

# Delphinidin, an Anthocyanidin in Pigmented Fruits and Vegetables, Protects Human HaCaT Keratinocytes and Mouse Skin Against UVB-Mediated Oxidative Stress and Apoptosis

Farrukh Afaq<sup>1</sup>, Deeba N. Syed<sup>1</sup>, Arshi Malik<sup>1</sup>, Naghma Hadi<sup>1</sup>, Sami Sarfaraz<sup>1</sup>, Mee-Hyang Kweon<sup>1</sup>, Naghma Khan<sup>1</sup>, Mohammad Abu Zaid<sup>1</sup> and Hasan Mukhtar<sup>1</sup>

Solar UV radiation, in particular its UVB component, is the primary cause of many adverse biological effects, the most damaging of which is skin cancer. Here, we assessed the photochemopreventive effect of delphinidin, a major anthocyanidin present in many pigmented fruits and vegetables, on UVB-mediated responses in human immortalized HaCaT keratinocytes and SKH-1 hairless mouse skin. We found that pretreatment of cells with delphinidin (1–20  $\mu\text{M}$  for 24 hours) protected against UVB (15–30  $\text{mJ}/\text{cm}^2$ , 24 hours)-mediated (i) decrease in cell viability and (ii) induction of apoptosis. Furthermore, we found that pretreatment of HaCaT cells with delphinidin inhibited UVB-mediated (i) increase in lipid peroxidation; (ii) formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG); (iii) decrease in proliferating cell nuclear antigen expression; (iv) increase in poly(ADP-ribose) polymerase cleavage; (v) activation of caspases; (vi) increase in Bax; (vii) decrease in Bcl-2; (viii) upregulation of Bid and Bak; and (ix) downregulation of Bcl-xL. Topical application of delphinidin (1 mg/0.1 ml DMSO/mouse) to SKH-1 hairless mouse skin inhibited UVB-mediated apoptosis and markers of DNA damage such as cyclobutane pyrimidine dimers and 8-OHdG. Taken together our results suggest that treatment of HaCaT cells and mouse skin with delphinidin inhibited UVB-mediated oxidative stress and reduced DNA damage, thereby protecting the cells from UVB-induced apoptosis.

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## INTRODUCTION

Human skin and particularly the epidermis, unlike all other organs, is continuously and directly exposed to numerous chemical and physical environmental stresses. Among these, solar UV radiation is the most ubiquitous damaging environmental factor (Bowden, 2004; Afaq *et al.*, 2005a). Exposure of solar UV radiation, particularly its UVB component, to humans causes many adverse effects that include erythema, hyperpigmentation, hyperplasia, immune suppression, photaging, and skin cancer (F'guyer *et al.*, 2003; Bowden, 2004; Matsumura and Ananthaswamy, 2004; Halliday, 2005; Afaq *et al.*, 2005a). UVB exposure to skin cells results in several types of DNA damage such as the formation of cyclobutane

pyrimidine dimers (CPDs), pyrimidine (6-4) pyrimidone photodimers, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Budiyanto *et al.*, 2000; Katiyar *et al.*, 2000; Cadet *et al.*, 2005). These effects of UVB radiation resulting in damaged DNA can initiate photocarcinogenesis. In addition, UV radiation produces reactive oxygen species (ROS) that can also damage DNA molecules and other lipid components, ultimately leading to carcinogenesis (F'guyer *et al.*, 2003; Bowden, 2004; Nishigori *et al.*, 2004). Studies have shown that excessive ROS production and/or their ineffective elimination is implicated in many cutaneous pathological processes (Katiyar *et al.*, 2001; F'guyer *et al.*, 2003).

Skin appears to be endowed with a variety of enzymatic as well as small molecular antioxidants that can inhibit oxidative damage (Afaq and Mukhtar, 2001). However, the antioxidant capability of the skin is often overwhelmed by excessive production of ROS. To overcome this imbalance, the use of antioxidant has been considered. In this regard, emphasis in defining novel botanical agents capable of ameliorating the adverse effects of ROS has become an important area of research. This is also because primary prevention approaches of skin cancer have proven inadequate in lowering the incidence of skin cancer, emphasizing the need to develop novel skin cancer chemopreventive

<sup>1</sup>Department of Dermatology, University of Wisconsin, Madison, Wisconsin, USA

Correspondence: Dr Hasan Mukhtar, Department of Dermatology, University of Wisconsin, Medical Sciences Center, Room No. B25, 1300 University Avenue, Madison, Wisconsin 53706, USA. E-mail: hmukhtar@wisc.edu

Abbreviations: CPD, cyclobutane pyrimidine dimer; LPO, lipid peroxidation; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; ROS, reactive oxygen species

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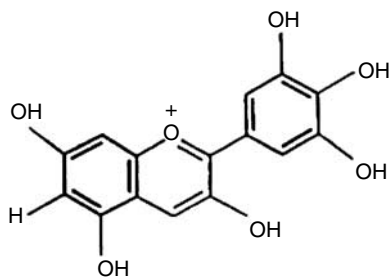


Figure 1. Structure of delphinidin.

agents. One approach to reduce its occurrence is through “Photochemoprotection”, which we define as “the use of agents capable of ameliorating the adverse effects of UVB on the skin”. Among many photochemoprotective agents, botanical antioxidants are showing promise. With a notion to protect humans against the adverse effects of UV radiation, in recent years there is increasing use of botanicals in skin care products. Recently, we have shown that anthocyanidin-rich pomegranate fruit extract inhibits UVB-mediated: (i) phosphorylation of mitogen-activated protein kinase; (ii) activation of IKK $\alpha$ ; (iii) degradation and phosphorylation of I $\kappa$ B $\alpha$ ; and (iv) nuclear translocation and phosphorylation of NF- $\kappa$ B at Ser<sup>536</sup> in normal human epidermal keratinocytes (Afaq *et al.*, 2005b). Among the six anthocyanidins present in pomegranate fruit extract, delphinidin (Figure 1) is the most abundant and is known to be present in many other pigmented fruits and vegetables like berries, dark grapes, egg plant, tomato, carrot, purple sweet potatoes, red cabbage, and red onion.

This study was designed to assess the photochemoprotective effects of delphinidin on UVB-mediated responses in human immortalized HaCaT keratinocytes under *in vitro* situation and SKH-1 hairless mouse model *in vivo* situation. Specifically, we determined the photochemopreventive effects of delphinidin on UVB-mediated oxidative stress, biomarkers of DNA damage and repair, and regulatory pathways for apoptosis such as caspases and Bcl-2 family of proteins.

## RESULTS

### Delphinidin treatment protects HaCaT cells from UVB-mediated decrease in cell viability

Initially we investigated the effect of delphinidin (1–20  $\mu$ M) against UVB-mediated decrease in cell viability. As expected, UVB (15–30 mJ/cm<sup>2</sup>) irradiation of HaCaT cells resulted in a decrease in cell viability (Figure 2a). This UVB-induced cell growth inhibition was significantly prevented by pretreatment of cells with delphinidin (1–20  $\mu$ M) in a dose-dependent manner as determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay (Figure 2a). Delphinidin treatment to the control cells showed no cytotoxic effects. For further experiments we selected a 10  $\mu$ M dose of delphinidin, as this dose provided substantial protection against UVB-mediated decrease in cell viability. UVB (15–30 mJ/cm<sup>2</sup>) irradiation resulted in distinct morphological changes in HaCaT cells, as the cells became round and detached from the surface of the plate, whereas pretreatment

of cells with delphinidin (10  $\mu$ M) prevented these morphological changes as assessed by phase contrast microscopy (Figure 2b).

### Delphinidin treatment protects HaCaT cells from UVB-mediated apoptosis

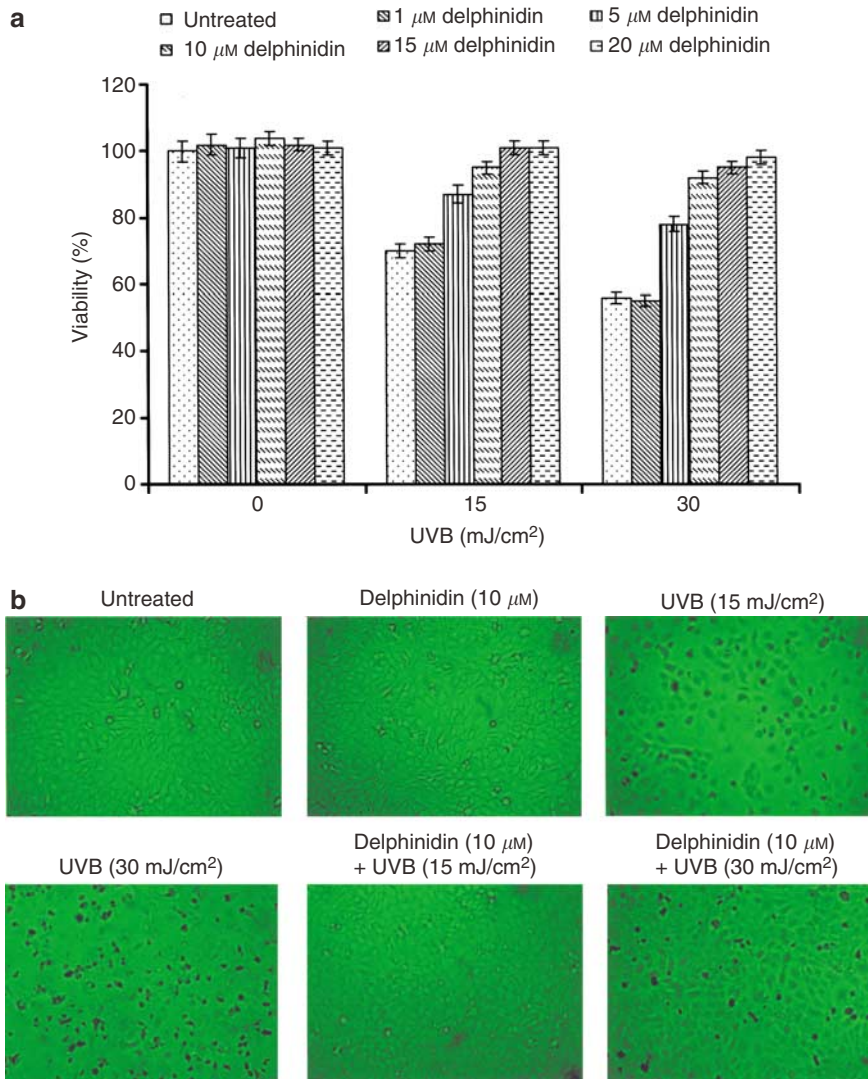
Next, we examined whether the protective effect of delphinidin on UVB-mediated decrease in cell viability was due to inhibition of apoptosis. The extent of apoptosis was quantified by flow cytometry analysis of delphinidin (10  $\mu$ M) treated and untreated cells exposed with 15 and 30 mJ/cm<sup>2</sup> doses of UVB. As shown by the data in Figure 3a, UVB irradiation of cells resulted in 11.88 and 25.72% apoptotic cells at 15 and 30 mJ/cm<sup>2</sup> of UVB, respectively. However, delphinidin pretreatment resulted in only 4.53 and 7.69% apoptotic cells at 15 and 30 mJ/cm<sup>2</sup> of UVB, respectively (Figure 3a). We also performed fluorescence microscopy after staining the cells with Annexin V and propidium iodide (PI) because this method identifies the apoptotic (green fluorescence) as well as necrotic (red fluorescence) cells. We found that pretreatment of HaCaT cells with delphinidin prevented UVB-mediated apoptosis (Figure 3b). Next, we examined whether delphinidin pretreatment could suppress poly(ADP-ribose) polymerase (PARP) cleavage. PARP is a 116 kDa protein that is cleaved into 85 kDa fragment during apoptotic cell death. UVB (15–30 mJ/cm<sup>2</sup>) irradiation of HaCaT cells resulted in cleavage of PARP protein 24 hours post-UVB exposure (Figure 3c). Western blot analysis and relative density of the bands suggest that pretreatment of cells with delphinidin (10  $\mu$ M) inhibited UVB-mediated cleavage of PARP (116 kDa) into 85 kDa fragment (Figure 3c and d).

### Delphinidin exerts strong antioxidant effect and inhibits UVB-mediated lipid peroxidation in HaCaT cells

In order to determine whether the protective effect of delphinidin against UVB-mediated apoptosis is due to its antioxidant activity, we performed trolox assay. Cells were pretreated with delphinidin (1–20  $\mu$ M) for 24 hours and cell lysates were prepared. Interestingly, we found that delphinidin possesses strong antioxidant activity as the trolox equivalent increased in cells treated with delphinidin in a dose-dependent manner when compared to untreated cells (Figure 4a). Next, we evaluated the effect of delphinidin on UVB-mediated lipid peroxidation (LPO), which is a well-accepted marker of oxidative stress. As shown by the data in Figure 4b, UVB irradiation of cells resulted in a significant induction of LPO (measured in terms of malondialdehyde production) by 122 and 186% at UVB doses of 15 and 30 mJ/cm<sup>2</sup>, respectively, when compared to untreated cells. Pretreatment of cells with delphinidin (10  $\mu$ M) significantly inhibited UVB-mediated increased LPO by 64 and 75% at UVB doses of 15 and 30 mJ/cm<sup>2</sup> respectively (Figure 4b).

### Delphinidin treatment inhibits UVB-mediated formation of 8-OHdG and degradation of proliferating cell nuclear antigen protein expression in HaCaT cells

UVB radiation induces oxidative damage in DNA, resulting in the formation of the adduct 8-OHdG. We therefore



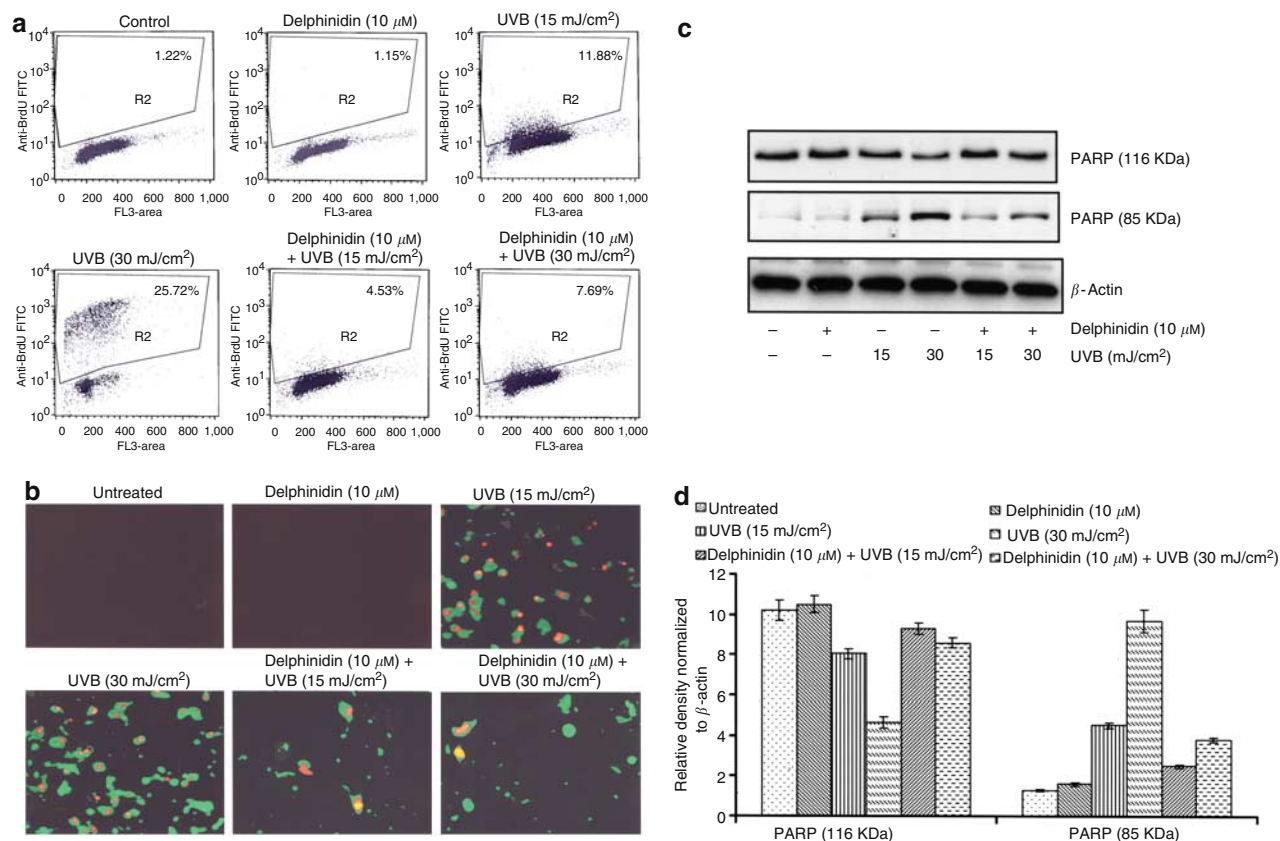
**Figure 2. Delphinidin treatment protects HaCaT cells against UVB-mediated decrease in cell viability.** HaCaT cells were treated with different doses of delphinidin (1, 5, 10, 15, and 20  $\mu$ M) for 24 hours after which the media was removed and cells were washed once with PBS and then fresh PBS was added and cells were then exposed to UVB (15–30 mJ/cm<sup>2</sup>) radiation. (a) At 24 hours after UVB irradiation, percent cell viability was assessed by MTT assay. Data shown are mean  $\pm$ SD of three separate experiments in which each treatment was repeated in 10 wells. \* $P$ <0.001 versus control, # $P$ <0.001 versus UVB. (b) Phase contrast microscopy, HaCaT cells were incubated without or with delphinidin (10  $\mu$ M) for 24 hours and then irradiated with UVB (15–30 mJ/cm<sup>2</sup>). A representative picture from three independent experiments with similar result is shown.

performed an immunocytochemical staining using antibody specific for 8-OHdG to study the effect of delphinidin on UVB-mediated formation of 8-OHdG. We found stronger and more extensive staining in the nuclei of UVB-irradiated cells, 24 hours post-UVB (15–30 mJ/cm<sup>2</sup>) exposure compared to those of nonirradiated cells (Figure 5a). However, treatment of cells prior to UVB irradiation resulted in a significant protection against UVB-mediated formation of 8-OHdG (Figure 5a). Proliferating cell nuclear antigen (PCNA) is an active nuclear protein involved in DNA replication, recombination and repair. We found that UVB (15–30 mJ/cm<sup>2</sup>) irradiation completely diminished PCNA protein expression compared to untreated cells. Pretreatment of HaCaT cells with

delphinidin significantly restored UVB-induced PCNA protein degradation (Figure 5b).

**Delphinidin treatment modulates UVB-mediated changes in Bcl-2 family of proteins in HaCaT cells**

Bcl-2 is a member of the large Bcl-2 family and protects cells from apoptosis. Studies have shown that the effect of Bcl-2 against apoptosis is influenced by Bax. Bcl-2 homodimer reveals antiapoptotic effect, whereas this effect is inhibited by Bax due to the formation of heterodimer complex of Bcl-2 and Bax protein. Thus, the ratio of both these proteins is critical for determining cell death or cell survival. UVB (15–30 mJ/cm<sup>2</sup>) irradiation of cells resulted in an increased expression of Bax protein and decreased expression of Bcl-2



**Figure 3. Delphinidin treatment protects HaCaT cells against UVB-mediated apoptosis.** HaCaT cells were treated with delphinidin (10  $\mu$ M) for 24 hours after which the media was removed and cells were washed once with PBS and then fresh PBS was added and cells were exposed to UVB (15–30 mJ/cm<sup>2</sup>) radiation. (a) Effects on UVB-mediated apoptosis as detected by flow cytometry. Cells showing fluorescence (R2) are considered as apoptotic and their percentage population is indicated. (b) Effects on UVB-mediated apoptosis as detected by fluorescence microscopy. The annexin-V-FLUOS staining kit was used for detection of apoptotic and necrotic cells. The kit uses a dual staining protocol in which apoptotic cells are stained with annexin V (green fluorescence) and the necrotic cells are stained with PI (red fluorescence). (c) Effects on UVB-mediated PARP cleavage. (d) Relative density was performed as described under “Materials and Methods”. Equal loading was confirmed by stripping the immunoblot and reprobing it for  $\beta$ -actin. The relative density of the bands was normalized to  $\beta$ -actin. Data shown are from representative experiments repeated thrice with similar results.

protein (Figure 6a). However, delphinidin treatment resulted in a significant decrease in proapoptotic protein Bax with concomitant increase in antiapoptotic protein Bcl-2 (Figure 6a) resulting in a shift in Bax/Bcl-2 ratio that does not favor apoptosis (Figure 6b). We also evaluated the effect of delphinidin treatment on other members of Bcl-2 family, viz. Bid and Bak (proapoptotic) and Bcl-xL (antiapoptotic). Western blot analysis data and relative density demonstrated that UVB irradiation of HaCaT cells resulted in a significant increase in the protein levels of Bid and Bad (proapoptotic) and a decrease in Bcl-xL (antiapoptotic) protein, and pretreatment of cells with delphinidin modulates these effects (Figure 6c). These results suggest that the protective effect of delphinidin against UVB-mediated apoptosis may be via modulation of Bcl-2 family members.

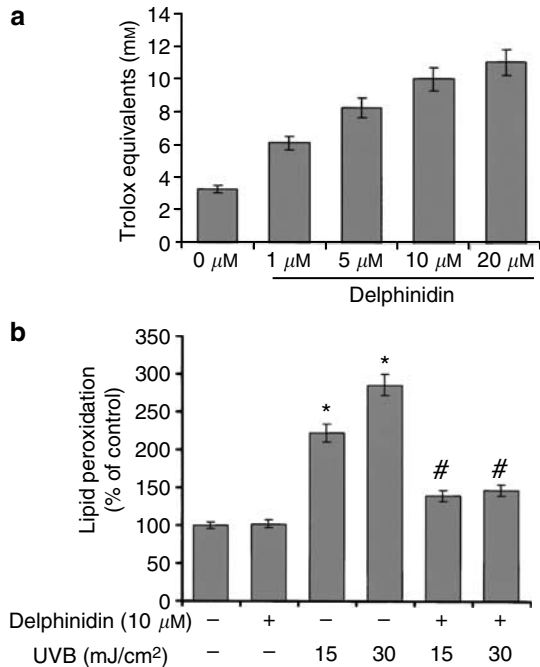
#### Delphinidin treatment modulates UVB-mediated activation of caspases in HaCaT cells

Exposure of cells to UVB induces activation of the proapoptotic protein including caspases. To further assess the effect of delphinidin on UVB-induced apoptosis, we

examined whether delphinidin treatment would protect UVB-mediated activation of caspases. As expected employing Western blot analysis we found that UVB irradiation resulted in a decrease in the protein expression of procaspase-3, -8, and -9, indicating that the pro form of caspase is cleaved into active form. Our results suggest that pretreatment of cells with delphinidin (10  $\mu$ M) inhibited UVB-mediated decrease in the protein expression of procaspase-3, -8, and -9 as demonstrated by relative density and Western blot analysis (Figure 7a and b).

#### Delphinidin treatment inhibits UVB-mediated formation of CPDs, 8-OHdG, and TUNEL-positive cells in SKH-1 hairless mouse skin

To investigate the relevance of our *in vitro* findings to *in vivo* situations, we determined the effect of topical application of delphinidin to SKH-1 hairless mouse on UVB-induced damage. It is known that both CPDs and 8-OHdG are formed in epidermal DNA after UVB irradiation and are considered as important biomarkers of DNA damage. Therefore, we first assessed the effect of delphinidin (both pretreatment and



**Figure 4. Delphinidin exerts strong antioxidant effect and inhibits UVB-mediated lipid peroxidation in HaCaT cells.** HaCaT cells were treated with different doses of delphinidin (1–20 μM) for 24 hours after which the media was removed and cells were harvested and cell lysates were prepared for antioxidant activity. (a) Effects on total antioxidant activity in cell lysate. The cell lysate was added to the ABTS radical cation decolorization assay. HaCaT cells were treated with delphinidin (10 μM) for 24 hours after which the media was removed and cells were washed once with PBS and then fresh PBS was added and cells were then exposed to UVB (15–30 mJ/cm<sup>2</sup>) radiation. Twenty-four hours post-UVB exposure; the cells were processed for lipid peroxidation as detailed in “Materials and Methods”. (b) Effects on UVB-mediated lipid peroxidation. Data shown are mean ± SD of three separate experiments. \*P < 0.001 versus control, #P < 0.001 versus UVB.

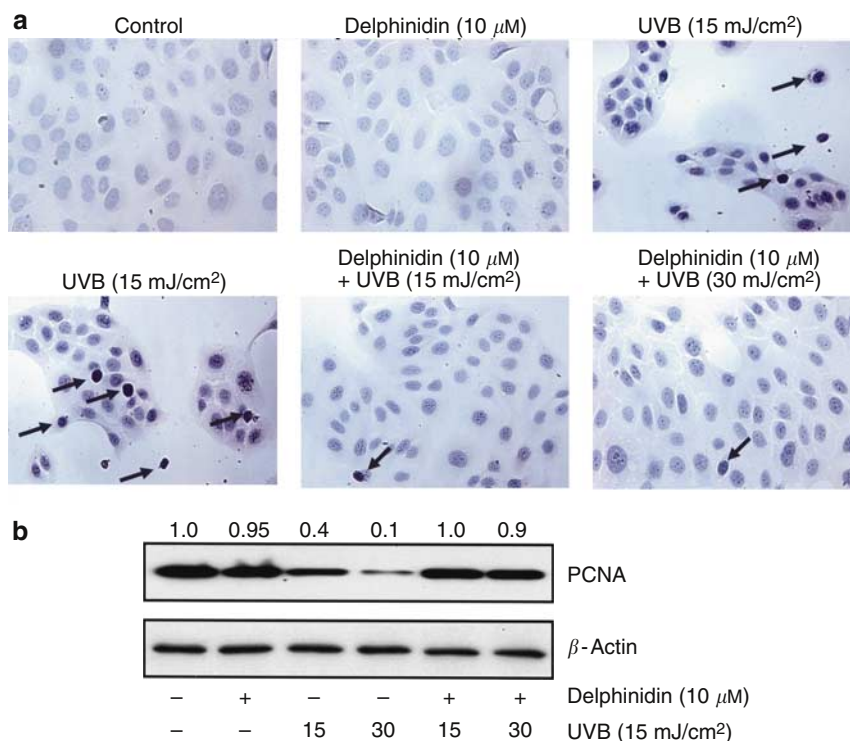
post-treatment) on UVB-mediated DNA damage in mouse epidermis by immunohistochemical analysis. We selected a time point of 1 hour post-UVB irradiation because at this time point maximum formation of CPDs and 8-OHdG was observed (Lu *et al.*, 1999; Gu *et al.*, 2005; Afaq *et al.*, 2006). As shown in Figure 8, UVB irradiation (180 mJ/cm<sup>2</sup>) to SKH-1 hairless mouse skin induced the formation of both CPDs (Figure 8c) and 8-OHdG (Figure 8h) when compared to their respective control groups (Figure 8a and f). Our data clearly demonstrate that topical application of delphinidin (both pretreatment and post-treatment) resulted in marked inhibition in the number of CPDs (Figure 8d and e) and 8-OHdG (Figure 8i and j) positive cells. Depending on the severity of the DNA damage after UVB irradiation, keratinocytes in the skin can undergo either DNA repair or apoptosis. Alternatively, UVB exposure leads to cutaneous neoplasia by imposing severe and sometimes irreparable damage. Therefore, we next assessed the effect of delphinidin (both pretreatment and post-treatment) on UVB-mediated apoptosis by TUNEL assay, 8 hours post-UVB irradiation. Our data suggest that UVB irradiation to SKH-1 hairless mouse skin

increased the number of apoptotic cells in the epidermis (Figure 8m) compared to control (Figure 8k) and delphinidin alone (Figure 8l) treated groups. Topical application of delphinidin (both pretreatment and post-treatment) inhibited UVB-induced formation of apoptotic cells as determined by TUNEL staining (Figure 8n and o).

**DISCUSSION**

In the United States alone, 1.2 million new cases of skin cancer are identified each year and this accounts for 40% of all new cancer cases diagnosed (Jemal *et al.*, 2005). Solar UVB radiation has been implicated as the main cause for skin cancer. The non-melanoma skin cancers comprising of basal cell carcinomas and squamous cell carcinomas are the most frequently diagnosed cutaneous malignancies. Therefore, additional efforts are needed to prevent skin cancers that result as a consequence of UVB exposure. Because skin cancer is a significant problem associated with mortality and morbidity, concerted efforts are needed to develop novel strategies for the prevention of UV-mediated damages. Photochemoprevention has been appreciated as a viable approach to reduce the occurrence of skin cancer and in recent years, the use of agents, especially botanical antioxidants, present in the common diet and beverages consumed by human population have gained considerable attention as photochemopreventive agents for human use. Many such agents have also found a place in skin care products. A case-control study in Australia demonstrated a significant inverse relationship between the risk of skin cancer and a high intake of vegetables, β-carotene, and vitamin C containing foods (Kune *et al.*, 1992). In the present study, we demonstrated that delphinidin protected HaCaT cells from UVB-mediated decrease in cell viability (Figure 2). Our results further suggest that pretreatment of cells with delphinidin inhibited UVB-mediated apoptosis as determined by flow cytometry, confocal microscopy, and PARP cleavage (Figure 3). Moreover, we found that UVB radiation to SKH-1 hairless mouse skin increased the number of apoptotic cells, and topical application of delphinidin (both pretreatment and post-treatment) inhibited UVB-induced apoptosis (Figure 8).

UV radiation results in an increased generation of ROS that overwhelms the antioxidant defense mechanisms of the target system. This condition of prooxidant/antioxidant disequilibrium is defined as “oxidative stress”. The epidermis is composed mainly of keratinocytes, which are rich in ROS detoxifying enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and in low-molecular-mass antioxidant molecules, and thus provide some natural protection against ROS (Afaq and Mukhtar, 2001). Skin spontaneously responds to increased ROS levels; however, this response may not be sufficient to prevent the progression of skin cancer. Our studies suggest that delphinidin possesses strong antioxidant activity (Figure 4a). UV radiation to the skin results in the formation of ROS that interact with proteins, lipids, and DNA (Halliday, 2005; Melnikova and Ananthaswamy, 2005). LPO in biological membranes is a free radical-mediated event and is regulated by the availability of



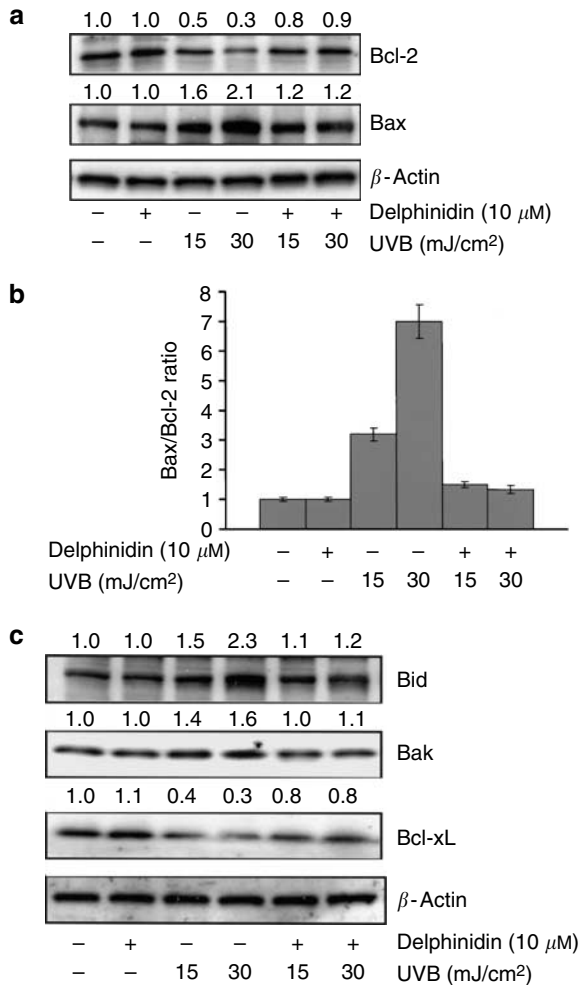
**Figure 5. Delphinidin treatment inhibits UVB-mediated formation of 8-OHdG and degradation of PCNA protein expression in HaCaT cells.** HaCaT cells were treated with delphinidin (10  $\mu$ M) for 24 hours after which the media was removed and cells were washed once with PBS and then fresh PBS was added and cells were then exposed to UVB (15–30 mJ/cm<sup>2</sup>) radiation. Twenty-four hours post-UVB exposure; the cells were processed for immunocytochemistry and Western blot analysis as detailed in “Materials and Methods”. (a) Effects on UVB-mediated formation of 8-OHdG as detected by immunocytochemistry. A representative picture from three independent experiments with similar results is shown. (b) Effects on UVB-mediated degradation of PCNA. Equal loading was confirmed by stripping the immunoblot and reprobing it for  $\beta$ -actin. The relative density of the bands was normalized to  $\beta$ -actin. The immunoblots shown here are representative of three independent experiments with similar results.

substrates in the form of polyunsaturated fatty acids and prooxidants that promote peroxidation (Katiyar *et al.*, 2001; Girotti and Kriska, 2004). LPO is highly detrimental to cell membrane structure and function, and its elevated level has been linked to damaging effects such as loss of fluidity, inactivation of membrane enzymes, increased cell membrane permeability to ions, and finally rupture of cell membrane leading to release of cell organelles (Brunet *et al.*, 2000; Cuzzocrea *et al.*, 2001). Thus, inhibition of LPO by delphinidin (Figure 4b) would result in reduction of the risk factors associated with UVB radiation.

UVB irradiation results in DNA modification both directly by forming CPDs and pyrimidine (6-4) pyrimidone photodimers, and indirectly via ROS that produces 8-OHdG (Budiyanto *et al.*, 2000; Katiyar *et al.*, 2000; Cadet *et al.*, 2005). 8-OHdG is a mutation-prone (G:C to T:A transversion) DNA base-modified product generated by ROS and photodynamic action (Hattori *et al.*, 1996). The transversion of G:C to T:A in UVB-induced skin cancers of mice and humans suggests that 8-OHdG plays an essential role in photocarcinogenesis (Basset-Seguín *et al.*, 1994; Nishigori *et al.*, 1994). Recent studies have shown that 8-OHdG formation in mice epidermis was reduced by super virgin olive oil painted immediately after UV radiation (Budiyanto *et al.*, 2000). The

formation of 8-OHdG after UVB exposure appears to be regulated by LPO and inflammation, and may play an important role in photocarcinogenesis. UVB induces oxidative damage in DNA resulting in the formation of the adduct 8-OHdG. The present study was undertaken to examine the effect of delphinidin against UVB-mediated DNA damage. Our results suggest reduced formation of UVB-induced 8-OHdG in HaCaT cells pretreated with delphinidin compared to UVB alone group (Figure 5a). Topical application of delphinidin (both pretreatment and post-treatment) to SKH-1 hairless mouse skin inhibited UVB-induced formation of CPDs and 8-OHdG (Figure 8). This indicates that the reduced formation of CPDs and 8-OHdG can be attributed to the scavenging activity of delphinidin against ROS (Figure 4a and 8).

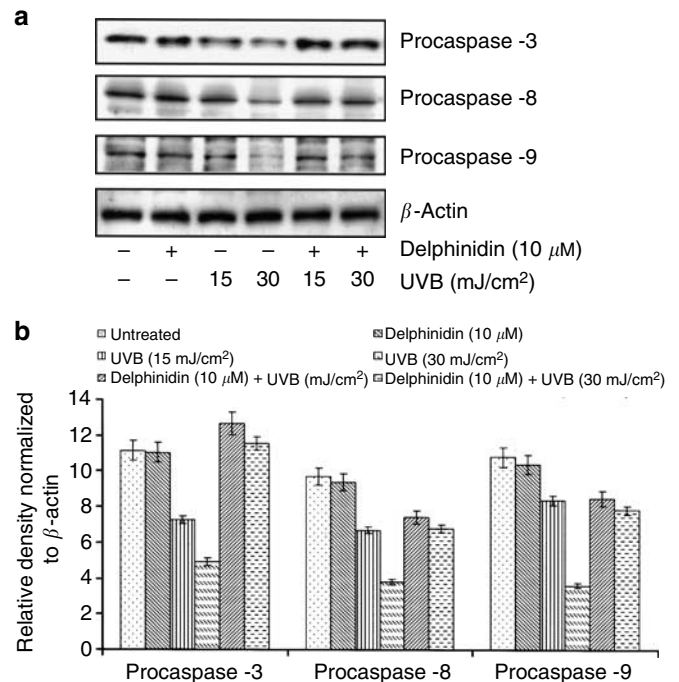
PCNA is an active nuclear protein and serve as a marker of DNA repair and indirectly as an indicator of UVB induced damage (Akbari *et al.*, 2004; Moore *et al.*, 2004; Constantin *et al.*, 2005). Studies have demonstrated that UVB irradiation completely diminished PCNA protein expression in mouse model of photocarcinogenesis. Topical application of lycopene significantly reversed UVB-induced PCNA inhibition (Fazekas *et al.*, 2003). Cells are equipped with extensive repair capacity for removing strand breaks, small base



**Figure 6. Delphinidin treatment modulates UVB-mediated changes in Bcl-2 family of proteins in HaCaT cells.** HaCaT cells were treated with delphinidin (10 μM) for 24 hours after which the media was removed and cells were washed once with PBS and then fresh PBS was added and cells were then exposed to UVB (15–30 mJ/cm<sup>2</sup>) radiation. Twenty-four hours post-UVB exposure, the cells were harvested and cell lysates were prepared and protein expression was determined. (a) Effects on UVB-mediated changes in Bax and Bcl-2 proteins expression. (b) Effects on UVB-mediated changes in Bax/Bcl-2 ratio. (c) Effects on UVB-mediated changes in Bid, Bak, and Bcl-xL proteins expression. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The relative density of the bands was normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results.

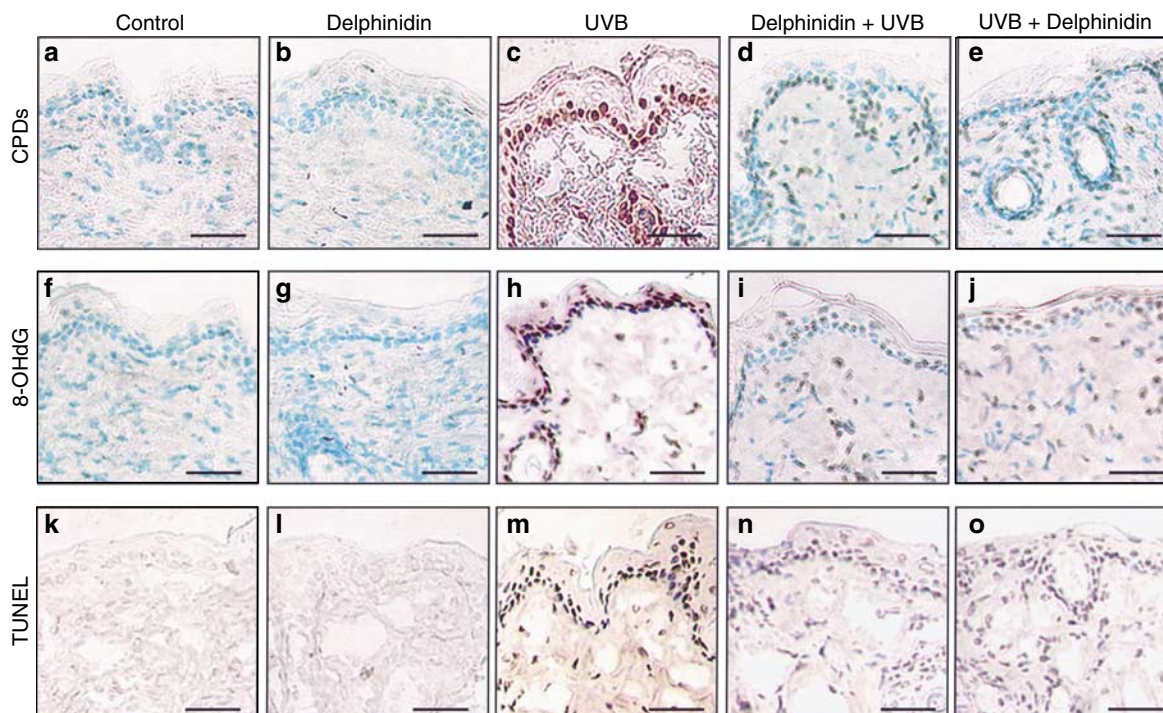
modifications (8-OHdG), and bulky lesions (CPDs). Our data suggest that pretreatment of HaCaT cells with delphinidin restored UVB-mediated decreased expression of PCNA (Figure 5b).

Apoptosis is a cell suicide process with characteristic morphological features that include nuclear membrane breakdown, chromatin condensation and fragmentation, and the formation of apoptosis (Thornberry and Lazebnik, 1998; Cory and Adams, 2002). Studies have shown that many apoptotic stimuli such as UV radiation, tumor necrosis factor alpha, Fas ligand, and chemotherapeutic agents induce cell death by activating caspases (Cryns and Yuan, 1998). Bcl-2 is



**Figure 7. Delphinidin treatment modulates UVB-mediated activation of caspases in HaCaT cells.** HaCaT cells were treated with delphinidin (10 μM) for 24 hours after which the media was removed and cells were washed once with PBS and then fresh PBS was added and cells were then exposed to UVB (15–30 mJ/cm<sup>2</sup>) radiation. Twenty-four hours post-UVB exposure, the cells were harvested and cell lysates were prepared and protein expression was determined. (a) Effects on UVB-mediated activation of caspases. (b) Relative density was performed as described under “Materials and Methods”. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The relative density of the bands was normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results.

a member of the large Bcl-2 family and protects cells from apoptosis. Bcl-2 is found on the cytoplasmic face of the outer mitochondrial membrane, nuclear envelope, and endoplasmic reticulum, and other Bcl-2 family members either reside on one or more of these membranes or congregate there during apoptosis (Kaufmann et al., 2003). Several studies have suggested that Bax and Bak appear to permeabilize the outer mitochondrial membrane, allowing efflux of apoptogenic proteins (Green and Reed, 1998; Martinou and Green, 2001; Newmeyer and Ferguson-Miller, 2003). Bax binds to the mitochondrial membrane and induces cytochrome c release that subsequently activates caspase-9 and caspase-3 leading to downstream apoptotic responses (Cory and Adams, 2002). The ratio between antiapoptotic (Bcl-2) and proapoptotic (Bax) has been suggested as a primary event in determining the susceptibility to apoptosis through maintaining the integrity of the mitochondria and inhibiting the activation of caspase cascade. Delphinidin treatment of HaCaT cells prior to UVB irradiation resulted in a significant decrease in Bax with concomitant increase in Bcl-2 resulting in a shift in Bax/Bcl-2 ratio that does not favor apoptosis (Figure 6a and b). Caspases are cysteine-dependent aspartic



**Figure 8. Topical application of delphinidin inhibits UVB-mediated formation of CPD, 8-OHdG, and TUNEL-positive cells in SKH1-hairless mouse skin.**

The groups of mice were either unexposed (control), treated topically on the dorsal skin with delphinidin (1 mg/100  $\mu$ l DMSO/mouse), exposed to UVB radiation (180 mJ/cm<sup>2</sup>), treated topically on dorsal skin with delphinidin (1 mg/100  $\mu$ l DMSO/mouse) and then 30 minutes later exposed to UVB radiation, exposed to UVB radiation and then treated immediately with delphinidin (1 mg/100  $\mu$ l DMSO/mouse) as described under "Materials and Methods". One and eight hours post-UVB irradiation, the animals were killed and skin biopsies were processed for staining. Immunohistochemical staining for (a-e) CPDs, (f-j) 8-OHdG, and (k-o) TUNEL was carried out as described under "Material and Methods". The staining shown here are representative data from different treatments. Bar = 25  $\mu$ m.

proteases that are activated by proteolytic cleavage into large and small subunits and form tetrameric complexes (Chang and Yang, 2000; Wang *et al.*, 2003). Proapoptotic caspases are grouped into initiator (caspase-8, -9, and -10) and executor (caspase-3, -6, and -7) caspases (Chang and Yang, 2000; Pirnia *et al.*, 2002; Milhas *et al.*, 2005). Although caspase activity is regulated primarily at the post-translational level, overexpression of caspases sensitizes cells for apoptosis. The present study suggests that the protective effects of delphinidin against UVB-mediated apoptosis may be via modulation of Bcl-2 family members (Figure 6) and inhibition of activation of caspases (Figure 7).

In summary, our results suggest that treatment of HaCaT cells and mouse skin with delphinidin inhibited UVB-mediated oxidative stress and reduced DNA damage, thereby protecting the cells from UVB-induced apoptosis. These effects of delphinidin are because of its strong antioxidant activity. Therefore, these results demonstrate that delphinidin has the potential to protect against the adverse effects of UVB radiation via modulation of Bcl-2 family members and inhibition of activation of caspases, which in turn may protect the cellular targets against UVB-induced damage. Our results provide basis for the photochemopreventive effect of delphinidin and suggest that it may be a useful agent against UVB-induced damage for human skin.

## MATERIALS AND METHODS

### Materials

Delphinidin (>98% pure) was purchased from Extrasynthase (Lyon, France). The mAbs and polyclonal antibodies for PARP (116 kDa), Bcl-2 Bax, Bak, Bcl-xL were obtained from Upstate Biotechnology (Lake Placid, NY). mAb for PARP (85 kDa) was purchased from Promega (Madison, WI). The other human reactive mAbs and polyclonal antibodies (Procaspase 3, 8, and 9) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-mouse or anti-rabbit secondary antibody horse-radish peroxidase conjugate was obtained from Amersham Life Science Inc. (Arlington Height, IL). Annexin-V-FLOUS staining kit was purchased from Roche Diagnostic Corporation (Indianapolis, IN). The DC BioRad Protein assay kit was purchased from BioRad Laboratories (Hercules, CA). DeadEnd™ Colorimetric TUNEL kit was procured from Promega (Madison, WI). Novex pre-cast Tris-Glycine gels were obtained from Invitrogen (Carlsbad, CA).

### Treatment of cells

The HaCaT cells were cultured in DMEM medium, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and were maintained at 95% humidity in 5% CO<sub>2</sub> environment at 37°C (approved by the Institutional Review Board). Delphinidin (dissolved in DMSO) was used for the treatment of cells. The final concentration of DMSO used was 0.1% (v/v) for each treatment. The cells

(70–80% confluent) were treated with delphinidin (10  $\mu\text{M}$ ) for 24 hours in DMEM medium after which the media was removed and cells were washed with phosphate-buffered saline (PBS) and then fresh PBS was added, and these delphinidin pretreated cells were irradiated with UVB (15–30  $\text{mJ}/\text{cm}^2$ ) with a custom designed Research Irradiation Unit (Daavlin, Bryan, OH) that consists of a fixture mounted on fixed legs. Mounted within the exposure unit are four UVB lamps and the exposure system is controlled using Daavlin Flex Control Integrating Dosimeters. In this system, dose units can be entered in  $\text{mJ}/\text{cm}^2$  for UVB; variations in energy output are automatically compensated for the delivery of the desired dose. Using this system, the cells were exposed to accurate dosimetry of UVB radiation. Twenty-four hours post-UVB exposure, cells were harvested and cell lysates were prepared.

### Cell viability

The effect of delphinidin or UVB (or both) on the viability of cells was determined by MTT assay and phase contrast microscopy. The cells were plated at  $1 \times 10^4$  cells per well in 200  $\mu\text{l}$  of complete culture medium containing 1, 5, 10, 15, and 20  $\mu\text{M}$  concentrations of delphinidin in 96-well microtiter plates for 24 hours at 37°C in a humidified chamber. Each concentration of delphinidin was repeated in 10 wells. After incubation for specified times at 37°C in a humidified incubator the cells were washed and exposed to UVB (15–30  $\text{mJ}/\text{cm}^2$ ). Twenty-four hours post-UVB exposure, MTT reagent (4  $\mu\text{l}$ , 5  $\text{mg}/\text{ml}$  in PBS) was added to each well and incubated for 2 hours. The microtiter plate containing the cells was centrifuged at 1,800 rpm for 5 minutes at 4°C. The MTT solution was removed from the wells by aspiration and the formazan crystals were dissolved in DMSO (150  $\mu\text{l}$ ). Absorbance was recorded on a microplate reader at 540 nm wavelength.

### Preparation of cell lysate

Following treatment of cells with delphinidin or UVB (or both), the medium was aspirated and the cells were washed twice in PBS (10  $\text{mM}$ , pH 7.4). The cells were incubated in 0.4 ml ice-cold lysis buffer (50  $\text{mM}$  Tris-HCl, 150  $\text{mM}$  NaCl, 1  $\text{mM}$  EGTA, 1  $\text{mM}$  EDTA, 20  $\text{mM}$  NaF, 100  $\text{mM}$   $\text{Na}_3\text{VO}_4$ , 0.5% NP-40, 1% Triton X-100, 1  $\text{mM}$  PMSF (pH 7.4)) with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA). The cells were then centrifuged at 14,000  $g$  for 25 minutes at 4°C and the supernatant (cell lysate) was collected, aliquoted, and stored at  $-80^\circ\text{C}$ .

### Western blot analysis

For Western blot analysis, 25–50  $\mu\text{g}$  of protein was resolved over 8–12% polyacrylamide gels and transferred onto a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer (5% nonfat dry milk, 1% Tween-20; in 20  $\text{mM}$  Tris-buffered saline, pH 7.6) for 1 hour at room temperature followed by incubation with appropriate mAb or polyclonal primary antibody in blocking buffer for 1 hour to overnight at 4°C. This was followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase (Amersham Life Sciences, Inc.) for 1 hour. The blot was then washed several times and detected by chemiluminescence (ECL kit, Amersham Life Sciences, Inc.) and autoradiography using

XAR-5 film obtained from Eastman Kodak Co. (Rochester, NY). Densitometric measurements of the band in Western blot analysis were performed using digitalized scientific software program UN-SCAN-IT (Silk Scientific Corporation, Orem, UT).

### Quantification of apoptosis

The effect of delphinidin or UVB (or both) on the extent of apoptosis was determined by TUNEL assay. The cells were treated with delphinidin (10  $\mu\text{M}$ ) for 24 hours after which the cells were washed and exposed to UVB (15 and 30  $\text{mJ}/\text{cm}^2$ ). Twenty four hours post-UVB exposure the cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and PI by use of an Apo-direct apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) as per the manufacturer's protocol. The labeled cells were analyzed by flow cytometry.

### Apoptosis detection by confocal microscopy

The Annexin-V-FLUOS staining kit was used for the detection of apoptotic and necrotic cells according to vendor's protocol. This kit uses a dual-staining protocol in which the apoptotic cells are stained with annexin V (green fluorescence), and the necrotic cells are stained with PI (red fluorescence). The fluorescence was measured by a Zeiss 410 confocal microscope (Thornwood, NY). Confocal images of green annexin-FITC fluorescence were collected using 488 nm excitation light from an argon/krypton laser, a 560 nm dichroic mirror, and a 514–540 nm bandpass barrier filter. Images of red PI fluorescence were collected using a 568 nm excitation light from the argon/krypton laser, a 560 nm dichroic mirror, and a 590 nm long pass filter.

### Measurement of antioxidant activity

To assess the antioxidant capacity of delphinidin (1–20  $\mu\text{M}$ ) in HaCaT cells, trolox antioxidant assay was performed using the trolox antioxidant assay kit from Cayman chemical (Ann Arbor, MI) following the vendor's protocol. Briefly, the assay relies on the ability of antioxidants in the cell lysates to inhibit the oxidation of ABTS<sup>R</sup> (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS<sup>R•+</sup> by metmyoglobin. The amount of ABTS<sup>R•+</sup> produced was monitored by reading the absorbance at 750 nm. The capacity of the antioxidants in the sample to prevent ABTS<sup>R</sup> oxidation was compared with that of trolox, a water-soluble tocopherol analogue and was quantified as millimolar trolox equivalents. The reaction was initiated by adding 40  $\mu\text{l}$  of hydrogen peroxide working solution (441  $\mu\text{M}$ ) to all the wells being used. The plate was covered and incubated at room temperature for 5 minutes and absorbance was read at 750 nm using a plate reader.

### Immunostaining for 8-OHdG

HaCaT cells, grown on cover slides and treated with delphinidin (10  $\mu\text{M}$ ) for 24 hours, washed with PBS and exposed to UVB (15 and 30  $\text{mJ}/\text{cm}^2$ ). Twenty four hours post-UVB exposure the cells were fixed in methanol at  $-20^\circ\text{C}$  for 5 minutes. Briefly, the fixed cells were incubated with primary antibody 8-OHdG (Chemicon International Inc., Temecula, CA) followed by secondary antibody conjugated with horseradish peroxidase. Cells were then treated with 3,3'-diaminobenzidine and counterstained with hematoxylin.

### LPO assay

After treatment with delphinidin or UVB (or both) cells were harvested and washed with PBS, and microsomal fraction was prepared as described earlier (Katiyar *et al.*, 2001). Briefly, cells were homogenized with a Polytron homogenizer in PBS buffer containing potassium chloride (1.19%, w/v) and centrifuged at 18,000g for 15 minutes at 4°C to prepare microsomal fraction (Katiyar *et al.*, 2001). The LPO assay was performed in microsomal fraction obtained from the different treatment groups. The generation of malondialdehyde was employed as a marker of LPO and estimated by the method of Wright *et al.*, (1981). Briefly, microsomal fraction (2.0 mg protein) was incubated for 1 hour at 37°C in the presence of ferric ions (1 mM) and ADP (5 mM) in Ca<sup>2+</sup>-free phosphate buffer (0.1 M; pH 7.4) containing MgCl<sub>2</sub> (0.1 mM). The reaction was terminated by addition of 0.6 ml of 10% (w/v) trichloroacetic acid followed by 1.2 ml of 0.5% (w/v) 2-thiobarbituric acid. The mixture was heated for 20 minutes at 90°C in a water bath. After cooling, the malondialdehyde levels were measured in the clear supernatant by recording absorbance at 532 nm. The final concentration of malondialdehyde generated during the reaction was calculated using a molar extinction coefficient of  $1.56 \times 10^5$  M/cm.

### Animals and treatment

Female SKH-1 hairless mice (6-weeks-old) obtained from Charles River Laboratories (Wilmington, MA) were used in this study. All procedures were performed in accordance with the guidelines of the Animal Care and Approved by Institutional Review Board. After their arrival in the animal facility, the animals were allowed to acclimatize for 1 week before the start of the experiments. The animals were fed Purina Chow diet and water *ad libitum*. The mice were maintained at standard conditions: temperature of 24 ± 2°C, relative humidity of 50 ± 10%, and 12 hours room light/12 hours dark cycle. For UVB irradiation, the mice were housed in specially designed cages where they were held in dividers separated by Plexiglas. Sixty-four female SKH-1 hairless mice, maintained as described, were divided into five groups of 16 animals each (except in control and delphinidin alone groups, where eight animals were used). The mice in the first group received a topical application of 100 µl DMSO alone, and those in the second group received 1 mg delphinidin in 100 µl DMSO/mouse. The mice in the third group received a topical application of 100 µl DMSO before a single UVB (180 mJ/cm<sup>2</sup>) exposure with a custom designed Research Irradiation Unit (Daavlin, Bryan, OH) as described above. The mice in the fourth group received a topical application of delphinidin (1 mg/100 µl DMSO/mouse), 30 minutes before UVB (180 mJ/cm<sup>2</sup>) exposure. The animals in the fifth group were exposed to UVB (180 mJ/cm<sup>2</sup>), and immediately following UVB irradiation, they received topical application of delphinidin (1 mg/100 µl DMSO/mouse). The mice were then killed at 1 and 8 hours post-UVB exposure and skin biopsies were harvested for immunohistochemical analysis of CPDs, 8-OHdG, and apoptotic cells. The selection of these time points are based on recently published studies (Lu *et al.*, 1999; Gu *et al.*, 2005; Afaq *et al.*, 2006).

### Immunohistochemical detection of CPDs and 8-OHdG

The skin biopsies were frozen in optimal cutting temperature compound under liquid nitrogen immediately after removal. The skin biopsies were stored at -80°C for further use. Briefly,

5-µm-thick frozen skin sections were thawed and fixed in cold acetone for 10 minutes and washed in PBS (pH 7.4). The sections were then kept in 70 mmol/l NaOH in 70% ethanol for 2 minutes to denature nuclear DNA followed by neutralization for 1 minute in 100 mmol/l Tris-HCl (pH 7.5) in 70% ethanol. Nonspecific binding sites were blocked by 30 minutes incubation at room temperature in PBS containing 10% goat serum. Sections were then incubated with mouse mAbs to 8-OHdG (Nikken SEIL Corporation, Japan), CPDs (Kamiya Biomedical Co., Seattle, WA). For negative control the sections were incubated without primary antibody. After overnight incubation at 4°C and three washes in PBS, the sections were incubated for 30 minutes with biotinylated rabbit anti-mouse link antibody and then for 30 minutes with streptavidin conjugated to horse radish peroxidase. After washing, sections were incubated with diaminobenzidine plus peroxidase substrate and counterstained with methyl green.

### TUNEL staining for apoptotic cells

Apoptotic cells were detected using the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI) using the manufacturer's protocol. Briefly, tissue sections were fixed in 4% paraformaldehyde, permeabilized with Proteinase K solution, re-fixed in 4% paraformaldehyde, and equilibrated with 100 µl of equilibration buffer. Sections were then labeled with TdT reaction mix by incubating at 37°C for 1 hour, after which the reaction was stopped using 2 × SSC. Hydrogen peroxide (0.3%) was used for quenching endogenous peroxidase activity followed by incubation with 100 µl streptavidin horseradish peroxidase (1:500) for 30 minutes at room temperature. Each step was followed by thorough washing with PBS. Finally, sections were stained with 3,3'-diaminobenzidine for 2–3 minutes and visualized under microscope using × 400 magnification for TUNEL-positive cells.

### Statistical analysis

The results are expressed as means ± SD. Statistical analysis of all the data between groups receiving UVB exposure alone and those with delphinidin treatment plus UVB exposure were performed by Student's *t*-test. The *P*-value < 0.05 was considered statistically significant.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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