Oral Chemoprevention of Skin Cancer in Mice by Benzophenone Sunscreens Dioxybenzone and Octabenzone in Drinking Water

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Abstract. *Background: Sunscreen compounds with added benefit of skin cancer prevention have both public and commercial interests. Our earlier study using the Epstein-Barr virus early antigen in vitro assay reported on skin cancer chemoprevention potential of benzophenone sunscreens. We now report the in vivo antitumor activity of two of the benzophenone sunscreens which tested positively in the in vitro assay, octabenzone (UV-1) and dioxybenzone (UV-2), in the two-stage mouse skin carcinogenesis model using (±)-(E)-4-methyl-2-[-(E)-hydroxyamino]-5-nitro-6-methoxy-3-hexanamide (NOR-1) as inducer and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as promoter. Materials and Methods: Pathogen-free, female hairless mice of HOS:HR-1 strain, 15 animals per control and test groups, were used. Skin tumors were induced by a single dose of NOR-1 (390 nmol in 100 μl of acetone). One week later, TPA (1.7 nmol in 100 μl of acetone) was applied to skin twice weekly for 20 weeks as tumor a promoter. The test compounds UV-I or UV-2 were administered at 0.0025% to mice through drinking water ad libitum, starting one week prior to and stopping one week after tumor initiation. All animals were examined weekly for the development of skin papillomas. Results: In both UV-1- and UV-2-treated mice, a two-week delay in tumor appearance, and significant inhibition ($p<0.001$) of tumor incidence (50% and 60%, respectively) and tumor burden (papilloma inhibition/mouse, 50% and 70%, respectively) were observed when compared to the positive control group. UV-2 (dihydroxy derivative) was a more potent inhibitor of skin tumor than UV-1 (monohydroxy derivative), which followed their antioxidant activity ranking. Conclusion: The results affirm the skin cancer chemoprevention potential of orally-ingested benzophenone sunscreens in mice and warrant studies in humans to validate synergistic protection achievable by complementation of oral and topical sunscreen usage.*

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Chemoprevention of cancer involves the use of natural or synthetic substances to suppress, delay, reverse, or prevent the development of cancer in humans (1-4). Advances in molecular genetics and tumor biology have unraveled valuable information about the genetic etiology and fundamental pathways involved in carcinogenesis. These molecular insights provide new therapeutic targets and unique opportunities to develop more effective methods of cancer management, including pre-symptomatic detection and prevention. A promising strategy of chemoprevention is identifying beneficial chemicals that humans are exposed to on a daily basis through diet, cosmetics, environment, *etc*., which may have potential for reducing tumor incidence, multiplicity and in some instances, a shift in the latency period (5, 6). Such events are more plausible if these exposures are effective in the prolonged cumulative promotional stage of cancer development, for example, during the long induction period of skin cancer by exposure to ultraviolet (UV) radiation from the sunlight and solar-simulating UV lamps in tanning salons (7, 8).

Skin cancer is the result of prolonged exposure to low-energy photons found in UV radiation which are generally not blocked by commonly used topical sunscreens. The energy transfer of these photons in the skin cells results in oxidative damage to photoprotective molecules and generates reactive oxygen species (ROS). These ROS lead to photoaging which is characterized by wrinkles, skin discoloration and eventually, cancer (9-12). Use of sunscreen and non-sunscreen (primarily of plant origin and vitamins) antioxidants to provide synergistic protection by their complementary topical and oral administrations has recently been proposed as a proactive strategy for combating skin cancer (11, 13-20).

Since 1996, we have conducted extensive studies with several plant-derived antioxidants and established their chemopreventive activity against skin carcinogenesis in several mouse models, when orally-administered in drinking water (20-23). Thus, red beetroot (*Beta vulgaris* L.) extract rich in betalain antioxidants showed significant reduction in 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted; DMBA-induced, UV-B (280-320 nm) light-promoted and (±)-(E)-4-methyl-2-[(E)-hydroxyamino]-5-nitro-6-methoxy-hexanamide (NOR-1)-induced, TPA-promoted skin tumors in mice when administered at a dose of 0.0025% in drinking water *ad libitum* (21, 22). Similarly, antioxidant-containing extract of *Lawsonia inermis* L. (henna) leaf powder and its pigment artifact, lawsone (a naphthoquinone derivative), were found to inhibit UV-B (280-320 nm)-induced, TPA-promoted skin tumor in mice when ingested at the dose of 0.0025% in drinking water (23).

Our recent investigation demonstrated the skin cancer chemoprevention potential of 14 commonly used sunscreen agents using the Epstein-Barr virus early antigen (EBV-EA) activation assay (24). This short-term *in vitro* method utilizes the EBV genome carrying human lymphoblastoid Raji cells treated with tumor promoter TPA and assesses the tumor-inhibitory effect of sunscreens on EBV-EA activation at the promotional stage of cancer development. Among the sunscreens studied, those belonging to the benzophenone chemical group (Figure 1), octabenzone (UV-1), dioxybenzone (UV-2), oxybenzone (UV-3) and sulsibenzone (UV-13) showed significant chemopreventive activity with relative ranking of UV-2 >UV-3 >UV-1 > UV-13.The dihydroxy derivative of benzophenone (UV-2) exhibited higher chemopreventive potential than the three monohydroxy derivatives, UV-1, UV-3 and UV-13, which correlated with their antioxidant activity profile.

In earlier studies with a wide variety of natural and synthetic chemopreventive substances, we observed a positive correlation between *in vitro* inhibition of EBV-EA activation and *in vivo* antitumor activity in several two-stage, experimental mouse skin carcinogenesis models (20-23, 25-27). Establishing such a correlation for benzophenone sunscreen agents would be of interest to both manufacturers and consumers of these commonly used products around the globe (28, 29). The comprehensive chemoprevention strategy of complementary oral and topical sunscreen use for control of short-term and long-term skin damages, including cancer, caused by prolonged exposure to UV radiation from the sun and tanning lamps would also be of immediate public health interest. One could envision the use of such an orally-active anti-skin cancer agent with proven human, environmental and ecological safety in public water supplies analogous to successful water fluoridation to control tooth decay initiated in the 1950s (30).

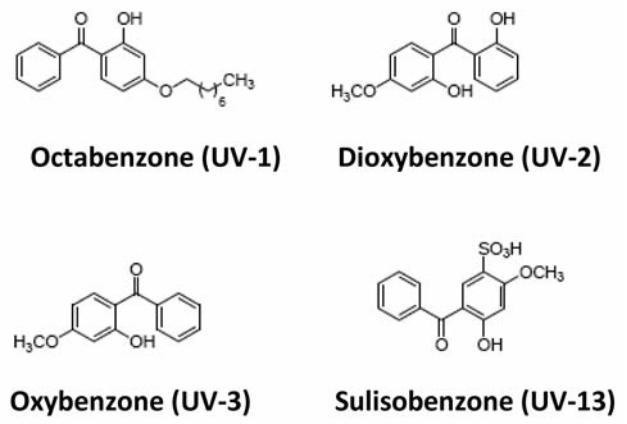

Figure 1. *Chemical structure of currently used benzophenone sunscreen agents.*

Accordingly, the objectives of this study were: to ascertain the *in vivo* antitumor activity of orally-administered UV-1 and UV-2 in the two-stage mouse skin carcinogenesis model utilizing NOR-1 as inducer and TPA as promoter of skin tumor; to evaluate potential correlation between the antioxidant property of UV-1 and UV-2 and their ability to promote anti-skin tumor activity in mice, and to examine the relevance of oral chemoprevention of skin cancer studies in mice to potential health benefits of complementary oral and topical sunscreen use in humans.

Materials and Methods

Chemicals. Acetone and TPA were procured from Wako Fine Chemical Industries, Osaka, Japan. NOR-1 was obtained from Dojindo Laboratories, Kumamoto, Japan. The sunscreens, octabenzone (UV-1) and dioxybenzone (UV-2) were purchased from Aldrich Chemical Company, Milwaukee, WI, USA. The UV number designation of benzophenones (UV-1, *etc.*, see Figure 1) follows that used in our earlier *in vitro* studies (24). All chemicals were of the highest purity available from the commercial sources indicated.

Animals. Female 6-week-old, pathogen-free, hairless mice of the HOS:HR-1 strain were purchased from Hoshino Animal

Table I. *Inhibition of NOR-1-induced, TPA-promoted skin tumors in mice by sunscreen agents octabenzone (UV-1) and dioxybenzone (UV-2).*

Group	Number of animals, start/end	Tumor incidence (%)*	Number of papillomas/ mouse (% decrease)
NOR-1 + TPA	15/15	100	10.2±1.2
NOR-1 + TPA+UV-1	15/15	60	5.1±1.3 (50%)
NOR-1 + TPA+UV-2	15/15	50	3.1±1.0 (70%)
TPA alone	15/15	0	0

*A two-week delay in tumor appearance was observed in both UV-1- and UV-2-treated mice compared to the NOR-1-treated positive control group of mice.

Factory, Saitama, Japan. The animals (5 per cage) were housed in a temperature-, humidity- and dark/light-controlled colony. They were fed *ad libitum* regular laboratory water and diet obtained from Oriental MF, Orient Yeast Company, Tokyo, Japan.

NOR-1-induced, TAP-promoted two-stage mouse skin carcinogenesis. The experimental procedure used has been described in detail elsewhere (22). Female, hairless HOS:HR-1 mice (six weeks old) in groups of 15 (housed five per cage) were used. One week prior to skin tumor initiation, 0.0025% of UV-1 and UV-2 were administered to two separate test groups of animals through drinking water *ad libitum*. The oral dosage of sunscreens was selected based on our earlier studies with other antioxidant compounds (20-23). The control group of animals received regular drinking water. Tumor was induced with a single dose of NOR-1 (390 nmol in 100 μl of acetone) applied topically on the back of all mice, both in control and test groups. One week after tumor initiation, oral feeding of UV-1 and UV-2 through drinking water in test groups was stopped and they resumed drinking regular water. Both control and test groups of animals were then topically treated with tumor promoter TPA (1.7 nmol in 100 μl of acetone) twice weekly for 20 weeks. All animals were weighed and examined weekly for the development of skin papillomas and the following were recorded: the week papillomas appeared, the number of mice bearing papillomas, the number of papillomas per mouse and animal survival. The mean water consumption per animal per day was also measured for both control and test groups.

Statistical analysis of skin tumor data. The data in Table I represent the mean±SD of 15 independent animal observations per control and test groups (n=15) with. Statistical significance between groups was calculated by analysis of variance.

Results

The benzophenone sunscreen agents, octabenzone (UV-1) and dioxybenzone (UV-2) which exhibited weak and moderate levels of antitumor promoting activity, respectively in the *in vitro* EBV-EA inhibition assay (24), were found to be effective inhibitors of skin tumor in the NOR-1- induced and TAP-promoted mouse model used in the present study. Thus, *ad libitum* oral administration of both UV-1 and UV-2 to experimental animals separately at a dose of 0.0025% in drinking water started one week prior to tumor initiation and stopped one week after tumor initiation, resulting in two-week delay in skin tumor appearance and significant inhibition ($p<0.001$) of tumor incidence (50% and 60%, respectively, see Table I) compared to the control group of mice. UV-1 and UV-2 treatment also significantly reduced ($p<0.001$) the average number of tumors formed per mouse, which were 5.1±1.3 and 3.1±1.0 papillomas/mouse, respectively, compared to 10.2±1.2 papillomas/mouse in the control group (50% and 70% inhibition, respectively), at the end of the 20-week experimental period (Table I).

There was no noticeable difference in body weight gain between the control and test groups of animals throughout the experimental period of 20 weeks. Additionally, the mean water consumption per animal per day was comparable in control and test groups. Thus, the mice of about 30 g average body weight consumed about 7 ml of water per day.

Discussion

While the cellular and molecular events that lead to UV-induced skin cancer are complex processes (10), they involve at least two distinct pathways that interact or converge (31, 32). One involves the action of UV radiation on target skin cells for neoplastic transformation and the other involves its effects on the host's immune system. Based on experimental studies, it has been proposed that the induction of skin cancer in humans may involve accumulated mutations caused by UV radiation from the sun and UV lamps used for indoor tanning. The UV absorbing sunscreens with SPF values of 15 or higher are capable of inhibiting p53 mutations with potential for protection against skin tumor development (33). We have based our investigations on the postulation that sunscreen compounds with antioxidant property may act as cellular free- radical scavengers and relieve oxidative stress involved in cancer-

causing mutagenic, inflammatory and immunosuppressive damages in skin cells resulting from prolonged, repeated exposure to UV radiation (24). With this strategy, we have successfully identified the skin cancer chemopreventive potential of a diverse group of plant-derived and synthetic antioxidants, such as beetroot (*Beta vulgaris* L.) (20-22) and henna (*Lawsonia inermis* L.) leaf (23) extracts, naphthoquinones (25) and polyphenols (26, 27).

Recently, the sunscreen compounds belonging to the benzophenone chemical category have been reported to have a scavenging effect on the oxidative stress of skeletal muscle cells (34). A plausible relationship between the antioxidant property of benzophenone sunscreens and their antitumor-promoting potential was observed in our earlier *in vitro* studies with EBV-EA activation (24). Thus, UV-2 (with two aromatic hydroxyl groups), a more potent antioxidant than the other three benzophenone sunscreens tested (UV-1, UV-3 and UV-13, all with one aromatic hydroxyl group, see Figure 1) exhibited the highest antitumor potential. In the present *in vivo* study in mice, UV-2 was also found to be a more potent inhibitor of skin papillomas than UV-1 (70% *vs.* 50%, Table I), which followed their antioxidant potency ranking. However, it should be noted that factor(s) other than or in addition to the antioxidant property may play a role in skin cancer chemoprevention activity of these mono- and dihydroxybenzophenone sunscreen agents.

Short-term ingestion of the benzophenone sunscreens, UV-1 and UV-2, during the tumor initiation period provided significant protection (>50%) against tumor initiation by NOR-1 in the present *in vivo* mouse skin carcinogenesis model employed. These two sunscreens also rendered protection against TPA-induced EBV-EA activation in our earlier *in vitro* study (24) which correlates with the promotional stage of skin carcinogenesis. Thus, we have demonstrated that the two benzophenone sunscreens tested are capable of inhibiting both initiation and promotional stages of carcinogenesis. UV radiation is known to be a complete carcinogen capable of initiation, promotion and progression, the three stages of skin carcinogenesis (10, 31). Our studies affirm that the benzophenone sunscreens (UV-1 and UV-2) have the ability to inhibit two of the three stages of skin carcinogenesis, namely initiation and promotional stages. Furthermore, the observed tumor-inhibitory activity of UV-1 and UV-2 sunscreens against the skin cancer inducer NOR-1, a nitric oxide (NO) donor, suggests they may play a role in the modulation of dermal NO synthase activity involved in UV radiation - induced skin carcinogenesis (35-38). Our earlier chemoprevention studies with extracts of beetroot and henna leaves utilizing NO as a tumor inducer have shown good correlation with their anticancer activity against skin carcinogenesis in mice exposed to UV-B light which was used both as initiator (with TPA as promoter) and promoter (with DMBA as initiator) when administered by either the oral or topical route (22, 23). Such correlation between chemoprevention of skin tumor in mice produced by NO and UV-B exposure has also been observed with sarcophine-diol, a dihydroxy derivative of marine natural product (39, 40).

Sunscreens provide protection against solar radiation by establishing a physical barrier between the skin and sunlight. Therefore, most sunscreens provide a protective effect when used topically and their systemic uptake is generally not significant (41). There appears to be no previous study on oral chemoprevention potential of antioxidant benzophenone suncreens in either animals or humans. Currently, a new trend in skin cancer prevention has emerged wherein the basal antioxidant threshold of the whole body is enhanced to increase its response to oxidative damage caused by exposure to UV radiation from the sun and tanning lamps (11-20). According to this line of thinking, oral ingestion of sunscreen compounds with potent antioxidant activity may have the potential for preventing the long-term effects of UV radiation, particularly photoaging and skin carcinogenesis. Such health benefits are expected to be achievable through small doses of sunscreens taken by the oral route. Thus, oral sunscreens complemented by traditional topical use could well become the future synergistic chemopreventive strategy for control of skin damage, including cancer, resulting from prolonged exposure to the sun and solar-simulating lamps used in tanning beds.

In the present study, the two benzophenone sunscreens (UV-1 and UV-2) were tested at the low oral dose of 0.0025% which corresponds to the consumption of about 7 ml of water per day by a mouse of about 30 g average body weight (equivalent to consuming approximately 400 mg of sunscreen/per day/70 kg person). Exposure to such levels of the two sunscreens is generally considered safe in humans (42, 43). UV-2 is among the oldest sunscreens in continued use since its introduction in the 1980s (44). Both the Food and Drug Administration in the US and the European Union approve its use in sunscreen and other cosmetic products, such as lip balm and anti-aging cream (45-47). UV-1 is suitable for use in sunscreen compositions as photoactive agent and approved for use as UV stabilizer in polyolefinic plastics used in food packaging. Their use in toothpaste and mouthwash to maintain general oral health by preventing dental plaque buildup and gingivitis, and in gum care products to treat periodontal disease is of current interest in dentistry (48, 49).

Conclusion

With the objective of identifying which of the currently used sunscreens have added benefit of protection against skin cancer, we have developed a reliable screening protocol consisting of the *in vitro* EBV-EA activation assay, as reported

earlier (24), followed by the *in vivo* confirmation test in the two-stage mouse skin cancer model utilizing NOR-1 as inducer and TPA as promoter of tumor, as outlined in the present study. Thus, using the above protocol, the benzophenone sunscreens, octabenzone (UV-1) and dioxybenzone (UV-2) were found to exhibit significant chemopreventive activity against mouse skin carcinogenesis which correlated with their antioxidant potency. Consequently, the benzophenone sunscreens may be considered as the latest addition to the new generation of orally active chemopreventive agents against skin carcinogenesis (11-20). Such oral use of sunscreens supplemented by their traditional topical application has the potential for comprehensive protection against both short-term and long-term skin damage caused by UV radiation from the sun and tanning salon lamps. In support of this postulation, recent preliminary clinical trials with several oral antioxidant formulations have provided encouraging results (13, 15, 16, 18, 19, 50).

The results from the present animal study affirm the skin cancer chemoprevention potential of orally administered benzophenone sunscreens. Recent epidemiological studies indicate reduced incidence of melanomas among topical sunscreen users (51-54). Further investigations, especially targeted epidemiological surveys, are warranted to ascertain whether such skin cancer reduction is demonstrable in populations around the globe who regularly use topical benzophenone sunscreens. Potential benefits of complementary oral ingestion and topical use of these sunscreens in achieving comprehensive chemoprevention of skin cancer caused by exposure to the sun and tanning lamps also needs to be investigated. In this context, our recent proposal of dietary supplementation with beetroot extract, an efficient inhibitor of skin carcinogenesis and currently approved for use in humans as red food color E162, to complement topically applied popular sunscreens with proven antiskin cancer activity is noteworthy (20-22).

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