Galangin (3,5,7-Trihydroxyflavone) Shields Human Keratinocytes from Ultraviolet B-Induced Oxidative Stress

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Abstract
Most skin damage caused by ultraviolet B (UVB) radiation is owing to the generation of reactive oxygen species. Phytochemicals can act as antioxidants against UVB-induced oxidative stress. This study investigated the protective effects of the flavone galangin against UVB-induced oxidative damage in human keratinocytes. Galangin efficiently scavenged free radicals and reduced UVB-induced damage to cellular macromolecules, such as DNA, lipids, and proteins. Furthermore, galangin rescued cells undergoing apoptosis induced by UVB radiation via recovering mitochondrial polarization and down-regulating apoptotic proteins. These results showed that galangin protects human keratinocytes against UVB radiation-induced cellular damage and apoptosis via its antioxidant effects.

Key Words: Apoptosis, Galangin, Human keratinocytes, Oxidative damage, Ultraviolet B

INTRODUCTION
Solar radiation can be divided into three main types based on its wavelength, namely, ultraviolet (UV), visible light, and infrared (Lyons and O’Brien, 2002). Of these, UV radiation is the most responsible for photoaging and skin cancer. UV radiation is subdivided into UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm). The ozone layer reflects UVC radiation, meaning only UVA and UVB reach the earth’s surface. UVB radiation is particularly absorbed by human skin and causes erythema, burns, immune suppression, and skin cancer (Park et al., 2013). Although UVA accounts for the majority of UV radiation that reaches the earth’s surface and can penetrate the skin deeper than UVB, it is weakly carcinogenic and causes aging and wrinkling of the skin (Yoshikawa et al., 1990; Donawho et al., 1996; Matsumura and Ananthaswamy, 2004).

UVB directly or indirectly damages skin cells via the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone(6-4) photoproducts or via the generation of reactive oxygen species (ROS), such as hydroxyl radicals (•OH), superoxide anions (•O2−), hydrogen peroxide (H2O2), and singlet oxygen (•O2) (Cunningham et al., 1985; Hattori et al., 1996; Meeran et al., 2008). UVB-exposed cells generate ROS by activating specific small molecules such as riboflavin, tryptophan, and porphyrin (Ikehata and Ono, 2011). The antioxidant defense system in cells balances ROS production; however, when levels of ROS are elevated, this antioxidant defense system is overwhelmed, resulting in oxidative stress. Uncontrolled release of ROS causes single- and double-strand DNA breaks and DNA-protein cross-linking (Caldwell et al., 2007). ROS also attack important cellular structural and functional molecules, such as lipids and proteins, causing the malfunction of cellular activities, finally leading to apoptosis, a process of programmed cell death (Tsui et al., 2008; Dhurrongvaraporn and Chanvorachote, 2013).

With the increased occurrence of skin cancers and other damaging effects of UVB exposure, the protection of skin from UVB-induced oxidative cellular damage has become a key consideration in the pharmaceutical industry. Phytochemicals are well-known for their protective effects against oxidative stress in the skin (Sumiyoshi and Kimura, 2009). Galangin (3,5,7-trihydroxyflavone, Fig. 1) is a type of flavonoid that is commonly found in Alpinia officinarum and Helichrysum aurantioides (Afolayan and Meyer, 1997; Ciolino and Yeh, 1999). Galangin has antibacterial (Cushnie and Lamb, 2005; 2006)
and antiviral (Afolayan et al., 1997) properties and suppresses breast tumor cell growth (So et al., 1996; Diffey, 2004). However, the cytoprotective effects of galangin against UVB-induced oxidative damage in human keratinocytes have not been studied. Therefore, the objective of this study was to investigate the protective effects of galangin against UVB-induced oxidative stress in human keratinocytes.

MATERIALS AND METHODS

Reagents
Galangin was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). (3,5,7-Trihydroxy-2-methoxy flavone) (galangin, 98%), 1,1-diphenyl-2-picrylhydrazyl (DPPH), N-acetyl cysteine (NAC), 5,5-dimethyl-1-pyrroline-N oxide (DMPO), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), and Hoechst 33342 were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) was purchased from Invitrogen (Carlsbad, CA, USA). Primary antibodies for Bax and Bcl-2 were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals and reagents were of analytical grade.

Cell culture and UVB exposure
The human keratinocyte cell line HaCaT was obtained from Amore Pacific Company (Yongin, Korea) and maintained at 37°C in an incubator with a humidified atmosphere of 5% CO2. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, streptomycin (100 µg/ml), and penicillin (100 units/ml). Cells were exposed to UVB at a dose of 30 mJ/cm2. The CL-1000M UV Crosslinker (UVP, Upland, CA, USA) was used as the UVB source and delivered a UVB energy spectrum of 280-320 nm.

Cell viability assay
The influence of galangin on cell viability was examined using the MTT assay. Cells were seeded in a 96-well plate at a density of 1×104 cells/ml. After 24 h, galangin was added to a final concentration of 20, 40, 80, or 100 µM and cells were incubated for 24 h. MTT stock solution (50 µl, 2 mg/ml) was added to each well to yield a final reaction volume of 200 µl. The supernatant was aspirated 4 h later and formazan crystals were dissolved in 150 µl of dimethylsulfoxide (DMSO). The absorbance at 540 nm was read using a spectrophotometer (Carmichael et al., 1987).

Detection of DPPH radicals
The ability of galangin to scavenge DPPH radicals was assessed. Cells in a 96-well plate were treated with 20, 40, 80, or 100 µM of galangin or 1 mM of NAC. DPPH dissolved in ethanol (0.1 mM) was added to each well to yield a total volume of 200 µl. After shaking for 3 h, unreacted DPPH was detected by measuring the absorbance at 520 nm using a spectrophotometer.

Detection of hydroxyl radicals
Hydroxyl radicals generated by the Fenton reaction (H2O2 + FeSO4) were reacted with DMPO. The resultant DMPO-·OH adducts were detected using an electron spin resonance (ESR) spectrometer (Li et al., 2004). The ESR spectrum was recorded 2.5 min after a phosphate buffer solution (pH 7.4) was mixed with 0.02 ml each of 0.3 M DMPO, 10 mM FeSO4, 10 mM H2O2, and 40 µM of galangin. The ESR spectrometer parameters were set as follows: central magnetic field, 336.8 mT; power, 1.00 mW; frequency, 9.4380 GHz; modulation width, 0.2 mT; amplitude, 600; sweep width, 10 mT; sweep time, 0.5 min; time constant, 0.03 sec; and temperature, 25°C.

Detection of intracellular ROS
DCF-DA fluorescence was detected to measure intracellular ROS generated by H2O2 or UVB (Rosenkranz et al., 1992). Cells were seeded at a density of 1.5×105 cells/ml and incubated at 37°C for 24 h. Galangin (40 µM) or NAC (1 mM) was added to each well. After 1 h, cells were treated with H2O2 (1 mM) or exposed to UVB. After 30 min, H2O2-treated cells were treated with DCF-DA (25 µM) and incubated for another 20 min. UVB-treated cells were incubated for 24 h, treated with DCF-DA (50 µM), and incubated for a further 30 min. Fluorescence of 2',7'-dichlorofluorescein (DCF) was detected and quantified using a PerkinElmer LS-58 spectrophotometer (PerkinElmer, Waltham, MA, USA). Intracellular ROS scavenging effect of galangin (%)=(absorbance of control cells- absorbance of galangin- or NAC-treated cells)/absorbance of control cells×100. Only H2O2-or UVB-treated cells were considered as controls.

Single cell gel electrophoresis (Comet assay)
DNA damage caused by oxidative stress was detected by the comet assay (Singh, 2000; Rajagopalan et al., 2003). Cells were seeded at a density of 1×105 cells/ml and incubated at 37°C for 24 h. Cells were treated with galangin (40 µM) and after 1 h, exposed to UVB (30 mJ/cm2). The cell suspension was collected and mixed with 120 µl of 0.7% low melting agarose (LMA) at 37°C. The mixture was spread on a fully frosted microscope slide pre-coated with 200 µl of 1% normal melting agarose. After this had solidified, a further 170 µl of LMA was applied to the slide. Slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 90 min at 4°C. Slides were then immersed in an unwinding buffer solution with an electrical field of 300 mV and 25 V for 20 min at room temperature. The slides were washed three times with neutralizing buffer (0.4 M Tris, pH 7.5) for 10 min each time and then washed with 70% ethanol for 5 min. Slides were stained with 70 µl of ethidium bromide and observed under a fluorescence microscope using an image analyzer (Kinetic Imaging, Komet 5.5, UK). The tail length and the percentage of fluorescence in comet tails were recorded for 50 cells per slide.

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Cells were seeded at a density of $1 \times 10^5$ cells/ml and incubated at 37°C for 24 h. Cells were treated with galangin (40 μM) and after 1 h, exposed to UVB (30 mJ/cm²), and incubated at 37°C for another 24 h. Lipid peroxidation was assayed by colorimetric determination of the amount of 8-isoprostane secreted into the culture medium by HaCaT keratinocytes (Beauchamp et al., 2002). A commercial enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) was used according to the manufacturer’s instructions.

**Protein carbonylation assay**

Cells were seeded at a density of $1 \times 10^5$ cells/ml and incubated at 37°C for 24 h. Cells were treated with galangin (40 μM) and after 1 h, exposed to UVB (30 mJ/cm²), and incubated at 37°C for another 24 h. The extent of protein carbonyl formation was determined using an OxiSelect™ Protein Carbonyl ELISA Kit from Cell Biolabs (San Diego, CA, USA) according to the manufacturer’s instructions.

**Nuclear staining with Hoechst 33342**

Cells were seeded at a density of $1 \times 10^5$ cells/ml and incubated at 37°C for 24 h. Cells were treated with galangin (40 μM) and after 1 h, exposed to UVB (30 mJ/cm²), and incubated at 37°C for another 24 h. The DNA-specific fluorescent dye Hoechst 33342 was added to each well and cells were incubated for 10 min at 37°C. Stained cells were visualized under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera. The degree of nuclear condensation was evaluated and apoptotic cells were counted.

**Analysis of mitochondrial membrane potential ($\Delta \psi_m$)**

JC-1 was added to each well and cells were incubated for 30 min at 37°C. Stained cells were washed with phosphate-buffered saline (PBS), coverslips were mounted onto microscope slides in mounting medium (DAKO, Carpinteria, CA, USA), and slides were examined using a confocal microscope. Microscopic images were collected using the Laser Scanning Microscope 5 PASCAL program (Carl Zeiss, Jena, Germany) (Cossarizza et al., 1993). In addition, mitochondrial membrane potential was analyzed by flow cytometry. Cells were harvested, washed, suspended in PBS containing JC-1 (10 μg/ml), incubated for 30 min at 37°C, and analyzed using a flow cytometer (Troiano et al., 2007).
Western blot analysis
Harvested cells were lysed by incubation on ice for 10 min in 150 μl of lysis buffer containing 120 mM NaCl, 40 mM Tris (pH 8), and 0.1% NP 40. The resultant cell lysates were centrifuged at 13,000 rpm for 5 min. Supernatants were collected and protein concentrations were determined. Aliquots were boiled for 5 min and electrophoresed on 12% SDS-polyacrylamide gels. Protein blots of the gels were transferred onto nitrocellulose membranes. The membranes were incubated with the appropriate primary antibodies (1:1,000) followed by horseradish peroxidase-conjugated anti-IgG secondary antibodies (1:5,000) (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Statistical analysis
All measurements were performed in triplicate and all values are expressed as means ± standard error. The results were subjected to an analysis of variance using Tukey’s test to analyze differences between means. In each case, a p-value of <0.05 was considered statistically significant.

RESULTS

Galangin attenuates UVB-induced ROS generation
The MTT assay showed that galangin was not toxic to HaCaT cells at any concentration used. Following the treatment with each of the concentrations of galangin tested, cell viability was more than 96% of that of control cells (Fig. 2A). Galangin showed DPPH radical-scavenging activity at concentrations of 20-100 μM compared to treatment with NAC (1 mM), a well-known antioxidant (Fig. 2B). Therefore, 40 μM was selected as the optimal concentration of galangin for further experiments. To assess the ability of galangin (40 μM) to scavenge hydroxyl radicals, ESR spectrometry was performed. In the Fenton reaction (Fe2++H2O2→Fe3++•OH+OH-), DMPO•OH adducts generated a signal of 2,915 in control cells and this was reduced to 1,803 in the presence of galangin (Fig. 2C). Next, the intracellular ROS scavenging activity of galangin was assessed using the DCF-DA fluorescent probe. Galangin scavenged 27% of ROS (versus 52% for NAC) in H2O2-treated cells and 27% of ROS (versus 22% for NAC) in UVB-treated cells (Fig. 2D). Taken together, these results show that galangin efficiently scavenges ROS.

Galangin significantly attenuates UVB-induced damage of cellular macromolecules
We next investigated whether galangin can protect macromolecules, such as DNA, lipids, and proteins, from UVB-induced oxidative damage. First, UVB-induced DNA damage and the protective effects of galangin were studied using the comet assay. Representative microscopy images indicating the length of comet tails and the percentage of fluorescence in the tails are shown in Fig. 3A. UVB treatment increased

Fig. 3. Galangin attenuates UVB-induced macromolecule damage. Cells were treated with 40 μM of galangin and exposed to UVB (30 mJ/cm²) 1 hour later. After 24 hours, (A) The comet assay was performed to assess DNA damage. Representative images and the percentage of cellular fluorescence within comet tails are shown. *Significantly different from control cells (p<0.05); #significantly different from UVB only-exposed cells (p<0.05). (B) Lipid peroxidation was assessed by measuring the concentration of 8-isoprostane in the conditioned medium. *Significantly different from control cells (p<0.05); #significantly different from UVB only-exposed cells (p<0.05). (C) Protein oxidation was determined by measuring the amount of carbonyls formed. *Significantly different from control cells (p<0.05); #significantly different from UVB only-exposed cells (p<0.05).
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The comet tail length and increased the percentage of fluorescence in the tail to 52%, indicating an increased level of DNA damage in keratinocytes, while galangin pretreatment significantly reduced this to 34%. UVB-induced cellular membrane damage was measured by detecting lipid peroxidation via colorimetric determination of the level of 8-isoprostane secreted into the culture medium by HaCaT cells. The concentration of 8-isoprostane was 219 pg/ml in the culture medium of UVB-exposed cells and 32 pg/ml in that of cells pretreated with galangin prior to UVB exposure (Fig. 3B). Finally, protein carbonylation was measured to assess the degree of protein damage. Oxidative stress usually modifies the amino acid side chains of proteins to carbonyl derivatives, which can be used to quantify protein damage caused by UVB-induced oxidative stress (Dalle-Donne, 2006). Protein carbonylation was higher in UVB-treated cells than in control cells, whereas this increase was significantly reduced in cells pretreated with galangin prior to UVB exposure (Fig. 3C). These findings confirm that galangin can protect cellular macromolecules from UVB-induced oxidative damage.

Galangin reduces UVB-induced apoptosis

The viability of HaCaT cells was assessed using the MTT assay (Fig. 4A). UVB exposure reduced the viability of keratinocytes to 60% in comparison to that of control cells. However, pretreatment with galangin increased cell viability to 76%, while pretreatment with the well-known antioxidant NAC increased cell viability to 88%. Next, cells were stained with Hoechst 33342 to visualize nuclear condensation and the formation of apoptotic bodies, which are characteristic of apoptosis. Normal nuclei were observed in control and galangin-treated cells, whereas notable nuclear condensation was found in UVB-exposed cells (Fig. 4B). The nuclear condensation was significantly lowered in galangin- and NAC-pretreated cells than in UVB only-exposed cells. Mitochondrial membrane permeability plays a crucial role in the mitochondria-mediated apoptosis pathway. To elucidate the mechanism underlying how galangin protects keratinocytes against UVB-induced apoptosis, changes in the mitochondrial membrane potential were assessed. UVB treatment strongly increased the level of green fluorescence caused by JC-1 monomers, indicat-
ing mitochondrial depolarization (Fig. 4C). However, galangin treatment prior to UVB exposure significantly reduced the intensity of green fluorescence, showing that galangin attenuated UVB-induced mitochondrial depolarization. To confirm these results, flow cytometric analysis of JC-1 was performed. As expected, the green fluorescence peak was increased in UVB-treated cells. However, galangin pretreatment notably reduced the peak of this fluorescence (Fig. 4D). Caspase-9, an initiator caspase, is cleaved to activate followed by the releasing cytochrome c from the depolarized mitochondria into the cytosol, cleaved caspase-9 propagates further activation of downstream apoptotic proteins which causes for cleavage of procaspase-3, activated caspase-3 triggers the apoptotic process (Adrain and Martin, 2001). To assess the caspase-9 and -3 expression levels, a western blot analysis was performed. UVB exposure resulted up-regulation of cleaved caspase-9 and -3 in HaCaT cells, interestingly galangin pretreatment significantly down-regulated the levels of cleaved caspase-9 and -3. The vulnerability of cells towards apoptosis is ultimately determined by the pro-apoptotic and anti-apoptotic proteins in the B-cell leukaemia/lymphoma-2 (Bcl-2) family (Basu and Haldar, 1998). Bcl-2 itself is consider as an anti-apoptosis protein while Bcl-2-associated x protein (Bax) is known as a pro-apoptotic protein in the Bcl-2 protein family (Wei et al., 2001) Therefore we next investigated the level of Bcl-2 and Bax proteins after galangin treatment (Fig. 4E). Galangin treatment prior to UVB exposure resulted up-regulation of Bcl-2 proteins whereas down-regulation of Bax proteins. These results indicate that galangin inhibits apoptotic process via establishing mitochondrial membrane polarity and regulating apoptotic proteins.

DISCUSSION

Chronic solar UVB exposure, also known as photoaging, is the most well understood skin aging mechanism (Nichols and Katiyar, 2010; Svbodova and Vostalova, 2010). Although the skin possesses a complex enzymatic and non-enzymatic defense system to protect it from adverse effects, prolonged UVB exposure can overwhelm this system. Solar UVB radiation has become one of the most common carcinogens in the environment and excessive UVB exposure leads to skin cancer. Meanwhile, due to the depletion of the stratospheric ozone
layer and climate change, the earth receives elevated levels of UV radiation (De Grujil et al., 2003; Diffey, 2004). UVB damages DNA directly via the formation of pyrimidine dimers and indirectly via ROS generation (Ichihashi et al., 2003). DNA is the chief information molecule in the cell; therefore, nuclear DNA must exist for the entire life time of the cell. DNA damage can critically affect cellular functions. ROS attack not only nucleic acids but also proteins and lipids, thereby interrupting cellular metabolism. In this regard, prevention of the adverse effects caused by exposure to harmful UV radiation has become a popular theme in the cosmetic and pharmaceutical industries. Phytochemicals are well-known for their abilities to protect the skin against harmful UV radiation. Galangin is a flavone whose antioxidant ability depends on the donation of hydrogen atoms to free radicals (Sim et al., 2007). In this study, galangin scavenged DPPH radicals and hydroxyl radicals (Fig. 2B, C). DCF-DA staining revealed that intracellular ROS generated via $H_2O_2$ and UVB (Fig. 2D) were removed by galangin. Flavonoid is known to possess antioxidant activity by donation of a hydrogen to radicals, depending on the substitution pattern of hydroxyl groups. Hydroxylated position is related with stability of the resulting phenoxyl radical by hydrogen donation or electron delocalization. The presence of 3-OH and 5-OH and C2-C3 double bond conjugated with the 4-oxo function increase antioxidant activity (Rice-Evans et al., 1996; Jung et al., 2005). In general, O-dihydroxy groups known as catechol structure in B ring increases radical scavenging activity, however it is reported that absence of catechol structure in the B ring like galangin is not always related with low antioxidant activity by compensating lack of catechol structure through combination C2-C3 double bond with the 3-OH and 4-keto group (Amic et al., 2003), demonstrating antioxidant effect as shown in our results.

UVB-induced ROS cause oxidative damage to DNA, lipids, and proteins in keratinocytes. Formation of CPDs and 6-4 photoproducts are considered as the main types of lesions caused by UVB radiation in DNA, and these adducts cause DNA strand breaks and induction of mutations (You et al., 2000). ROS especially hydroxyl radicals abstract allylic hydroxyl hydrogen forming carbon centered lipid radicals which will rapidly react with oxygen to form lipid peroxy radicals, interestingly these peroxy radicals are capable of abstract hydrogens from another lipid molecules generating lipid radicals initiating chain reactions (Ayala et al., 2014). ROS attack proteins and cause reversible and/or irreversible modifications, such as protein carboxylation, formation of adducts with lipid peroxidation products and protein protein cross-linking (Perlui et al., 2010). Formation of protein carboxyls could be either by oxidative cleavage of proteins or by direct oxidation of lysine, arginine, proline, and threonine residues (Sander et al., 2002). The comet assay showed that galangin significantly suppressed DNA strand breaks induced by UVB (Fig. 3A). Detection of 8-isoprostone released by cells into the extracellular environment is an accurate means of assaying lipid peroxidation. Galangin-pretreated cells showed notably lower levels of 8-isoprostane in the culture medium than UVB alone-treated cells (Fig. 3B), suggesting that galangin attenuated UVB-induced lipid peroxidation in skin cells. Proteins are oxidized by ROS and formation of protein carbonyls is a hallmark of oxidative stress. Our results illustrated that the level of carbonyls was significantly attenuated in cells pretreated with galangin prior to UVB exposure (Fig. 3C).

At elevated concentration of ROS overwhelms the antioxidant defense system and interrupts cellular metabolism. In response to DNA damage, cells can undergo apoptosis (Barzilai and Yamamoto, 2004).

Recent studies show that UVB induced cell death is basically governed through apoptotic pathway (Ji et al., 2012). In addition to DNA damage and cell cycle arrest by UVB induced oxidative stress, following the membrane damage via lipid peroxidation and protein carbonylation, ROS weaken the inner cellular structures especially mitochondrial membranes which causes to release cytochrome c into the cytosol and activates apoptotic proteins (Kulms et al., 2002). Galangin pre-treatment significantly increased the viability of UVB-exposed cells (Fig. 4A). Hoechst 33342 staining revealed that galangin pretreatment effectively reduced the formation of apoptotic bodies and DNA condensation induced by UVB exposure (Fig. 4B). Furthermore, we investigated the impact of galangin pre-treatment on mitochondria-mediated apoptosis via JC-1 staining. Confocal microscopy (Fig. 4C) and flow cytometric (Fig. 4D) analyses confirmed that galangin restored mitochondrial membrane polarization. These data showed that galangin reduced apoptosis of UVB-radiated cells by establishing mitochondrial polarization. Pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 regulate the release of cytochrome c into the cytosol (Atan et al., 1999). Caspases are synthesized in their inactive forms in the cells and activated in respond to apoptotic signals (Thornberry and Lazebnik, 1998). Released cytochrome c into the cytosol binds to apoptotic protease activating factor 1, which then activates caspase-9 by cleaving procaspase-9 (Bossey-Wetzel et al., 1998). Cleaved caspase-9 activates caspase-3 and which then triggers downstream caspase cascades to execute apoptosis (Soengas et al., 1999). Our results elucidated that galangin suppressed the expression of Bax, cleaved caspase-9 and -3 while increased the expression of Bcl-2 protein level (Fig. 4E).

In summary, our results confirmed that galangin possesses antioxidant properties and it interferes intrinsic pathway of apoptosis via down regulating key apoptotic proteins, which it protected HaCaT cells from UVB-induced oxidative damage.

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