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A dicyanotriterpenoid induces cytoprotective enzymes and reduces multiplicity of skin tumors in UV-irradiated mice

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Abstract

Inducible phase 2 enzymes constitute a primary line of cellular defense. The oleanane dicyanotriterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile (TP-225) is a very potent inducer of these systems. Topical application of TP-225 to SKH-1 hairless mice increases the levels of NAD(P)H-quinone acceptor oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) and protects against UV radiation-induced dermal thickening. Daily topical treatments of 10 nmol of TP-225 to the backs of mice that were previously subjected to low-level chronic UVB radiation (30 mJ/cm^2/session, twice a week for 17 weeks), led to 50% reduction in multiplicity of skin tumors. In addition, the total tumor burden of squamous cell carcinomas was reduced by 5.5-fold. The identification of new agents for protection against UV radiation-induced skin cancer and understanding of their mechanism(s) of action is especially important in view of the fact that human skin cancers represent a significant source of increasing morbidity and mortality.

Keywords: 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile; Heme oxygenase 1; NQO1; SKH-1 hairless mouse

Phase 2 proteins are essential for defense against electrophiles and oxidants. They are expressed at low basal levels, but can be markedly elevated by various small molecules (inducers). This induction is a promising strategy for protection against chronic diseases [1]. Inducers modify highly reactive cysteine residues of the cellular protein sensor Keap1 rendering it unable to target its partner, transcription factor Nrf2, for ubiquitination and proteasomal degradation. Consequently, Nrf2 undergoes nuclear translocation, binds to the upstream regulatory regions of phase 2 genes (AREs, antioxidant response elements), and activates their transcription [2–4].

Michael acceptors are a major class of inducers [5] and their potencies parallel their reactivities with sulfhydryl agents [6]. Plants contain a wide variety of secondary metabolites that are Michael acceptors, e.g., cinnamates, curcuminoids, flavonoids, avicins. The triterpenoids oleanolic and ursolic acid have weak anti-inflammatory and anti-carcinogenic properties [7]. Many triterpenoids have been synthesized in an effort to improve their anti-inflammatory potencies [8,9]. Indeed, the Michael acceptor-containing 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and CDDO-imidazole inhibit potently the

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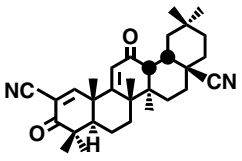
γ-interferon-induced synthesis of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), block proliferation, promote differentiation, and induce apoptosis. In search for potential molecular target(s), CDDO was found to induce phase 2 enzymes, e.g., heme oxygenase 1, NAD(P)H-quinone acceptor oxidoreductase 1 (NQO1), and enzymes of glutathione biosynthesis [10]. Furthermore, there is a close correlation spanning 6 orders of magnitude of concentrations between the potencies of a large series of triterpenoids in elevating NQO1, and in suppressing iNOS activation [11]. The dicyanotriterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile (TP-225) (Fig. 1) is the most potent compound in this series leading us to hypothesize that small quantities of TP-225 could induce cytoprotective defenses *in vivo* and inhibit tumor development. We used a "high-risk" model of skin carcinogenesis in SKH-1 hairless mice [12,13] in which the animals were first chronically exposed to low doses of UV radiation (30 mJ/cm^2, twice a week for 17 weeks) and, after completion of the irradiation schedule, received daily topical treatments of TP-225.

Materials and methods

Cell cultures and NQO1 assay. Cells were maintained in 5% CO_2 at 37 °C in the following media: α-MEM supplemented with 10% heat- and charcoal-treated FBS (Hepa1c1c7), DMEM supplemented with 10% heat-inactivated FBS (RAW 264.7), Iscove's MEM supplemented with 10% heat-inactivated FBS (mouse embryo fibroblasts), or EMEM supplemented with 8% heat-, charcoal-, and Chelex-treated FBS and 50 μM $CaCl_2$ (308 keratinocytes). For evaluation of inducer activity of TP-225, cells (10,000 per well for Hepa1c1c7, mouse embryo fibroblasts and 308 keratinocytes, or 50,000 cells per well for RAW 264.7) were grown for 24 h in 96-well plates, then exposed to serial dilutions of TP-225 for 48 h, and lysed in 0.08% digitonin. Activity of NQO1 was determined by the Prochaska test [14].

Animals. Experiments were in compliance with the National Institutes of Health Guidelines and approved by the Johns Hopkins University Animal Care and Use Committee. Female SKH-1 hairless mice (6 weeks old, obtained from Charles River, Wilmington, MA) were kept on a 12-h light/12-h dark cycle, 35% humidity, and given free access to water and pelleted AIN 76A diet that does not contain any phase 2 inducers (Harlan TekLad, Madison, WI). The animals were acclimatized for 1 week before beginning the radiation schedule.

Short-term topical treatment with TP-225 and processing of skin samples. Mice ($n = 3$) were treated topically with a single dose of: (i) 50 μl of acetone containing 50 nmol of TP-225, applied to ~4-cm^2 patch on the caudal area of the back, and (ii) 50 μl of acetone, applied to the rostral area. The animals were euthanized 24 h later. A second group ($n = 4$) received three doses of 10 nmol TP-225, at 24-h intervals. Twenty-four hours after the last dose, the mice were irradiated with a single dose of UVB light (150 mJ/cm^2) and euthanized 20 h later. Two identical segments (1.5 × 1 cm) of skin within each treated area were removed: one was frozen in liquid N_2 for analysis of NQO1 activity; the other was submerged in Optimal Cutting Temperature compound (Tissue-Tek; Sakura Fineteck, Torrance, CA) and frozen at −80 °C for histological cryosectioning. Frozen samples (100 mg) were pulverized in liquid N_2 and homogenized in 1 ml of 0.25 M sucrose/10 mM Tris–HCl, pH 7.4. After centrifugation at 14,000*g* for 30 min at 4 °C, aliquots of the clear supernatants were used for determination of protein concentration and NQO1 activity.



2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile

(TP-225)

Fig. 1. Chemical structure of TP-225.

Immunohistochemistry. Frozen tissue blocks were sectioned (Microm International HM 505 E microtome cryostat) at −25 °C and the 10-μm cryosections were mounted on microscope slides. Sections were immunoprobed using primary antibodies against NQO1 (1:200 dilution; a generous gift from John Hayes, University of Dundee, Scotland) and HO-1 (1:1000 dilution; Stressgen, Victoria, Canada), followed by FITC-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD). A Leica MZ FL III microscope equipped with an excitation filter at 470/40 nm and a barrier filter of 525/50 nm was used to visualize the FITC fluorescence.

UV-irradiation of mice and treatment with TP-225. The UV lamps (FS72T12-UVB-HO, National Biological Corporation, Twinsburg, OH) used to irradiate the animals emit UVB (280–320 nm, 65% of total energy) and UVA (320–375 nm, 35% of total energy) radiation. The dose was quantified with a UVB Daavlin Flex Control Integrating Dosimeter and calibrated with an IL-1400 radiometer (International Light, Newburyport, MA). The animals were irradiated for 17 weeks on Tuesdays and Fridays with a dose of 30 mJ/cm^2/session. One week later, they were divided into three groups of 30. The animals in each group were treated topically with 100 μl of acetone solutions containing either 10 nmol (high dose), or 1 nmol TP-225 (low dose), or 100 μl of acetone, 5 days a week for 13 weeks. Tumors (defined as lesions >1 mm in diameter) and body weights were recorded weekly. Tumor volumes were determined ($v = 4\pi r^3/3$) by measuring the height, length, and width, and using the average of the three as the diameter. All mice were euthanized on the same day. Dorsal skins were dissected (2.5 × 5 cm), stapled to a card, photographed, and fixed in 10% phosphate-buffered formalin at 4 °C for 24 h, and subsequently dehydrated in ascending concentrations (80, 95, and 100%) of ethanol, cleared in xylene, and embedded in paraffin. Five-micron sections were made, deparaffinized, rehydrated, stained with hematoxylin and eosin, and examined with a Zeiss microscope attached to a Nikon DXM 1200 digital camera for image acquisition.

Statistical analysis. All values are means ± 1 SD, unless otherwise noted. The differences between groups in the average number of skin tumors per mouse were determined by Student's *t*-test (with unequal variance). Weekly weights and changes in weight were subject to ANOVA by using STATA 7.0 (Stata Corporation, College Station, TX).

Results and discussion

Induction of NQO1 by TP-225

We previously identified the dicyanotriterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile (TP-225) (Fig. 1) as an exceedingly potent inducer of NQO1 in murine Hepa1c1c7 cells and inhibitor of γ-IFN-dependent induction of iNOS in primary and established cultures of murine macrophages [11]. Here we report that TP-225 is exceptionally potent in elevating NQO1 activity in a dose-dependent manner in various mouse cell lines, e.g., hepatoma cells (Hepa1c1c7), macrophages (RAW 264.7), embryo fibroblasts (MEF), and keratinocytes (308) (Fig. 2). The CD values (Concentration that Doubles the specific activity

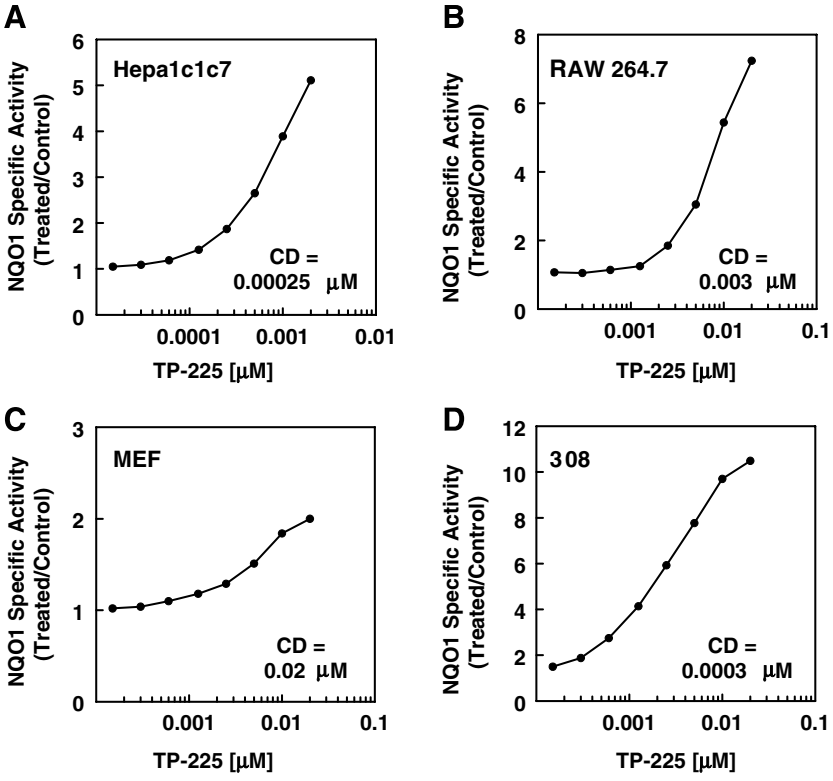


Fig. 2. Induction of NQO1 as a function of concentration by TP-225 in murine cells: (A) Hepa1c1c7; (B) RAW 264.7; (C) embryonic fibroblasts (MEF); and (D) 308 keratinocytes. Cells were plated in 96-well plates and 24 h later were exposed to serial dilutions of TP-225 for a further 48 h. NQO1 activity is expressed as mean ratios of treated over control specific activities using eight replicate wells for each inducer concentration. The standard deviation for all points was less than 10%. Shown are the CD values, i.e., the concentrations that double the NQO1 specific activity.

of NQO1) for TP-225 varied among the cell lines and were in the range of 0.02 μM (in MEF)–0.0003 μM (in Hepa1c1c7 and 308 keratinocytes). Thus, in Hepa1c1c7 cells, which we and others routinely use as a highly quantitative screening bioassay for potential inducers and chemoprotective agents [14,15], TP-225 is ~1000-fold more potent than sulforaphane, the most potent naturally-occurring inducer known. The magnitude of induction is especially striking in 308 keratinocytes, in which >10-fold induction was observed without any apparent cytotoxicity at the highest concentration tested (0.02 μM).

Induction of cytoprotective enzymes by TP-225 in mouse skin

The ability of TP-225 to induce cytoprotective enzymes in the skin of female SKH-1 hairless mice was evaluated next. The NQO1 activity was substantially elevated (1.5-fold at 24 h, $P = 0.017$) in homogenates of skin to which the triterpenoid was applied (Fig. 3A). Immunohistochemical analysis confirmed increase of NQO1 protein levels in the epidermis (Fig. 3B). The protein levels of heme oxygenase 1 (HO-1) were also elevated. Notably, the strongest induction was in the epidermis and the epithelial cells within the hair follicles.

We next examined whether UVB radiation could affect the basal and inducible levels of NQO1 in skin. A single exposure to UVB radiation (150 mJ/cm^2) did not affect the enzyme activity ($P = 0.608$) (Fig. 3C). Three daily topical applications of 10 nmol of TP-225, at 24-h intervals, led to a marked increase (2.7-fold, $P = 0.019$) in activity in skin of mice that were exposed to UVB radiation. Thus, the effects of single and multiple doses of TP-225 on induction of NQO1 are reminiscent of the inducer effects of single and multiple topical applications of sulforaphane-containing broccoli sprout extracts to the skin of both mice and humans [13,16], suggesting that, although structurally unrelated, both compounds share common molecular target(s) and mechanism(s) of action. This conclusion is further supported by previous findings that: (i) sulforaphane and TP-225 bind and chemically modify highly reactive cysteine residues of the inducer sensor Keap1 and compete with other inducers of cytoprotective proteins for binding to Keap1 [2,11,17], and (ii) the inducer activities of both compounds depend on functional Nrf2, the major transcription factor responsible for phase 2 gene expression [11,18].

One of the consequences of UV radiation is increase in skin thickness which, at early time points, is largely due to vasodilation and edema [19]. Indeed, histological examination of skin sections from mice that were exposed to a single dose of UV radiation (150 mJ/cm^2) revealed that their dermal thickness was increased by ~75% compared to nonirradiated control mice (Fig. 3D). The dermal thickness of the back skin of control mice was not significantly

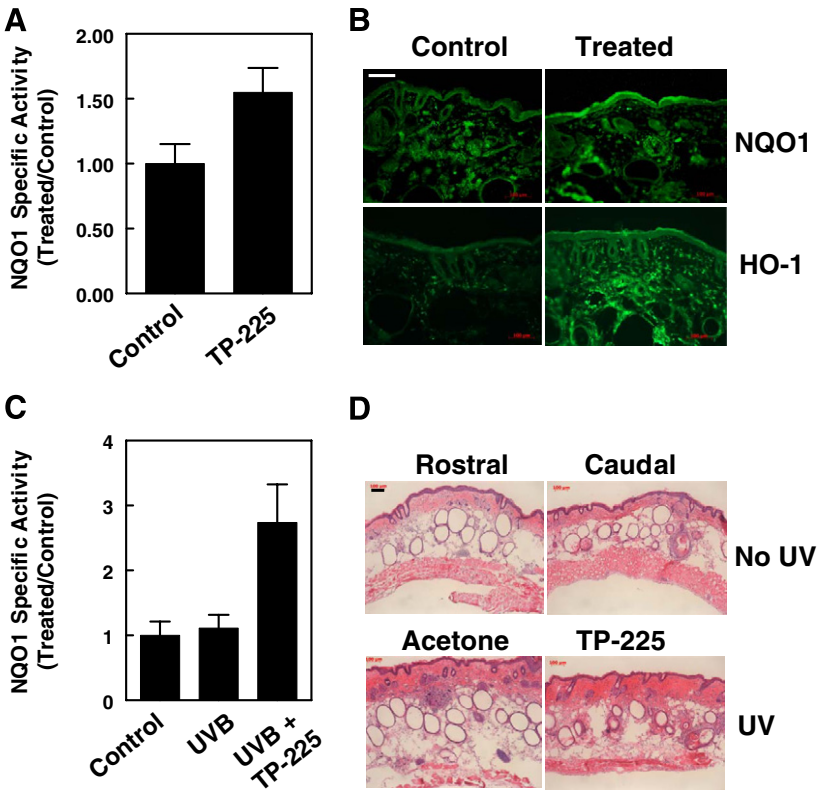


Fig. 3. Induction of cytoprotective proteins in mouse skin by topical application of TP-225. (A and B) The backs of SKH-1 hairless mice ($n = 3$) were treated topically with a single dose of: (i) 50 μl of acetone, applied to the rostral area (control), and (ii) 50 nmol of TP-225 (in 50 μl of acetone), applied to the caudal area. The animals were euthanized 24 h later. NQO1 specific activity (A) was measured in total skin homogenate supernatants. Fresh frozen sections (10 μm thickness) of skin were immunostained with specific antibodies to detect protein levels of NQO1 or HO-1 (B) Scale bar: ~100 μm. (C and D) The mouse backs ($n = 4$) received topically 3 doses, every 24 h, of: (i) 10 nmol of TP-225 (in 50 μl of acetone), applied to the caudal area, and (ii) 50 μl of acetone, applied to the rostral area. Twenty-four hours after the last application, the animals were exposed to UVB light (150 mJ/cm^2). They were euthanized 20 h after irradiation. NQO1 activity (C). Hematoxylin and eosin staining (D). Scale bar: ~100 μm.

different between the rostral and the caudal areas, 468 ± 73 μm and 422 ± 47 μm, respectively. In UV-irradiated mice (that were also treated with acetone), the dermal thickness was substantially increased and was 817 ± 42 μm. Treatment with TP-225 led to substantial protection and the dermal thickness was 656 ± 27 μm, significantly thinner than the acetone-treated control area ($P = 0.00009$). Of note, the last dose of TP-225 was applied 24 h prior to radiation and this compound does not absorb in the UVB region (290–320 nm), thus excluding the possibility of UV light-absorbing effects.

Topical application of TP-225 reduces the multiplicity of tumors in UVB-irradiated SKH-1 hairless mice

SKH-1 hairless mice were exposed to UVB radiation (30 mJ/cm^2) twice weekly for 17 weeks. We then applied daily (5 days a week for 13 weeks) acetone solutions of TP-225 containing either 1 nmol (low dose) or 10 nmol (high dose) of the triterpenoid to their backs. The control animals received vehicle (acetone). Although there was a significant difference in animal weight among the three groups ($F = 13.99$, $P < 0.0001$), the week-to-week differences in weight were not significantly different ($F = 0.42$, $P = 0.66$) (Fig. 4A). Thus, the body weights at the onset of the experiment (before radiation) were: 22.5 ± 0.3 g for the control group, 22.4 ± 0.3 g for the low dose-treated, and 23.2 ± 0.8 g for the high dose-treated group. At the end of the experiment (30 weeks later), the respective body weights were: 32.1 ± 0.5 g, 32.6 ± 1.5 g, and 34.5 ± 1.4 g.

The earliest lesions were observed 1 week after radiation was terminated. At this time point, 4, 2, and 1 mice of the control, low dose-treated, and high dose-treated groups, respectively, developed their first tumor. Overall, there was no significant difference in tumor incidence (Fig. 4B). Tumor multiplicity was identical for the control mice and for those treated with the low dose of TP-225. In contrast, there was a significant ~50% reduction in tumor multiplicity for the animals receiving the high dose of triterpenoid (Fig. 4C). Thus, at week 7 tumor multiplicity was 0.6 ± 0.3, 0.6 ± 0.3, and 0.2 ± 0.1 tumors per mouse for the control, low dose-treated, and high dose-treated groups, respectively. At the end of the study (13 weeks post-radiation), tumor multiplicities were 1.3 ± 0.3 for the 10 nmol of TP-225 group and 2.4 ± 0.6 tumors per mouse for the control group. This effect of the high dose TP-225 treatment was highly significant both with

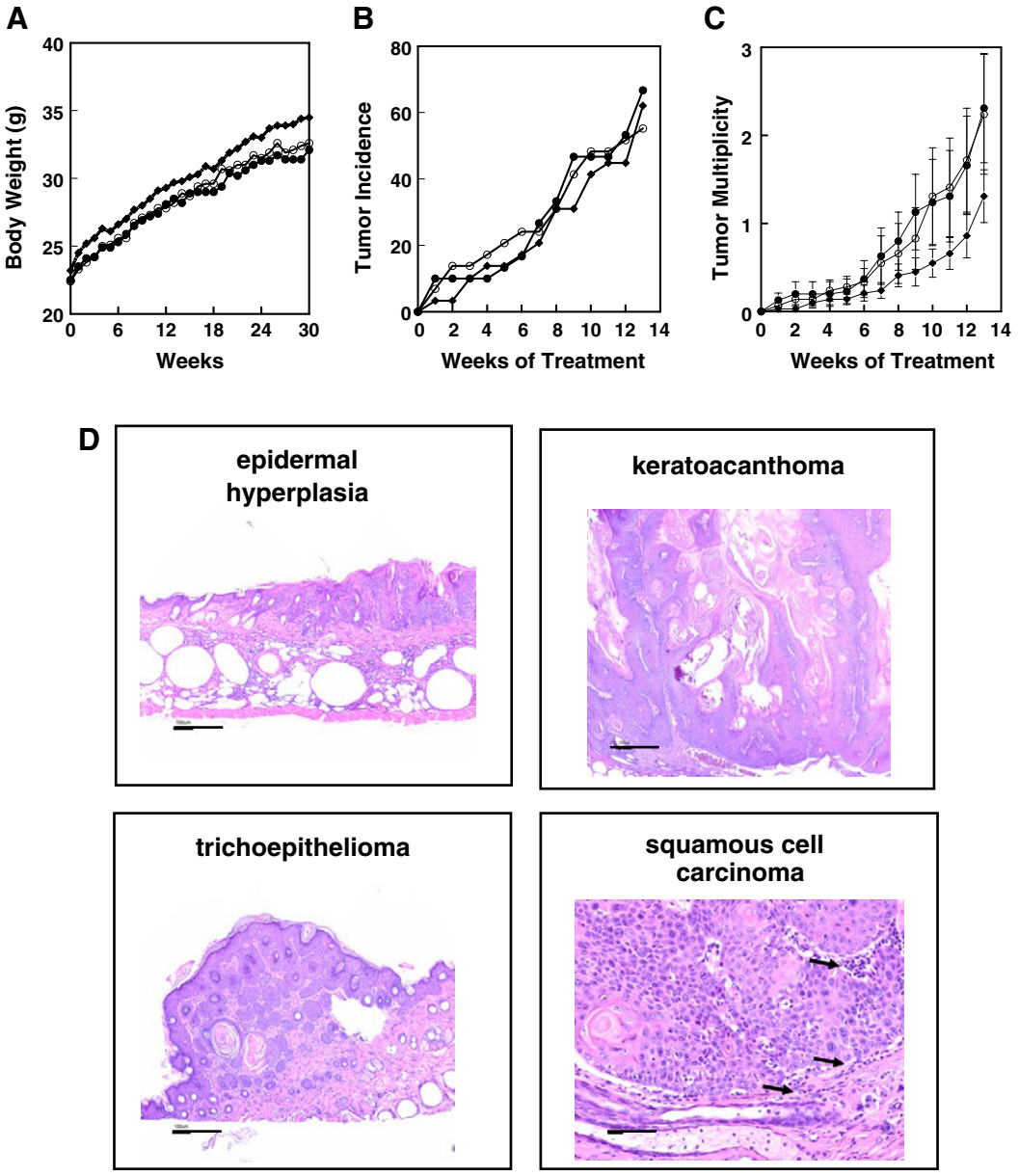


Fig. 4. Topical application of TP-225 reduces multiplicity of skin tumors in previously irradiated hairless mice. (A) SKH-1 hairless mice were irradiated with UVB light (30 mJ/cm^2/session) twice a week for 17 weeks. After completion of irradiation (time 0 on graphs B and C), the backs of the animals were treated daily topically with either 100 μl of acetone (●), 1 nmol of TP-225 in 100 μl of acetone (○), or 10 nmol of TP-225 in 100 μl of acetone (■). The appearance of tumors was monitored weekly, tumors were mapped, counted, and their volumes determined. The number of tumors per mouse (multiplicity) is expressed as average values ± SEM based on the total number of animals at risk. (D) Histopathology of tumors. Examples are shown of pre-malignant or malignant epithelial lesions which included epidermal hyperplasia, keratoacanthoma, trichoepithelioma, and squamous cell carcinoma *in situ* with mixed inflammatory infiltrates (arrows). Scale bars: ~300 μm for epidermal hyperplasia and trichoepithelioma, ~600 μm for keratoacanthoma, and ~100 μm for squamous cell carcinoma.

($P < 0.0001$) and without ($P < 0.0002$) inclusion of tumor-free animals in the analysis.

Topical application of TP-225 reduces the appearance of premalignant lesions and the volume of malignant tumors

Histopathology of sectioned tissues distinguished proliferative epithelial lesions (potential premalignant lesions for squamous cell carcinomas) from mesenchymal lesions or pilary cysts and revealed that ~90% were epithelial lesions (epidermal hyperplasia, keratoacanthoma and trichoepithelioma tumors) (Fig. 4D). Five animals from the control group and four animals from the high dose-treated group had malignant tumors (squamous cell carcinoma *in situ*). Furthermore, proliferative epithelial lesions were consistently associated with a mixed inflammatory infiltrate that varied in severity (Fig. 4D, arrows). The effect of treatment was mostly on the appearance of pre-malignant tumors (Table 1). The total tumor burden (i.e., the sum of the volumes) of the malignant tumors was substantially reduced

Table 1
Effect of TP-225 on UV radiation-induced skin tumors in SKH-1 hairless mice

Types of Tumors and Sizes	Total number of tumors in group (% of tumors)	
	Solvent control	TP-225 (10 nmol/day)
Total number of tumors (30 mice per group)	72	38
Size of tumors		
Tumors < 5 mm^3	62 (86)	30 (79)
Tumors > 5 mm^3	10 (14)	8 (21)
Histopathology		
Premalignant tumors	65 (90)	33 (87)
Malignant (squamous cell carcinoma)	7 (10)	5 (13)
Tumor Burden		
Total burden of premalignant tumors (mm^3)	139	182
Total burden of malignant tumors (mm^3)	137	25

by the treatment. Thus, the total tumor burden of the lesions that were diagnosed as "carcinoma *in situ*" was 137 mm^3 for the control and 25 mm^3 for the high dose-treated groups. However, perhaps due to the small number of malignant tumors and the large variation in their volumes, the difference in malignant tumor volume per tumor did not reach statistical significance ($P = 0.07$).

Concluding remarks

Deficiencies in functional NQO1 (gene deletions or null polymorphisms) have been associated with higher susceptibility to carcinogenesis of the skin in mice [20] and humans [21]. Induction of HO-1 has been implicated in the protection of the skin against UV radiation-induced immunosuppression [22] and inflammation [23], two processes with substantial contributions to the development of skin cancer. Xu et al. [24] reported that the levels of HO-1 are much lower in the skin of *nrf2*-knockout mice compared to their wild-type counterparts, and *nrf2*-knockouts are much more susceptible to chemically-induced skin carcinogenesis and cannot be protected by sulforaphane. Curiously, the levels of Nrf2 and HO-1 were undetectable in skin tumors from mice of either genotype.

The selection of TP-225 as a chemoprotective agent in this study was based on mechanistic understanding of its ability to induce potently cytoprotective enzymes and anti-inflammatory responses in various cell lines and in the mouse skin. The "high risk" hairless mouse model was chosen based on its relevance to humans both in terms of chronic exposure to UV radiation early in life and low radiant dose comparable to human sun exposures. Because UV radiation is the principal cause of premalignant actinic keratosis and skin cancer, the most common malignancy in humans, identification of new chemoprotective agents with defined mechanism(s) of action is of high importance.

Acknowledgments

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