Carcinogenesis vol.25 no.8 pp.1449–1458, 2004
doi:10.1093/carcin/bgh151

Chemopreventive activity of parthenolide against UVB-induced skin cancer and its mechanisms

Yen-Kim Won[1], Choon-Nam Ong[1], Xianglin Shi[2] and Han-Ming Shen[1,3]

[1]Department of Community, Occupational, and Family Medicine, Faculty of Medicine, National University of Singapore, 16 Medical Drive, Singapore 117597, Republic of Singapore and [2]Health Effects Laboratory Division, National Institutes of Occupational Safety and Health, Morgantown, WV 26505, USA

[3]To whom the correspondence should be addressed
Email: cofshm@nus.edu.sg

Parthenolide (PN) is a major sesquiterpene lactone of feverfew (*Tanacetum parthanium*) with known anti-inflammatory activity. However, the anticancer effects of PN have not been well studied. In the present investigation, we examined the cancer chemopreventive property of PN using a combination of *in vivo* and *in vitro* approaches. We first tested the anticancer effect of PN in UVB-induced skin cancer model. Mice fed with PN (1 mg/day) showed a delayed onset of papilloma incidence, a significant reduction in papilloma multiplicity (papilloma/mouse) and sizes when compared with the UVB-only group. To our surprise, neither PN nor the known cyclooxygenase (COX)-2 inhibitor celecoxib inhibit UVB-induced COX-2 expression and epidermal prostaglandin E_2 (PGE_2) production. We next investigated the molecular mechanism(s) involved in its anticancer effects using cultured JB6 murine epidermal cells. Non-cytotoxic concentrations of PN significantly inhibited UVB-induced activator protein-1 DNA binding and transcriptional activity. In addition, PN pre-treatment also inhibited c-Jun-N-terminal kinase (JNK) and p38 kinase activation. More importantly, we found that impaired AP-1, JNK and p38 signaling led to the sensitization of JB6 cells to UVB-induced apoptosis. Data from our study for the first time confirm the anticancer property of PN in an animal model, and provide evidence that the inhibitory effects on AP-1 and mitogen-activated protein kinases serve as one of the underlying mechanisms for the cancer chemopreventive property of PN.

Introduction

Feverfew (*Tanacetum parthenium*) has been used as a herbal medicine for the treatment of fever, arthritis and migraine in Asia and Europe for centuries. The crude extracts of this herb are known to have anti-microbial and anti-inflammatory properties (1,2). The principal active component in feverfew is the sesquiterpene lactone parthenolide (PN) that contains a highly electrophilic α-methylene-γ-lactone ring and an epoxide residue capable of interacting rapidly with nucleophilic sites of biological molecules (Figure 1) (3). At present, there is some preliminary evidence showing the anticancer property of PN. For instance, PN is a potent inhibitor of DNA synthesis and cell proliferation in a number of cancer cell lines (4–6). Patel and co-workers (7) have reported that PN sensitizes breast cancer cells to the chemotherapeutic agent paclitaxel via its inhibitory effect on nuclear factor kappa B (NF-κB). Recently, Wen and colleagues (8) demonstrated that PN-induced apoptosis involves caspase activation and mitochondria dysfunction in hepatoma cells. To date, the anticancer property of PN has not been studied using *in vivo* animal models.

Skin cancer is the most common type of cancer among Caucasians (9). Clinically, skin cancers are classified into two major groups: malignant melanoma and non-melanoma skin cancer (NMSC). Numerous studies have demonstrated UVB as the major etiological factor for NMSC (10–12), which is derived from skin epithelial cells and can be further categorized into squamous cell carcinoma and basal cell carcinomas (13). The mechanism(s) of UVB-induced skin cancer have not been fully understood. Several transcriptional factors including activator protein-1 (AP-1), NF-κB, nuclear factor of activated T cells (NF-AT), and signal transducers and activators of transcription (STATs) have been linked to the tumor-promoting ability of UVB (14–17). AP-1 is an important nuclear transcription factor involved in many cellular functions such as cell proliferation, cell death, cell survival and differentiation (18). The mammalian AP-1 complex consists of either homodimers or heterodimers of Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), Jun dimerization partners (JDP1 and JDP2) and the closely related activating transcription factors (ATF2, LRF1/ATF3 and B-ATF) (19). Depending on the composite, the resulting AP-1 complex can then bind to and transactivate either the TPA-response element or the cAMP responsive elements to regulate the transcription of many genes involved in cell proliferation, apoptosis, metastasis and cellular metabolism (14,20,21). One important aspect of AP-1 function is its role in tumor promotion based on the fact that viral and cellular Jun or Fos can cause malignant transformation in fibroblasts (22,23). Gene products promoting invasion and metastasis are also under AP-1 regulation (22). Furthermore, inhibition of UVB-induced skin cancer

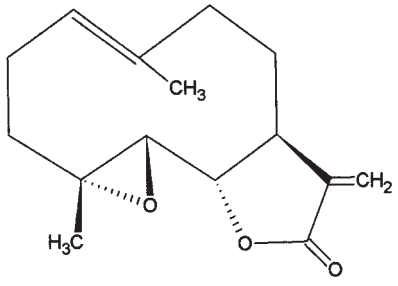

Fig. 1. Chemical structure of PN.

Abbreviations: AP-1, activator protein-1; COX, cyclooxygenase; JNK, c-Jun-N-terminal kinase; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor kappa B; PGE_2, prostaglandin E2; PN, parthenolide; UVB, ultraviolet-B light.

has been shown to be mediated through suppression on AP-1 transactivation (24).

It has been well documented that PN is a potent inhibitor of NF-κB signaling pathway, which contributes to its anti-inflammatory activity (25–27). In contrast, relatively little is known with regards to the effects of PN on other signaling molecules such as AP-1 and their involvements in its anti-cancer activity. The main objective of the present study is to systematically investigate the cancer chemopreventive property of PN using a combination of both *in vivo* animal model and *in vitro* cell culture study. We first tested the anticancer property of PN in UVB-induced skin cancer model using female SKH-1 hairless mice. To determine the mechanism(s) involved in the anticancer effect of PN, we further investigated its effect on AP-1 and mitogen-activated protein kinase (MAPK) signaling cascade in JB6 murine epidermal cells. Data from our study for the first time demonstrate the anticancer property of PN in an animal model, and provide evidence that the inhibitory effects on AP-1 and MAPK serve as one of the underlying mechanisms for the cancer chemopreventive property of PN.

Materials and methods

Chemicals and reagents

PN (97% pure) was purchased from Biomol (Plymouth Meeting, PA). Celecoxib was kindly provided by Pfizer (New York, NY). Anti p-c-Jun-N-terminal kinase (JNK) p-p38, p38, p-Erk, Erk, p-c-Jun, c-Jun, p-ATF-2 and ATF-2 polyclonal antibodies were purchased from Cell Signaling (Beverly, MA). Secondary antibodies (horseradish peroxidase conjugated goat anti-mouse IgG and goat anti-rabbit IgG) and enhanced chemiluminescence substrate were from Pierce (Rockford, IL). [γ-^{32}P]ATP was obtained from Perkin-Elmer (Boston, MA). SP600125 was purchased from Calbiochem (San Diego, CA). Other common chemicals were from Sigma-Aldrich (St Louis, MO).

Animals and treatment

Female SKH-1 hairless mice were purchased from Charles River Laboratories (Wilmington, MA), and they were 8–9 weeks of age at the beginning of the experiments. The experiments were conducted following the rules and regulations by the Animal Welfare Committee of the university. All mice were housed in the Animal Holding Unit under climate-controlled environment with a 12-h light/dark cycle. Mice (5 mice/cage) were allowed free access to food pellets and water placed inside the food chamber on top of the cage cover. The various treatments and UVB irradiation schemes were summarized in Figure 2. Briefly, mice were placed on specifically prepared food pellets containing DMSO, PN or celecoxib 1 week prior to UVB treatment and throughout the study. The dosage of PN was calculated based on our preliminary acute toxicity study (data not shown). We also included a known cyclooxygenase (COX)-2 inhibitor celecoxib as a positive control (28). Both compounds were first dissolved in DMSO, diluted with sunflower oil, and then coated onto food pellets. These food pellets were prepared weekly and stored at 4°C once coated. Fresh food pellets were supplied three times a week and the amount of consumption was recorded at the same time. UVB was delivered through a bank of FS24 lamps (Light Sources, Orange, CT) with spectral irradiance of 280–400 nm, 80% of which in the UVB region (280–320 nm) and with a peak at ~313 nm. The emitted UVB dose was quantified using a phototherapy radiometer (International Light, Newburyport, MA) equipped with IL SED 240 detector. Mice were exposed to an initial dose of 100 mJ/cm^2 of UVB (5 days/week). A weekly increment of 50 mJ/cm^2 was applied until a maximal dose of 200 mJ/cm^2 was reached. The UVB treatment was continued for 25 weeks. A negative control group (no PN treatment and no UVB exposure, $n = 5$) was included. Papilloma (lesion >1 mm) were examined and recorded weekly. Papilloma incidence (% of mice with one or more papilloma) and papilloma multiplicity (no. of papilloma/mouse) were determined after the termination of the UVB treatment. All mice were humanely killed at the end of the 25-week study. Papillomas were surgically removed, paraffin embedded and cut into 4 μm sections in preparation for immunohistochemistry staining. The dorsal skins of mice were also removed, snap-frozen in liquid nitrogen, and then stored at −80°C until further analysis.

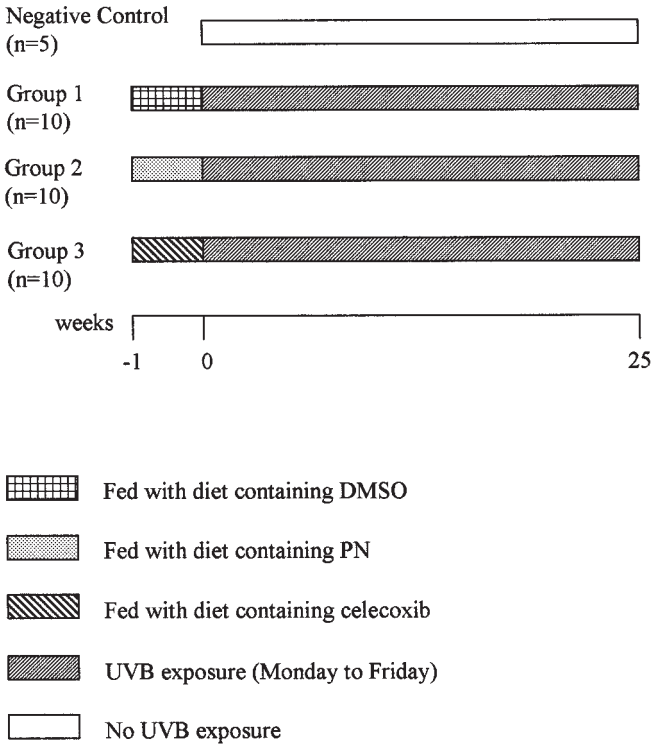

Fig. 2. Schematic representation of the chemopreventive aspect of PN or celecoxib against UVB-induced skin cancer in SKH-1 hairless mice. Mice were placed on diet containing 2% DMSO (group 1), 1 mg/day PN (group 2) or 3 mg/day celecoxib (group 3) 1 week prior to the initiation of UVB exposure and was maintained throughout the study. Mice were exposed to an initial dose of 100 mJ/cm^2 of UVB (5 days/week), with an increment of 50 mJ/cm^2 per week until a maximal dose of 200 mJ/cm^2 was reached. The UVB treatment was continued for 25 weeks. A negative control group with no UVB exposure was also included.

Immunohistochemistry staining

The immunostaining was done using VECTOR® M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instruction. Sections were deparaffinized, permeabilized and then incubated with anti-Cox-2 monoclonal antibody (1:500 dilution) (Transduction Laboratories, Los Angeles, CA). Sections were visualized with 3,3′-diaminobenzidine through avidin–biotin–horseradish peroxidase system. Slides were counter-stained with hematoxylin-1 (Sigma-Aldrich).

Prostaglandin E_2 (PGE_2) determination

Skin samples were homogenized and purified using a Sep-Pak® Vac C-18 cartridge (Waters, Milford, MA). PGE_2 in skin samples were measured using monoclonal enzyme immunoassay kit (Cayman, Ann Arbor, MI) according to manufacturer's protocol.

Cell culture and UVB exposure

JB6 murine epidermal cells and JB6 cells stably transfected with an AP-1 luciferase reporter plasmid have been reported previously (29,30). All cells were cultured in MEM supplemented with 5% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2/air atmosphere. Cells were seeded in 6-well plates or 60 mm cultural dishes and starved with MEM containing 0.5% FBS for 24 h after reaching 80% confluence. After pre-treatment with medium containing various chemicals at designated concentrations, cells were washed with PBS once and then exposed to UVB in fresh PBS. Cells were returned to incubator with the addition of the previous culture medium until time of collection.

Determination of cell death and apoptosis

Cell viability was measured via LDH leakage (31), while apoptosis was examined using propodium iodide (PI) staining for DNA content analysis (sub-G_1 cells) and DNA ladder formation as reported previously (32).

Nuclear extracts preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to published method with modifications (26). JB6 cells were scraped after the designated treatment, washed with ice-cold PBS twice and then re-suspended in 120 μl of Buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, protease inhibitor cocktail). After 15 min of incubation on ice, Nonidet P-40 was added (final concentration 0.3%) and tubes were vortexed vigorously. The lysates were centrifuged at 2000 *g* for 10 min at 4°C. The resulting nuclear pellet was re-suspended in 40 ml of Buffer B (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 450 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitor cocktail) and incubated on ice for 30 min. The nuclear extracts were collected after 15 min of centrifugation at 20 000 *g* at 4°C. Protein concentration was quantified using Bio-Rad protein assay kit (Hercules, CA). All samples were kept at −70°C until assay. The DNA binding reaction mixture contained 5 μg of nuclear extract, 5× Buffer C (100 mM HEPES, pH 7.9, 20% glycerol, 1 mM DTT and 300 mM KCl), 2 μg poly(dI–dC), 2 μg BSA and was incubated for 30 min on ice. ^{32}P-Labeled AP-1 oligonucleotide (5′-CGCTTGATGAGTCGACCGGAA-3′, 3′-GCGAACTACTCAGTCGGCCTT-5′) was then added in a total volume of 20 μl. The DNA–protein complexes were resolved in 5% polyacrylamide gel using a vertical gel electrophoresis apparatus (Gibco-BRL, Gaithersburg, MD). Gels were then dried and exposed to an X-ray film (Kodak) at −70°C overnight.

Western blot

Cells were scraped after the designated treatment and washed with ice-cold PBS twice. Pellets were re-suspended in lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, protease inhibitor cocktail), sonicated on ice for 15 s, and centrifuged at 10 000 *g* for 10 min at 15°C. Thirty micrograms of protein were separated on 10% SDS–polyacrylamide gel in Mini-Protein II system (Bio-Rad). Following electrophoresis the protein was transferred to a PVDF membrane (Millipore, Bedford, MA) and subsequently hybridized with various antibodies. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station.

AP-1 transactivation assay

JB6 cells stably transfected with an AP-1 luciferase reporter plasmid were subjected to designated treatments. At 6 h post-UVB irradiation, the cell lysates were collected after the addition of cell lysis buffer (Promega). Luciferase activity was measured using a luciferase assay kit (Promega). The relative light units were then determined in a luminometer (Lumi-one, Trans Orchid, Tampa, FL) for 15 s after a 5-s delay time.

Statistical analysis

All numeric data are presented as means ± standard deviations (SD) and analyzed using one-way ANOVA with Student–Newman–Keuls multiple comparisons as post-hoc test (SPSS 11.5). Part of the results from the animal study was analyzed using Fisher exact probability test. A P value <0.05 is considered as statistically significant.

Results

The chemopreventive activity of PN against UVB-induced skin cancer

In this study, the anticancer property of PN was first tested in a UVB-induced mouse skin cancer model, which was established based on published methods (28,33). No papilloma was detected in the negative control group throughout the study. As shown in Figure 3A, PN (1 mg/mouse/day) or celecoxib (3 mg/mouse/day) significantly delayed the onset of papilloma incidence comparing it with the UVB-only group: the UVB-only group reached 100% tumor incidence at week 13, while PN- or celecoxib-fed groups achieved full tumor incidence at weeks 18 and 20, respectively. Papilloma multiplicity (number of papilloma/mouse) was also significantly reduced in mice fed with either PN or celecoxib (Figure 3B): PN treatment decreased the papilloma multiplicity by 30%, comparable with that of celecoxib (40%). In addition, differences in the severity of tumor growth and the size distribution of papilloma among different groups were also noted (Table I): papillomas observed in the group fed with PN were of smaller size (73% papillomas of 1–2 mm in diameter) as compared with that of the control (59%). Furthermore, immunohistochemistry

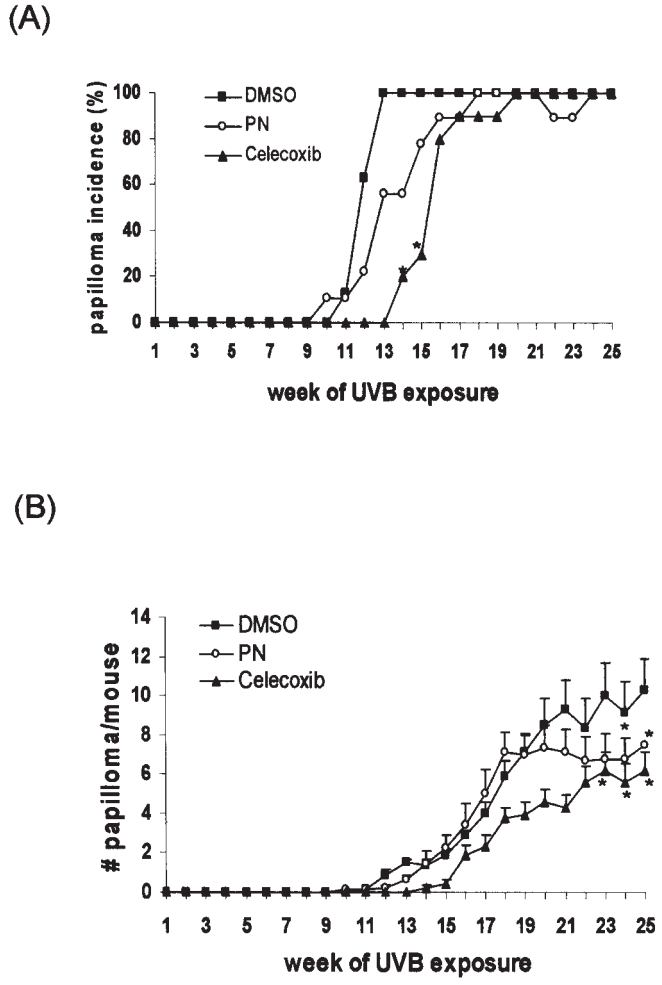

Fig. 3. Inhibitory effects of PN and celecoxib on UVB-induced papilloma incidence and yield in SKH-1 mice. Mice were placed on diets containing PN or celecoxib ($n = 10$) 1 week prior to the beginning of UVB irradiation (5 times/week) and the number of papillomas were counted weekly. (**A**) Papilloma incidence was calculated as the percentage of mice having tumors in each treatment group and analyzed using Fisher exact probability test. (**B**) Papilloma yield was calculated as the mean values of papillomas/mouse ± SD for each treatment group, and analyzed using one-way ANOVA with Student–Newman–Keuls multiple comparisons test on data from the last three weeks of the study. Asterisks indicate significant difference between the control and treated groups ($P < 0.05$).

Table I. Size distribution of papillomas

Treatment group	*n*	% Small papillomas (1–2 mm)[a]	% Big papillomas (≥3 mm)
Control (UVB only)	10	59.00 ± 10.52	41.0 ± 9.32
PN + UVB	10	73.67 ± 6.54[b]	26.33 ± 5.34[b]
Celecoxib + UVB	10	65.90 ± 6.15	34.10 ± 11.1

[a]Means ± SD.
[b]$P < 0.05$ compared with the UVB-only control group using one-way ANOVA with Student–Newman–Keuls multiple comparisons test.

staining (Figure 4) showed both PN and celecoxib treatments markedly inhibited the UVB-induced hyperplastic response (increased thickness of the epidermis) as compared with the control group. In addition, the effect of celecoxib appears to be more profound, and this observation is basically consistent with the data in Figure 3. Taken together, here for the first

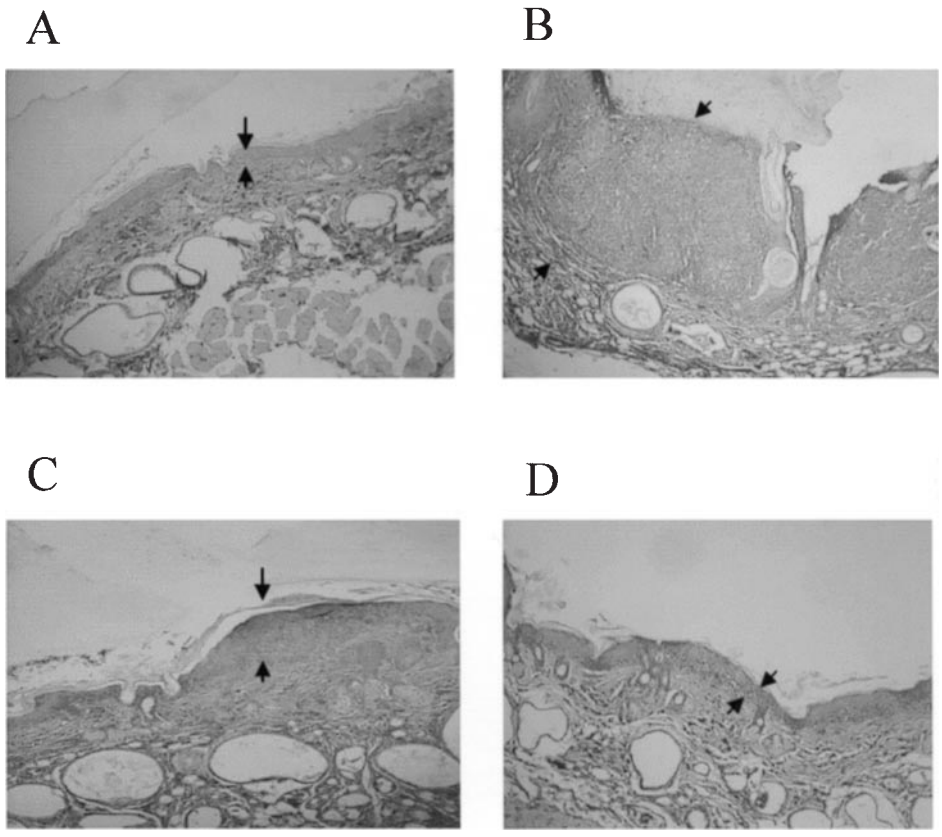

Fig. 4. Effects of PN and celecoxib on UVB-induced hyperplasia (area between the arrows). Mice were placed on a diet containing (**B**) DMSO, (**C**) 1 mg/day PN and (**D**) 3 mg/day celecoxib. A negative control group on normal diet with no UVB exposure was also included (**A**). Mice were placed on this diet 1 week prior to and were continued throughout the 25 weeks of UVB exposure (5 times/week). At the end of the study, dorsal skins of the mice were removed and formalin fixed. Cut sections (4 μm) were processed for hematoxylin and eosin staining, and photomicrographed at 400×.

time we provide evidence that PN possesses strong chemopreventive property against UVB-induced skin cancer.

COX-2 and UVB-induced skin cancer

It has been relatively well known that COX-2 over-expression is implicated in UVB-induced skin cancer (34–36), and COX-2 inhibitors are known to be effective in preventing UVB-induced skin cancer (28,33). To our surprise, in the present study we found that COX-2 is unlikely to be the direct molecular target of either PN or celecoxib. As shown in Figure 5, UVB exposure led to COX-2 over-expression in skin tissue determined by immunohistochemistry, while either PN or celecoxib failed to suppress this reaction (Figure 5A). A consistent pattern of changes was also found for the PGE_2 level in the skin tissue: UVB exposure markedly enhanced the PGE_2 level, while no reduction was found with either PN or celecoxib treatment (Figure 5B). Therefore, it is believed that the chemopreventive activity of PN or celecoxib might be independent of COX-2 in our experimental system.

PN sensitizes mouse epidermal cells to UVB-induced apoptosis

In order to elucidate the mechanism(s) involved in the chemopreventive property of PN against UVB-induced skin cancer, a mouse epidermal cell line JB6 was used in the subsequent *in vitro* study. We first tested the cytotoxicity of PN on this cell line and the LD_{50} was estimated at 8.5 μM (data not shown). Next, we investigated whether PN has any synergistic or antagonistic effect on UVB-induced apoptosis. High dosage of UVB is capable of inducing apoptosis in JB6 cells (data not shown) and our preliminary experiment showed that 50 mJ/cm^2 UVB was the lowest observed effect level with marginal cytotoxic effect to cells. Pre-treating cells with non-cytotoxic concentrations of PN (2.5 and 5 μM) greatly enhanced UVB (50 mJ/cm^2)-induced cell death as measured by LDH leakage (Figure 6A) and percentage of sub-G_1 cells determined by DNA content analysis (Figure 6B). Similar results were also found in DNA gel electrophoresis showing DNA fragmentation, a hallmark of apoptosis (Figure 6C). Based on the common understanding that removal of damaged cells via apoptosis is an important anti-oncogenic process (37–39), it is thus believed that such sensitization by PN may serve as one of the underlying mechanisms for its chemopreventive property against UVB-induced skin cancer.

PN inhibits AP-1 DNA binding and transcriptional activity induced by UVB

Among various UV-elicited signaling pathways, it has been well documented that AP-1 is an important transcription factor mainly involved in cell survival and proliferation (18). It has been reported that UVB induces DNA binding activity of AP-1 in JB6 cells (40). As shown in Figure 7A, 50 mJ/cm^2 UVB vastly induced the AP-1 binding activity in JB6 cells. Pre-treatment with PN (2.5–5 μM) effectively suppressed the UVB-induced DNA binding capability of AP-1. The effect of

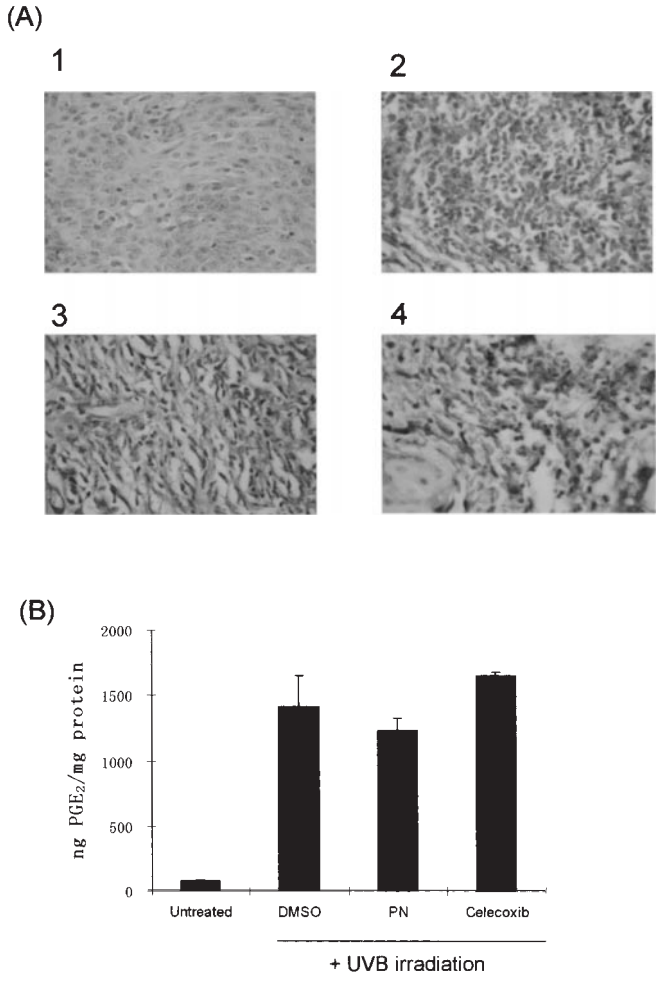

Fig. 5. (**A**) Immunohistochemistry staining of COX-2 in mouse skin samples. Skin samples were stained for COX-2 at the end of the study protocol. Images of (1) negative control, (2) DMSO, (3) PN, (4) celecoxib-treated groups were photomicrographed at 1000×. COX-2 staining gives a brown reaction product. (**B**) PGE_2 levels in untreated or UVB-irradiated mice fed with DMSO-, PN- or celecoxib-containing diets. PGE_2 was extracted from the skin samples at the end of the study protocol and analyzed by enzyme immunoassay described in Materials and methods. Data were presented in means ± SD.

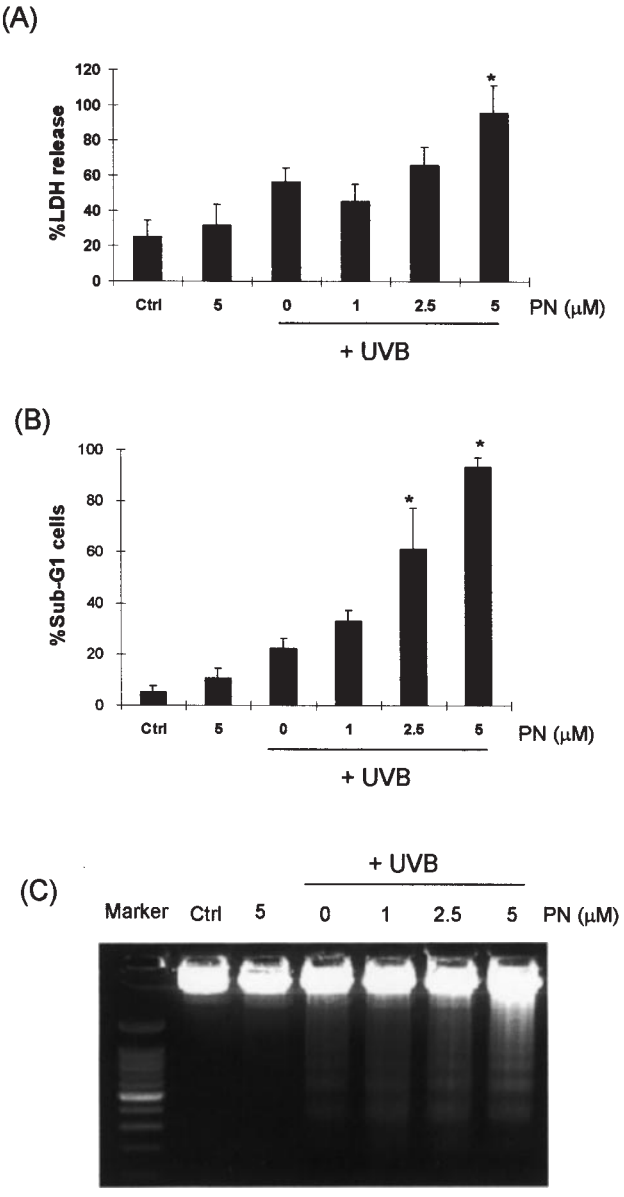

Fig. 6. PN sensitizes cells to UVB-induced apoptosis. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN for 2 h, and then subjected to UVB irradiation of 50 mJ/cm^2. The viability of cells was determined by (**A**) LDH leakage, (**B**) DNA content analysis for sub-G_1 cells and (**C**) DNA fragmentation at 24 h post-UVB irradiation as described in Materials and methods. DNA fragments were resolved by electrophoresis at 50 V on 1.5% agarose gels impregnated with ethidium bromide, detected by UV transillumination and analyzed using Kodak Image Station 440.

PN on AP-1 transcriptional activity was also examined in JB6 cells stably transfected with an AP-1 luciferase reporter plasmid. Cells were pre-treated with PN (1–5 μM) for 2 h and followed by UVB irradiation (50 mJ/cm^2). Being consistent with our findings in EMSA, significant reduction of AP-1 luciferase activity was observed with higher concentrations of PN (2.5–5 μM) (Figure 7B).

PN inhibits UVB-induced phosphorylation of c-Jun and ATF-2

Both c-Jun and ATF-2 are important components of AP-1 complex (19). It has been reported previously that UV-induced AP-1 DNA binding is independent of new protein synthesis, and both Jun and ATF-2 activation occur as a result of post-translational modification involving changes in the phosphorylation states (41–43). Here we further investigated whether PN suppresses UVB-induced AP-1 activation via inhibition of the phosphorylation states of its key components. As shown in Figure 8A, pre-treatment with PN (5 μM) completely blocked UVB-induced c-Jun phosphorylation at both Ser63 and Ser73 sites. No changes in total protein level for c-Jun were detected in cells treated with either UVB and/or PN. Similar inhibition on UVB-induced ATF-2 phosphorylation was also observed with PN pre-treatment (Figure 8B). Therefore, it is believed that PN suppresses UVB-elicited AP-1 activation via reduced phosphorylation levels of its key components such as c-Jun and ATF-2.

PN blocks UVB-induced MAPK pathways

JNK and p38 are two main groups of MAPKs that are directly involved in UVB-elicited AP-1 activation (44,45). c-Jun and

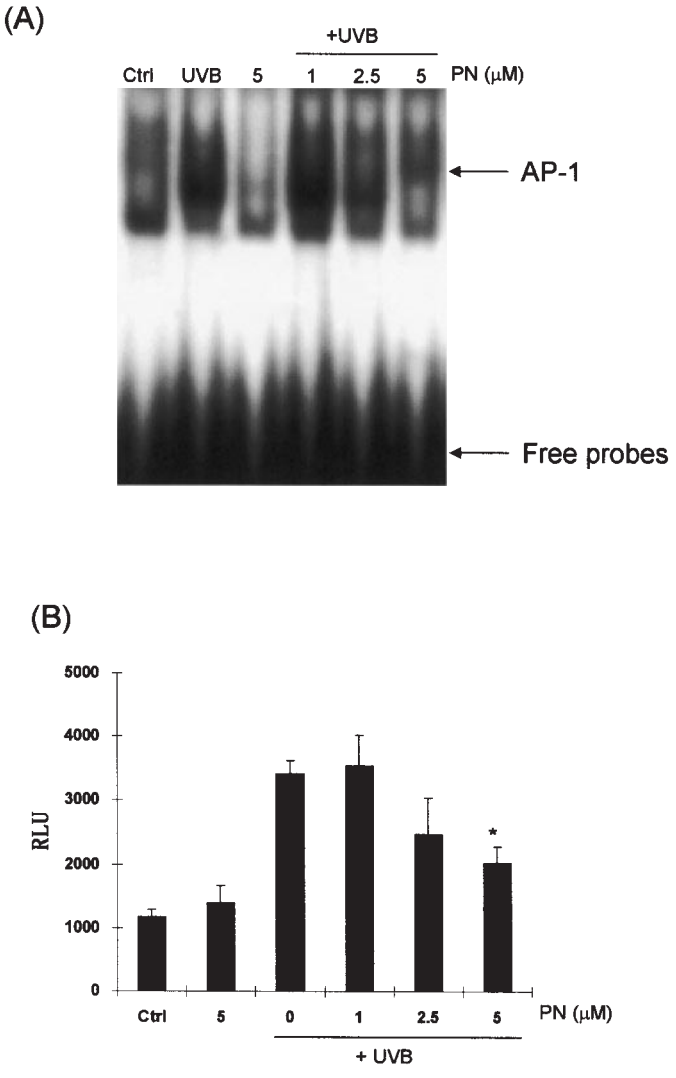

Fig. 7. PN inhibits UVB-induced AP-1 activation. (**A**) PN inhibits the UVB-induced DNA binding activity of AP-1. JB6 cells were pre-treated with 1, 2.5 or 5 μM of PN for 2 h, followed by UVB irradiation with 50 mJ/cm^2. Control cells were mock irradiated. Cells were harvested 6 h after UVB irradiation. Five micrograms of nuclear extracts were subjected to EMSA as described in the Materials and methods. (**B**) PN inhibits the UVB-induced transcriptional activity of AP-1. JB6 cells stably transfected with an AP-1 luciferase reporter plasmid were pre-treated with 1, 2.5 or 5 μM of PN for 2 h, followed by UVB irradiation with 50 mJ/cm^2. Control cells were mocked irradiated. Cells were harvested 6 h after UVB irradiation. Luciferase activity was determined as described in the Materials and methods. Data were presented as means ± SD from three independent experiments and analyzed using one-way ANOVA with Student–Newman–Keuls multiple comparisons test. An asterisk indicates statistical significance with a P-value <0.05.

ATF-2 serve as direct downstream targets for JNK and p38, respectively. In order to further understand the mechanisms involved in the inhibitory effect of PN on UVB-induced AP-1 activation, we examined whether PN acts on UVB-elicited JNK and p38 activation. Pre-treatment with PN (5 μM) completely blocked UVB-induced JNK phosphorylation without affecting the total JNK protein level (Figure 9A). A similar inhibitory effect on UVB-induced p38 phosphorylation was also observed in cells pre-treated with PN (Figure 9B). Therefore, it is believed that PN suppresses UVB-mediated AP-1 activation through its inhibitory effects on JNK and p38.

PN sensitizes UVB-induced apoptosis via JNK and p38

It is still controversial with regards to the exact role of JNK and p38 in UV-induced apoptosis. In mouse embryonic fibroblasts, JNK has been shown to be required for UV-induced cell death (46). On the contrary, there is evidence indicating that JNK and p38 act as cell survival/anti-apoptotic mechanism in UV-treated cells (47–49). As shown above (Figure 6), PN effectively sensitizes UVB-induced apoptosis in JB6 cells. Notably, similar sensitization was also found in cells pre-treated with SP600125 (a specific inhibitor of JNK) or SB203580 (a specific inhibitor of p38), although to a less extent (Figure 10). The effectiveness and specificity of these two inhibitors were confirmed by the significant reduction in phosphorylation of their respective targets (Figures 8 and 9). Data from this experiment thus provide indirect evidence that PN may sensitize UVB-induced apoptosis via its inhibitory effect on JNK and p38. It appears that both JNK and p38 contribute to the cell survival mechanisms in UVB-treated JB6 cells, based on the observation that PN as a dual-inhibitor of JNK and p38 are much more effective than the individual inhibitor for sensitizing UVB-induced apoptotic cell death.

Discussion

Previous studies have demonstrated PN as a potent inhibitor of DNA synthesis and cell proliferation in a number of cancer cell lines (4–6). More recently, PN has been reported to be a potent apoptosis inducer in human cancer cells (8). Furthermore, it has been shown that PN sensitizes breast cancer cells to chemotherapeutic agent paclitaxel (7). However, the anticancer potential of PN has not been tested on animal models. Here for the first time we provide clear evidence that PN possesses chemopreventive property against UVB-induced skin cancer in female SKH-1 hairless mice: (i) PN treatment delayed the onset of tumor incidence from week 13 to week 18 (Figure 3A); (ii) PN reduced tumor multiplicity by 30% (Figure 3B); (iii) smaller papilloma sizes were found in mice treated with PN (Table I); and (iv) PN treatment reduced the hyperplastic response of the epidermis induced by UVB irradiation (Figure 4). The chemopreventive capability of PN is found to be as effective as the pharmaceutical COX-2 inhibitor celecoxib in preventing UVB-induced photocarcinogenesis (28,33).

COX-2 is the inducible subtype of the enzyme COX, which converts free arachidonic acid to PGEs (50). The fact that UVB up-regulates COX-2 and PGE production suggested COX-2 may contribute to photocarcinogenesis (34,51). Preliminary findings from our laboratory revealed a positive correlation between cytotoxic response to PN and COX-2 expression (unpublished data), which leads us to hypothesize that PN may exert its effect in a COX-2-dependent manner. However, our results showed that neither treatment of PN nor the specific COX-2 inhibitor celecoxib alters COX-2 expression or PGEs production induced by UVB (Figure 5), suggesting that COX-2 is unlikely to be the direct molecular target for PN and celecoxib. This finding is in concordance with a previous report in which long-term oral treatment of celecoxib has no effect on UVB-induced expression of COX-2 (28). Furthermore, *in vitro* studies also demonstrated that celecoxib induces apoptosis and cell cycle arrest in a COX-2-independent manner (52–54). In contrast, a recent study reported that topical treatment of celecoxib is as effective as the oral route in inhibiting UVB-induced skin cancer while also suppressing PGE_2 production

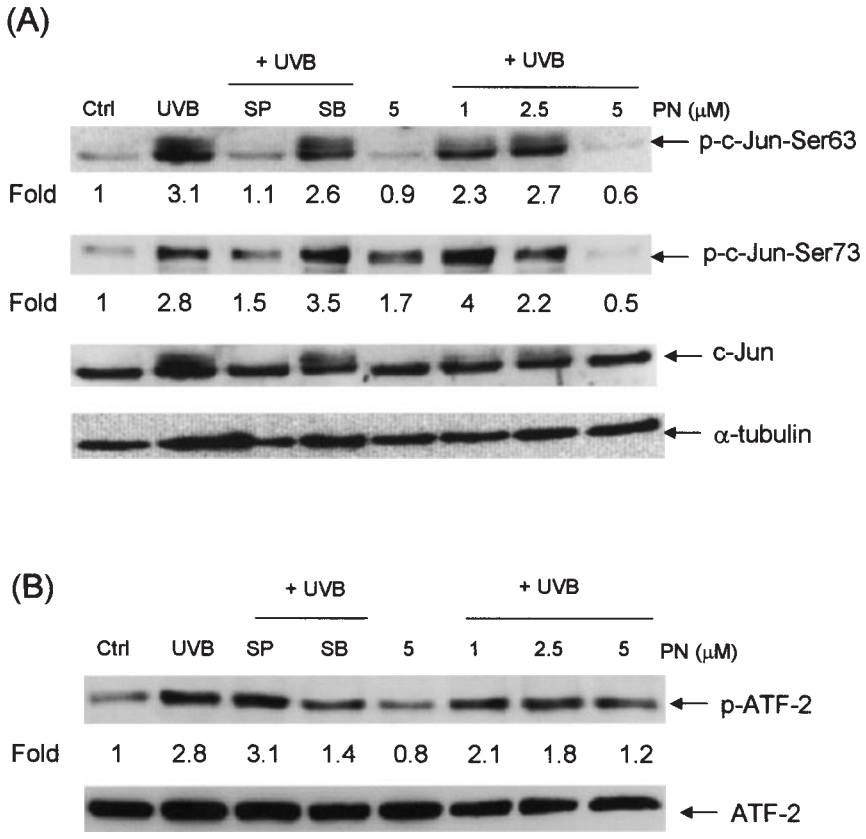

Fig. 8. PN inhibits the UVB-induced phosphorylations of (**A**) c-Jun and (**B**) ATF-2. JB6 cells were pre-treated with 1, 2.5 and 5 μM of PN, 20 μM of SP600125, or 10 μM SB203580 for 2 h, and then subjected to UVB irradiation of 50 mJ/cm^2. Cells were harvested 2 h after UVB irradiation. Thirty micrograms of proteins were separated on 10% SDS–polyacrylamide gels and the subsequent membranes were hybridized with anti-p-c-Jun and p-ATF-2 antibodies. α-Tubulin was blotted as loading control. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station 440.

induced by UVB (55). It remains to be determined whether such a difference in PGE_2 suppression by celecoxib is due to the different routes of administration applied.

Nuclear transcriptional factors such as AP-1, NF-κB, NF-AT and STATs have been reported to be involved in the UVB-induced signaling pathway (14–17). In order to further understand the possible mechanism(s) contributing to the observed chemopreventive property of PN, we investigated the effects of PN on AP-1 and the related MAPKs signaling cascade using JB6 murine epidermal cells. AP-1 plays a key role in pre-neoplastic to neoplastic transformation in cell culture and the blockage of AP-1 activities has been shown to inhibit cell transformation (56). Moreover, AP-1 has been shown to regulate apoptosis, with both pro-apoptotic and anti-apoptotic function (18). It appears that the exact outcome of AP-1 manipulations is highly tissue- and developmental stage-specific. In the present study, we demonstrated that PN pre-treatment inhibits the UVB-induced DNA binding and transcriptional activity of AP-1 (Figure 7). Two of the major components of AP-1 complex, c-Jun and ATF-2, can activate AP-1 via post-translational modification involving changes in the phosphorylation states (42–44). In response to UV, c-Jun is rapidly phosphorylated at both Ser63 and Ser73 sites along with ATF-2. Our data showed that PN subdues UVB-induced AP-1 transactivation via blockage of c-Jun and ATF-2 phosphorylations (Figure 8). The outcome of these series of inhibitions seems to lead to massive sensitization to UVB-induced apoptosis (Figure 6), suggesting an anti-apoptotic role of AP-1. Such anti-apoptotic function of AP-1 and c-Jun has been reported previously. Wisdom and co-workers (47) noted that c-Jun protects cells from UV-induced apoptosis, and phosphorylation of c-Jun on Ser63 and Ser73 is required for such protection. Moreover, Ivanov and colleagues (57) demonstrated that c-Jun protects cells against UV-induced cell death via co-operation with STAT3 to suppress transcription of Fas.

The UV-activated signal transduction pathway is primarily mediated by MAPKs. Mammals express at least four distinctly regulated groups of MAPK: Erks, JNK, p38 and Erk5 (58). All MAPKs are activated by dual phosphorylation on threonine and tyrosine at T-X-Y motifs within the activation loop. Once activated, they translocate to the nucleus and phosphorylate target transcription factors. In response to UV, key MAPKs such as JNK and p38 are activated, which in turn phosphorylate c-Jun at Ser63 and Ser73 sites and ATF-2, respectively (42,43). It is rather controversial in regards to the exact role of JNK in UV-mediated apoptosis. For instance, JNK has been shown to be required for UV-induced apoptotic cell death in mouse embryonic fibroblasts (46), while other studies demonstrated the anti-apoptotic role of JNK in UV-induced apoptosis (47,57). Here we showed that the inhibition of JNK leads to sensitization of JB6 cells to UVB-induced apoptosis, suggesting an anti-apoptotic role of JNK in our system (Figure 10). In general, the exact role of JNK in apoptosis varies depending on a number of factors that include the nature of the stimuli, cell type, the duration of activation and more importantly, the

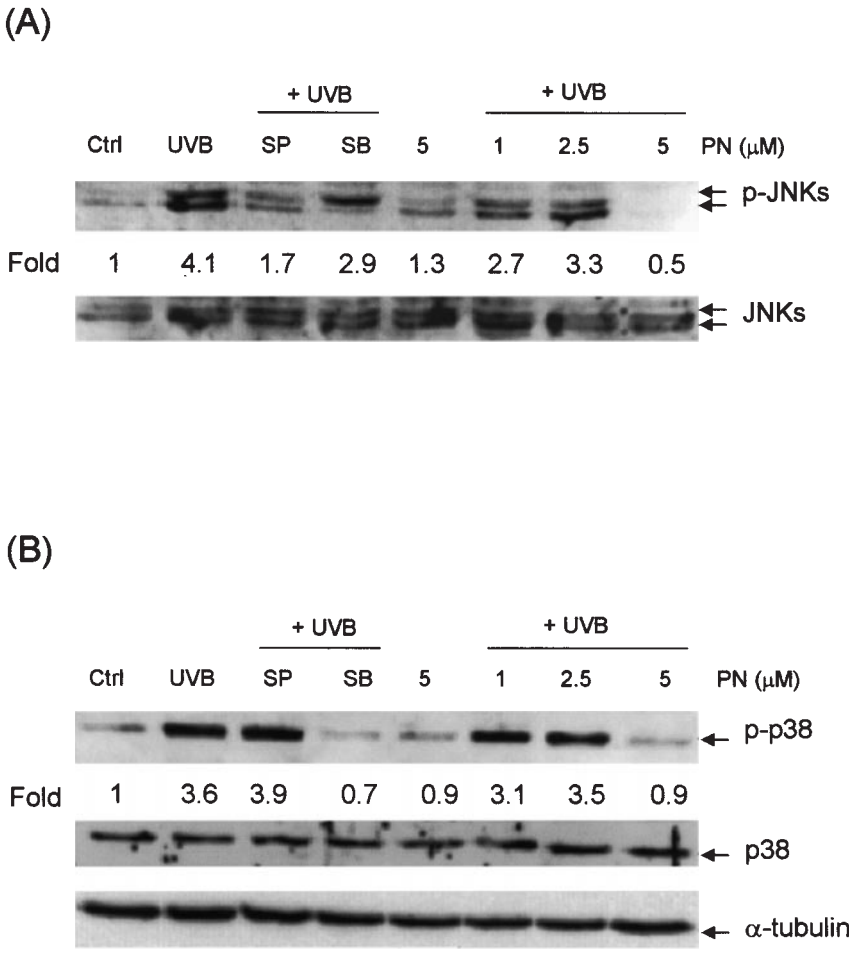

Fig. 9. PN inhibits the UVB-induced phosphorylation of (**A**) JNK and (**B**) p38. JB6 cells were pre-treated with 1, 2.5 and 5 μM of PN, 20 μM of SP600125, or 10 μM SB203580 for 2 h, and then subjected to UVB irradiation of 50 mJ/cm^2. Cells were harvested 2 h after UVB irradiation. Thirty micrograms of proteins were separated on 10% SDS–polyacrylamide gels and the subsequent membranes were hybridized with anti-p-JNK, JNK, p-p38, p38 antibodies. α-Tubulin was blotted as loading control. The blots were detected using the enhanced chemiluminescence method (Pierce) and analyzed using Kodak Image Station 440.

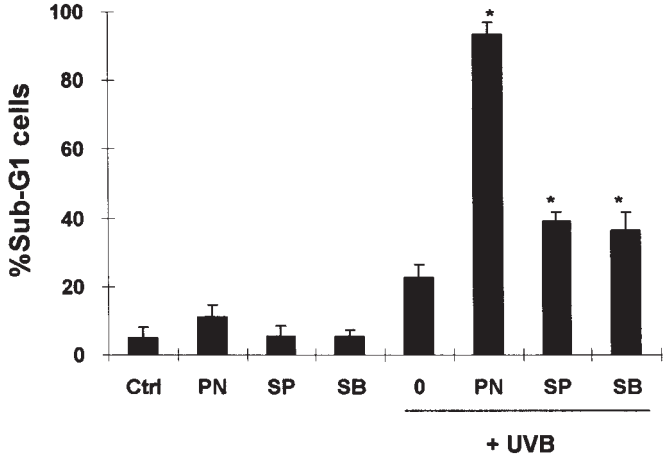

Fig. 10. PN, SP600125 and SB203580 sensitize cells to UVB-induced apoptosis. JB6 cells were pre-treated with PN (5 μM), SP600125 (20 μM) or SB203580 (10 μM) for 2 h, and then subjected to UVB irradiation of 50 mJ/cm^2. The viability of cells was determined using PI staining in sub-G_1 assay at 24 h post-UVB treatment. Data were presented as means ± SD from three independent experiments and analyzed using one-way ANOVA with Student–Newman–Keuls multiple comparisons test. An asterisk indicates statistical significance with a P-value <0.05.

interaction of other signaling pathways such as NF-κB and the AKT-PI3K cascades (59–62). The involvement of those cell survival signaling pathways in PN-mediated cell death sensitization remains to be investigated further.

In the present study, we also noted the potent inhibitory effect of PN on UVB-induced p38 activation (Figure 9), and p38 inhibition leads to sensitization of cells to UVB-induced apoptosis (Figure 10), implying an anti-apoptotic role of p38 in UVB-induced apoptotic cell death. Although it is still controversial regarding the exact role of p38 in apoptosis, p38 has been reported to protect cells from UV-induced apoptosis through down regulation of NF-κB activity and Fas expression (48). Some other mechanisms have also been proposed to be involved in the anti-apoptotic function of p38. For instance, the p38 MAPK inhibitor SB203580 can trigger a significant, Ras-independent activation of c-Raf in certain cell lines in the concentration range of 8–25 μM (63,64). Activated Raf may phosphorylate and inactivate Bad, a pro-apoptotic member of the Bcl-2 family of proteins, or up-regulate the transcription of pro-survival genes to prevent cytochrome *c* release and subsequently apoptosis (65). Furthermore, Raf-1 has been shown to promote cell survival by antagonizing apoptosis signal-regulating kinase 1, an important mediator of apoptotic signaling (66). In the present study, we demonstrated PN as a dual

inhibitor of both JNK and p38 in UVB-treated cells (Figure 9). Incidentally, the PN-elicited sensitization of JB6 cells to UVB-induced apoptosis is twice as strong as with the individual JNK and p38 inhibitor (Figure 10). These findings suggest that both JNK and p38 contribute to the cell survival mechanisms in UVB-treated cells.

UV exposure could result in direct or indirect DNA damage, and the damage is normally repaired by a nucleotide excision repair mechanism (67). Most of the irreparable DNA-damaged cells will be eliminated through apoptosis, as evident in skin with the appearance of sunburn cells (38). However, not all cells with irreparable DNA damage will undergo apoptosis. DNA lesions that are not repaired or incorrectly repaired may lead to mutations and subsequently carcinogenesis. Therefore, the removal of damaged cells through sensitizing cells to apoptosis denotes an effective mean in preventing carcinogenesis process (37–39).

In summary, our data for the first time demonstrated the chemopreventive property of PN against UVB-induced skin cancer in female SKH-1 mice. Using JB6 murine epidermal cells *in vitro*, we also found the synergistic effect of PN and UVB in sensitizing cells to apoptosis. Such sensitization appears to be mediated through inhibition on AP-1, JNK and p38 signaling pathways and may contribute to the anticancer activity of PN.

Acknowledgements

The authors would like to thank S.Y.Zhang, M.Zhao, J.Su, Y.B.Ong, M.T.Tham and B.L.Lee for technical assistance; and Dr A.Seow and Ms B.C.Tai for their advice in statistical analysis. Y.K.Won is supported by a research scholarship from the National University of Singapore. This work is supported by a research grant from the National Medical Research Council (NMRC 0645/2000, Singapore).

References

1. Brown,A.M.G., Edwards,C.M., Davey,M.R., Power,J.B. and Lowe,K.C. (1997) Pharmacological activity of feverfew [*Tanacetum parthenium* (L.) Schultz-Bip.]: assessment by inhibition of human polymorphonuclear leukocyte chemiluminescence *in-vitro*. *J. Pharm. Pharmacol.*, **49**, 558–561.
2. Jain,N.K. and Kulkarni,S.K. (1999) Antinociceptive and anti-inflammatory effects of *Tanacetum parthenium* L extract in mice and rats. *J. Ethnopharmacol.*, **68**, 251–259.
3. Macias,F.A., Galindo,J.C., Castellano,D. and Velasco,R.F. (1999) Sesquiterpene lactones with potential use as natural herbicide models (I): trans,trans-germacranolides. *J. Agric. Food Chem.*, **47**, 4407–4414.
4. Woynarowski,J.M. and Konopa,J. (1981) Inhibition of DNA biosynthesis in HeLa cells by cytotoxic and antitumor sesquiterpene lactones. *Mol. Pharmacol.*, **19**, 97–102.
5. Hall,I.H., Williams,W.L,Jr., Grippo,A.A., Lee,K.H., Holbrook,D.J. and Chaney,S.G. (1988) Inhibition of nucleic acid synthesis in P-388 lymphocytic leukemia cells in culture by sesquiterpene lactones. *Anticancer Res.*, **8**, 33–42.
6. Ross,J.J., Arnason,J.T. and Birnboim,H.C. (1999) Low concentrations of the feverfew component parthenolide inhibit *in vitro* growth of tumor lines in a cytostatic fashion. *Planta Med.*, **65**, 126–9.
7. Patel,N.M., Nozaki,S., Shortle,N.H. *et al.* (2000) Paclitaxel sensitivity of breast cancer cells with constitutively active NF-κB is enhanced by IκBa super-repressor and parthenolide. *Oncogene*, **19**, 4159–4169.
8. Wen,J., You,K.R., Lee,S.Y., Song,C.H. and Kim,D.G. (2002) Oxidative stress-mediated apoptosis. The anticancer effect of the sesquiterpene lactone parthenolide. *J Biol. Chem.*, **277**, 38954–38964.
9. Parker,S.L., Tong,T., Bolden,S. and Wingo,P.A. (1997) Cancer Statistics, 1997. *CA Cancer J. Clin.*, **47**, 5–27.
10. Jung,E.G. (1991) Photocarcinogenesis in the skin. *J. Dermatol.*, **18**, 1–10.
11. Brash,D.E. (1997) Sunlight and the onset of skin cancer. *Trends Genet.*, **13**, 410–414.
12. Yamawaki,M., Katiyar,S.K., Anderson,C.Y., Tubesing,K.A., Mukhtar,H. and Elmets,C.A. (1997) Genetic variation in low-dose UV-induced suppression of contact hypersensitivity and in the skin photocarcinogenesis response. *J. Invest. Dermatol.*, **6**, 716–721.
13. de Gruijl,F.R. (1999) Skin cancer and solar UV radiation. *Eur. J. Cancer*, **35**, 2003–2009.
14. Angel,P., Szabowski,A. and Schorpp-Kistner,M. (2001) Function and regulation of AP-1 subunits in skin physiology and pathology. *Oncogene*, **20**, 2413–2423.
15. Siebenlist,U., Brown,K. and Franzoso,G. (1995). NF-κB: a mediator of pathogen and stress responses. In Baeuerle,P.A. (ed.) *Inducible Gene Expression*. Birkhauser, Boston, pp. 93–141.
16. Huang,C., Mattjus,P., Ma,W.Y., Rincon,M., Chen,N.Y., Brown,R.E. and Dong,Z. (2000) Involvement of nuclear factor of activated T cells activation in UV response. Evidence from cell culture and transgenic mice. *J. Biol. Chem.*, **275**, 9143–9149.
17. Zhang,Y., Liu,G. and Dong,Z. (2001) MSK1 and JNKs mediate phosphorylation of STAT3 in UVA-irradiated mouse epidermal JB6 cells. *J. Biol. Chem.*, **276**, 42534–42542.
18. Shaulian,E. and Karin,M. (2002) AP-1 as a regulator of cell life and death. *Nature Cell Biol.*, **4**, E131–136.
19. Karin,M., Liu,Z. and Zandi,E. (1997) AP-1 function and regulation. *Curr. Opin. Cell Biol.*, **9**, 240–246.
20. Jochum,W., Passegue,E. and Wagner,E.F. (2001) AP-1 in mouse development and tumorigenesis. *Oncogene*, **20**, 2401–2412.
21. Ozanne,B.W., McGarry,L., Spence,H.J., Johnston,I., Winnie,J., Meagher,L. and Stapleton,G. (2000) Transcriptional regulation of cell invasion: AP-1 regulation of a multigenic invasion programme. *Eur. J. Cancer*, **36**, 1640–1648.
22. Lamb,R.F., Hennigan,R.F., Turnbull,K., Katsanakis,K.D., Mackenzie,E.D., Birnie,G.D. and Ozanne,B.W. (1997) AP-1 mediated invasion requires increased expression of the hyaluronan receptor CD44. *Mol. Cell Biol.*, **17**, 963–976.
23. Suzuki,T., Murakami,M., Onai,N., Fukuda,E., Hashimoto,Y., Sonobe,M.H., Kameda,T., Ichinose,M., Miki,K. and Iba,H. (1994) Analysis of AP-1 function in cellular transformation pathways. *J. Virol.*, **68**, 3527–3535.
24. Barthelman,M., Chen,W., Gensler,H.L., Huang,C., Dong,Z. and Bowden,G.T. (1998) Inhibitory effects of perillyl alcohol on UVB-induced murine skin cancer and AP-1 transactivation. *Cancer Res.*, **58**, 711–716.
25. Bork,P.M., Schmitz,M.L., Kuhnt,M., Escher,C. and Heinrich,M. (1997) Sesquiterpene lactone containing Mexican India medicinal plants as potent inhibitors of transcription factor NF-κB. *FEBS Lett.*, **402**, 85–90.
26. Hehner,S.P., Heinrich,M., Bork,P.M., Vogt,M., Ratter,F., Lehmann,V., Schulze-Osthoff,K., Droge,W. and Schmitz,M.L. (1998) Sesquiterpene lactones specifically inhibit activation of NF-κB by preventing the degradation of IκB-α and IκB-β. *J. Biol. Chem.*, **273**, 1288–1297.
27. Hehner,S.P., Hofmann,T.G., Droge,W. and Schmitz,M.L. (1999) The anti-inflammatory sesquiterpene lactone parthenolide inhibits NF-κB by targeting the IκB kinase complex. *J. Immunol.*, **163**, 5617–5623.
28. Fischer,S.M., Lo,H.H., Gordon,G.B., Seibert,K., Kelloff. G., Lubet,R.A. and Conti,C.J. (1999) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor and indomethacin against ultraviolet light-induced skin carcinogenesis. *Mol Carcinog.*, **25**, 231–240.
29. Ding,M., Shi,X., Dong,Z., Chen,F., Lu,Y., Castranova,V. and Vallyathan,V. (1999) Freshly fractured crystalline silica induces activator protein-1 activation through ERKs and p38 MAPK. *J. Biol. Chem.*, **274**, 30611–30616.
30. Ding,M., Shi,X., Lu,Y., Huang,C., Leonard,S., Roberts,J., Antonini,J., Castranova,V. and Vallyathan,V. (2001) Induction of activator protein-1 through reactive oxygen species by crystalline silica in JB6 cells. *J. Biol. Chem.*, **276**, 9108–9114.
31. Shen,H.M., Yang,C.F. and Ong,C.N. (1999) Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG2 cells. *Int. J. Cancer*, **81**, 820–828.
32. Yang,C.F., Shen,H.M. and Ong,C.N. (2000) Ebselen induces apoptosis in HepG (2) cells through rapid depletion of intracellular thiols. *Arch. Biochem. Biophys.*, **374**, 142–152.
33. Pentland,A.P., Schoggins,J.W., Scott,G.A., Khan,K.N. and Han,R. (1999) Reduction of UV-induced skin tumors in hairless mice by selective COX-2 inhibition. *Carcinogenesis*, **20**, 1939–1944.
34. Buckman,S.Y., Gresham,A., Hale,P., Hruza,G., Anast,J., Masferrer,J. and Pentland,A.P. (1998) Cox-2 expression is induced by UVB exposure in human skin: implications for the development of skin cancer. *Carcinogenesis*, **19**, 723–729.

35. Chan,G., Boyle,J.O., Yang,E.K. *et al*. (1999) Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. *Cancer Res*., **59**, 991–994.
36. Athar,M., An,K.P., Morel,K.D., Kim,A.L., Aszterbaum,M., Longley,J., Epstein,E.H. and Bickers,D.R. (2001) Ultraviolet B (UVB)-induced Cox-2 expression in murine skin: an immunohistochemical study. *Biochem. Biophys. Res. Commun*., **280**, 1042–1047.
37. Hildesheim,J., Bulavin,D.V., Anver,M.R., Alvord,W.G., Hollander,M.C., Vardanian,L. and Fornace,A.J.,Jr (2002) Gadd45a protects against UV irradiation-induced skin tumors and promotes apoptosis and stress signaling via MAPK and p53. *Cancer Res*., **62**, 7305–7315.
38. Kulms,D. and Schwarz,T. (2002) Molecular mechanisms involved in UV-induced apoptotic cell death. *Skin Pharmacol. Appl. Skin Physiol*., **15**, 342–347.
39. D'Errico,M., Teson,M., Calcagnile,A., De Santis,L.P., Nikaido,O., Botta,E., Zambruno,G., Stefanini,M. and Dogliotti,E. (2003) Apoptosis and efficient repair of DNA damage protect human keratinocytes against UVB. *Cell Death Differ*., **10**, 754–756.
40. Huang,C., Ma,W.Y., Hanenberger,D., Cleary,M.P., Bowden,G.T. and Dong,Z. (1997) Inhibition of ultraviolet B-induced activator protein-1 (AP-1) activity by aspirin in AP-1-luciferase transgenic mice. *J. Biol. Chem*., **272**, 26325–26331.
41. Buscher,M., Rahmsdorf,H.J., Litfin,M., Karin,M. and Herrlich,P. (1988) Activation of the c-fos gene by UV and phorbol ester: different signal transduction pathways converge to the same enhancer element. *Oncogene*, **3**, 301–311.
42. Devary,Y., Gottlieb,R.A., Lau,L.F. and Karin,M. (1991) Rapid and preferential activation of the c-jun gene during the mammalian UV response. *Mol. Cell. Biol*., **11**, 2804–2811.
43. van Dam,H., Wilhelm,D., Herr,I., Steffen,A., Herrlich,P. and Angel,P. (1995) ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J*., **14**, 1798–1811.
44. Adler,V., Polotskaya,A., Kim,J., Dolan,L., Davis,R., Pincus,M. and Ronai,Z. (1996) Dose rate and mode of exposure are key factors in JNK activation by UV irradiation. *Carcinogenesis*, **17**, 2073–2076.
45. Chen,W. and Bowden,G.T. (2000) Role of p38 mitogen-activated protein kinases in ultraviolet-B irradiation-induced activator protein 1 activation in human keratinocytes. *Mol. Carcinog*., **28**, 196–202.
46. Tournier,C., Hess,P., Yang,D.D., Xu,J., Turner,T.K., Nimnual,A., Bar-Sagi,D., Jones,S.N., Flavell,R.A. and Davis,R.J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science*, **288**, 870–874.
47. Wisdom,R., Johnson,R.S. and Moore,C. (1999) c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. *EMBO J*., **18**, 188–197.
48. Ivanov,V.N. and Ronai,Z. (2000) p38 protects human melanoma cells from UV-induced apoptosis through down-regulation of NF-kappaB activity and Fas expression. *Oncogene*, **19**, 3003–3012.
49. Chouinard,N., Valerie,K., Rouabhia,M. and Huot,J. (2002) UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53. *Biochem. J*., **365**, 133–145.
50. Marnett,L.J. (2000) Cyclooxygenase mechanisms. *Curr. Opin. Chem. Biol*., **4**, 545–552.
51. Grewe,M., Trefzer,U., Ballhorn,A., Gyufko,K., Henninger,H. and Krutmann,J. (1993) Analysis of the mechanism of ultraviolet (UV) B radiation-induced prostaglandin E2 synthesis by human epidermoid carcinoma cells. *J. Invest. Dermatol*., **101**, 528–531.
52. Elder,D.J., Halton,D.E., Hague,A. and Paraskeva,C. (1997) Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin. Cancer Res*., **3**, 1679–1683.
53. Grosch,S., Tegeder,I., Niederberger,E., Brautigam,L. and Geisslinger,G. (2001) COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. *FASEB J*., **15**, 2742–2744.
54. Waskewich,C., Blumenthal,R.D., Li,H., Stein,R. Glodenberg,D.M. and Burton,J. (2002) Celecoxib exhibits the greatest potency amongst cyclooxgenase (COX) inhibitors for growth inhibition of COX-2 negative hemaopoietic and epithelial cell lines. *Cancer Res*., **62**, 2029–2033.
55. Wilgus,T.A., Koki,A.T., Zweifel,B.S., Kusewitt,D.F. and Rubal,P.A. (2003) Inhibition of cutaneous ultraviolet light B-mediated inflammation and tumor formation with topical celecoxib treatment. *Mol. Carcinog*., **38**, 49–58.
56. Dong,Z., Birrer,M.J., Watts,R.G., Matrisian,L.M. and Colburn,N.H. (1994) Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc. Natl Acad. Sci. USA*, **91**, 609–613.
57. Ivanov,V.N., Bhoumik,A., Krasilnikov,M., Raz,R., Owen-Schaub,L.B., Levy,D., Horvath,C.M. and Ronai,Z. (2001) Cooperation between STAT3 and c-jun suppresses Fas transcription. *Mol. Cell*., **7**, 517–528.
58. Chang,L. and Karin,M. (2001) Mammalian MAP kinase signaling cascades. *Nature*, **410**, 37–40.
59. Davis,R.J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell*, **103**, 239–252.
60. Lamb,J.A., Ventura,J.J., Hess,P., Flavell,R.A. and Davis,R.J. (2003) JunD mediates survival signaling by the JNK signal transduction pathway. *Mol. Cell*., **11**, 1479–1489.
61. Lin A. (2003) Activation of the JNK signaling pathway: breaking the brake on apoptosis. *Bioessays*, **25**, 17–24.
62. Molton,S.A., Todd, D.E. and Cook,S.J. (2003) Selective activation of the c-Jun N-terminal kinase (JNK) pathway fails to elicit Bax activation or apoptosis unless the phosphoinositide 3′-kinase (PI3K) pathway is inhibited. *Oncogene*, **22**, 4690–4701.
63. Hall-Jackson,C.A., Goedert,M., Hedge,P. and Cohen,P. (1999) Effect of SB 203580 on the activity of c-Raf *in vitro* and *in vivo*. *Oncogene*, **18**, 2047–2054.
64. Kalmes,A., Deou,J., Clowes,A.W. and Daum,G. (1999) Raf-1 is activated by the p38 mitogen-activated protein kinase inhibitor, SB203580. *FEBS Lett*., **444**, 71–74.
65. Bonni,A., Brunet,A., West,A.E., Datta,S.R., Takasu,M.A. and Greenberg,M.E. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science*, **286**, 1358–1362.
66. Chen,J., Fujii,K., Zhang,L., Roberts,T. and Fu,H. (2001) Raf-1 promotes cell survival by antagonizing apoptosis signal-regulating kinase 1 through a MEK-ERK independent mechanism. *Proc. Natl Acad. Sci. USA*, **98**, 7783–7788.
67. Thoma,F. (1999) Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair. *EMBO J*., **18**, 6585–6598.

Received January 1, 2004; revised and accepted March 9, 2004