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ORIGINAL PAPER

Inhibition of UVA-induced apoptotic signaling pathway by polypeptide from *Chlamys farreri* in human HaCaT keratinocytes

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Abstract Chronic UVA irradiation has been reported to induce photoaging and photocarcinogenesis. UVA is a potent inducer of reactive oxygen species (ROS), which can induce various biological processes, including apoptosis. Polypeptide from *Chlamys farreri* (PCF) is a novel marine active material isolated from the gonochoric Chinese scallop *C. farreri*. In our previous studies, PCF was found to be an effective antioxidant inhibiting UVA-induced ROS production and a potential inhibitory agent for UVA-induced apoptosis in the human keratinocyte cell line HaCaT. The intracellular mechanisms of how PCF protects HaCaT cells from UVA-induced apoptosis are not understood. Thus, we here investigate the effect of PCF on UVA-induced intracellular signaling of apoptosis. Pretreatment with the ROS scavenger *N*-acetylcysteine (NAC), the p38 MAPK inhibitor SB203580 or the caspase-3 inhibitor Ac-DEVD-CHO was found to effectively prevent UVA-induced apoptosis, indicating that ROS, p38 MAPK and caspase-3 play important roles in apoptosis. H_2O_2-induced apoptosis was attenuated by PCF, suggesting that PCF plays its anti-apoptotic role through its antioxidant activity. In addition, PCF treatment inhibited UVA-induced p38 MAPK activation and caspase-3 activation, as assayed by Western blot analysis and flow cytometry, respectively. Our results suggest that PCF attenuates UVA-induced apoptosis through a reduction of ROS generation and diminished p38 MAPK and caspase-3 activation.

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Introduction

Ultraviolet (UV) irradiation has deleterious effects on human skin, including sunburn, immune suppression, cancer, and photoaging [1]. The UV spectrum can be divided into three wavelength ranges, UVA (320–400 nm), UVB (280–320) and UVC (200–280 nm) [2, 3]. Of the solar radiation that reaches the surface of the earth, 90–99% is comprised of UVA and 1–10% is comprised of UVB. The ozone layer absorbs a large portion of UVB and all of the UVC wavelengths [3]. UVA penetrates more deeply into the skin than UVB, reaching not only the epidermis but also the dermis. Approximately 10–20% of the carcinogenic dose of sunlight is derived from UVA. Chronic UVA irradiation alone has been reported to induce photoaging and to lead to the formation of tumors in hairless mouse models [4, 5].

UV irradiation is a potent inducer of various reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide (H_2O_2) and hydroxyl radicals [6]. ROS have been shown to induce various biological processes, including apoptosis or programmed cell death [7].

The MAPK are a family of proline-directed Ser/Thr kinases composed of ERK1/2, JNK and p38 kinase. Various extracellular stimuli including UV irradiation activate MAPKs. p38 MAPK appears to play a major role in apoptosis, cytokine production, transcriptional regulation, and cytoskeletal reorganization [8]. It has been shown that p38 MAPK plays an important role in UV-induced apoptosis [9–11].

The caspase family of cysteine proteases plays a key role in apoptosis and inflammation [12]. Caspase-3 is a key protease that is activated during the early stages of apoptosis. It is central to the execution of apoptosis in response to UV exposure [13].

One approach to protecting humans from the harmful effects of UV irradiation is to use active photoprotectives. In recent years, naturally occurring compounds have gained considerable attention as protective agents. For instance, vitamins C, E, and β-carotene have been incorporated into many skin care products. Polypeptide from *Chlamys farreri* (PCF), Mr = 879, is a novel marine active material isolated from gonochoric Chinese scallop *C. farreri*. As an octapeptide, PCF consists of Pro, Asn, Ser, Thr, Arg, Hyl, Cys, and Gly. Studies from our laboratory have indicated that PCF could inhibit production of ROS and apoptosis of HaCaT cells after UVA exposure [14]. Based on the important roles of ROS, p38 MAPK and caspase-3 in UV-induced apoptosis, in the present study, we investigated the possibility that PCF inhibited UVA-induced apoptosis in HaCaT cells through effects on ROS, p38 MAPK and caspase-3.

Materials and methods

Materials

Cell culture materials were purchased from GIBCO-BRL. The p38 MAPK inhibitor SB203580 (dissolved in DMSO) was purchased from Calbiochem (Darmstadt, Germany). Caspase-3 inhibitor Ac-DEVD-CHO (dissolved in DMSO) and anti-p38 MAPK antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-p38 MAPK antibody and anti-β-actin antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-active caspase-3 monoclonal antibody was purchased from BD Pharmingen. *N*-acetylcystein (NAC) was obtained from Sigma. PCF (Yellow Sea Fishery Research Institute, China) was purified and analyzed by HPLC, dissolved in sterile deionized water, and stored at 4°C. The PCF purity used in this study is more than 96%. All the other chemicals used were of the highest grade commercially available.

Cell culture and UVA treatment

The spontaneously immortalized human keratinocyte cell line HaCaT, kindly provided by Dr. Ding Boxiao (Yonsei University, Korea), was cultured in 95% air, 5% CO_2 at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin. Cells were grown to 80–90% confluence and placed in serum-free DMEM overnight prior to irradiation. UVA exposure was performed with UVA lamps (Beijing Normal University, China) and last 2 h to reach the fluence 10 J/cm^2. PCF, NAC, SB203580 and Ac-DEVD-CHO were added in medium 2, 2, 1 and 1 h, respectively, before irradiation. During irradiation, medium was discarded and cells were placed in PBS. Control cells were mock-irradiated in PBS. After irradiation, medium was exchanged and cells were cultured normally.

DNA fragmentation assay

DNA fragmentation is a marker of cell apoptosis [15]. The pattern of DNA fragmentation was analyzed by agarose gel electrophoresis 18 h after irradiation. Briefly, 1×10^6 cells were centrifuged at 1,000*g* for 10 min. Cell pellets were resuspended in 500 µl cell lysis buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.5, 10 mM EDTA, 0.5% SDS, 500 mg l^{-1} proteinase K) and incubated overnight at 50°C. After incubation, the cell lysate was extracted with phenol/chloroform/isopropyl alcohol (25:24:1, v/v). DNA was precipitated with sodium acetate and ethanol at −20°C overnight, and then washed with 70% ethanol. DNA pellets were dissolved in 1× TE buffer and were incubated with RNase A (20 µg l^{-1}) at 37°C for 30 min. DNA samples were separated by horizontal electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

Hoechst 33258 staining

We also conducted Hoechst 33258 staining for the identification of apoptotic nuclei. HaCaT cells were collected and fixed, washed twice with PBS and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Apoptosis, Hoechst Staining Kit, Beyotime Biotechnology, China). Stained nuclei were observed under a fluorescence microscope (Leica DBI 4000 B). The number of cells containing apoptotic nuclei among the blue fluorescent protein expressing cells was counted via fluorescence microscopy. In each group, five microscopic fields were selected randomly and more than seven hundreds cells were counted. Apoptotic cell death was then calculated as a percentage of apoptotic cells over the total blue fluorescent protein-positive cells. Three independent experiments were done.

Western blot analysis

Total cellular protein was extracted in lysis buffer (20 mM Tris–HC1, pH 7.5, 150 mM NaC1, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg l^{-1} leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cells were scraped and centrifuged at 10,000*g* at 4°C for 10 min. Protein levels were quantified using a bicinchoninic acid assay (Beyotime Biotechnology, China). For Western analysis, 40 µg of protein were resolved on a 12% SDS-polyacrylamide gel. The

protein was then transferred to a nitrocellulose membrane. The membrane was then blocked with 5% BSA in TBST at room temperature for 1 h and subsequently incubated with rabbit anti-phospho-p38 (1:200) or anti-β-actin (1:1,000) antibodies overnight at 4°C. Goat anti-rabbit secondary antibodies were diluted at 1:2,000 in 5% BSA/TBST and were incubated with membranes for 1 h at room temperature. Membranes were washed three times for 5 min each in TBST between antibody incubations. Protein bands were visualized using the ECL Western blotting kit (Pufei Biotechnology, Shanghai, China). The membranes were reprobed with anti-p38 antibody after the phospho-p38 blots were stripped. The densities of sample bands were determined using a fluorescence scanner and analyzed with Quantity One analysis software (Bio-Rad, USA).

Flow cytometric analysis of caspase-3 activity

Caspase-3 is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. Active caspase-3 proteolytically cleaves and activates other caspases, as well as relevant targets in the cytoplasm. Caspase-3 activity was detected by flow cytometry analysis using the anti-active caspase-3 monoclonal antibody, which specifically recognizes the active form of caspase-3. Briefly, cells were washed twice in PBS, fixed using 4% polyoxymethylene for 30 min and then permeabilized using 1% TritonX-100 for 10 min at room temperature, before they were washed twice with PBS. Cells were stained with anti-active caspase-3 antibody for 30 min at room temperature in the dark. Following incubation with the antibody, cells were washed in wash buffer, resuspended in wash buffer and analyzed by flow cytometry (FACSVantage SE, BD Biosciences).

Statistical analysis

Data were expressed as means ± SD. The statistical significance was assessed by one-way analysis of variance (ANOVA). The level of differences among groups was analyzed by Student–Newman–Keuls' test. Differences were considered to be statistically significant with $P < 0.05$.

Results

Induction of apoptosis in UVA-irradiated HaCaT cells is demonstrated by the appearance of a DNA ladder (Fig. 1, lane 2) at 18 h after irradiation. Pretreatment with 1.42, 2.84, or 5.69 mM PCF for 2 h prior to irradiation markedly attenuated UVA-induced apoptosis (Fig. 1, lanes 3–5). The ROS scavenger NAC also demonstrated anti-apoptotic activity (Fig. 1, lane 6), suggesting that UVA-induced

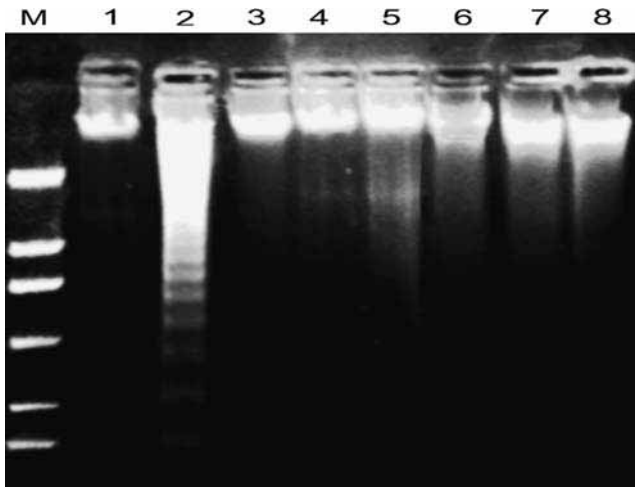

Fig. 1 Effect of PCF, NAC, SB203580 or Ac-DEVD-CHO on UVA-induced DNA-fragmentation in HaCaT cells. *M* marker (2,000, 1,000, 750, 500, 250, 100 bp); *lane 1* HaCaT cells were mock-irradiated (control); *lane 2* HaCaT cells were irradiated with 10 J/cm^2 UVA; *lanes 3–6* HaCaT cells were pretreated with 1.42, 2.84, 5.69 mM PCF or 5 mM NAC for 2 h prior to UVA irradiation, respectively; *lanes 7–8* HaCaT cells were pretreated with 10 μM SB203580 or 100 μM Ac-DEVD-CHO for 1 h prior to UVA irradiation respectively. After irradiation, cells were further incubated for 18 h, and DNA-fragmentation were detected by agarose gel electrophoresis. Results were representative of three independent experiments

apoptosis in HaCaT cells is mediated by ROS. To investigate the relationship between antioxidant activity and anti-apoptotic effect, the effect of PCF on H_2O_2-induced apoptosis was examined, using chromatin condensation as a marker for apoptosis. As shown in Fig. 2, pretreatment with 1.42 and 5.69 mM PCF for 2 h prior to H_2O_2 treatment also prevented H_2O_2-induced apoptosis. These data support the view that the protective effect of PCF on UVA-induced apoptosis is mediated by its effects on ROS.

As shown in Fig. 3, lane 2, UVA irradiation strongly stimulates the expression of phosphorylated p38 MAPK, which is the active form of the kinase, while total levels of p38 MAPK are not affected. To address the contribution of p38 MAPK to induce apoptosis in HaCaT cells, we examined the effect of the specific p38 MAPK inhibitor SB203580 on UVA-induced apoptosis. Preincubation of HaCaT cells with SB203580 effectively prevented UVA-induced apoptosis (Fig. 1, lane 7) and significantly blocked p38 MAPK activation induced by UVA (Fig. 3, lane 7). These results indicate that UVA-induced p38 MAPK activation contributes to the induction of the apoptotic cascade.

To investigate whether the protective effect of PCF on UVA-induced apoptosis is related to p38 MAPK, we treated HaCaT cells with different concentrations of PCF before UVA exposure and then tested for phosphorylated p38 MAPK. As shown in Fig. 3, lanes 4–6, when cells were preincubated with PCF for 2 h, the levels of phosphorylated

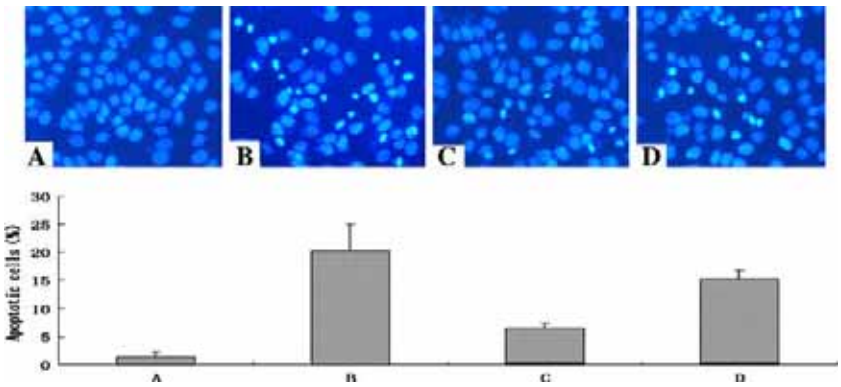

Fig. 2 Effect of PCF on H_2O_2-induced apoptosis in HaCaT cells. *A* control, *B* HaCaT cells were incubated with 10 mM H_2O_2 for 10 min, *C* and *D* HaCaT cells were pretreated with 5.69 or 1.42 mM PCF for 2 h prior to H_2O_2 incubation, respectively. After H_2O_2 incubation, cells were further incubated for 24 h, and apoptotic cells were detected by Hoechst 33258 staining. Indicated are mean and SD of three independent experiments

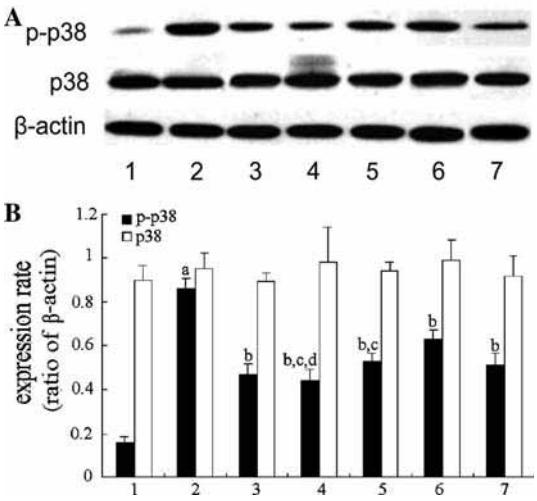

Fig. 3 Effect of PCF, NAC and SB203580 on UVA-induced p38 MAPK activation in HaCaT cells. **a** *Lane 1* HaCaT cells were mock-irradiated (control), *lane 2* HaCaT cells were irradiated with 10 J/cm^2 UVA, *lanes 3–6* HaCaT cells were pretreated with 5 mM NAC, 5.69, 2.84 or 1.42 mM PCF for 2 h prior to UVA irradiation respectively, *lane 7* HaCaT cells were pretreated with 10 μM SB203580 for 1 h prior to UVA irradiation, respectively. Cells were harvested 0.5 h after irradiation, and p38 MAPK activation were detected by Western Blot. Results shown are representative of three independent experiments. **b** Quantification of **a** results. Results were expressed as the ratio of expression level of phosphorylated p38 MAPK or total p38 MAPK over β-actin. $^aP < 0.05$ compared with control, $^bP < 0.05$ compared with UVA irradiation group, $^cP < 0.05$ compared with 1.42 mM PCF + UVA group, $^dP < 0.05$ compared with 2.84 mM PCF + UVA group

p38 MAPK were concentration-dependently decreased. These results suggest that PCF inhibits UVA-induced apoptosis through inhibition of p38 MAPK activation. NAC also attenuates phosphorylation of p38 MAPK, indicating the possible role of oxidative stress in UVA-induced p38 MAPK activation.

To determine whether caspase-3 activation is important for UVA-induced apoptosis, HaCaT cells were pretreated with the caspase-3 specific inhibitor Ac-DEVD-CHO prior to UVA exposure. As shown in Fig. 1, lane 8, the UVA-induced apoptosis was significantly inhibited, suggesting that UVA-induced apoptosis is caspase-3-dependent.

In order to evaluate the effects of PCF on UVA-induced caspase-3 activation, caspase-3 activity was measured by flow cytometry with anti-active caspase-3 monoclonal antibody. As shown in Fig. 4, caspase-3 activity increased significantly in UVA-treated HaCaT cells compared with untreated cells. When cells were preincubated with different concentrations of PCF, caspase-3 activity decreased in a dose-dependent manner. These results suggest that caspase-3 is involved in the anti-apoptotic effect of PCF on UVA-induced apoptosis. The inhibitory effect of NAC on caspase-3 activity indicates the relationship of oxidative stress and caspase-3 activity.

Discussion

UVA is known for its photo-oxidative effects, generating ROS, which can affect several cellular functions. Antioxidants can reduce the production of ROS. For example, the known antioxidant (−)-epigallocatechin-3-gallate (EGCG) was found to inhibit UV-induced apoptosis of human epidermal keratinocytes in vivo and in vitro [16]. It has also been reported that treatment of HaCaT cells with antioxidant cyanidin-3-*O*-β-glucopyranoside (C-3-G) before UVA irradiation inhibited ROS formation and apoptosis [17]. PCF was found to have potent antioxidant activity [18, 19] and inhibitory effect on UVA-induced apoptosis [14] in our previous studies, but its anti-apoptotic mechanisms were not understood.

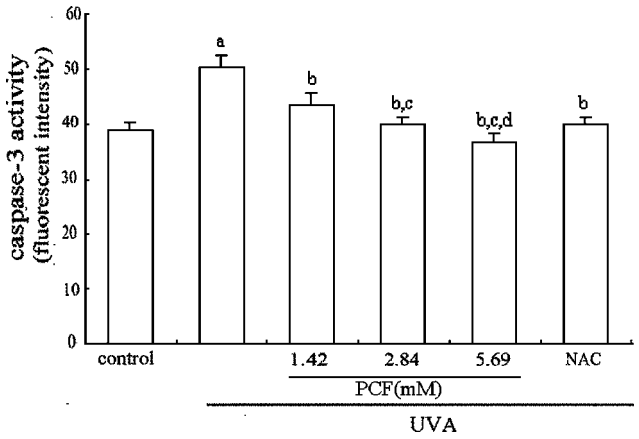

Fig. 4 Effect of PCF and NAC on UVA-induced caspase-3 activation in HaCaT cells. HaCaT cells were pretreated with 1.42, 2.84, 5.69 mM PCF, or 5 mM NAC for 2 h prior to UVA irradiation (10 J/cm^2). After irradiation, cells were incubated for 12 h, and caspase-3 activation were detected by flow cytometry. Results shown are representative of three independent experiments. $^{a}P < 0.05$ compared with control, $^{b}P < 0.05$ compared with UVA irradiation group, $^{c}P < 0.05$ compared with 1.42 mM PCF + UVA group, $^{d}P < 0.05$ compared with 2.84 mM PCF + UVA group

NAC has been identified as an antioxidant for its ability to supply cysteine for the synthesis of glutathione (GSH), which is depleted by ultraviolet irradiation [20]. It is also associated with the scavenging of ROS [21]. Although the efficacy of NAC in protecting Jurkat cells against apoptosis produced by UVC irradiation has been reported [22], this study observed the protective effects of NAC on UVA-induced apoptosis in HaCaT cells. These results indicate that ROS play an important role in mediating the apoptotic effect of UVA irradiation. Here, we show that H_2O_2 and UVA-induced apoptosis can be efficiently attenuated by PCF, suggesting that impairment of ROS formation during UVA irradiation is responsible for the anti-apoptotic effect of PCF.

A number of studies have confirmed that ROS significantly contribute to UV-induced signal transduction. Particularly, UV irradiation-induced activation of p38 MAPK, which is involved in a stress-induced signaling pathway, involves ROS to a significant extent [23–25]. So it was of interest to examine the protective effect of PCF on UVA-induced apoptosis through p38 MAPK. In this study we observed p38 MAPK activation in HaCaT cells at a UVA dose of 10 J/cm^2. The role of p38 MAPK in UV-induced apoptosis is controversial, appearing to be dependent upon cell type and the nature of UV irradiation (wavelength and dose). Sustained p38 MAPK activation contributes to the UVB-induced apoptosis by mediating the release of mitochondrial cytochrome *c* into the cytosol in HaCaT cells [10]. In contrast, Bachelor et al. [26] describe a role for p38 MAPK in the survival of UVA-irradiated human keratinocytes through the post-transcriptional regulation of the anti-apoptotic Bcl-2 family member, Bcl-X_L. In this study, we showed that SB203580 effectively prevented UVA-induced apoptosis, suggesting that in our system, activation of p38 MAPK results in apoptosis. SB203580 effectively inhibited phosphorylation of p38 MAPK induced by UVA and PCF attenuated p38 MAPK activation dose-dependently. These results suggest that PCF inhibited UVA-induced apoptosis through its effects on p38 MAPK.

Caspase-3 is synthesized in cells as an inactive 32 kDa precursor, which is proteolytically processed into the 17 and 12 kDa subunits of the mature caspase-3 during apoptosis. In line with another report [27], we presented here strong evidence that apoptosis induced by UVA was a typical caspase-3-dependent process. Pretreatment of HaCaT cells with the caspase-3 inhibitor Ac-DEVD-CHO drastically inhibited UVA-induced apoptosis. Pretreatment of HaCaT cells with PCF attenuated UVA-induced activation of caspase-3 in a dose-dependent manner. Therefore, it is likely that PCF plays its anti-apoptotic role by inhibition of caspase-3 activation.

Peus et al. [23] reported that antioxidant ascorbic acid strongly blocked p38 MAPK activation by UVB, suggesting the key role of ROS in p38 MAPK response to UVB. In this study, pretreatment with NAC also inhibited UVA-induced p38 MAPK phosphorylation and caspase-3 activation. Therefore, the inhibitory effect of PCF on p38 MAPK and caspase-3 activation may partly depend on its property as a ROS scavenger. Furthermore, previous studies have shown that a p38 MAPK inhibitor inhibits UV-induced activation of caspase-3 [13]. Thus, the PCF-mediated inhibition of UVA-induced activation of caspase-3 may be related to the inhibition of p38 MAPK activation.

Taken together, our observations suggest that ROS, p38 MAPK, and caspase-3 are involved in the inhibitory effect of PCF on UVA-induced apoptosis of HaCaT cells. Furthermore, PCF may affect the ROS/p38/caspase-3 signaling pathway in UVA-induced apoptosis. PCF could be optimized as a prophylactic agent to prevent the formation of sunburn cells and protect the natural barrier function of the skin. However, we should consider the two sides about the inhibition of apoptosis by PCF: It is good, because PCF reduced the UVA damage of the cells; but it is bad, if the cells survive despite DNA damage.

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