

Effect of Caffeine on the ATR/Chk1 Pathway in the Epidermis of UVB-Irradiated Mice

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Abstract

Administration of caffeine was shown in earlier studies to enhance UVB-induced apoptosis and inhibit UVB-induced carcinogenesis in hairless SKH-1 mice. Here, we describe a potential mechanism for these *in vivo* effects. A single irradiation of mouse skin with UVB activated the ataxia-telangiectasia mutated- and Rad3-related (ATR) pathway, causing a severalfold increase in keratinocytes with phospho-Chk1 (Ser³⁴⁵) and a marked decrease in mitotic keratinocytes with cyclin B1 compared with baseline. When given in the drinking water for 1 to 2 weeks before UVB, caffeine (0.4 mg/mL) markedly inhibited the UVB-induced phosphorylation of Chk1 on Ser³⁴⁵ and caused premature expression of cyclin B1 in the epidermis. Normal keratinocytes had delayed mitotic entry for >10 h following UVB. Caffeine administration reduced this mitotic delay to only 4 h and caused markedly increased apoptosis by 6 to 10 h after UVB. p53 knockout mice were used to determine the role of p53 in these processes. Irradiation with UVB markedly decreased the number of mitotic keratinocytes with cyclin B1 in p53 knockout mice, and topical caffeine immediately after UVB abrogated this response and increased UVB-induced apoptosis severalfold. These effects of caffeine in knockout mice were substantially greater than in wild-type mice. The ability of caffeine to promote the deletion of p53^{-/-} keratinocytes may be relevant to its inhibitory effect on UVB-induced skin cancer. Our studies indicate that administration of caffeine enhances the removal of DNA-damaged cells by inhibiting the ATR-mediated phosphorylation of Chk1 and prematurely increasing the number of cyclin B1-containing cells that undergo lethal mitosis. [Cancer Res 2008;68(7):2523-9]

Introduction

In earlier studies, we showed an inhibitory effect of p.o. administration of green or black tea on the formation of UVB-induced keratoacanthomas and squamous cell carcinomas in SKH-1 hairless mice (1, 2). The regular teas were more effective than the decaffeinated teas, adding back caffeine to the decaffeinated teas restored their inhibitory activities, and administration of caffeine alone strongly inhibited tumor formation (3, 4). These results indicated that caffeine is a major component of tea responsible for its inhibitory effect on UVB-induced tumor

formation in mice. Mechanistic studies indicated that p.o. administration of green tea or black tea to tumor-bearing mice enhanced apoptosis in skin tumors and inhibited their growth (5, 6). P.o. administration of green tea or caffeine for 2 weeks before a single irradiation with UVB enhanced UVB-induced increases in epidermal wild-type p53 protein and apoptosis in the epidermis of SKH-1 mice (7). In addition, topical application of caffeine immediately after a single irradiation with UVB also enhanced UVB-induced apoptosis, but this treatment had only a small stimulatory effect on UVB-induced increases in wild-type p53 in the epidermis (8). In this study, caffeine was given immediately after UVB to prevent a possible sunscreen effect. P.o. or topical administration of caffeine had a selective proapoptotic effect on UVB-treated skin and did not affect apoptosis in the epidermis of normal non-UVB-treated skin (7, 8). In additional studies, we found that topical application of caffeine to p53^{-/-} or bax^{-/-} mice enhanced UVB-induced apoptosis by a p53- and bax-independent mechanism (9).

Cell culture studies indicate that DNA damage activates a wild-type p53-dependent G₁ checkpoint and a p53-independent G₂ checkpoint. Activation of these checkpoints inhibits cell division and allows time for DNA repair before mitosis, thereby preventing the propagation of DNA-damaged cells. Cell culture studies have also shown that treatment of DNA-damaged cells with caffeine overrides the G₂ checkpoint, thereby preventing cells from arresting in G₂, and this effect of caffeine results in attempted replication of the DNA-damaged cells that results in cell death (10-13).

Cell culture studies indicate that cyclin B1 accumulates during the S and G₂ phases of the cell cycle, and these changes are associated with an increase in cyclin B1 mRNA levels (14) and a decrease in protein degradation (15-18). Treatment of the cells with a DNA-damaging agent, such as hydroxyurea or UV, decreases the level of cyclin B1 as well as mRNA for cyclin B1, and these treatments prevent entry into mitosis by both an ataxia-telangiectasia mutated (ATM)/ATM- and Rad3-related (ATR)-dependent and an ATM/ATR-independent mechanism (15).

Because most (but not all) cell culture studies suggest the importance of the ATR/Chk1/cyclin B1 signal transduction pathway for the effects of caffeine in overcoming the DNA damage-induced G₂ cell cycle arrest (see Discussion), we hypothesize that a key component of the proapoptotic effect of caffeine in the epidermis of UVB-treated mice is inhibition of the UVB→ATR→Chk1→cdc25c→cdc2→cdc2/cyclin B1 pathway that normally results in a decrease in cdc2/cyclin B1 activity (increased phospho-cdc2/cyclin B1 and decreased dephospho-cdc2/cyclin B1) and an arrest before mitosis. In the presence of caffeine, this arrest should be abrogated (cdc2/cyclin B1 level should be increased despite UVB) and cells should proceed into mitosis prematurely leading to p53-independent chromatin condensation and cell death (13, 19), probably by mitotic catastrophe followed by apoptosis, as

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suggested by Brown and Attardi (20) and by others (see Fig. 1; refs. 21–23). The results described here provide support for this hypothesis by indicating that administration of caffeine stimulates UVB-induced apoptosis, inhibits UVB-induced phosphorylation of Chk1 on Ser³⁴⁵, and curtails the UVB-induced decrease in mitotic cells with cyclin B1 in the epidermis that occurs shortly after UVB irradiation.

Materials and Methods

Exposure of mice to UV and the preparation of serial sections of skin. Female SKH-1 hairless mice, 6 to 8 wk of age, were fed a Purina Laboratory Chow 5001 diet and irradiated with UV lamps (FS72T12-UVB-HO; National Biological Corp.) that emit UVB (280–320 nm; 75–80% of total energy) and UVA (320–375 nm; 20–25% of total energy) as described in our earlier studies (1). Skin samples (20 mm long; 5 mm wide), which also contained associated dermis, were taken from the middle of the back and placed in 10% phosphate-buffered formalin at 4°C for 18 to 24 h followed by washing and storing in 75% ethanol until the samples were processed for histology as described earlier (7).

All histologic and immunohistochemical determinations were performed with 400-fold magnification and scored blind by two investigators (Y-P.L. and Y-R.L.), who evaluated coded samples randomly. Good agreement was obtained between the two investigators, and the mean value obtained from the examination of multiple fields by each investigator was determined for each mouse and used in the calculation of mean \pm SE for the mice in each group. Each microscope field (40-fold magnification) was approximately equivalent to a 0.5-mm length of epidermis.

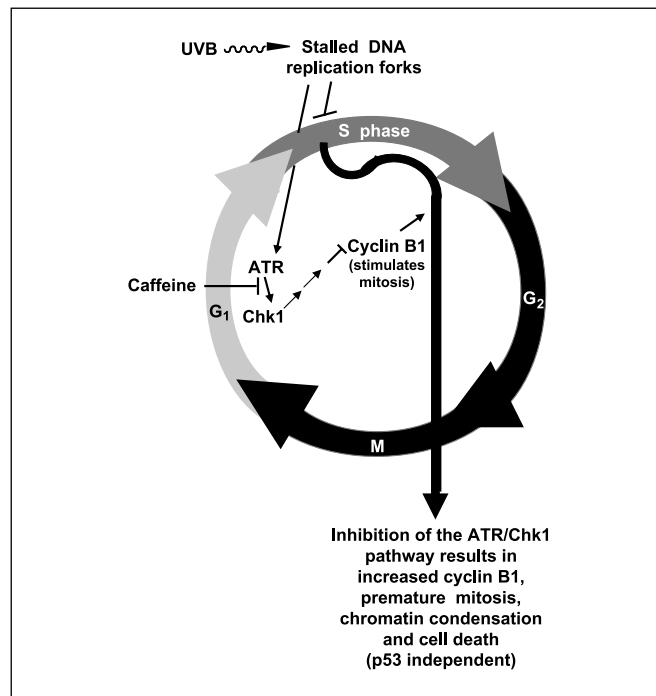


Figure 1. Proposed effects of UVB and caffeine on the ATR/Chk1/cyclin B1 pathway and premature chromatin condensation. The signal that DNA synthesis is not complete (and that chromatin condensation and mitosis should not start) is sent by ATR after it is recruited to stalled replication forks that have been coated by replication protein A. ATR-mediated phosphorylation of Chk1 results in a decreased level and function of cyclin B1, which delays mitotic entry and prevents premature chromatin condensation and mitosis. Inhibition of the ATR/Chk1 pathway in UVB-treated cells should result in a premature increase in cyclin B1 and premature chromatin condensation, mitosis, and cell death, probably by mitotic catastrophe followed by apoptosis, as suggested by Brown and Attardi (20). Adapted with permission from Nghiem et al. (13).

Measurement of apoptotic and mitotic cells in the epidermis.

Identification of apoptotic sunburn cells was based morphologically on cell shrinkage and nuclear condensation attributable to fragmentation of the cells (24, 25). Earlier studies showed that sunburn cells are indeed apoptotic cells (26). Apoptotic sunburn cells were identified in the epidermis by their intensely eosinophilic cytoplasm and small, dense nuclei, which were observed in H&E-stained histologic sections of the skin using light microscopy. 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.

Cells undergoing mitosis were determined as described earlier (6). Mitotic cells were determined by observing (a) chromosome condensation together with breakdown of the nuclear envelope, (b) alignment of the chromosomes on the spindle equator, (c) separation of sister chromatids, and (d) movement to their respective spindle poles. Two separate nonadjacent skin sections from each mouse were analyzed, and an average value for the mitotic index and percentage of apoptotic cells was calculated.

Phospho-Chk1 and cyclin B1 immunostaining in the epidermis. The antibody used for the immunohistochemical detection of phospho-Chk1 (Ser³⁴⁵) was from Santa Cruz Biotechnology (cat. no. sc-17922), and the antibody used for the immunohistochemical measurement of cyclin B1 was from Abcam (cat. no. ab72).

Skin sections were stained by the Biotin-Streptavidin Amplified System (alkaline phosphatase-conjugated streptavidin) using StrAviGen Super Sensitive Universal immunostaining kit purchased from Biogenex, with some modifications. Paraffin sections were first treated with 0.01 mol/L sodium citrate buffer (pH 6.0) in a microwave oven at high temperature for 10 min for phospho-Chk1 or cyclin B1 staining. The sections were then incubated with a protein block (normal goat serum) for 10 min at room temperature. The sections were incubated with phospho-Chk1 antibody (1:100 dilution) or cyclin B1 antibody (1:500 dilution) for 1 h at room temperature. The samples were then incubated with a biotinylated anti-rabbit secondary antibody for 5 min at 37°C followed by incubation with conjugated streptavidin solution for 5 min at 37°C. Color development was achieved by incubation with New Fuchsin Substrate Pack (containing 0.6 mg/mL levamisole solution; Biogenex) for 20 min at room temperature. The slides were then counterstained with hematoxylin and dehydrated, and coverslips were added for permanent mounting.

A positive reaction was shown as a brown precipitate in the cells. The percentage of phospho-Chk1 or cyclin B1-positive cells in the epidermis (combined basal and suprabasal layers) was calculated from the number of phospho-Chk1-stained (nuclear staining) or cyclin B1-stained (staining in nucleus and cytoplasm) positive cells per 100 cells counted from the entire 20-mm length of epidermis for each skin section.

Measurement of phospho-Chk1 (Ser³⁴⁵), phospho-Chk2 (Thr⁶⁸), cyclin B1, and phospho-cyclin B1 (Ser¹⁴⁷) in the epidermis by Western blots. Dorsal skin samples were removed and immediately placed in a buffer solution containing 75 mmol/L dibasic sodium phosphate and monobasic potassium phosphate (pH 7.7) at 52°C for 20 s. The samples were then submerged immediately in an ice bath containing the same buffer for 1 min. The epidermis was scraped from the dermis and placed in 1.2 mL of lysis buffer containing 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride (Cell Signaling Technology, Inc.). The epidermis was sonicated five times (5 s each time) at 4°C. Samples were centrifuged at 17,800 × g for 20 min at 4°C. Equal amounts of supernatant protein (40 μg) were separated by SDS-PAGE (4% stacking and 4–15% gradient) and electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked in 5% nonfat milk in PBS-Tween 20 for 1 h and incubated with phospho-Chk1 (Ser³⁴⁵) antibody (1:250 dilution), phospho-Chk2 (Thr⁶⁸) antibody (1:150 dilution), and cyclin B1 antibody (1:150 dilution). Blots were washed in PBS-Tween 20 and then incubated with a 1:1,500 dilution of peroxidase-conjugated secondary antibody (Amersham) in PBS-Tween 20 for 1 h at room temperature. Blots

were again washed in PBS-Tween 20 and then developed by enhanced chemiluminescence (Amersham).

The antibody used for the measurement of phospho-Chk1 (Ser³⁴⁵) by Western blot was obtained from Cell Signaling Technology (cat. no. 2341), and the antibody used for the measurement of phospho-Chk2 (Thr⁶⁸) was obtained from Rockland Immunochemicals, Inc. (cat. no. 600-401-280). The antibody used for the measurement of cyclin B1 by Western blots was obtained from Santa Cruz Biotechnology (cat. no. sc-752), and the antibody used for the measurement of phospho-cyclin B1 (Ser¹⁴⁷) by Western blots was obtained from Cell Signaling Technology (cat. no. 4131).

Results

Inhibitory effect of administration of caffeine or caffeine sodium benzoate on UVB-induced increase in the level of phospho-Chk1 (Ser³⁴⁵) in the epidermis of SKH-1 mice. In recent studies, we identified caffeine sodium benzoate as a complex of caffeine and sodium benzoate that is more active than caffeine at enhancing UVB-induced apoptosis (27). Our hypothesis predicts that administration of caffeine or caffeine sodium benzoate will inhibit UVB-induced activation of the ATR/Chk1 pathway by inhibiting the phosphorylation of Chk1 at Ser³⁴⁵, thereby leading to increased cyclin B1, premature chromatin condensation, premature mitosis, and cell death in DNA-damaged cells (see Fig. 1).

P.o. administration of caffeine (0.4 mg/mL, 2.1 mmol/L) or caffeine sodium benzoate (2.1 mmol/L) in the drinking water to SKH-1 mice for 1 week before exposure to UVB (30 mJ/cm²) markedly inhibited the UVB-induced increase in the level of phospho-Chk1 (Ser³⁴⁵) at 6 h after UVB, but there was little or no effect on the level of phospho-Chk2 (Thr⁶⁸) as measured by Western blots (Fig. 2). Administration of caffeine or caffeine sodium benzoate had little or no effect on the level of phospho-Chk1 (Ser³⁴⁵) in the absence of UVB (Fig. 2). Densitometry measurements on Western blots from the epidermis in three separate experiments indicated that treatment with UVB plus caffeine or UVB plus caffeine sodium benzoate decreased the level of phospho-Chk1 (Ser³⁴⁵) by an average of 82% and 99%, respectively, compared with animals that received only UVB irradiation at 6 h after UVB (Fig. 2B).

In a time course study, p.o. administration of caffeine (0.4 mg/mL in the drinking water) to SKH-1 mice for 2 weeks increased UVB-induced apoptosis at 6 to 10 h after UVB (Fig. 3A) and inhibited the UVB-induced increase in the number of phospho-Chk1 (Ser³⁴⁵)-positive cells at 6 to 16 h after UVB (Fig. 3B).

Effect of administration of caffeine or caffeine sodium benzoate on UVB-induced changes in cyclin B1 and mitotic cells with cyclin B1 in the epidermis of SKH-1 mice. We evaluated the possibility that administration of caffeine enhanced UVB-induced apoptosis through abrogation of the cell cycle blockade at the G₂-M checkpoint by elevating epidermal cyclin B1 prematurely, thereby causing premature and lethal mitosis in the epidermis of UVB-treated animals (see Fig. 1). Irradiation of control mice with UVB (30 mJ/cm²) in the absence of caffeine resulted in a 38% decrease in the number of cyclin B1-positive cells (nuclear staining) at 6 h after UVB, and there was an even greater decrease in the number of mitotic cells with cyclin B1 (Fig. 3C and D). Irradiation with UVB (30 mJ/cm²) decreased the number of epidermal mitotic cells with cyclin B1 by 61% at 2 h after UVB, 62% at 4 h after UVB, 75% at 6 h after UVB, and 75% at 10 h after UVB (Fig. 3D), which allowed time for DNA repair before increased mitosis that occurred at later times after UVB exposure. P.o.

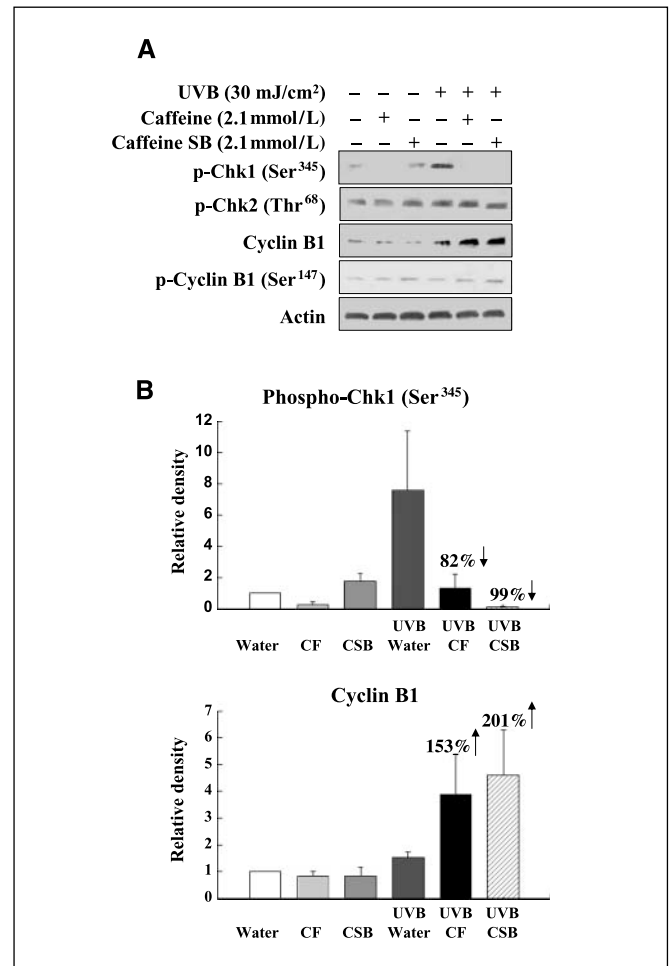


Figure 2. Effects of p.o. administration of caffeine or caffeine sodium benzoate on the levels of phospho-Chk1 (Ser³⁴⁵) and cyclin B1 in UVB-treated epidermis of SKH-1 mice: Western blot studies. Female SKH-1 mice were treated p.o. with caffeine (0.4 mg/mL, 2.1 mmol/L) or caffeine sodium benzoate (Caffeine SB; 2.1 mmol/L) as their sole source of drinking fluid for 1 wk. The animals were then treated with UVB (30 mJ/cm²) and killed 6 h later. A, phospho-Chk1 (Ser³⁴⁵), phospho-Chk2 (Thr⁶⁸), cyclin B1, and phospho-cyclin B1 (Ser¹⁴⁷) were determined by Western blot. B, densitometry results from three independent experiments. The antibodies used for the detection of these proteins are described in Materials and Methods.

administration of caffeine (0.4 mg/mL) to SKH-1 mice for 2 weeks before UVB irradiation blocked the normal UVB-induced decrease in cyclin B1 staining cells and mitotic cells with cyclin B1 at 6 to 10 h after UVB as measured by immunohistochemistry (Fig. 3C and D). An example of a cell undergoing mitosis that was also stained for cyclin B1 is shown in Fig. 4.

Our results show that p.o. administration of caffeine for 2 weeks before UVB irradiation increased the number of cyclin B1-stained cells in UVB-treated mice compared with control mice irradiated with UVB, and the time course for this effect of caffeine administration on cyclin B1 was similar to the time course for the inhibitory effect of caffeine on the UVB-induced increase in phospho-Chk1 (Ser³⁴⁵; Fig. 3B and C) and for the time course for the stimulatory effect of caffeine on UVB-induced apoptosis (Fig. 3A). Enhanced chromatin condensation (attempted mitosis) of DNA-damaged cells before repair and at an inappropriate early stage of the cell cycle would be expected to enhance cell death (Fig. 1). Our results indicate that administration of caffeine

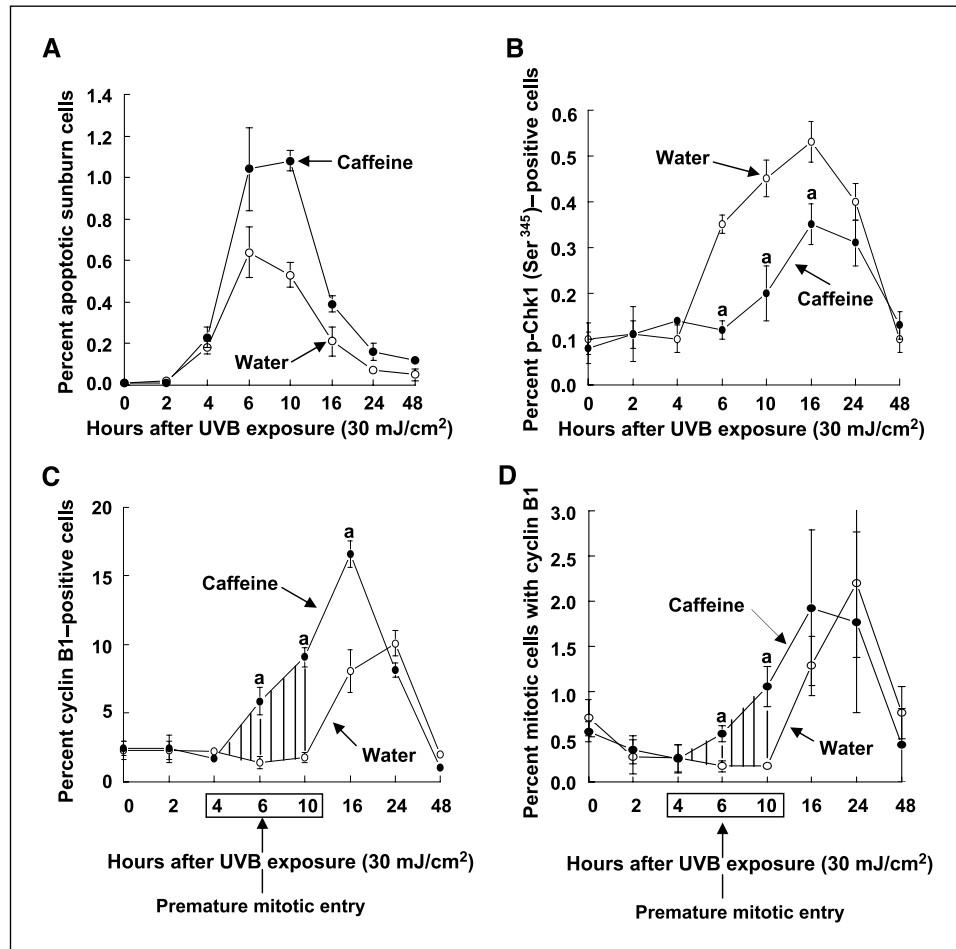


Figure 3. Time course for the effects of p.o. administration of caffeine on UVB-induced changes in the percentage of apoptotic sunburn cells, phospho-Chk1 (Ser³⁴⁵)-positive cells, cyclin B1-positive cells, and mitotic cells with cyclin B1 in the epidermis of SKH-1 mice: immunohistochemical studies. Female SKH-1 mice were treated p.o. with caffeine (0.4 mg/mL, 2.1 mmol/L) in the drinking water for 2 wk. Control mice received only water. The animals were then irradiated with UVB (30 mJ/cm²) and killed at the indicated times. The percentage of phospho-Chk1 (Ser³⁴⁵) and cyclin B1-positive cells were determined immunohistochemically. The antibodies used for the detection of these proteins are described in Materials and Methods. Cells with both mitosis (measured by morphology) and cyclin B1 (measured by immunohistochemistry) were also determined. Points, mean of five mice; bars, SE. Shaded areas, premature mitotic entry by epidermal cells from caffeine-treated mice. a, P < 0.01.

overcame the prolonged UVB-induced decrease in the percentage of mitotic cells with cyclin B1 that normally occurred after UVB (Fig. 3D). This effect of caffeine was associated with an increased level of cyclin B1 and enhanced early and inappropriate mitosis in the caffeine-treated mice.

It was of interest that the total number of mitotic cells in the epidermis after UVB or in the epidermis of mice pretreated with caffeine before UVB irradiation closely resembled the number of mitotic cells with cyclin B1 (data not presented). These results indicate that most mitotic epidermal cells in this study also had elevated cyclin B1.

P.o. administration of caffeine or caffeine sodium benzoate to SKH-1 mice for 1 week before exposure to UVB increased the level of cyclin B1 at 6 h after UVB when compared with the level of cyclin B1 in control mice irradiated with UVB as measured by Western blots at 6 h after UVB (Fig. 2). This treatment with caffeine or caffeine sodium benzoate, however, had little or no effect on the level of phospho-cyclin B1 (Ser¹⁴⁷; Fig. 2). Densitometry measurements on Western blots from the epidermis in three separate experiments as shown in Fig. 2 indicate that treatment with UVB plus caffeine or UVB plus caffeine sodium benzoate increased the level of cyclin B1 by an average of 153% and 201%, respectively, above the level of cyclin B1 in the epidermis of animals that received only UVB irradiation.

In another study, topical application of caffeine or caffeine sodium benzoate to SKH-1 mice immediately after irradiation with UVB increased by approximately 2- to 4-fold the number of cyclin

B1-positive cells in the epidermis at 6 h after UVB (compared with UVB alone) as measured by immunohistochemistry (Table 1). There was little or no effect of UVB irradiation alone or with topical applications of caffeine or caffeine sodium benzoate immediately after UVB on the number of phospho-cyclin B1 (Ser¹⁴⁷)-positive cells in the epidermis (data not shown).

Comparison of the effects of p.o. administered caffeine on the time course for UVB-induced changes in apoptosis, phospho-Chk1 (Ser³⁴⁵), cyclin B1, and mitotic cells with cyclin B1 in the epidermis of SKH-1 mice. We observed that caffeine-induced changes in apoptosis, phospho-Chk1 (Ser³⁴⁵), and cyclin

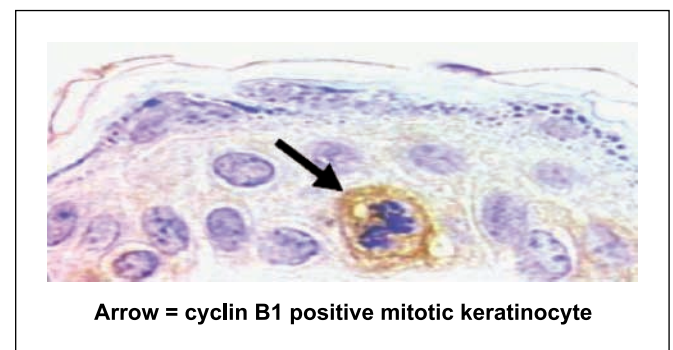


Figure 4. Illustration of a cyclin B1-positive keratinocyte. This figure shows a representative mitotic cell with cyclin B1 staining taken from a mouse treated with caffeine and UVB as described in Fig. 3.

Table 1. Effects of topical application of caffeine or caffeine sodium benzoate on the level of cyclin B1 in the epidermis of SKH-1 mice at 6 h after UVB

	Percent cyclin B1-positive cells		
	Vehicle control	Caffeine	Caffeine sodium benzoate
No UVB	11.3 ± 3.7	7.5 ± 2.6	9.4 ± 3.4
UVB	9.9 ± 3.1	21.8 ± 4.0*	44.4 ± 6.3 [†]

NOTE: Female SKH-1 mice (three per group) were treated topically with 100 μ L of acetone/water (9:1) or with 6.2 μ mol of caffeine or caffeine sodium benzoate in 100 μ L of acetone/water (9:1) immediately after 30 mJ/cm² of UVB and 0.5 and 2 h later. The animals were killed 6 h after UVB. Cyclin B1-positive cells in frozen sections of the epidermis were determined immunohistochemically. Each value represents the mean \pm SE.

* $P < 0.10$.

[†] $P < 0.01$.

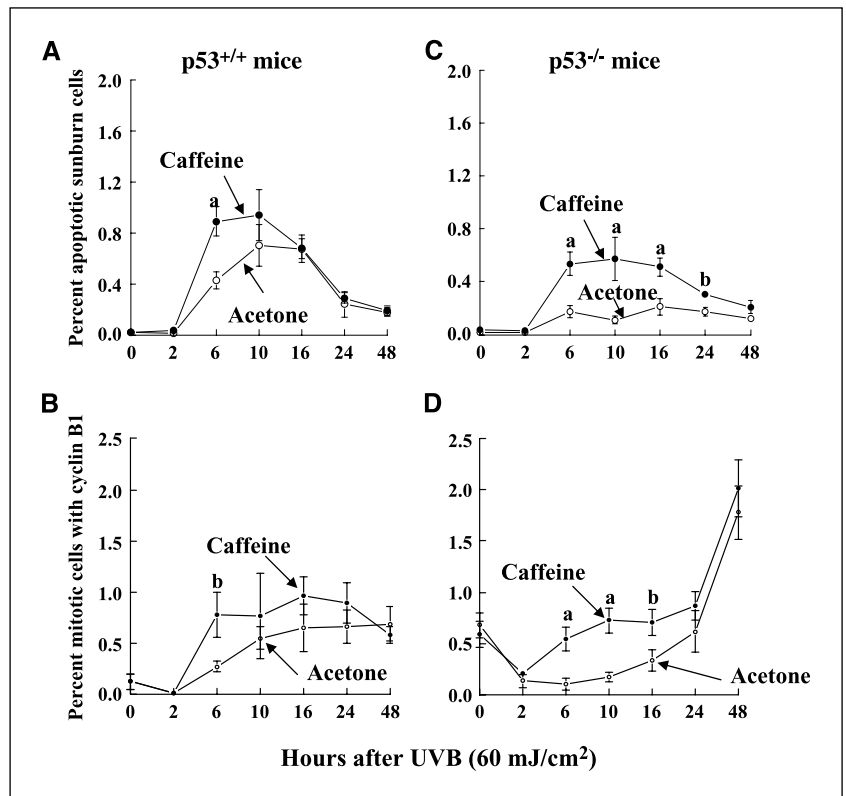
B1 (relative to UVB-treated control animals) all started at 6 h after UVB, and the greatest differences between UVB-treated control animals and UVB plus caffeine-treated animals occurred between 6 and 10 h after UVB (Fig. 3). Although the caffeine-induced increase in apoptosis in UVB-irradiated mice was maximal between 6 and 10 h after UVB and then declined (Fig. 3A), caffeine continued to affect UVB-induced changes in phospho-Chk1 (Ser³⁴⁵) and cells with cyclin B1 beyond 10 h (Fig. 3B and C). Decreases in apoptosis before the peak effects of caffeine on phospho-Chk1 and

cyclin B1 may occur because of an antiapoptotic response after 10 h (such as an increase in E2F1, activated AKT, or survivin).

Treatment of SKH-1 mice with caffeine enhances UVB-induced apoptosis by both enhancing the level of p53 (7) and by a p53-independent pathway involving premature mitosis (Figs. 3 and 5). p53 is not required for caffeine-induced premature mitosis after UVB irradiation as indicated in p53 knockout mice (Fig. 5). It is important to note that the presence of a peak of cyclin B1-positive cells does not by itself lead to apoptosis, but rather, cyclin B1-positive cells that arise before 16 h after UVB (premature increase in cyclin B1 and premature mitosis) is what leads to apoptosis in caffeine-treated mice. In UVB plus water-treated mice, there is no increase in cyclin B1-positive cells (mitotic cells with cyclin B1) before 16 h (Fig. 3C and D). This indicates a functional "replication checkpoint." At 16 h, the water-treated mice had keratinocytes that were undergoing mitosis as indicated by cyclin B1 expression; yet, because they had 16 h to recover from UVB damage, there was no corresponding increase in apoptosis (Fig. 3A, apoptosis is markedly declining by 16 h). In contrast, caffeine caused "premature mitosis" (visible at 6 and 10 h after UVB), and it is this early mitosis that then led to increased apoptosis in the caffeine-treated mice. The fact that the caffeine-treated mice went on to have a peak in cyclin B1-positive cells at 16 h but did not lead to apoptosis was because these cells began mitosis later and had had sufficient time to recover from UVB damage.

The data described in Fig. 3 at early time intervals after UVB irradiation provide support for our hypothesis that caffeine abrogates the UVB-induced blockade of the cell cycle at the G₂-M checkpoint by inhibiting the ATR/Chk1 pathway and prematurely elevating cyclin B1 and mitosis, thereby resulting in cell death (shown in Fig. 1).

Figure 5. Time course for the effects of topical application of caffeine on UVB-induced changes in the percentage of apoptotic sunburn cells, cyclin B1-positive cells, and mitotic cells with cyclin B1 in the epidermis of p53 knockout mice: immunohistochemical studies. Male p53^{+/+} or p53^{-/-} mice (five per group) were treated topically with 100 μ L of acetone/water (9:1) or with 6.2 μ mol of caffeine in 100 μ L of acetone/water (9:1) immediately after 60 mJ/cm² of UVB and 0.5 and 2 h later as described earlier (9). The animals were killed at several times after UVB. Stored paraffin blocks from the earlier study (9) were used for determining the percentage of cyclin B1-positive cells and mitotic cells with cyclin B1 in the epidermis as described in Fig. 3. Data for apoptotic sunburn cells were reported earlier (9) and are included here for comparison with the cyclin B1 data. Points, mean; bars, SE. a, $P < 0.01$; b, $P < 0.05$.



Effects of caffeine on the time course for UVB-induced changes in apoptosis and mitotic cells with cyclin B1 in the epidermis of p53 knockout mice. In an earlier study, we found that topical application of caffeine enhanced UVB-induced apoptosis in p53^{-/-} C57BL/6J mice (9). In the present study, we used stored paraffin blocks from our earlier study to determine the time course for the effect of caffeine on UVB-induced changes in the percentage of mitotic cells with cyclin B1 (Fig. 5D). The results indicate a dramatic UVB-induced decrease in the percentage of mitotic cells with cyclin B1 in p53^{-/-} mice between 2 and 16 h after UVB, and this decrease was abrogated starting at 6 h after UVB in mice that were treated topically with caffeine immediately after UVB irradiation (Fig. 5D), indicating a p53-independent effect of caffeine in enhancing the number of mitotic cells with cyclin B1 at early times after UVB irradiation. The time course for the stimulatory effect of caffeine administration on the percentage of mitotic cells with cyclin B1 in p53 knockout mice treated with UVB paralleled the time course for the formation of apoptotic sunburn cells (Fig. 5C and D). The results also indicate that the magnitude of the caffeine effect is greater in p53^{-/-} mice than in their littermate wild-type mice (Fig. 5A and B versus C and D).

Discussion

In earlier studies, we found that p.o. administration of caffeine inhibited UVB-induced carcinogenesis (3) and enhanced UVB-induced apoptosis by p53-dependent and p53-independent mechanisms (7–9). In the present study, we investigated the effect of caffeine administration on UVB-induced activation of the p53-independent ATR/Chk1 pathway that normally inhibits mitosis at the G₂-M checkpoint after exposure to UVB, thereby allowing the cells time to repair their damaged DNA.

The mechanisms and molecular targets for the proapoptotic effect of caffeine after DNA damage have been investigated in cultured cell lines. A recent study indicated that ATR is an important proapoptotic target for caffeine in human osteosarcoma cells (13), and loss of p53 function sensitized these cells to premature chromatin condensation caused by the ATR inhibitor caffeine (13, 19). These investigators found that activation of ATR after DNA damage prevented premature chromatin condensation via Chk1 protein kinase regulation, and caffeine abrogated this effect, which resulted in premature chromatin condensation (13). Other studies indicated that caffeine directly disrupts the ATR/Chk1 checkpoint pathway (28). The ATR protein has higher affinity for DNA in UVB-damaged cells than for undamaged DNA, and damaged DNA stimulates the kinase activity of ATR to a significantly higher level than undamaged DNA (29).

Although the present study and others suggest that caffeine overcomes the G₂ checkpoint block by inhibiting the ATR-dependent phosphorylation of Chk1, other mechanisms may also play a role. A recent study indicated that although caffeine inhibited ATM and ATR kinase activity *in vitro*, studies done with cultured cells unexpectedly indicated that treatment with 2 to 8 mmol/L caffeine in combination with hydroxyurea stimulated ATR-dependent phosphorylation of Chk1 at Ser³⁴⁵ (30). One possible interpretation of this paradoxical result is that inhibition of ATR by caffeine causes stalled replication forks to convert to more severe forms of damage such as double-strand breaks, paradoxically augmenting damage signaling (31). In a recent study, 5 mmol/L caffeine enhanced apoptosis in mitotic checkpoint-

arrested HeLa cells by mechanisms that seem to be independent of the ATR/Chk1/cyclin B1 signal transduction pathway (32). Inhibition of p21-activated PAK1, with an antiapoptotic function, was identified as a possible contributor to caffeine-induced apoptosis (32). It is important to point out that most cell culture studies used millimolar concentrations of caffeine that are 50- to 100-fold higher than what is achievable after p.o. administration of caffeine to mice or humans, although these concentrations are achievable after topical application.

In a recent study, we found that topical applications of caffeine to mice with patches of epidermal cells with one or more UVB-induced p53 mutations enhanced the elimination of these patches of p53-mutant cells (33). Interestingly, caffeine administration had selectivity and enhanced the elimination of cells with a p53 mutation on both alleles to a greater extent than cells with a p53 mutation on only one allele (34). These *in vivo* results are consistent with prior *in vitro* data that suggest a greater sensitivity to cell death by caffeine or ATR inhibition in p53-defective cells than in p53 wild-type cells (13).

Our results provide the first demonstration of an *in vivo* effect of caffeine administration on the ATR/Chk1/cyclin B1 pathway in animals, and this effect parallels the stimulatory effect of caffeine administration on UVB-induced apoptosis. In the present study, caffeine in the drinking water (0.4 mg/mL) given for 2 weeks before UVB treatment inhibited UVB-induced phosphorylation of epidermal Chk1 and abrogated the UVB-induced decrease in mitotic cells with cyclin B1 as well as the decrease in total mitotic cells. This is consistent with a model in which caffeine promotes premature mitosis and apoptosis through blocking ATR function as shown in the model in Fig. 1. Based on this model, we anticipated that caffeine-induced increases in epidermal cyclin B1 may be associated with changes in cyclin B1 phosphorylation. However, this was not observed using an antibody that can detect phosphorylation at Ser¹⁴⁷. It seems that the phosphorylation of cyclin B1 at positions other than Ser¹⁴⁷ plays an important role in the control of the cell cycle as shown by the work of Yang et al. (35). Cyclin B1 sites that are phosphorylated to promote nuclear import include Ser⁹⁴, Ser⁹⁶, Ser¹⁰¹, and Ser¹¹³ (35); however, phosphospecific antibodies have not been developed to investigate this process.

We explored the possibility that the effects of caffeine on UVB-induced carcinogenesis were mediated by inhibition of phosphodiesterase and an increased level of cyclic AMP (cAMP). Topical applications of 125 nmol of cAMP twice a day, 5 days a week for 21 weeks, to UVB-initiated high-risk mice had no effect on the percent of mice with tumors or the number of tumors per mouse but tumor size was increased (data not presented). In additional studies, treatment of mice with topical applications of dibutyryl cAMP or other compounds that increase cAMP had no effect on UVB-induced carcinogenesis (36). These studies indicate that alterations in cAMP levels that could occur after caffeine administration are not important for the inhibitory effect of caffeine on UVB-induced carcinogenesis.

The dose of p.o. caffeine used in the present study (0.4 mg/mL in the drinking water) as well as in earlier studies where caffeine inhibited UVB-induced carcinogenesis gave an average plasma level of ~16 μmol/L (37), which is similar to that observed in people drinking three to five cups of coffee per day, and some coffee drinkers had much higher plasma levels of caffeine (38). Epidemiology studies suggest that coffee drinkers and tea drinkers have a lower risk of skin cancer (39–41). Whether drinking these

caffeine-containing beverages modifies UVB-induced apoptosis and the ATR/Chk1 pathway in humans is unknown. Studies from our laboratory showed that administration of caffeine or caffeine sodium benzoate has sunscreen activity, increases the elimination of DNA-damaged cells by apoptosis, and inhibits UVB-induced carcinogenesis in SKH-1 mice (3, 4, 7, 8, 27, 42). Topical applications of caffeine or caffeine sodium benzoate to UVB-pretreated high-risk mice inhibited carcinogenesis in the absence of further UVB treatment (4, 27, 42), and administration of caffeine also enhanced apoptosis in the tumors (42). The results of these studies suggest that caffeine and caffeine sodium benzoate may be mechanistically novel agents for inhibiting sunlight-induced skin cancer.

In summary, the present study indicates that administration of caffeine to SKH-1 mice inhibits UVB-induced phosphorylation of Chk1 and prematurely increases the number of mitotic cells with cyclin B1 that are likely to go on to apoptosis. These effects are associated with an increase in UVB-induced apoptosis and inhibition of UVB-induced carcinogenesis.

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References

1. Wang ZY, Huang MT, Ferraro T, et al. Inhibitory effect of green tea in the drinking water on tumorigenesis by ultraviolet light and 12-*O*-tetradecanoylphorbol-13-acetate in the skin of SKH-1 mice. *Cancer Res* 1992;52:1162-70.
2. Wang ZY, Huang MT, Lou YR, et al. Inhibitory effects of black tea, green tea, decaffeinated black tea, and decaffeinated green tea on ultraviolet B light-induced skin carcinogenesis in 7,12-dimethylbenz[*a*]anthracene-initiated SKH-1 mice. *Cancer Res* 1994;54:3428-35.
3. Huang MT, Xie JG, Wang ZY, et al. Effects of tea, decaffeinated tea, and caffeine on UVB light-induced complete carcinogenesis in SKH-1 mice: demonstration of caffeine as a biologically important constituent of tea. *Cancer Res* 1997;57:2623-9.
4. Lou YR, Lu YP, Xie JG, Huang MT, Conney AH. Effects of oral administration of tea, decaffeinated tea, and caffeine on the formation and growth of tumors in high-risk SKH-1 mice previously treated with ultraviolet B light. *Nutr Cancer* 1999;33:146-53.
5. Wang ZY, Huang MT, Ho CT, et al. Inhibitory effect of green tea on the growth of established skin papillomas in mice. *Cancer Res* 1992;52:6657-65.
6. Lu YP, Lou YR, Xie JG, Yen P, Huang MT, Conney AH. Inhibitory effect of black tea on the growth of established skin tumors in mice: effects on tumor size, apoptosis, mitosis and bromodeoxyuridine incorporation into DNA. *Carcinogenesis* 1997;18:2163-9.
7. Lu YP, Lou YR, Li XH, et al. Stimulatory effect of oral administration of green tea or caffeine on ultraviolet light-induced increases in epidermal wild-type p53, p21(WAF1/CIP1), and apoptotic sunburn cells in SKH-1 mice. *Cancer Res* 2000;60:4785-91.
8. Lu YP, Lou YR, Li XH, et al. Stimulatory effect of topical application of caffeine on UVB-induced apoptosis in mouse skin. *Oncol Res* 2002;13:61-70.
9. Lu YP, Lou YR, Peng QY, Xie JG, Conney AH. Stimulatory effect of topical application of caffeine on UVB-induced apoptosis in the epidermis of p53 and Bax knockout mice. *Cancer Res* 2004;64:5020-7.
10. Walters RA, Gurley LR, Tobey RA. Effects of caffeine on radiation-induced phenomena associated with cell-cycle traverse of mammalian cells. *Biophys J* 1974;14:99-118.
11. Rowley R, Zorch M, Leeper DB. Effect of caffeine on radiation-induced mitotic delay: delayed expression of G₂ arrest. *Radiat Res* 1984;97:178-85.
12. Zampetti-Bosseler F, Scott D. The effect of caffeine on X-ray-induced mitotic delay in normal human and ataxia-telangiectasia fibroblasts. *Mutat Res* 1985;143:251-6.
13. Nghiem P, Park PK, Kim Y, Vaziri C, Schreiber SL. ATR inhibition selectively sensitizes G₁ checkpoint-deficient cells to lethal premature chromatin condensation. *Proc Natl Acad Sci U S A* 2001;98:9092-7.
14. Hwang A, Maity A, McKenna WG, Muschel RJ. Cell cycle-dependent regulation of the cyclin B1 promoter. *J Biol Chem* 1995;270:28419-24.
15. Florensa R, Bachs O, Agell N. ATM/ATR-independent inhibition of cyclin B accumulation in response to hydroxyurea in nontransformed cell lines is altered in tumour cell lines. *Oncogene* 2003;22:8283-92.
16. Fang G, Yu H, Kirschner MW. Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G₁. *Mol Cell* 1998;2:163-71.
17. Zachariae W, Schwab M, Nasmyth K, Seufert W. Control of cyclin ubiquitination by CDK-regulated binding of Hct1 to the anaphase promoting complex. *Science* 1998;282:1721-4.
18. Lukas C, Sorensen CS, Kramer E, et al. Accumulation of cyclin B1 requires E2F and cyclin-A-dependent rearrangement of the anaphase-promoting complex. *Nature* 1999;401:815-8.
19. Nghiem P, Park PK, Kim Y, Desai BN, Schreiber SL. ATR is not required for p53 activation but synergizes with p53 in the replication checkpoint. *J Biol Chem* 2002;277:4428-34.
20. Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer* 2005;5:231-7.
21. Castedo M, Kroemer G. Mitotic catastrophe: a special case of apoptosis. *J Soc Biol* 2004;198:97-103.
22. Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 2004;23:2825-37.
23. Niida H, Tsuge S, Katsuno Y, Konishi A, Takeda N, Nakanishi M. Depletion of Chk1 leads to premature activation of Cdc2-cyclin B and mitotic catastrophe. *J Biol Chem* 2005;280:39246-52.
24. Ziegler A, Jonason AS, Leffell DJ, et al. Sunburn and p53 in the onset of skin cancer. *Nature* 1994;372:773-6.
25. Young AR. The sunburn cell. *Photodermatol* 1987;4:127-34.
26. Brash DE. Sunlight and the onset of skin cancer. *Trends Genet* 1997;13:410-4.
27. Lu YP, Lou YR, Xie JG, et al. Caffeine and caffeine sodium benzoate have a sunscreen effect, enhance UVB-induced apoptosis, and inhibit UVB-induced skin carcinogenesis in SKH-1 mice. *Carcinogenesis* 2007;28:199-206.
28. Kumagai A, Guo Z, Emami KH, Wang SX, Dunphy WG. The *Xenopus* Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts. *J Cell Biol* 1998;142:1559-69.
29. Unsal-Kacmaz K, Makhov AM, Griffith JD, Sancar A. Preferential binding of ATR protein to UV-damaged DNA. *Proc Natl Acad Sci U S A* 2002;99:6673-8.
30. Cortez D. Caffeine inhibits checkpoint responses without inhibiting the ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) protein kinases. *J Biol Chem* 2003;278:37139-45.
31. Brown EJ, Baltimore D. Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes Dev* 2003;17:615-28.
32. Gabrielli B, Chau YQ, Giles N, Harding A, Stevens F, Beamish H. Caffeine promotes apoptosis in mitotic spindle checkpoint-arrested cells. *J Biol Chem* 2007;282:6954-64.
33. Lu YP, Lou YR, Liao J, et al. Administration of green tea or caffeine enhances the disappearance of UVB-induced patches of mutant p53 positive epidermal cells in SKH-1 mice. *Carcinogenesis* 2005;26:1465-72.
34. Kramata P, Lu YP, Lou YR, et al. Effect of administration of caffeine or green tea on the mutation profile in the p53 gene in early mutant p53-positive patches of epidermal cells induced by chronic UVB-irradiation of hairless SKH-1 mice. *Carcinogenesis* 2005;26:1965-74.
35. Yang J, Song H, Walsh S, Bardes ES, Kornbluth S. Combinatorial control of cyclin B1 nuclear trafficking through phosphorylation at multiple sites. *J Biol Chem* 2001;276:3604-9.
36. Zajdela F, Latarjet R. Ultraviolet light induction of skin carcinoma in the mouse; influence of cAMP modifying agents. *Bull Cancer* 1978;65:305-13.
37. Conney AH, Zhou S, Lee MJ, Xie JG, Yang CS, Lou Y. Stimulatory effect of oral administration of tea, coffee or caffeine on UVB-induced apoptosis in the epidermis of SKH-1 mice. *Toxicol Appl Pharmacol* 2007;224:209-13.
38. de Leon J, Diaz FJ, Rogers T, et al. A pilot study of plasma caffeine concentrations in a US sample of smoker and nonsmoker volunteers. *Prog Neuropsychopharmacol Biol Psychiatry* 2003;27:165-71.
39. Jacobson BK, Bjelke E, Kvale G, Heuch I. Coffee drinking, mortality, and cancer incidence: results from a Norwegian prospective study. *J Natl Cancer Inst* 1986;76:823-31.
40. Hakim IA, Harris RB, Weisgerber UM. Tea intake and squamous cell carcinoma of the skin: influence of type of tea beverages. *Cancer Epidemiol Biomarkers Prev* 2000;9:727-31.
41. Rees JR, Stukel TA, Perry AE, Zens MS, Spencer SK, Karagas MR. Tea consumption and basal cell and squamous cell skin cancer: results of a case-control study. *J Am Acad Dermatol* 2007;56:781-5.
42. Lu YP, Lou YR, Xie JG, et al. Topical applications of caffeine or (-)-epigallocatechin gallate (EGCG) inhibit carcinogenesis and selectively increase apoptosis in UVB-induced skin tumors in mice. *Proc Natl Acad Sci U S A* 2002;99:12455-60.