

## Caffeine and caffeine sodium benzoate have a sunscreen effect, enhance UVB-induced apoptosis, and inhibit UVB-induced skin carcinogenesis in SKH-1 mice

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**Topical application of caffeine sodium benzoate (caffeine-SB) immediately after UVB irradiation of SKH-1 mice enhanced UVB-induced apoptosis by a 2- to 3-fold greater extent than occurred after the topical application of an equimolar amount of caffeine. Although topical application of caffeine-SB or caffeine enhanced UVB-induced apoptosis, both substances were inactive on non-UVB-treated normal skin. Topical application of caffeine-SB or caffeine (each has UVB absorption properties) 0.5 h before irradiation with a high dose of UVB decreased UVB-induced thymine dimer formation and sunburn lesions (sunscreen effect). Caffeine-SB was more active than an equimolar amount of caffeine in exerting a sunscreen effect. In additional studies, caffeine-SB strongly inhibited the formation of tumors in UVB-pretreated 'high-risk mice' and in tumor-bearing mice, and the growth of UVB-induced tumors was also inhibited. Caffeine-SB and caffeine are the first examples of compounds that have both a sunscreen effect and enhance UVB-induced apoptosis. Our studies suggest that caffeine-SB and caffeine may be good agents for inhibiting the formation of sunlight-induced skin cancer.**

### Introduction

In earlier studies, we demonstrated an inhibitory effect of oral administration of caffeine on the formation of ultraviolet B (UVB)-induced non-malignant and malignant skin tumors (keratoacanthomas and squamous cell carcinomas) in SKH-1 hairless mice (1,2). Mechanistic studies indicated that oral administration of caffeine for 2 weeks prior to a single irradiation with UVB enhanced UVB-induced increases in apoptosis in the epidermis of SKH-1 mice (3). In addition, topical application of caffeine immediately after a single irradiation with UVB also enhanced UVB-induced apoptosis in the epidermis (4). Oral administration or topical application

**Abbreviations:** AUC, area under the curve; caffeine-SB, caffeine sodium benzoate; UVB, ultraviolet B.

of caffeine did not affect apoptosis in normal non-UVB-treated skin (3,4).

In recent studies, we treated SKH-1 mice with UVB twice a week for 20 weeks. These mice have no tumors, but they have a high risk of developing tumors during the next several months ('high-risk mice') (2). Oral administration of caffeine to these 'high-risk mice' inhibited the subsequent formation of skin tumors (2). Topical applications of caffeine to these 'high-risk mice' also inhibited tumorigenesis and selectively increased apoptosis in the tumors but not in non-tumor areas of the epidermis (5).

In the present study, we explored the effects of several commonly used caffeine complexes on UVB-induced apoptosis and searched for complexes more active than caffeine as stimulators of UVB-induced apoptosis. We identified caffeine sodium benzoate (caffeine-SB) as a highly active agent when given immediately after UVB irradiation.

We also explored the effect of caffeine and caffeine-SB as potential sunscreens when given before UVB irradiation, and we found that topical application of caffeine or caffeine-SB prior to UVB irradiation decreased the formation of UVB-induced thymine dimers. Our studies identified caffeine and caffeine-SB as compounds that have both a sunscreen effect and enhance UVB-induced apoptosis.

### Materials and methods

#### *Chemicals and animals*

Acetone (HPLC grade) and 10% phosphate-buffered formalin were obtained from Fisher Scientific (Springfield, NJ). Dermabase cream was purchased from Paddock Laboratories (Minneapolis, MN). It is a washable, oil-in-water emulsion base that contains: purified water, mineral oil, petrolatum, cetostearyl alcohol, propylene glycol sodium lauryl sulfate, isopropyl palmitate, imidazolidinyl urea, methylparaben and propylparaben.

Caffeine (>99% purity), sodium benzoate and caffeine nicotinate were obtained from the Sigma Chemical (St Louis, MO). Caffeine-SB, caffeine citrate and caffeine triiodide were purchased from MP Biomedicals (Aurora, OH).

Female SKH-1 hairless mice (6–7 weeks old) were purchased from the Charles River Breeding Laboratories (Kingston, NY), and the animals were kept in our animal facility for at least 1 week before use. Mice were given water and Purina Laboratory Chow 5001 diet from Ralston Purina (St Louis, MO) *ad libitum*, and they were kept on a 12 h light/12 h dark cycle.

#### *Exposure of mice to UVB and the preparation of skin sections*

The UV lamps used (FS72T12-UVB-HO; National Biological; Twinsburg, Ohio) emitted UVB (280–320 nm; 75–80% of total energy) and UVA (320–375 nm; 20–25% of total energy). The dose of UVB was quantified with a UVB Spectra 305 dosimeter (Daevlin, Byran, OH). The radiation was further calibrated with a model IL-1700 research radiometer/photometer (International Light, Neburgport, MA).

For studies on the effects of caffeine or caffeine complexes on UVB-induced apoptosis, female SKH-1 mice (7–8 weeks old, 5 mice/group) were treated topically with caffeine or a caffeine complex immediately after a single irradiation with UVB (30 mJ/cm<sup>2</sup>), and the animals were killed 6 h later. Our previous studies indicated that 6–10 h after UVB exposure is the peak time for the formation of UVB-induced apoptotic sunburn cells (3,4).

For studies on the effects of caffeine or caffeine-SB on UVB-induced thymine dimer formation, female SKH-1 mice (7–8 weeks old, 5 mice/group)

were treated topically with 100 mg Dermabase cream, 4.8% caffeine or 8.0% caffeine-SB in 100 mg of Dermabase cream 0.5 h before a single irradiation with UVB (180 mJ/cm<sup>2</sup>). The animals were killed just before UVB exposure and at 0.5, 2, 6, 10, 16, 24 and 48 h after UVB.

For studies on the effects of caffeine or caffeine-SB on UVB-induced sunburn skin lesions, female SKH-1 mice (7–8 weeks old; 5 mice/group) were treated topically with 100 mg Dermabase cream, 4.8% caffeine or 8.0% caffeine-SB in 100 mg Dermabase cream 0.5 h before UVB (180 mJ/cm<sup>2</sup>), and these treatments were repeated 24 h later. The area (mm<sup>2</sup>) and the intensity of red color (erythema) in UVB-induced skin lesions were estimated 5 days after the first UVB treatment. The sunburn lesion index was calculated as the lesion area multiplied by the arbitrary intensity: 0, no lesion; 1, barely detectable red lesion; 2, moderate red lesion; and 3, bright red lesion.

Skin samples, ~2 cm length and 0.5 cm width, were taken from the middle of the back, stapled flat to a plastic sheet and placed in 10% phosphate-buffered formalin at 4°C for 18–24 h. The skin samples were then dehydrated in ascending concentrations of ethanol (80, 95 and 100%), cleared in xylene, and embedded in Paraplast (Oxford Labware, St Louis, MO). Serial sections of skin of 4 μm thickness containing the epidermis and dermis were made, deparaffinized, rehydrated with water and used for regular hematoxylin–eosin (H&E) or immunohistochemical staining.

For cancer prevention studies, female SKH-1 mice (7–8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice a week for 20 weeks and UVB treatment was stopped. These tumor-free mice with a high risk of developing skin tumors ('high-risk mice') were randomized and divided into five groups (30 mice/group). Each group was treated topically on the back with 100 mg Dermabase cream, caffeine (1.2 or 3.6%) in Dermabase cream, or an equimolar amount of caffeine-SB (2.0 or 6.0%) in Dermabase cream once a day, 5 days a week for 17 weeks. Tumors on the treated areas of the mice were counted every 2 weeks. All mice were killed at 2 h after the last application of caffeine or caffeine-SB and all the tumors were taken and characterized by histological examination.

For studies on the treatment of tumor-bearing mice, female SKH-1 mice (7–8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice weekly for 20 weeks and UVB irradiation was stopped. These mice developed skin tumors gradually and at 13 weeks after stopping UVB, mice with skin tumors were equally divided into two groups (20 mice/group) according to the body weight, number of tumors per mouse and tumor volume per mouse. One group of mice was treated topically with 100 mg Dermabase cream and another group of mice was treated with 4% caffeine-SB in Dermabase cream once a day 5 days a week for 8 weeks. The number and size of the tumors in the animals were recorded every 2 weeks. All mice were sacrificed after 8 weeks of treatment with Dermabase cream or caffeine-SB in Dermabase cream.

For histopathological examination, the animals were killed and the dorsal skins were taken to include each of the grossly observed masses in the treated areas of the mice. The skins were stapled flat to a plastic sheet and placed in 10% phosphate-buffered formalin at 4°C for 24 h. The skin samples were then dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in Paraplast. Sections of skin of 4 μm thickness were made, deparaffinized, rehydrated with water and used for regular H&E staining. The counting and characterization of all tumors were done blind with respect to treatment group as described previously (2,6).

#### Measurement of apoptotic sunburn cells

Identification of apoptotic sunburn cells in the epidermis was based morphologically on cell membrane shrinkage and nuclear condensation of keratinocytes attributable to fragmentation of the cells (7,8). Earlier studies demonstrated that sunburn cells are indeed apoptotic cells (9). Apoptotic sunburn cells were identified in the epidermis by their intensely eosinophilic cytoplasm and small, dense nuclei, which were observed in H&E-stained histological sections of the skin under 400-fold magnification using a light microscope. The percentage of apoptotic sunburn cells in the epidermis (basal plus suprabasal layers) was calculated from the number of these cells per 100 cells counted from the entire 20-mm length of epidermis for each skin section.

#### Thymine dimer detection *in situ*

Thymine dimers in epidermal cells were detected by a horseradish peroxidase (HRP)-labeled monoclonal anti-thymine dimer antibody (KAMIYA Biomedical Company, Seattle, WA) and visualized using streptavidin-peroxidase and 3,3'-diaminobenzidine which stains thymine dimer-containing nuclei a dark brown as we described previously (8). Briefly, endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 10 min. The slides were then incubated with 0.125% trypsin for 10 min at 37°C. After rinsing in deionized water,

tissue sections were incubated with 1 N HCl for 30 min, followed by incubation with goat serum for 10 min. The sections were covered with mouse monoclonal anti-thymine dimer antibody for 90 min, and then incubated with conjugated streptavidin solution for 5 min at 37°C. Color development was achieved by incubation with a substrate solution containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide. The slides were counterstained in Mayer's hematoxylin (Sigma Chemical), cleared with xylene, mounted with a coverslip and scored under a light microscope. The percentage of thymine dimer positive cells in the epidermis for each mouse was calculated from the number of stained thymine dimer positive cells per 100 cells counted from the entire 20-mm length of epidermis for each skin section.

#### Measurement of tumor size

Tumor volume was determined by measuring the 3D size (height, length and width) of each mass. The average of the three measurements was used as the diameter. The radius (*r*) was determined, and the volume was calculated by: Volume = 4π*r*<sup>3</sup>/3 (5).

#### Statistical analysis

The Student's *t*-test was used for simple comparisons of two groups. The analysis of variance (ANOVA) model with Dunnett's adjustment was used for comparisons of multiple treatment groups with a common control group.

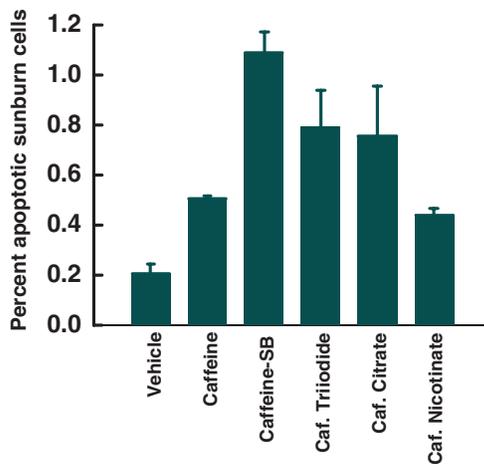
The area under the curve (AUC) was used to compare the response variables over time between treatment groups, and the peak response values over time between treatment groups. The responses were percent apoptotic sunburn cells, percent thymine dimer positive cells and the ratio of these two percentages. The test of difference of AUC between two treatment groups was based on Bailer's method (10), with which the trapezoidal rule was used to estimate the AUC. The peak time of the response was estimated by the time at which the observed mean response was maximum. The ANOVA model was used for the comparison of the peak responses among the three treatment groups. If the differences among the three treatment groups were significant, pair-wise comparisons between two groups were conducted using Tukey's multiplicity adjustment. All tests were conducted at the 5% significance level. Using the Bonferroni correction, pair-wise comparisons of AUCs of the three groups were significant at the 5% level when the *P*-value was <0.0167 (= 0.05/3).

## Results

### *Effects of topical application of caffeine or caffeine complexes immediately after UVB irradiation on UVB-induced increases in epidermal apoptotic sunburn cells in SKH-1 mice*

In earlier studies, we found that topical application of caffeine immediately after UVB irradiation stimulated UVB-induced apoptosis and the peak time for the formation of UVB-induced apoptotic sunburn cells is 6–10 h after UVB exposure (4). In the present study, we found that topical application of certain caffeine complexes immediately after UVB irradiation was more active than application of caffeine in stimulating UVB-induced apoptosis in the epidermis (Figure 1). Topical applications of caffeine (6.2 μmol) in 90% acetone/10% water immediately after UVB irradiation and 30 and 120 min later stimulated UVB-induced apoptosis by 148% at 6 h after UVB whereas applications of equimolar amounts of caffeine-SB, caffeine triiodide or caffeine citrate enhanced UVB-induced apoptosis by 434, 287 and 271%, respectively (Figure 1). Caffeine nicotinate was somewhat less active than caffeine. In a separate experiment, dissolving caffeine and sodium benzoate in 90% acetone/10% water resulted in a solution that was less active than caffeine-SB and had about the same activity as caffeine at enhancing UVB-induced apoptosis (data not presented).

In additional studies, we found that a single topical application of caffeine or caffeine-SB in Dermabase cream (an oil-in-water emulsion used in humans) was more effective than application in a 90% acetone/10% water solution for enhancing UVB-induced apoptosis (data not



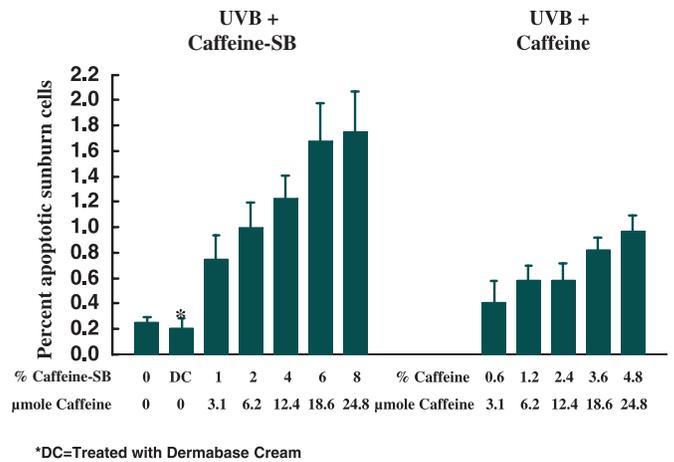
**Fig. 1.** Effects of topical application of caffeine or different caffeine complexes immediately after UVB irradiation on UVB-induced increases in apoptosis in the epidermis of SKH-1 mice. Female SKH-1 mice (5 mice/group) were exposed to UVB (30 mJ/cm<sup>2</sup>) once followed immediately by treatment with caffeine, caffeine-SB, caffeine triiodide (Caf. Triiodide), caffeine citrate (Caf. Citrate) or caffeine nicotinate (Caf. Nicotinate) (6.2 μmol in 100 μl of 90% acetone/10% water), and another two applications were at 0.5 and 2 h after UVB. The mice were killed at 6 h after UVB, and apoptotic sunburn cells in the epidermis were determined morphologically. Each value represents the mean ± SE from five mice.

presented). We prepared various concentrations of caffeine (0.6, 1.2, 2.4, 3.6 or 4.8%) and equimolar concentrations of caffeine-SB (1.0, 2.0, 4.0, 6.0 or 8.0%) in Dermabase cream. A single topical application of 100 mg Dermabase cream containing caffeine or caffeine-SB to the skin of SKH-1 mice immediately after UVB strongly enhanced UVB-induced apoptosis in the epidermis whereas Dermabase cream alone was inactive (Figure 2). Caffeine-SB was ~2- to 3-times more active than caffeine in stimulating UVB-induced apoptosis (Figure 2).

*Effects of topical application of caffeine or caffeine-SB 0.5 h before UVB irradiation, on UVB-induced thymine dimer formation and apoptotic sunburn cells in the epidermis of SKH-1 mice*

**Thymine dimer formation.** Since our UV lamps emit light predominately between 280 and 320 nm (see Materials and methods section) and caffeine and caffeine-SB have appreciable UVB absorption between 260 and 300 nm (with a peak at ~273 nm), we studied the time course for the effects of pretreatment with caffeine or caffeine-SB on UVB-induced thymine dimer formation in the epidermis. In this study, female SKH-1 mice were treated topically with 100 mg Dermabase cream, 4.8% caffeine (24.8 μmol) or 8.0% caffeine-SB (24.8 μmol) in Dermabase cream 0.5 h before a single irradiation with UVB (180 mJ/cm<sup>2</sup>). The animals were killed just before UVB exposure and at 0.5, 2, 6, 10, 16, 24 and 48 h after UVB. Thymine dimer positive cells in the epidermis were detected by immunohistochemistry. The results indicated a strong inhibitory effect of pretreatment of the mice with caffeine or caffeine-SB on UVB-induced thymine dimer formation at each time interval after UVB irradiation (Figure 3A).

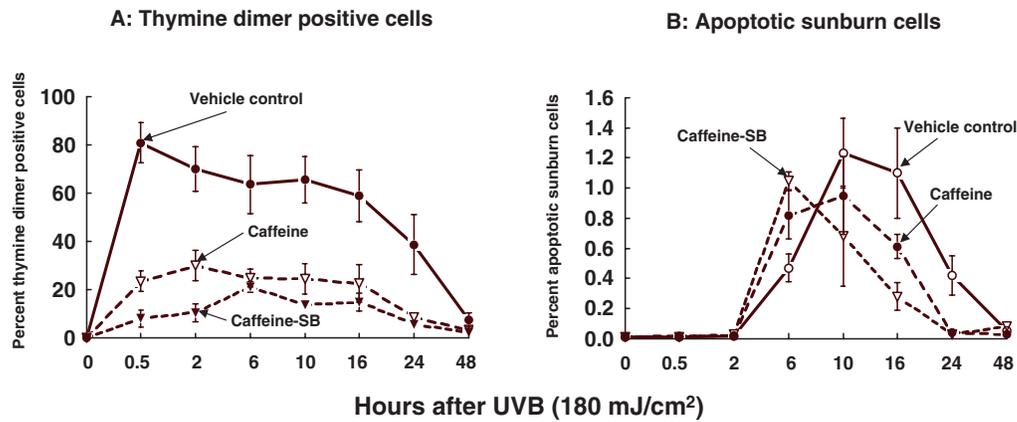
The values for the area under the curve (AUC) for percent thymine dimer positive cells × h (0 → 48 h) after UVB irradiation in the Dermabase cream control, caffeine and



**Fig. 2.** Dose-response for effects of topical application of caffeine or caffeine-SB in Dermabase cream immediately after UVB irradiation on UVB-induced apoptosis in the epidermis of SKH-1 mice. Female SKH-1 mice (7–8 weeks old, 5 mice/group) were treated topically once with (A) 100 mg Dermabase cream per mouse containing 3.1, 6.2, 12.4, 18.6 or 24.8 μmol of caffeine (0.6–4.8%) or (B) 100 mg Dermabase cream containing equimolar amounts of caffeine-SB (1.0–8.0%) immediately after irradiation with 30 mJ/cm<sup>2</sup> of UVB. The control animals were either untreated or treated with Dermabase cream (DC) alone immediately after irradiation with 30 mJ/cm<sup>2</sup> of UVB. All animals were killed 6 h after UVB. Apoptotic sunburn cells in the epidermis were determined morphologically. Animals without UVB treatment had 0.01 ± 0.01% apoptotic cells in their epidermis. Each value represents the mean ± SE from five mice.

caffeine-SB groups were 1982 ± 225, 673 ± 71 and 413 ± 41 (mean ± SE), respectively. The AUC (0 → 48 h) for the Dermabase cream group was significantly different from the AUC for the caffeine or caffeine-SB groups ( $P < 0.0001$ ). The difference in AUC between the caffeine and caffeine-SB groups was also significant ( $P = 0.0016$ ). Topical application of caffeine or caffeine-SB 0.5 h prior to UVB exposure decreased the AUC (0 → 48 h) for thymine dimer positive cells by 66 or 79%, respectively. It was of interest that the peak values for thymine dimer positive cells for the Dermabase cream control, caffeine and caffeine-SB groups occurred at 0.5, 2 and 6 h, respectively (Figure 3A).

**Apoptotic sunburn cells.** Pretreatment of SKH-1 mice with caffeine or caffeine-SB 0.5 h before irradiation with UVB increased the percentage of apoptotic sunburn cells at 6 h after UVB irradiation (compared with vehicle and UVB treatment) even though the thymine dimer level was decreased (Figure 3B). This was not observed at later time intervals (Figure 3B). The AUC values (from 0 to 48 h after UVB) for the ratio of the percent apoptotic sunburn cells/percent thymine dimer positive cells for the Dermabase cream control, caffeine and caffeine-SB groups were 62.0 ± 10.8, 83.0 ± 14.7 and 123.5 ± 20.0 (mean ± SE), respectively, indicating that pretreatment with caffeine or caffeine-SB enhanced the ratio of apoptotic sunburn cells/thymine dimer positive cells by 34 and 99%, respectively. The AUC ratio (0 → 48 h) for the Dermabase cream group was significantly different from the AUC ratio for the caffeine-SB group ( $P = 0.0068$ ). Examination of the ratios of apoptotic sunburn cells/thymine dimer positive cells between 0.5 and 10 h after UVB (times prior to and during maximum apoptosis response) revealed that pretreatment of the mice with caffeine or caffeine-SB increased these ratios by >100% at each time



**Fig. 3.** Effect of topical application of caffeine or caffeine-SB 0.5 h before UVB irradiation on UVB-induced formation of thymine dimers and apoptotic cells in the epidermis of SKH-1 mice. Female SKH-1 mice (7–8 weeks old) were treated topically with 100 mg Dermabase cream, or with 4.8% caffeine or 8.0% caffeine-SB in Dermabase cream 0.5 h before a single irradiation with UVB (180 mJ/cm<sup>2</sup>). The animals were killed just before or at 0.5, 2, 6, 10, 16, 24 or 48 h after UVB. (A) thymine dimer positive cells and (B) apoptotic sunburn cells. Each value represents the mean  $\pm$  SE from five mice.

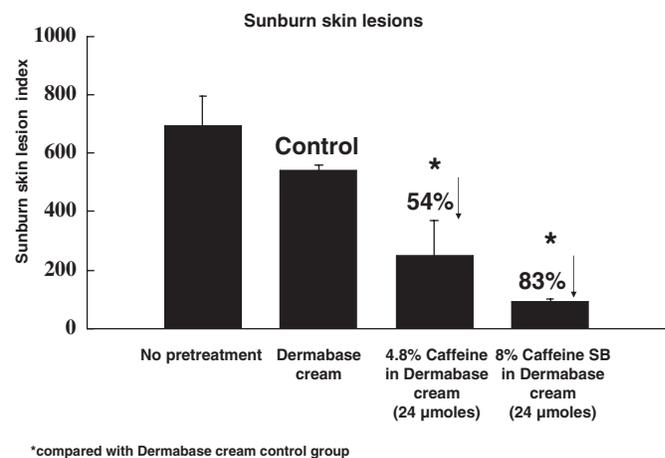
interval studied. Overall, the data suggest that pretreatment of mice with caffeine or caffeine-SB, 0.5 h before UVB irradiation, has a potent sunscreen effect and also enhances apoptosis when corrected for thymine dimers.

#### *Effects of topical application of caffeine or caffeine-SB, 0.5 h before UVB irradiation on UVB-induced sunburn skin lesions in the epidermis of SKH-1 mice*

In a separate study, we pretreated mice topically with 100 mg Dermabase cream or with 4.8% caffeine or 8.0% caffeine-SB (equimolar amounts of caffeine) in Dermabase cream 0.5 h prior to a high dose of UVB (180 mJ/cm<sup>2</sup>) on two consecutive days, and sunburn skin lesions were evaluated 5 days after the first UVB treatment. The results showed that pretreatment of the mice with caffeine inhibited UVB-induced formation of 'skin sunburn lesions' by 54% when compared with the Dermabase cream control and that pretreatment with an equimolar amount of caffeine-SB had an even stronger inhibitory effect (83% inhibition, Figure 4) when compared with the Dermabase cream vehicle control. Histology studies indicated that topical application of caffeine or caffeine-SB, 0.5 h prior to each irradiation with UVB, had a strong inhibitory effect on UVB-induced epidermal hypertrophy and other UVB-induced changes observed at 5 days after the first irradiation with UVB (data not presented). The results of our studies indicated that pretreatment of mice with caffeine or caffeine-SB had a strong protective effect on UVB-induced sunburn lesions.

#### *Effects of topical applications of caffeine or caffeine-SB on the formation of UVB-induced skin tumors in SKH-1 mice previously treated with UVB for 20 weeks ('high-risk mice')*

SKH-1 mice were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice a week for 20 weeks, and UVB treatment was stopped. These tumor-free mice with a high risk of developing papillomas, keratoacanthomas and squamous cell carcinomas during the following several months in the absence of further treatment with UVB ('high-risk mice') were randomized and divided into five groups (30 mice/group). Each group was treated topically on the back with 100 mg of Dermabase cream or with caffeine (1.2 or 3.6%) in Dermabase cream or an equimolar amount of caffeine-SB (2.0 or 6.0%) in Dermabase

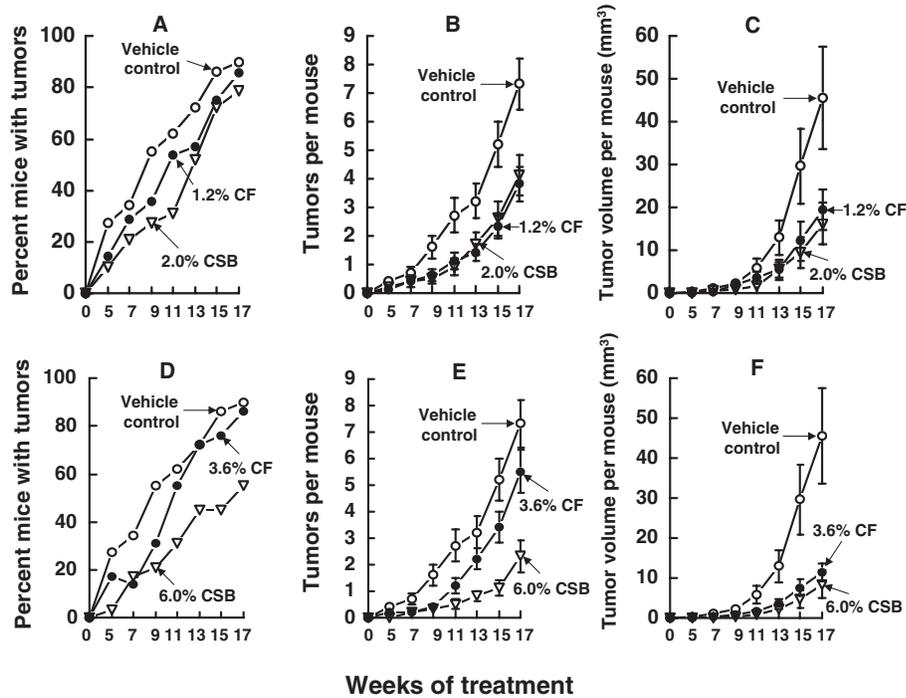


**Fig. 4.** Effect of topical application of caffeine or caffeine-SB 0.5 h before UVB irradiation on UVB-induced sunburn skin lesions. Female SKH-1 mice (7–8 weeks old; 5 mice/group) were treated topically with 100 mg Dermabase cream, or with 4.8% caffeine or 8.0% caffeine-SB in Dermabase cream 0.5 h before UVB (180 mJ/cm<sup>2</sup>), and these treatments were repeated 24 h later. The area (mm<sup>2</sup>) and the intensity of red color in UVB-induced skin lesions were estimated 5 days after the first UVB treatment. The sunburn lesion index was calculated as the lesion area (mm<sup>2</sup>) multiplied by the arbitrary intensity: 0, no lesion; 1, barely detectable red lesion; 2, moderate red lesion; and 3, bright red lesion. Each value is the mean  $\pm$  SE from five mice.

cream once a day 5 days a week for 17 weeks. Tumors on the treated areas of the mice were counted every 2 weeks, and all the tumors were characterized by histological examination.

The results showed that although a similar chemopreventive activity was observed when the 'high-risk mice' were treated with a low dose of caffeine or an equimolar dose of caffeine-SB (Figure 5A–C), the caffeine-SB was more active than caffeine when a high equimolar dose was used (Figure 5D–F).

Histological examination of all tumors from the mice that received the high dose of caffeine (3.6%) or the equimolar high dose of caffeine-SB (6.0%) revealed that the number of squamous cell carcinomas per mouse was decreased by 77 and 88%, respectively, and the number of non-malignant tumors (papillomas + keratoacanthomas) per mouse was



**Fig. 5.** Inhibitory effect of topical application of caffeine or caffeine-SB on the formation of skin tumors in 'high-risk SKH-1 mice' previously treated with UVB. Female SKH-1 mice (7–8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice weekly for 20 weeks, and these tumor-free mice with a high risk of developing skin tumors were treated topically with 100 mg Dermabase cream with caffeine (CF: 1.2 or 3.6%), or with an equimolar amount of caffeine-SB (CSB: 2.0 or 6.0%) in Dermabase cream once a day 5 days a week for 17 weeks. Tumors on the treated areas of the mice were counted every 2 weeks. All the mice were killed at 2 h after the last application of caffeine or caffeine-SB and all the tumors were taken and characterized by histological examination. Each value is the mean  $\pm$  SE.

decreased by 56 and 75%, respectively. Topical application of 6.0% caffeine-SB inhibited by 76% the total number of tumors per mouse (both malignant and non-malignant tumors) and this effect is significantly greater than that for the 3.6% caffeine group (57% inhibition,  $P < 0.05$ ) (Table I). Topical applications of 3.6% caffeine in Dermabase cream decreased the volume of squamous cell carcinomas per mouse by 97%, and the volume of non-malignant tumors per mouse was decreased by 81%. Topical applications of an equimolar concentration of 6.0% caffeine-SB decreased the volume of squamous cell carcinomas per mouse by 93% and the volume of non-malignant tumors per mouse was decreased by 88% (Table II). In conclusion, the results of our study indicate that topical applications of caffeine or caffeine-SB in Dermabase cream to 'high-risk mice' previously exposed to UVB markedly inhibited the formation and decreased the size of both malignant and non-malignant skin tumors.

#### *Effect of topical applications of caffeine-SB on the growth of UVB-induced skin tumors in tumor-bearing SKH-1 mice*

In a pilot study, female SKH-1 mice (7–8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice weekly for 20 weeks, and UVB irradiation was stopped. These mice developed skin tumors gradually, and 13 weeks after the last irradiation with UVB, the mice with skin tumors were equally divided into two groups (20 mice/group with an equal number of tumors per mouse and total tumor volume per mouse). One group of mice was treated topically with 100 mg of Dermabase cream and another group of mice was treated with 4% caffeine-SB in Dermabase cream once a day 5 days a week for 8 weeks. The number and size of the tumors were

recorded every 2 weeks, and all mice were sacrificed after 8 weeks of treatment. The results indicated that topical applications of caffeine-SB in Dermabase cream inhibited the formation of new tumors by 45%, and the increase in tumor size per mouse was inhibited by 65% (Figure 6).

## Discussion

It was more than 30 years ago that Zajdela and Latarjet (11) found an inhibitory effect of topical applications of caffeine on UVB-induced tumorigenesis in mice, and they provided evidence that caffeine was inhibiting UVB-induced carcinogenesis by a cAMP-independent mechanism (12). In more recent studies, we demonstrated an inhibitory effect of orally administered caffeine on UVB-induced carcinogenesis when caffeine was administered during the course of UVB treatment (1) or in UVB-pretreated mice after stopping UVB (2). In additional studies, we demonstrated that topical application of caffeine immediately after a single irradiation of SKH-1 mice with UVB enhanced UVB-induced epidermal apoptosis (4). In the present study, we found that topical application of caffeine-SB immediately after UVB exposure was 2- to 3-fold more potent than caffeine at enhancing UVB-induced apoptosis, and caffeine citrate was also more active than caffeine. It is not known if the enhanced activity of the caffeine complexes was because of enhanced absorption of caffeine into epidermal cells or because of strong intrinsic biological activity of these complexes. It is of interest that intravenous injections of caffeine-SB have been used for the treatment of headaches in patients given spinal anesthesia (13), and caffeine-SB has also been used to treat respiratory

**Table 1.** Inhibitory effect of topical applications of caffeine or caffeine-SB in Dermabase cream on the formation of skin tumors in 'high-risk SKH-1 mice' previously treated with UVB

Treatment	No. of mice	Squamous cell papillomas		Keratoacanthomas		Total non-malignant tumors		Squamous cell carcinomas		Total tumors	
		Percent of mice with tumors	Tumors/mouse	Percent of mice with tumors	Tumors/mouse	Percent of mice with tumors	Tumors per mouse	Percent of mice with tumors	Tumors/mouse	Percent of mice with tumors	Tumors/mouse
Dermabase	28	7	0.11 ± 0.08	96	11.36 ± 1.37	96	11.46 ± 1.39	50	0.93 ± 0.22	96	12.39 ± 1.53
3.6% Caffeine	29	3 (-57)	0.07 ± 0.07 (-36)	86 (-10)	5.00 ± 0.92 <sup>a</sup> (-56)	86 (-10)	5.07 ± 0.93 <sup>a</sup> (-56)	17 <sup>a</sup> (-66)	0.21 ± 0.09 <sup>a</sup> (-77)	86 (-10)	5.28 ± 0.93 <sup>a</sup> (-57)
6% Caffeine-SB	27	0 (-100)	0.00 ± 0.00 (-100)	74 <sup>b</sup> (-23)	2.89 ± 0.64 <sup>a,c</sup> (-75)	74 <sup>b</sup> (-23)	2.89 ± 0.64 <sup>a,c</sup> (-75)	11 <sup>a</sup> (-78)	0.11 ± 0.06 <sup>a</sup> (-88)	74 <sup>b</sup> (-23)	3.00 ± 0.69 <sup>b</sup> (-76)

Female SKH-1 mice (7–8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice weekly for 20 weeks and UVB was stopped. These tumor-free mice were treated topically with 100 mg Dermabase cream 3.6% caffeine or 6.0% caffeine-SB (equimolar concentration) in 100 mg Dermabase cream once a day 5 days a week for 17 weeks as described in Figure 4. The mice were sacrificed at 2 h after the last application of caffeine or caffeine-SB. All tumors were characterized by histopathology studies. Each value is the mean ± SE, and the numbers in parentheses represent percent decrease when compared with the Dermabase cream group.

<sup>a</sup>*P* < 0.01; <sup>b</sup>*P* < 0.05 (comparison with the Dermabase cream control).

<sup>c</sup>*P* < 0.05 (comparison with the 3.6% caffeine group).

depression associated with overdoses of central nervous system depressant drugs or respiratory failure due to electric shock (13). Caffeine citrate has been used primarily to treat idiopathic headache and for the short-term treatment of apnea in premature infants (14).

Since both caffeine and caffeine-SB absorb UVB light, we investigated the possibility that these compounds have sunscreen activity. Indeed, application of both compounds prior to UVB irradiation inhibited UVB-induced formation of thymine dimers in DNA, and these treatments also inhibited the formation of UVB-induced sunburn lesions. The data described in the present report as well as in our earlier reports (3–5) suggest that both caffeine and caffeine-SB inhibit UVB-induced carcinogenesis by functioning both as a sunscreen (by decreasing DNA damage), as well as by enhancing apoptosis in DNA damaged cells. To the best of our knowledge, caffeine and caffeine-SB are the first examples of substances that have both a sunscreen effect and enhance UVB-induced apoptosis. Although topical application of caffeine or caffeine-SB, 0.5 h prior to UVB irradiation, markedly decreased the level of thymine dimers in DNA at all time intervals after UVB irradiation, we unexpectedly observed a gradual increase in the level of thymine dimers in DNA between 0.5–6 h post-UVB in the caffeine and caffeine-SB groups (Figure 3A). The time for the peak level of epidermal thymine dimers for control, caffeine and caffeine-SB-treated animals was 0.5, 2 and 6 h after exposure to UVB, respectively. The reason for this effect is unknown, but it may be related to an inhibitory effect of caffeine and caffeine-SB on the repair of thymine dimers in DNA. The ability of caffeine to inhibit DNA repair in cultured cells is well-known (15–17), but its effect on DNA repair *in vivo* in animals is not known.

Mechanistic studies indicate that topical applications of caffeine can enhance UVB-induced apoptosis in mouse skin by a p53- and bax- independent process (18), and caffeine also abrogates the ATR-dependent G<sub>2</sub>/M checkpoint thereby enhancing premature chromatin condensation and cell death in cultured cells (19). Preliminary studies in our laboratory indicate that administration of caffeine inhibits UVB-induced phosphorylation of Chk1 (Ser 345) and enhances premature mitosis and cell death in UVB-irradiated mouse epidermis.

The use of conventional sunscreens that inhibit UVB-induced DNA damage is a major recommendation for inhibiting sunlight-induced skin cancer in humans (20–23), and the topical application of T4 endonuclease V - containing liposomes to enhance the repair of UVB-induced DNA damage in the skin has also been studied (24,25). The topical application of caffeine or caffeine-SB is an alternative approach for inhibiting sunlight-induced skin cancer. Topical application of caffeine or caffeine-SB enhances UVB-induced apoptosis and has a sunscreen effect. In addition, topical applications of caffeine stimulates apoptosis in pre-neoplastic lesions in the skin and in tumors (5,26). We anticipate that caffeine-SB will have a similar effect.

Administration of caffeine orally or by injection inhibits carcinogenesis in several animal models, but carcinogenesis is enhanced in some animal models (reviewed in ref. 27). For instance, oral administration of caffeine to rats inhibits 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced breast cancer (28) and 2-acetylaminofluorene-induced liver cancer (29), but PhIP-induced colon cancer

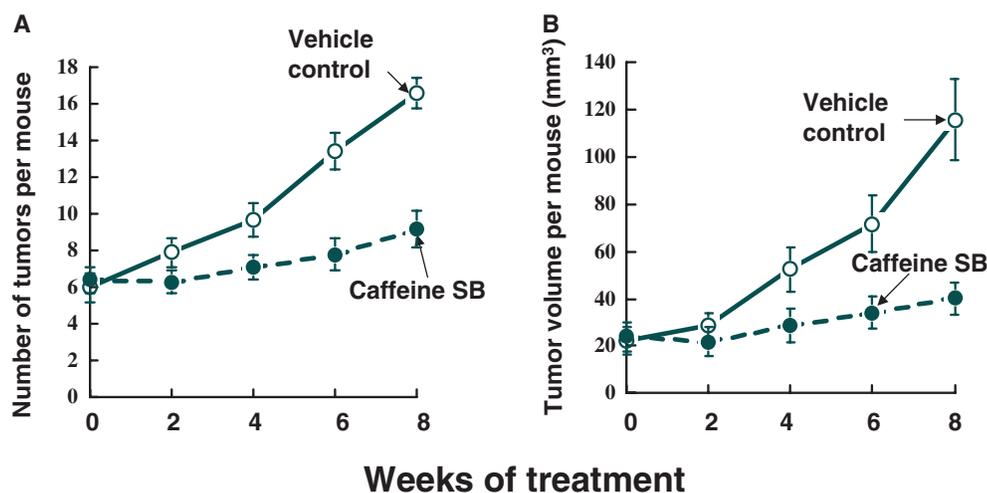
**Table II.** Inhibitory effect of topical application of caffeine or caffeine-SB in Dermabase cream on the growth of tumors in 'high-risk SKH-1 mice' previously treated with UVB

Treatment	No. of mice	Squamous cell papillomas (tumor volume/mouse) (mm <sup>3</sup> )	Keratoacanthomas (tumor volume/mouse) (mm <sup>3</sup> )	Total non-malignant tumors (tumor volume/mouse) (mm <sup>3</sup> )	Squamous cell carcinomas (tumor volume/mouse) (mm <sup>3</sup> )	Total tumors (tumor volume/mouse) (mm <sup>3</sup> )
Dermabase	28	3.1 ± 2.8	48.9 ± 10.6	52.0 ± 11.9	68.0 ± 24.5	120.0 ± 33.0
3.6% Caffeine	29	0.9 ± 0.9 (-71)	9.1 ± 2.2 <sup>a</sup> (-81)	10.0 ± 2.6 <sup>a</sup> (-81)	2.2 ± 1.3 <sup>a</sup> (-97)	12.3 ± 2.7 <sup>a</sup> (-90)
6% Caffeine-SB	27	0 (-100)	6.2 ± 3.9 <sup>a</sup> (-87)	6.2 ± 3.9 <sup>a</sup> (-88)	4.7 ± 2.8 <sup>a</sup> (-93)	10.9 ± 4.7 <sup>a</sup> (-91)

Female SKH-1 mice (7–8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice weekly for 20 weeks and UVB was stopped. These tumor-free mice were treated topically with 100 mg Dermabase cream, 3.6% caffeine or 6.0% caffeine-SB (equimolar concentration) in 100 mg Dermabase cream once a day 5 days a week for 17 weeks as described in Figure 5. The mice were sacrificed at 2 h after the last application of caffeine or caffeine-SB. All tumors were characterized by histopathology studies and the size of each tumor was determined.

Each value is the mean ± SE for total tumor volume per mouse, and the numbers in parentheses represents percent decrease when compared with the Dermabase cream control.

<sup>a</sup>*P* < 0.01 (comparison with the Dermabase cream control).



**Fig. 6.** Effect of topical applications of caffeine-SB on the growth of UVB-induced skin tumors in tumor-bearing SKH-1 mice. Female SKH-1 mice (7–8 weeks old) were irradiated with UVB (30 mJ/cm<sup>2</sup>) twice weekly for 20 weeks and UVB irradiation was stopped. These mice developed skin tumors gradually, and at 13 weeks after stopping UVB, mice with skin tumors were equally divided into two groups according to the number of tumors, tumor volume and body weight (20 mice/group). One group of mice was treated topically with 100 mg Dermabase cream only and another group of mice was treated topically with 4% caffeine-SB in Dermabase cream once a day 5 days a week for 8 weeks. The number (A) and size of the tumors (B) were recorded every 2 weeks. The mice were sacrificed after 8 weeks of treatment. Each value is the mean ± SE.

and DMBA-induced breast cancer are enhanced (28,30,31). A recent epidemiology report indicating an association between coffee drinking and a decreased risk of liver cancer in humans (32,33) is of considerable interest, and confirmatory studies are needed.

In summary, the results of the present study indicate that topical applications of caffeine or caffeine-SB to UVB-pretreated 'high-risk mice' inhibits skin carcinogenesis (keratoacanthomas and squamous cell carcinomas), and tumor growth in these animals is also inhibited (Tables I and II; Figures 5 and 6). The results of the present study and our earlier work (3–5) suggest that topical applications of caffeine or caffeine-SB either before or after exposure to sunlight may inhibit sunlight-induced keratoses and squamous cell carcinomas in humans. Clinical trials are needed to determine the effectiveness and safety of topical applications of caffeine or caffeine-SB alone or together with conventional sunscreens on sunlight-induced skin cancer in humans.

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