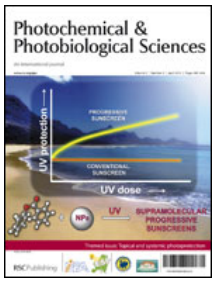

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Dietary glucoraphanin-rich broccoli sprout extracts protect against UV radiation-induced skin carcinogenesis in SKH-1 hairless mice

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Feeding broccoli sprout extracts providing daily doses of 10 μmol of glucoraphanin to SKH-1 hairless mice with prior chronic exposure to UV radiation (30 mJ cm^{-2} of UVB, twice a week, for 17 weeks) inhibited the development of skin tumors during the subsequent 13 weeks; compared to the controls, tumor incidence, multiplicity, and volume were reduced by 25, 47, and 70%, respectively, in the animals that received the protective agent.

Nonmelanoma skin cancers represent the most common malignancies in humans and are a cause for significant morbidity and mortality. In the USA, more than 1 million new cases are diagnosed each year.[1] UV radiation, the principal carcinogen that contributes to the development of nonmelanoma skin cancer, triggers a wide array of pathological events, such as direct and indirect DNA damage, inflammation, and immunosuppression. It is therefore not surprising that there is substantial interest in developing strategies to control and prevent photodamage as means to protect against the development of skin cancer. In addition to sunscreen products, both topical and dietary agents, many of plant origin, are currently being developed to achieve this goal. Examples include alkaloids (*e.g.*, caffeine and sanguinarine), carotenoids (*e.g*, β-carotene, lutein), flavonoids (*e.g.*, epigallocatechin 3-gallate, epicatechin, genistein, silibinin), melatonin, and vitamin E.[2-7]

Studies conducted more than 30 years ago indicated that an antioxidant-supplemented diet, *e.g.*, containing butylated hydroxytoluene (BHT), or disulfiram, significantly protected against UV radiation-induced skin carcinogenesis in mice.[8] In addition, topical or dietary administration of BHT or butylated hydroxyanisole (BHA) inhibited the phorbol ester-dependent induction of ornithine decarboxylase (an early indicator of tumor promotion) in mouse epidermis.[9] Coincidentally, it was found that the same molecules (BHT, BHA, and disulfiram) induce cytoprotective proteins (*e.g.*, glutathione *S*-transferases, NAD(P)H: quinone oxidoreductase 1 [NQO1]) in rodents and protect against chemical carcinogenesis.[10] We therefore hypothesized that induction of cytoprotective proteins could be an effective strategy for protection against skin cancer.

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The isothiocyanate sulforaphane was isolated from broccoli extracts as the principal inducer of NQO1, a representative cytoprotective enzyme.[11] Sulforaphane was subsequently shown to protect against carcinogenesis in more than 10 different animal models involving various carcinogens and target organs.[12] Using a model of UV radiation-induced skin carcinogenesis in which SKH-1 hairless mice are rendered high-risk to tumor development by chronic exposure to low doses of UV radiation comparable to human outdoor exposures,[2] we found that daily topical applications of broccoli sprout extracts containing 1 μmol of sulforaphane, beginning after completion of the irradiation schedule, reduced by ~50% the incidence, multiplicity, and volume of skin tumors.[13] *The aim of the present study was to establish whether protection could be achieved by dietary means.*

We used broccoli sprout extracts as a delivery vehicle for glucoraphanin, the glucosinolate precursor of sulforaphane. In order to prepare and standardize the extracts, 3-day-old broccoli sprouts (*Brassica oleracea italica* cv. DeCicco) were grown by a commercial green-sprouter (Sprouters Northwest; Kent, WA). Glucosinolates were extracted with boiling water and lyophilized. The resulting powder contained 185 μmol of glucoraphanin per gram. Glucoraphanin was the predominant glucosinolate in the powder as determined both by HPLC[14] and by the cyclocondensation reaction[15] after myrosinase-catalyzed conversion to isothiocyanate. The extracts were mixed with powdered diet (AIN 76A, Harlan TekLad, Madison, WI) to obtain 10 μmol of glucoraphanin per 3 g of diet.

Because sulforaphane (and not its glucosinolate precursor) is the active chemical entity that leads to the transcriptional induction of cytoprotective genes, it was important to establish that glucoraphanin is converted to sulforaphane *in vivo* when fed to the animals. Thus, a metabolic study was first undertaken. Female SKH-1 hairless mice (6–8 weeks old) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were acclimatized in our animal facility for 2 weeks before the start of the experiment. The animals were kept on a 12-h light/12-h dark cycle, 35% humidity, and given free access to water and food. Two groups of mice ($n = 5$) were placed in metabolic cages and the excreted urine was collected every 24 h. Following a baseline period of 3 days on powdered control diet, the animals from one of the groups were fed broccoli sprout powder containing the equivalent of 10 μmol of glucoraphanin per 3 g of diet. Urine was collected every 24 h for 4 more days. No isothiocyanates or isothiocyanate metabolites (dithiocarbamates) were detected in urine of the animals at the onset of the experiment, or in mice that were fed control diet throughout the experiment. For the animals that received the broccoli sprout diet, the mean

24-h urinary dithiocarbamate excretion was 0.761 ± 0.085 μmol (or 7.61 ± 0.85% of the 10 μmol dose).

During the skin carcinogenesis experiment, 60 SKH-1 hairless mice were exposed to UV radiation (30 mJ cm^{-2} of UVB) twice a week for 17 weeks. Radiation was provided by 6 UVB lamps (FS72T12-UVB-HO, National Biological Corporation, Twinsburg, OH) emitting UVB (280–320 nm, 65% of total energy) and UVA (320–375 nm, 35% of total energy). After completion of the irradiation schedule, the animals were divided into two groups of 30. The first group received powdered inducer-free AIN 76A diet (control). The animals in the second group were fed with the same diet that was supplemented with standardized broccoli sprout extracts containing 10 μmol of glucoraphanin per 3 g of diet. Formation of tumors (defined as lesions larger than 1 mm in diameter) was recorded weekly.

Body weights were monitored weekly (Fig. 1A). The average body weights (mean ± SD) at the onset of the experiment were: 22.7 ± 0.7 g for the control and 23.2 ± 0.7 g for the treated group. During the period of irradiation (17 weeks), the animals were fed pelleted diet, and at the end of irradiation their respective body weights were essentially identical, *i.e.*, 29.9 ± 0.7 g and 29.6 ± 1.2 g. In order to incorporate the freeze-dried broccoli sprout extract into the diet with minimal risk of modifying the active ingredients, the animals were fed powdered AIN 76A into which broccoli sprout extract was mixed. The animals thus had to be changed from pelleted to powdered diet at the time treatment was initiated. Strikingly, as soon as the diet of the animals was changed from pellets to powder, all mice started gaining weight much more rapidly, in agreement with our previous report in which we noted that in comparison with mice consuming pellets, powder-fed mice gained weight more rapidly and developed tumors much faster.[16] At the end of the experiment, the body weights were: 39.0 ± 1.5 g for the control and 38.7 ± 3.0 g for the treated animals. Notably, there was no significant difference in weight gain between the control and the treated groups at any time point.

Treatment with broccoli sprout extract resulted in protection against the carcinogenic effects of UV radiation. Thus, 2 weeks after the end of the irradiation schedule (which was 1 week after

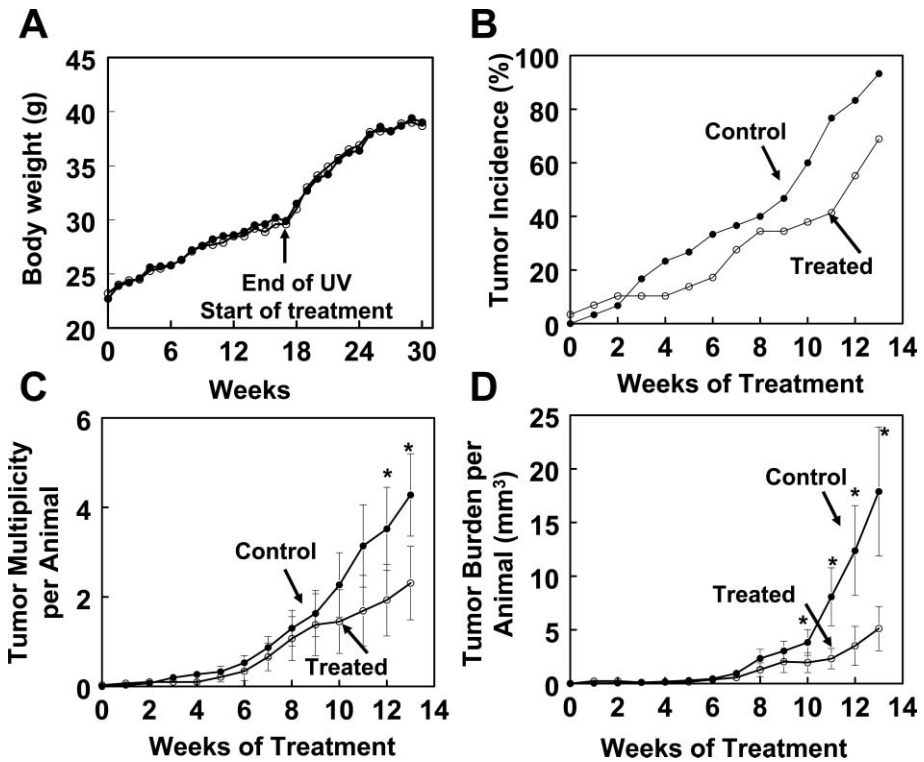

Fig. 1 Inhibition of UVB radiation-induced skin carcinogenesis in high-risk mice by dietary broccoli sprout extracts. SKH-1 hairless mice fed pelleted AIN 76A diet were exposed to UV radiation (30 mJ cm^{-2} per session of UVB) twice a week for 17 weeks and then divided into two groups of 30 animals each. During the subsequent 13 weeks, the mice from each group received either powdered AIN 76A diet (control, filled circles), or freeze-dried broccoli sprout powder containing 10 μmol of glucoraphanin per 3 grams of diet (treated, open circles). Body weights were monitored weekly and are shown in **(A)**. Weekly weights and changes in weight were evaluated by ANOVA, and were not significantly different. Note that the weight gain of the mice profoundly increased once the diet was changed from pellets to powder. Tumor incidence **(B)**, multiplicity **(C)**, and volume **(D)** were evaluated weekly. Average values ± 1 SE are shown. Tumor incidence represents the percent of tumor-bearing mice within a group. There were significant differences ($p < 0.0002$) when incidence data were plotted as a survivor function (Kaplan–Meier) followed by a log-rank test for equality. The differences between groups in the average number of skin tumors per mouse were determined by Student's *t* test (with unequal variance). Tumor multiplicity represents the average number of tumors per mouse. Tumor volumes were determined by measuring the height, length, and width of each mass that was larger than 1 mm in diameter. The average of the three measurements was used as the diameter, from which the radius and the volume were calculated ($v = 4\pi r^3/3$). Tumor volume per mouse represents the average of the sum of the volumes of all tumors in a group divided by the number of animals in that group. ANOVA and Kaplan-Meier log rank tests were performed using STATA 7.0 (Stata Corporation, College Station, TX). Statistically significant differences are indicated by an asterisk (*).

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treatment with protector was started) one and two mice from the treated and the control groups, respectively, developed their first tumor (Fig. 1B). Nine weeks post-irradiation 50% of the control animals had tumors, while it took three more weeks for 50% of the treated animals to develop tumors. At the end of the experiment, 93% of the control animals had tumors and only 2 mice out of 30 were tumor-free. Tumor incidence was reduced by 25% in the animals receiving the protective agent, and 9 out of 29 mice were tumor-free. Kaplan–Meier survival analysis, followed by a log-rank test for equality of survivor function showed that the difference between treated and control groups was highly significant ($\chi^2 = 13.98$; $p < 0.0002$). The effect of treatment on tumor multiplicity was even greater; a 47% reduction (Fig. 1C). Thus, while the animals in the control group had an average of 4.3 tumors per mouse, the number of tumors per mouse was 2.3 for the treated group. The difference between groups became significant at the 95% level at week 12 ($p < 0.05$, by ANOVA). Total tumor volume (expressed in mm^3) per mouse was also affected by the treatment (Fig. 1D). Feeding with the protective agent resulted in ~70% reduction in tumor volume with a significant difference between the control and the treated groups at time points beyond 9 weeks.

It is noteworthy that in this model the weight gain of the animals has a profound effect on tumor development.[16] Although the mice in our experiments had free access to food, once switched from a pelleted to a powdered diet of identical composition they consumed higher quantities of food and gained weight much more rapidly than when they were fed pellets. Since there was no difference in body weight between the control and the treated groups, the reduction in tumor incidence, multiplicity, and volume can be attributed entirely to the presence of broccoli sprout extract powder in the diet.

It was important to establish whether the long-term feeding with glucoraphanin-rich broccoli sprout extracts affected the rate of conversion of glucoraphanin to sulforaphane. To this end, 5 mice from each group were placed in metabolic cages during the last week of the carcinogenesis experiment. Urine was collected every 24 h. As expected, no dithiocarbamates were detected in urine of animals from the control group. In the treated group, the mean 24-h urinary dithiocarbamate excretion was 0.725 ± 0.223 μmol, (or 7.25 ± 2.23% of the 10 μmol daily dose), in full agreement with the rate of conversion during the short-term (4-day) pre-intervention feeding study.

The histopathology of skin tumors induced by chronic UV radiation in SKH-1 hairless mice has been well characterized.[2,17] Lesions range histologically, from epidermal hyperplasia to squamous cell carcinoma. We stratified tumors based only upon size, using a binary classification of "large" (volume > 10 mm^3) and "small" (volume < 10 mm^3). This analysis revealed that the strongest effect of treatment was on the large (and presumably malignant) tumors. Thus, there were 12 large tumors in the control group. In contrast, there were only 2 large tumors in the treated group. The number of small tumors was decreased by ~40% in the treatment group: 66 tumors compared to 112 tumors in the control group. Thus, inclusion of broccoli sprout extract in the diet had a profound effect on: (i) the appearance of small tumors, and (ii) the progression to large tumors. This finding is different from the effect of topical application of sulforaphane-containing broccoli sprout extracts which reduced the number of small, but not of large tumors,[13] perhaps reflecting the differences in modes of delivery of the protective agent.

The exact mechanism(s) by which feeding glucoraphanin-rich broccoli sprout extracts protect against UV radiation-induced skin carcinogenesis in this model are presently unknown and are likely to be multiple. In addition to induction of cytoprotective proteins, sulforaphane inhibits pro-inflammatory pathways and, at higher concentrations, causes cell cycle arrest and apoptosis, properties which could collectively contribute to inhibition of tumor development.[12] Sulforaphane is also protective against the development of UVB-induced squamous cell carcinoma when applied topically to the mouse skin during the period of irradiation,[18] and either before,[19] after or during[20] application of 7,12-dimethylbenz[*a*]anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse models of chemical skin carcinogenesis. Inhibition of the UV radiation-induced activation of AP-1,[18] activation of Nrf2,[19] and inhibition of TPA-induced ornithine decarboxylase activity[20] all factor into the protective mechanisms of sulforaphane. In the present study it is clear that, because the animals received the protective agent after the completion of the irradiation schedule, the inhibitory effect of sulforaphane is on tumor progression, and not on tumor initiation. This model is therefore highly relevant to humans, because most individuals in the general population are exposed to UV radiation as children and decrease their exposures in adulthood, and most nonmelanoma skin cancers take many years (and often decades) to become malignant, during which time there is an opportunity to intervene with tumor development. Furthermore, the presence of broccoli and broccoli sprouts in the human diet allows the use of extracts of this plant as safe and convenient delivery vehicles for the administration of glucoraphanin and sulforaphane to humans. Notably, 3-day-old broccoli sprouts grown from selected seeds contain much more uniform and ~50 times higher inducer activity than do mature plants.[21] We have conducted a randomized, placebo-controlled, double-blind clinical Phase 1 study of safety, tolerance, and pharmacokinetics of broccoli sprout extracts containing either glucosinolates (principally glucoraphanin) or isothiocyanates (principally sulforaphane), and found no evidence of systematic, clinically significant, adverse events that could be attributed to ingestion of the sprout extracts.[22]

It should be pointed out that in mice we observe a consistent conversion to sulforaphane following oral dosing of glucoraphanin, such that the mean 24-h urinary dithiocarbamate excretion is ~5–10% of the dose. In sharp contrast, in humans there appears to be a very high interindividual variability in the excretion rates, ranging from 1 to 45% of the administered dose.[23] Such interindividual variability will undoubtedly affect the protective efficiency, as suggested by the inverse association that was observed for excretion of dithiocarbamates and two urinary biomarkers, namely aflatoxin-DNA adducts (a biomarker for aflatoxin exposure) and phenanthrene tetraols (biomarkers for exposure to the hydrocarbon air pollutant phenanthrene) in a study population in Qidong, People's Republic of China.[23] The reasons for this interindividual variation remain unknown, but probably include: (i) differences in the composition of the microflora of the gastrointestinal tract that determine the extent of hydrolysis of glucoraphanin to sulforaphane, (ii) polymorphisms in glutathione transferases that affect metabolism of sulforaphane, (iii) other factors that influence bioavailability, biotransformation,

and excretion. Detailed understanding of these factors is mandatory for the design of future human intervention studies.

In conclusion, dietary administration of glucoraphanin-rich broccoli sprout extracts reduces tumor incidence, multiplicity, and volume in SKH-1 hairless mice that were previously chronically exposed to UV radiation. Because UV radiation is the most ubiquitous environmental carcinogen and because broccoli sprouts are already available and consumed by humans, these findings can be directly translated to human populations.

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