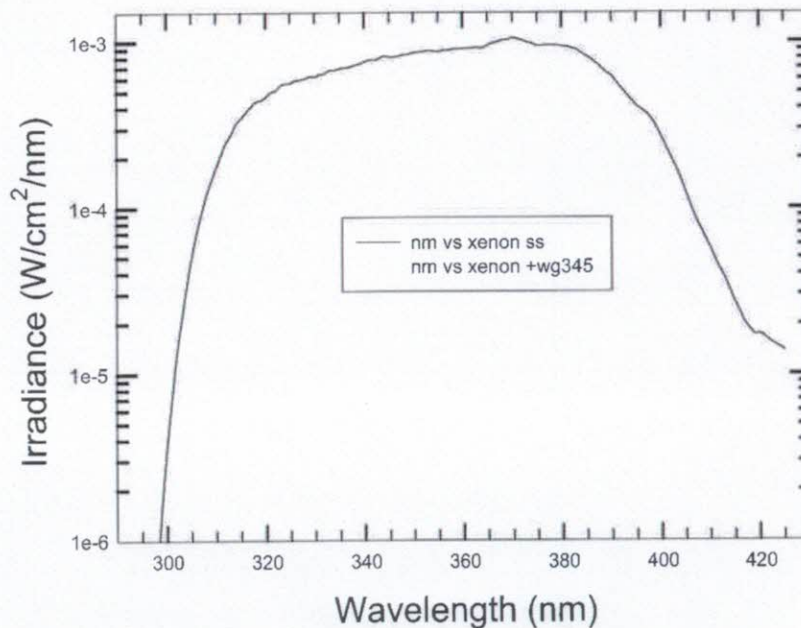


Figure 1. Spectral output of 1,600-watt solar simulator used in this work (Solar Light Co. Philadelphia, PA). Solid line, used in this study, shows the normal spectrum of this lamp (mainly ultraviolet B (UVB) + ultraviolet A (UVA) wavelengths). Dashed line shows spectrum obtained by inserting an additional 1.0 cm Schott WG 345 filter, resulting in removal of the UVB wavelengths.

Three groups of 10 mice were treated with AC-11 at 0.5%, 1.5%, and 3.0% in AC-11, formulated in a non-irritating, dye-free, perfume-free, and fragrance-free vanishing cream vehicle. Ten (10) mice were treated with vehicle only and 10 mice treated with

neither vehicle nor AC-11. Each concentration of AC-1 was applied daily to the backs of each mouse prior to irradiation for the duration of irradiation. Controls included “no treatment” and “vehicle only” in the presence and absence of UV. Following cessation of irradiation, animals were allowed to rest for an additional three months and then re-examined for clinical signs of (pre) cancerous lesions (see below).

After establishing the minimal erythema dose of solar-simulating radiation (SSR), study mice were initially exposed to 0.9 X the minimal erythema dose (MED) 5 times a week for 2 weeks. The dose was then increased by 20% (of the MED) every two weeks for a total of 77 irradiation days. The total dose of solar-simulating radiation was 738 J/cm². The primary parameters measured were the effectiveness of AC-11 at preventing the development of actinic keratoses.

Tumor progression was assessed according to the following 0-4 scale with the assigned score: mild erythema (0); intense macular erythema (0); light scaling accompanying erythema (1); firm scaling, palpable keratosis (2); raised palpable plaque, corresponding to early malignant development (3); and extensive tumor development (4). Mild erythema and intense macular erythema are not considered precancerous and were thus assigned a tumor progression score of zero.

Tumor progression scores were reported as weighted averages (means) ± standard errors and compared using Kruskal-Wallis one-way analysis of variance. A p value < 0.05 was considered significant. Specific parameter of DNA repair were not evaluated e.g., (amount of reparation enzyme(s), cyclobutane pyrimidine dimers (CPDs,) inflammation marker(s) due to the focus of this study on histology. Other studies have evaluated these DNA repair metrics [16].

Histologic examination with routine H & E was carried out immediately after cessation of UV radiation. (Pre) cancerous lesions exhibited epidermal and stratum corneum hyperplasia, and dermal alterations from a “basket weave” to a “laminated” architecture, which grew more prominent with increasing chronic irradiation.

Results

Clinical Results: Of the 50 mice exposed to solar simulating radiation, 5 were biopsied as a representative sample (one from each of the different AC-11 concentrations used, one from the vehicle-only group, and one from the untreated controls), for histological examination and not included in the assessment of clinical response. Other animals were not evaluable for reasons not related to the study or the test material due to premature death, some of missing mice had suffered strokes and died for unknown reasons (a common drop out rate in mouse studies). The cause of the premature death was could not be determined. In total, 21 AC-11-treated and 14 vehicle-only controls or untreated controls were evaluable.

Both *Table 1* and *Figure 2* indicate that AC-11 retarded, but did not stop, the progression of carcinogenesis. Immediately after irradiation (day 77), the percentage of animals that had a more severe clinical response was skewed to the animals that did not receive topical AC-11. Following a total cumulative dose of 738 J/cm², 85.7% all of the irradiated control animals, which did not receive AC-11 had precancerous actinic keratosis (AK)-type lesions (2+) or early squamous cell carcinoma (SCC) (3+), in comparison with 47.7 % of AC-11-treated animals. -Immediately following a total cumulative dose of 738 J/cm², 85.7 of the irradiated control animals had precancerous AK-type lesions (2+) or early SCC (3+), in comparison with 47.7 % of AC-11-treated animals. AC-11 had precancerous actinic keratosis (AK)-type lesions (2+) (64.3% versus 42.9%). More importantly, the percent of animals with 3+ reactions was substantially higher in controls than in AC-11 treated animals (21.4% versus 4.8%). There were no significant differences between the AC-11 groups, deemed a single group in *Table 1*. A significant (p<0.02) difference was noted in the mean tumor progression scores when controls (2.07 ± 0.62) were compared to AC-11-treated animals (1.52 ± 0.60; *Figure 1*). There was no difference in mean tumor progression scores when responses to the different concentrations AC-11 were compared. There was no difference in mean tumor progression scores when controls treated with vehicle-only were compared to untreated controls, suggesting that the vehicle had no significant effect on tumor progression.

Table 1. Response to solar-simulating radiation at day 77 in hairless mice treated with or without topical AC-11. The total cumulative dose was 738 J/cm².

| Clinical Response Category** | Controls* (n=14) | | AC-11† (n=21) | |
|------------------------------|------------------|---------|---------------|---------|
| | Count | Percent | Count | Percent |
| E1 | 0 | 0.0 | 0 | 0.0 |
| E2 | 0 | 0.0 | 0 | 0.0 |
| 1+ | 2 | 14.3 | 11 | 52.4 |

| | | | | |
|----|---|------|---|------|
| 2+ | 9 | 64.3 | 9 | 42.9 |
| 3+ | 3 | 21.4 | 1 | 4.8 |
| 4+ | 0 | 0.0 | 0 | 0.0 |

* Controls included 7 mice that were treated with vehicle only and 7 that were untreated.

** See "Methods" section for explanation of symbols

† AC-11 was applied topically in concentrations of 0.5%, 1.5%, and 3.0% to 7, 6, and 8 evaluable animals, respectively. Varying concentration of AC-11 were used to see what level of AC-11 was needed to prevent the development of actinic keratoses and squamous cell cancers.

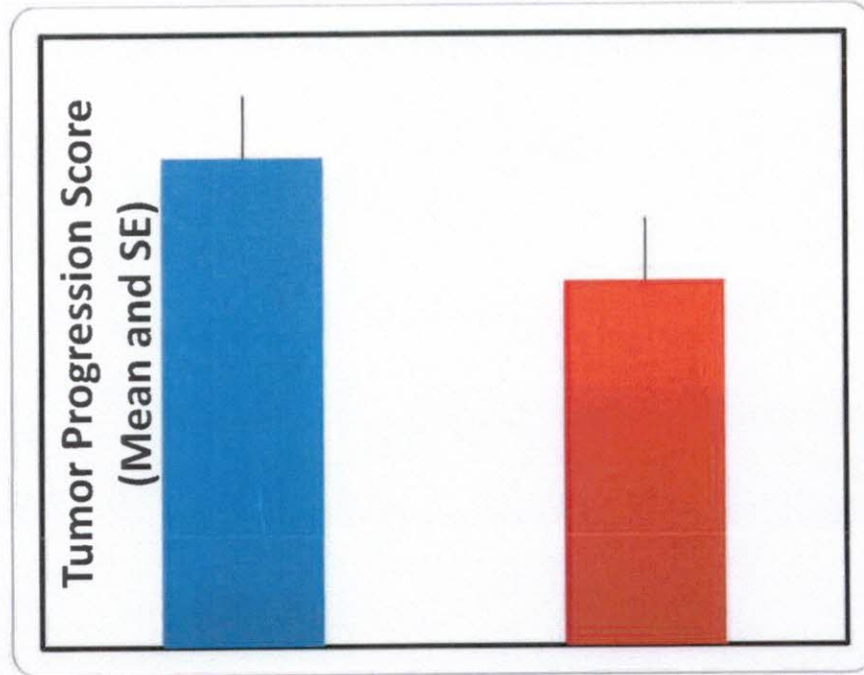


Figure 2. Mean (and standard error) tumor progression scores in hairless mice exposed to a cumulative dose of 738 J/cm² of solar-simulating radiation administered over 77 days. Control animals (n=14) included untreated and vehicle-only-treated animals. Topical application of AC-11 (total n=21) included animals treated with 0.5%, 1.5%, and 3.0% concentrations of AC-11. Tumor progression scores were assigned as follows: 4+ = extensive tumor development; 3+ = early malignancies (raised palpable plaques); 2+ = firm scaling, palpable keratosis; 1+ = light scaling with erythema. This was a clinical assessment buttressed by subsequent histological evaluation. The erythema responses, E1 and E2 are not considered precancerous. Mice were examined one day after cessation of UV irradiation.

Histologic Results: The epidermis of normal hairless mouse skin is three layers thick. Normal collagen has a basket-weave pattern and is eosinophilic (Figure 3a). Un-irradiated mouse skin treated with vehicle only for 77 days have an identical pattern to that of untreated un-irradiated hairless mouse skin (Figure 3b).

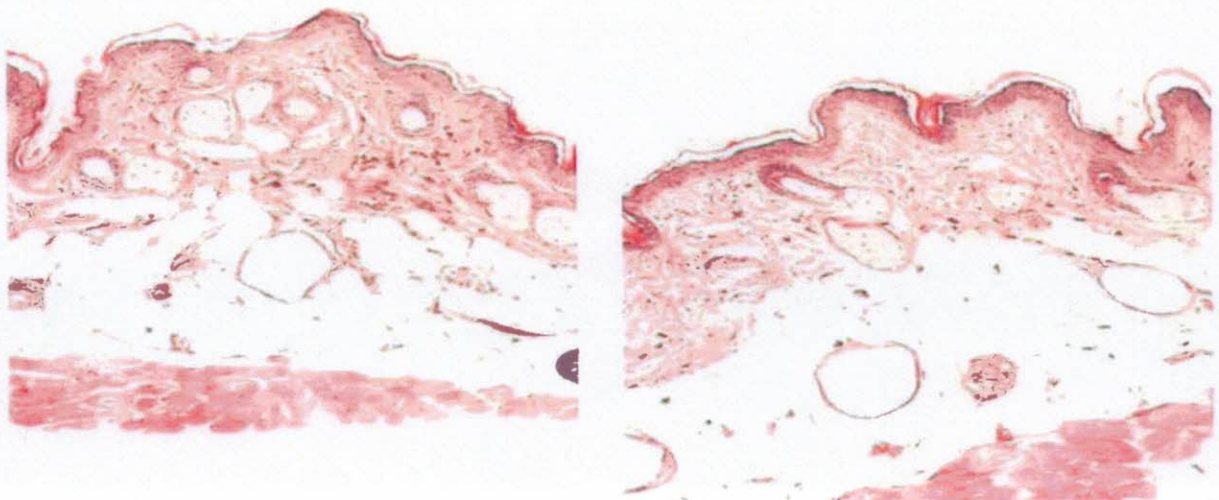


Figure 3a. Untreated hairless mouse skin. Normal mouse skin has three layers of epidermis. Collagen bundles have a "basket-weave" pattern. **Figure 3b.** Mouse treated with vehicle only. No apparent changes from normal can be seen. (10x H&E staining).

Irradiation with solar – simulating UV for 77 days according to the protocol described in "methods" resulted in 1+ – 3+ responses (table 1) Figure 4a is a histological section from a mouse exhibiting a 3+ response (see above). There is noticeable hyperplasia in the epidermis and stratum corneum, as well as a moderate change in architecture of dermal collagen from a "basket-weave" to a birefringent laminated structure, characteristic of UV damage. The presence of vehicle appears to marginally attenuate all of these responses to UV alone (Figure 4b). There was a very slight protective effect of 3.0% AC-11 against solar-simulating UVA (Figure 5). The absorption and transmission of AC-11 as thin film in de-ionized water is shown in Figure 6, demonstrating that AC-11 does not block UVB and has an insignificant effect on blocking UVA. Thus spectra showing absorption and transmission of AC-11 absorbs UVA to an insignificant extent but typically UVA will break down AC-11 molecules, AC-11 absorbs electromagnetic radiation at a peak of 200 nm¹⁶ and marginal effect on blocking UVA can not be compared to stabilized UVA blocker e.g. drometrizole trisiloxane or ecamsule. Here the AC-11 was not reapplied but still had positive benefit on preventing the development of actinic keratoses which suggests AC-11 did not act as a UVA blocker but enhanced DNA repair and this is why AC-11 treated mice had fewer keratoses and squamous cell cancers during the treatment period.

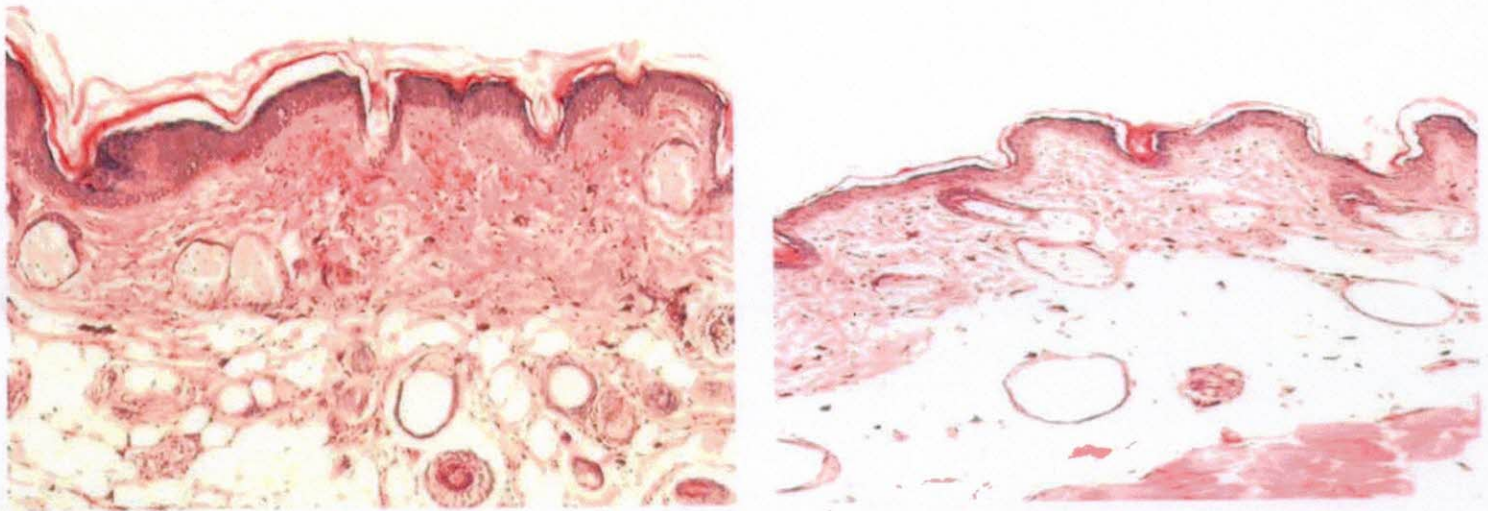


Figure 4a: Histologic section from mouse with clinical 3+ response. There is noticeable hyperplasia in the epidermis and stratum corneum. Along with significant change in the architecture of dermal collagen from a "basket – weave" to a birefringent laminated architecture, characteristic of UV damage. These changes are attenuated by the vehicle (**Figure 4b.**)

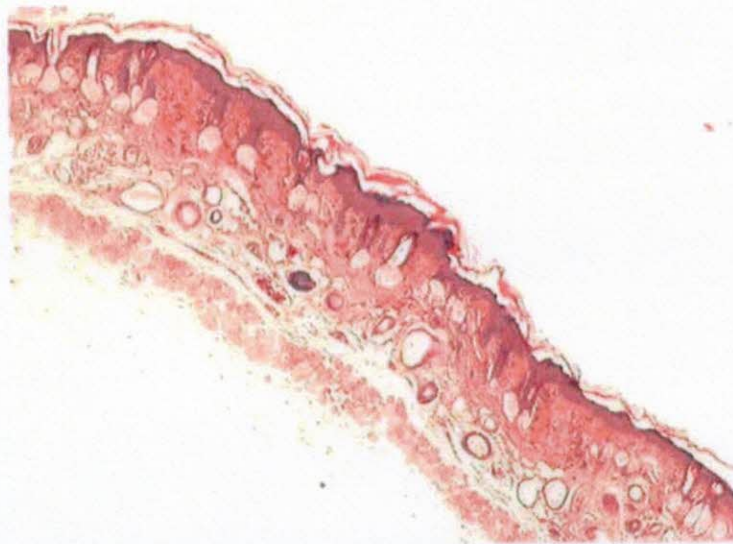


Figure 5. There is a modest protective effect of 3.0% AC - 11 against solar-simulating UV (Compare with figure 3a).

Three months after cessation of exposure to UVA radiation, the lesions in all but three of the 14 animals that had been chronically exposed to UV irradiation had progressed to papillomas and frank squamous cell carcinomas (+4 responses). This suggested that application of AC-11 slowed the development of cancers, but did not seem to suppress the overall development of tumors when application of AC-11 was stopped. This implies if AC-11 if used indefinitely it would stop the development of actinic keratoses and squamous cell carcinomas

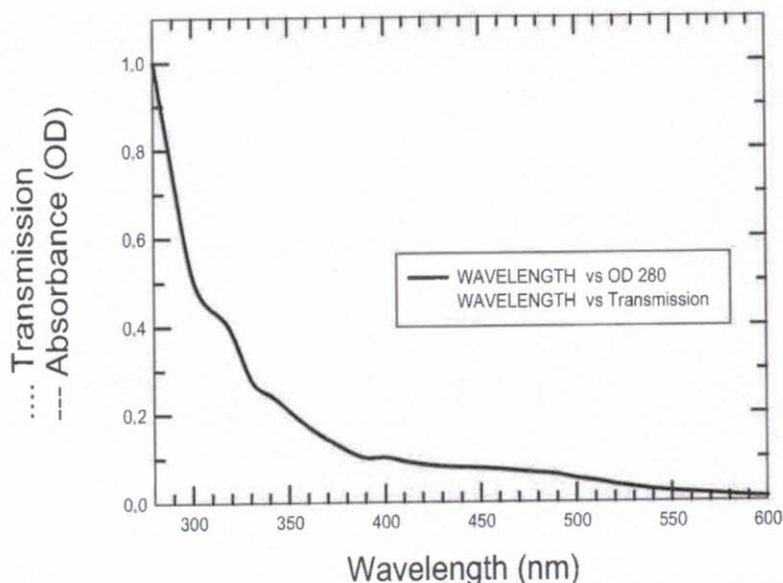


Figure 6. Absorption and Transmission of AC-11 as thin film* in deionized water. [AC-11] = 40 mg/ml
 *Three drops of above solution were dried overnight onto a methacrylate disposable UV grade 1.0 cm cuvette (Sigma Chemical Co.).

Solid line: Absorbance (O.D.)
 Dotted line: Transmission T, related by $T = 10^{-OD}$

Discussion

Both Table 1 indicate that AC-11 slowed, but did not stop, the progression of carcinogenesis; however, three months after stopping irradiation and use of AC-11, almost all of 37 the animals had developed papillomas or tumors. Results obtained in the hairless mouse model of UV-induced carcinogenesis suggest that topical AC-11 may be an effective adjunct to the standard precautions of limiting sun exposure and the application of sunscreens to minimize the risk of non-melanoma skin cancer if it is applied as a treatment at time of sun exposure or to cells with precancerous or cancerous growths thereafter. There is much literature that attests to the importance of free radical scavenging in AC-11 therapy of pre cancers and cancers [7-17].

The data contained in our experiments reported herein suggests that topical application of AC-11 can significantly retard the development of skin cancers if these are detected as precancerous lesions, for it to prevent cancers it must be used indefinitely something we did not do in this study. AC-11 does not block UVB and has an insignificant effect on blocking UVA [16]. It is not clear if this effect of decreasing actinic keratoses is by up regulation of DNA repair enzymes anti-inflammatory, or effects on NFkB. Further testing of AC-11 is warranted to determine how this treatment may be made more available and efficacious [16,17].

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