Published in final edited form as:

Photochem Photobiol. 2012 ; 88(5): 1135–1140. doi:10.1111/j.1751-1097.2011.01050.x.

Silibinin is a Potent Sensitizer of Ultraviolet A Radiation-induced Oxidative Stress and Apoptosis in Human Keratinocyte HaCaT Cells

Sreekanth Narayanapillai¹, Chapla Agarwal^{1,2}, Cynthia Tilley¹, and Rajesh Agarwal^{1,2,*}

¹Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Denver, Aurora, Colorado, United States of America

²University of Colorado Cancer Center, Aurora, Colorado, United States of America

Abstract

Ultraviolet A radiation (UVA, 315–400nm), which constitutes approximately 95% of the UV irradiation in natural sunlight reaching earth surface, is a major environmental risk factor associated with human skin cancer pathogenesis. UVA is an oxidizing agent that causes significant damage to cellular components through the release of reactive oxygen species (ROS) and leads to photoaging and photocarcinogenesis. Here we investigate the effect of silibinin, the flavonolignan from *Silybum marianum*, on UVA-induced ROS and cell death in human keratinocyte cell line HaCaT. In addition, the effect of silibinin on UVA-induced intracellular ROS-mediated endoplasmic reticulum (ER) stress was also analyzed. UVA irradiation resulted in ROS production and apoptosis in HaCaT cells in a dose-dependent manner, and the ROS levels and apoptotic index were found to be elevated significantly when the cells were treated with 75 μ M silibinin for 2h before UVA exposure. When the cells were pretreated with 10mM N-acetyl cysteine (NAC), the enhancement of UVA-induced apoptosis by silibinin was compromised. Furthermore, we found that silibinin enhances ER stress-mediated apoptosis in HaCaT cells by increasing the expression of CHOP protein. These results suggest that silibinin may be beneficial in the removal of UVA-damaged cells and the prevention of skin cancer.

Introduction

Solar ultraviolet (UV) radiation is the major environmental etiologic factor in the development of non-melanoma skin cancer, which is the most frequently diagnosed neoplastic condition in humans (1, 2). A radiation (UVA) has been considered less carcinogenic and mutagenic because of its limited direct DNA damage potential as the constituent nucleotide bases do not absorb significantly at wavelengths above 320 nm (3). However, UVA constitutes about 95% of the total UV irradiation (4) and comprises approximately 10–20% of the carcinogenic fraction in natural sunlight (5). UVA can penetrate the epidermal layer as well as the underlying dermis and lead to the development of human skin cancer (6, 7). At the cellular level, UVA-induced damage occurs mainly via oxidative processes and UVA is considered as the most important source of oxidative stress in human skin (8). Exposure of skin to UVA results in the generation of large quantities of intracellular reactive oxygen species (ROS), which directly or indirectly affects various cell signaling pathways and also mediates various UV-induced cutaneous reactions including inflammation, photosensitivity, and carcinogenesis (1, 9). Uncontrolled release of ROS is involved in the pathogenesis of a number of human skin disorders including cutaneous

*Corresponding author: Rajesh.Agarwal@ucdenver.edu.

neoplasia by inducing oxidative DNA damage including base damage, single and double-strand breaks, DNA-protein crosslinking etc. (10, 11). UVA radiation has also been shown to induce photoaging and squamous cell carcinoma in mice (12–14), and chronic UVA exposure induces malignant transformation and apoptosis resistance in human keratinocyte HaCaT cells (15).

Several studies have demonstrated ROS generation as an important mechanism behind UVA-induced apoptosis, and the failure in removal of UV-damaged cells leads to tumor development (5, 13, 16, 17). Therefore, apoptotic elimination of UV-damaged epidermal keratinocytes is an efficient and ideal protective mechanism for the prevention of skin cancer (18). It has been reported that the oxidative stress-induced damage can lead to endoplasmic reticulum (ER) stress and can also upregulate the ER stress responsive gene GRP78 (19, 20). Recent studies have also shown that environmental dose of UV radiation causes unfolded protein response (UPR) and ER stress (21) as well as the induction of damage responsive transcription factor CHOP/GADD153 in the epidermis (22).

Various phytochemicals have been shown as potent chemopreventive agents against UV-induced skin damage and photocarcinogenesis. Silibinin, a naturally occurring polyphenolic flavanolignan from *Silybum marianum* has been shown as a photodamage sensor which protects or enhances apoptosis in cultured human keratinocytes depending on the extent of damage (23). The protective role of silibinin against UVB-induced DNA damage and carcinogenesis has been well demonstrated in cultured keratinocytes and animal models; however, its effect on the UVA-induced changes in the skin has not been well examined. In this study, we used the human keratinocyte cell line, HaCaT to analyze the effect of silibinin on UVA-induced decrease in cell viability and apoptosis along with associated mechanisms. Our data provide evidence that pretreatment of HaCaT cells with silibinin before UVA exposure significantly enhances UVA-induced apoptosis in a ROS and ER stress-dependent manner, and thereby, accelerates the removal of UVA-damaged cells. These findings suggest the usefulness of silibinin as a potent chemopreventive agent against UVA-induced skin damage and cancer.

Materials and Methods

Reagents and antibodies

Rabbit polyclonal cleaved caspase-3, human-specific cleaved PARP, GRP78 and mouse monoclonal CHOP were purchased from Cell Signaling Technology (Beverly, MA); IR800 or IR700 fluorescent dye-labeled anti-mouse and anti-rabbit IgGs were from LI-COR Biosciences (Lincoln, NE). Silibinin and all other reagents were from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

Cells and UVA treatment

The immortalized human keratinocyte cell line HaCaT was cultured in DMEM supplemented with 10% fetal bovine serum and 100 u/ml of penicillin/streptomycin (Gibco BRL, Grand Island, NY) under standard conditions. For all treatments, cells were grown to 80% confluence, treated with DMSO or silibinin in DMSO for 2h, and then exposed to UVA. In some cases, cells were pre-treated with NAC before UVA exposure for 2h, or with other inhibitors immediately after UVA exposure as specified in the results and figure legends. Before UVA irradiation, media was removed from culture plates; cells were washed with phosphate-buffered saline twice and then covered with a thin layer of phosphate-buffered saline followed by UVA irradiation. Control cultures were identically processed but not irradiated. The UVA light source was a bank of four F20T12/BL/HO PUVA bulbs equipped with a UVA Spectra 305 Dosimeter (Daavlin Co., Bryan, OH),

providing a peak emission at 365 nm as monitored with a SEL 033 photodetector attached to an IL 1400 Research Radiometer (International Light, Newburyport, MA)

Trypan blue dye exclusion assay

HaCaT cells were plated at a cell density of 5,000/cm² in 60-mm culture plates under standard culture conditions. Next day, silibinin/NAC pretreated or DMSO treated cells were exposed to UVA at different doses. At the end of desired treatment times (6–24 h), cells were harvested by trypsinization, stained with Trypan blue (Gibco BRL, Grand Island, NY) and counted for live and dead cells using a hemocytometer.

Western immunoblotting

Following the desired treatments, cell lysates were prepared in non-denaturing lysis buffer (10mM Tris-HCl, 150mM NaCl, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.2mM sodium orthovanadate, 0.5% NP-40, 5 U/ml aprotinin) and protein concentration in the lysates was determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). For immunoblot analyses, 60µg of protein per sample was denatured in 2X SDS-PAGE sample buffer, resolved on Tris/glycine gels, transferred onto nitrocellulose membranes and probed with specific primary antibody followed by appropriate IR800 or IR700 dye-labeled secondary antibody, and visualized using an Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

Apoptosis assay by annexin V and propidium iodide (PI) staining

For quantitative apoptotic cell death, HaCaT cells were plated in 60 mm dishes, treated with DMSO/silibinin for 2h and exposed to the desired doses of UVA as indicated. After 16h of incubation, cells were collected, stained with Annexin V and PI (Molecular Probes) following the manufacturer's protocol and analyzed immediately by flow cytometry at the FACS Analysis Core Facility of the University of Colorado Cancer Center.

Measurement of ROS production

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2',7'-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF). HaCaT cells were cultured in 24-well plates, pre-treated with NAC and/or silibinin treated as indicated, UVA-irradiated, washed with PBS and incubated with 20µM DCFH-DA for 20min at 37°C. Fluorescence intensity per each well was detected using a multi-functional microplate reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 480nm and at an emission wavelength of 530nm.

Statistical analysis

Statistical significance of differences between control and treated samples was determined using Student's *t* test (SigmaStat 3.5, Systat Software, Inc., Richmond, CA). The values of *P* < 0.05 were considered significant. The data in all cases are representative of at least 2 independent experiments.

Results

UVA induces apoptosis in HaCaT cells in a dose dependent manner

To determine the effect of UVA irradiation on viability, HaCaT cells were exposed to increasing doses of UVA (2.5, 5 and 10J/cm²), and cell viability was analyzed as a function of time (6, 12, 18, and 24 h) by Trypan blue exclusion assay. We found that exposure to 5J/cm² and 10J/cm² doses of UVA results in significant decrease in cell viability at 18h and 24h time points (Fig. 1A) in a dose-dependent manner. UVA at 5J/cm² dose decreased the

cell viability by 29% ($P<0.05$) and 36% ($P<0.001$) at 18h and 24h respectively, whereas 10J/cm² UVA decreased the cell viability by 37% ($P<0.001$) and 48% ($P<0.001$) at 18h and 24h, respectively, as compared to the DMSO-treated control cells. Next, we determined whether UVA treatment leads to the decrease in cell viability by inducing apoptosis in HaCaT cells. The cells were exposed to increasing doses of UVA and after 24h, cell lysates were prepared and subjected to western blotting for the well established apoptosis markers, cleaved caspase-3 and cleaved PARP. As shown in Fig. 1B, UVA exposure at 5J/cm² and 10J/cm² doses resulted in significant activation of caspase-3 and PARP cleavage within 24h. We also analyzed the time kinetics of caspase-3 activation and PARP cleavage in HaCaT cells following 5J/cm² UVA exposure and found their expression significantly elevated after 18h and 24h of irradiation (Fig. 1C). Next, we quantified the apoptotic cell percentage in the total cell population by flow cytometric analysis of annexin V/PI stained cells. Results showed that 5J/cm² and 10J/cm² doses of UVA induce 20% ($P<0.001$) and 31% ($P<0.001$) apoptosis, respectively, after 16h of irradiation (Fig. 1D). Overall, these results clearly showed that UVA exposure decreases viability of HaCaT cells by inducing apoptosis, and accordingly we selected the 5J/cm² dose of UVA for further experiments.

Silibinin pretreatment enhances UVA-induced apoptosis

HaCaT cells were pretreated with DMSO or silibinin for 2h followed by sham irradiation or exposure to 5J/cm² UVA. At 24h post-UVA exposure, both adherent and floating cells were collected and Trypan blue dye exclusion assay was carried out. As shown in Fig. 2A, treatment with 75μM silibinin alone for 24h did not significantly affect the viability of HaCaT cells. The 5J/cm² UVA irradiation resulted in 34% ($P<0.005$) decrease in cell viability after 24h of irradiation. More interestingly, when the cells were treated with 75μM silibinin before UVA exposure, the cell viability was decreased by 54% ($P<0.001$) as compared to the untreated control cells or by 31% ($P<0.001$) as compared to UVA irradiated cells. In the next set of experiments, we examined the effect of silibinin pretreatment on UVA-induced expression of apoptosis markers such as cleaved caspase-3 and cleaved PARP by western blotting. As shown in Fig. 2B, silibinin pretreatment significantly enhanced caspase-3 activation and PARP cleavage in HaCaT cells at 18h and 24h time points. These results clearly indicated that silibinin pretreatment enhances UVA induced cell death by augmenting apoptosis.

Silibinin augments UVA-induced apoptosis in HaCaT cells by enhancing ROS generation

Since UVA has been proven as a potent inducer of various ROS and oxidative stress in different cell types, our next aim was to analyze whether UVA-induced ROS leads to apoptosis in HaCaT cells and how silibinin pretreatment modulates UVA-induced ROS. As shown in Fig. 3A, 5J/cm² UVA induced significant amount of intracellular ROS in HaCaT cells at 0.5, 1 and 2h time points as assessed by the oxidative conversion of dichlorofluorescein diacetate to fluorescent dichlorofluorescein. Even though 2h treatment with 75μM silibinin was not found to alter the ROS levels as compared to DMSO-treated control cells, the cells which were treated with silibinin prior to UVA irradiation showed further increase in ROS levels, in comparison with UVA alone treated cells. Interestingly, when HaCaT cells were pretreated with the ROS scavenger, NAC, UVA failed to induce a significant increase in intracellular ROS levels in DMSO treated as well as silibinin pretreated cells. Since it was clear that silibinin pretreatment increases UVA-induced ROS levels in HaCaT cells, in the next set of experiments we sought to determine the correlation between elevation in ROS and cell viability. From the results of Trypan blue dye exclusion assay, we found that UVA-mediated decrease in cell viability was restored when the cells were pretreated with 10mM NAC and that in NAC-pretreated cells, silibinin failed to enhance UVA-induced cell death within 24h (Fig. 3B). These observations confirmed the involvement of ROS in UVA-induced death response and its augmentation by silibinin in

HaCaT cells. To further confirm the involvement of ROS in UVA-induced apoptosis we carried out western blot analyses for cleaved caspase-3 and cleaved PARP using protein extracts from UVA irradiated HaCaT cells with or without silibinin and/or NAC pretreatment. The results revealed that UVA alone or in combination with silibinin pretreatment could not lead to the expression of the apoptosis markers within 24h, when the cells were pretreated with 10mM NAC (Fig. 3C). These findings were further confirmed by flow cytometric analysis of AnnexinV/PI stained HaCaT cells after various treatments as indicated in Fig. 3D. We observed that NAC pretreatment could significantly bring down apoptotic cell death in UVA alone treated or silibinin+UVA treated HaCaT cells and these observations substantiated the role of ROS in silibinin-mediated enhancement of UVA-induced apoptosis.

Silibinin enhances UVA-induced apoptosis by upregulating ER-stress

To further investigate the role of ROS in UVA-induced apoptosis, we analyzed the expression of ER-stress marker GRP78 and the CHOP protein by western blotting. A 5J/cm² UVA exposure increased the expression of GRP78 in DMSO treated or silibinin pretreated HaCaT cells after 24h of irradiation. It was also observed that the expression level of CHOP/GADD153 transcription factor, which is involved in ER stress mediated apoptosis, was significantly elevated in UVA exposed cells. Pretreatment of HaCaT cells with 75μM silibinin for 2h before UVA exposure was found to further increase the expression of CHOP protein in these cells, suggesting that silibinin pretreatment enhances UVA-induced ER-stress mediated apoptosis (Fig. 4A). This observation was further confirmed by treating the cells with 10mM NAC, which was shown to inhibit UVA-induced ROS in the earlier set of experiments. In line with the previous observations, we found that NAC pretreatment abolishes UVA-induced overexpression of GRP78 and CHOP, indicating that ROS-induced and oxidative stress mediated apoptosis mediated by CHOP is the major mechanism of cell death in HaCaT cells following UVA irradiation. To provide further evidence for the involvement of ROS-dependent ER-stress in UVA-induced apoptosis and its enhancement by silibinin, we treated HaCaT cells with a chemical inhibitor of GRP78 accumulation and ER stress called Piericidin A. As shown in Fig. 4B, treatment with Piericidin A markedly reduced the expression of GRP78, CHOP, cleaved caspase-3 and cleaved PARP in DMSO/silibinin pretreated cells after 24h of UVA exposure.

Discussion

In the past, most of the studies on the prevention and treatment of UV-induced skin cancer had given a traditional emphasis on the UVB component, as the UVA part was considered less mutagenic and less relevant in skin tumorigenesis. However, recent studies indicate that UVA also plays a pivotal role with detectable fingerprint mutations in human skin cancers (6). UVA induces photo-oxidative effects leading to the generation of ROS which has been linked with apoptosis as well as protein- and DNA-damage potential (24). The role of ROS in UVA-induced apoptosis was also confirmed by several studies using various antioxidants (25–27). In addition, UVA has been shown to deplete the intracellular non-enzymatic antioxidant, glutathione (28). Therefore, agents which can enhance the ROS levels and oxidative stress in the UVA-damaged cells can accelerate apoptotic cell death and thereby remove the damaged cell to reduce the risk of skin cancer development.

In the present study, we assessed the apoptosis inducing potential of UVA in HaCaT cells by western blotting for apoptosis markers as well as by AnnexinV/PI staining, and provide the evidence for the pivotal role of ROS generation in the dose-dependent apoptosis induction by UVA.. Importantly, in the cells pre-exposed to 75μM silibinin, both UVA-induced ROS levels and the apoptotic cell death was found to be significantly higher, supporting our assumption that silibinin enhances UVA-induced apoptosis by elevating oxidative stress.

These results are not in line with a previous report showing that post-treatment with flavonolignans from *Silybum marianum* moderates apoptosis in UVA-irradiated HaCaT cells by eliminating ROS (29). We, however, also observed similar protective role of silibinin against UVA-induced apoptosis when added to the medium post-UVA exposure (data not shown). These results suggest that silibinin has differential effect on UVA-caused ROS generation and apoptosis in HaCaT cells depending on its pre- versus post-UVA exposure treatment.

Our hypothesis that UVA-mediated oxidative stress plays a pivotal role in apoptosis was further proven with the observation that pretreatment with NAC, which can induce cellular glutathione synthesis and reduce UVA-induced oxidative stress (30), alleviated UVA-induced ROS generation and subsequent apoptosis. Since ROS production is known to induce protein aggregation and misfolding, and ER stress results from the accumulation of misfolded proteins and unfolded protein response (31), the elevated levels of GRP78, which is the protective chaperone protein associated with ER stress, clearly suggests the involvement of ER stress in UVA-induced apoptosis. In addition, the significant upregulation of CHOP/GADD153 protein, which is one of the components of the ER stress-mediated apoptosis pathway, by silibinin pretreatment in UVA-exposed HaCaT cells, indicate the augmentation of ER stress mediated apoptosis by silibinin in these cells. Since CHOP is reported to downregulate the antiapoptotic Bcl-2 protein and induce the translocation of proapoptotic Bax protein to the mitochondria (32, 33), the upregulation of CHOP by silibinin can be considered as an important mechanism by which it enhances UVA-induced apoptosis. Furthermore, the observation that NAC pre-treatment down-regulates both GRP78 and CHOP expression confirmed the occurrence of ROS-induced ER stress in HaCaT cells following UVA irradiation. The involvement of ER stress in UVA-induced apoptosis was further evidenced where a chemical inhibitor of GRP78 and ER stress Plericidin A, completely abolished UVA-induced apoptotic responses.

In summary, our findings suggest the use of silibinin as a potent sensitizer of UVA-induced apoptosis via increasing the ROS formation and ER stress and thus it will lead to the rapid elimination of UVA-damaged cells before their malignant transformation. Our proposed overall molecular mechanisms by which silibinin enhance UVA-induced apoptosis in HaCaT cells are illustrated in Fig. 5. In addition, our data establish correlations between UVA-induced ROS generation and ER stress-mediated apoptosis in HaCaT cells and warrant further *in vivo* studies to decipher the effect of silibinin on UVA-induced apoptosis in a preclinical setting.

Acknowledgments

This work was supported by USPHS RO1 grant CA140368 from NCI.

References

1. Ichihashi M, Ueda M, Budiyanto A, Bito T, Oka M, Fukunaga M, Tsuru K, Horikawa T. UV-induced skin damage. *Toxicology*. 2003; 189:21–39. [PubMed: 12821280]
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA: a cancer journal for clinicians*. 2011; 61:69–90. [PubMed: 21296855]
3. de Grujil FR. Photocarcinogenesis: UVA vs. UVB radiation. *Skin Pharmacol Appl Skin Physiol*. 2002; 15:316–320. [PubMed: 12239425]
4. Parisi AV, Wong JC. An estimation of biological hazards due to solar radiation. *J Photochem Photobiol B*. 2000; 54:126–130. [PubMed: 10836542]

5. Li JL, Liu N, Chen XH, Sun M, Wang CB. Inhibition of UVA-induced apoptotic signaling pathway by polypeptide from *Chlamys farreri* in human HaCaT keratinocytes. *Radiation and environmental biophysics*. 2007; 46:263–268. [PubMed: 17487501]
6. Agar NS, Halliday GM, Barnetson RS, Ananthaswamy HN, Wheeler M, Jones AM. The basal layer in human squamous tumors harbors more UVA than UVB fingerprint mutations: a role for UVA in human skin carcinogenesis. *Proc Natl Acad Sci U S A*. 2004; 101:4954–4959. [PubMed: 15041750]
7. Bachelor MA, Bowden GT. UVA-mediated activation of signaling pathways involved in skin tumor promotion and progression. *Semin Cancer Biol*. 2004; 14:131–138. [PubMed: 15018897]
8. Steenvoorden DP, van Henegouwen GM. The use of endogenous antioxidants to improve photoprotection. *J Photochem Photobiol B*. 1997; 41:1–10. [PubMed: 9440308]
9. Bickers DR, Athar M. Oxidative stress in the pathogenesis of skin disease. *The Journal of investigative dermatology*. 2006; 126:2565–2575. [PubMed: 17108903]
10. Black HS. ROS: a step closer to elucidating their role in the etiology of light-induced skin disorders. *The Journal of investigative dermatology*. 2004; 122:xiii–xiv. [PubMed: 15175053]
11. Briganti S, Picardo M. Antioxidant activity, lipid peroxidation and skin diseases. What's new. *J Eur Acad Dermatol Venereol*. 2003; 17:663–669. [PubMed: 14761133]
12. de Laat A, van der Leun JC, de Gruijl FR. Carcinogenesis induced by UVA (365-nm) radiation: the dose-time dependence of tumor formation in hairless mice. *Carcinogenesis*. 1997; 18:1013–1020. [PubMed: 9163689]
13. Nishigori C. Cellular aspects of photocarcinogenesis. *Photochem Photobiol Sci*. 2006; 5:208–214. [PubMed: 16465307]
14. Sterenborg HJ, van der Leun JC. Tumorigenesis by a long wavelength UV-A source. *Photochemistry and photobiology*. 1990; 51:325–330. [PubMed: 2356228]
15. He YY, Pi J, Huang JL, Diwan BA, Waalkes MP, Chignell CF. Chronic UVA irradiation of human HaCaT keratinocytes induces malignant transformation associated with acquired apoptotic resistance. *Oncogene*. 2006; 25:3680–3688. [PubMed: 16682958]
16. Valencia A I, Kochevar E. Ultraviolet A induces apoptosis via reactive oxygen species in a model for Smith-Lemli-Opitz syndrome. *Free Radic Biol Med*. 2006; 40:641–650. [PubMed: 16458195]
17. de Gruijl FR. Photocarcinogenesis: UVA vs UVB. *Methods Enzymol*. 2000; 319:359–366. [PubMed: 10907526]
18. Reagan-Shaw S, Breur J, Ahmad N. Enhancement of UVB radiation-mediated apoptosis by sanguinarine in HaCaT human immortalized keratinocytes. *Mol Cancer Ther*. 2006; 5:418–429. [PubMed: 16505117]
19. Gomer CJ, Ferrario A, Rucker N, Wong S, Lee AS. Glucose regulated protein induction and cellular resistance to oxidative stress mediated by porphyrin photosensitization. *Cancer Res*. 1991; 51:6574–6579. [PubMed: 1835901]
20. Lee AS. The glucose-regulated proteins: stress induction and clinical applications. *Trends Biochem Sci*. 2001; 26:504–510. [PubMed: 11504627]
21. Mera K, Kawahara K, Tada K, Kawai K, Hashiguchi T, Maruyama I, Kanekura T. ER signaling is activated to protect human HaCaT keratinocytes from ER stress induced by environmental doses of UVB. *Biochem Biophys Res Commun*. 2010; 397:350–354. [PubMed: 20513357]
22. Anand S, Chakrabarti E, Kawamura H, Taylor CR, Maytin EV. Ultraviolet light (UVB and UVA) induces the damage-responsive transcription factor CHOP/gadd153 in murine and human epidermis: evidence for a mechanism specific to intact skin. *The Journal of investigative dermatology*. 2005; 125:323–333. [PubMed: 16098044]
23. Dhanalakshmi S, Mallikarjuna GU, Singh RP, Agarwal R. Dual efficacy of silibinin in protecting or enhancing ultraviolet B radiation-caused apoptosis in HaCaT human immortalized keratinocytes. *Carcinogenesis*. 2004; 25:99–106. [PubMed: 14555614]
24. He YY, Huang JL, Block ML, Hong JS, Chignell CF. Role of phagocyte oxidase in UVA-induced oxidative stress and apoptosis in keratinocytes. *The Journal of investigative dermatology*. 2005; 125:560–566. [PubMed: 16117799]
25. Chung JH, Han JH, Hwang EJ, Seo JY, Cho KH, Kim KH, Youn JI, Eun HC. Dual mechanisms of green tea extract (EGCG)-induced cell survival in human epidermal keratinocytes. *The FASEB*

journal: official publication of the Federation of American Societies for Experimental Biology. 2003; 17:1913–1915.

26. Dou M, Han Y, Han Z, Chen X, Wang Y, Tan J, Wang C, Hou Y. Inhibitory effect of polypeptide from *Chlamys farreri* on UVA-induced apoptosis in human keratinocytes. *Investigational new drugs*. 2004; 22:391–398. [PubMed: 15292709]
27. Tarozzi A, Marchesi A, Hrelia S, Angeloni C, Andrisano V, Fiori J, Cantelli-Forti G, Hrelia P. Protective effects of cyanidin-3-O-beta-glucopyranoside against UVA-induced oxidative stress in human keratinocytes. *Photochemistry and photobiology*. 2005; 81:623–629. [PubMed: 15701043]
28. Schneider LA, Dissemmond J, Brenneisen P, Hainzl A, Briviba K, Wlaschek M, Scharffetter-Kochanek K. Adaptive cellular protection against UVA-1-induced lipid peroxidation in human dermal fibroblasts shows donor-to-donor variability and is glutathione dependent. *Archives of dermatological research*. 2006; 297:324–328. [PubMed: 16328346]
29. Svobodova A, Zdarilova A, Maliskova J, Mikulkova H, Walterova D, Vostalova J. Attenuation of UVA-induced damage to human keratinocytes by silymarin. *Journal of dermatological science*. 2007; 46:21–30. [PubMed: 17289350]
30. Emonet-Piccardi N, Richard MJ, Ravanat JL, Signorini N, Cadet J, Beani JC. Protective effects of antioxidants against UVA-induced DNA damage in human skin fibroblasts in culture. *Free radical research*. 1998; 29:307–313. [PubMed: 9860045]
31. Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxidants & redox signaling*. 2007; 9:2277–2293. [PubMed: 17979528]
32. Matsumoto M, Minami M, Takeda K, Sakao Y, Akira S. Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. *FEBS letters*. 1996; 395:143–147. [PubMed: 8898082]
33. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Molecular and cellular biology*. 2001; 21:1249–1259. [PubMed: 11158311]

Figure 1. UVA irradiation decreases cell viability by inducing apoptosis in HaCaT cells

(A) HaCaT cells were sham irradiated or irradiated with different doses of UVA, both floating and adherent cells were collected at different time points as indicated and subjected to Trypan blue dye exclusion assay as detailed in 'Materials and Methods'. '#' indicates $P < 0.05$, '*' indicates $P < 0.001$ and 'NS' indicates not statistically significant. B) HaCaT cells were sham irradiated or irradiated with different doses of UVA, lysates were prepared after 24h of UVA-exposure and Western blotting was done for cleaved caspase-3 and cleaved PARP. (C) HaCaT cells were sham irradiated or irradiated with 5J/cm² UVA, harvested after 6, 12, 18 or 24h of irradiation, lysates were prepared and western blotting was done for cleaved caspase-3 and cleaved PARP. Expression of β-Actin is shown as loading control. (D) HaCaT cells were sham irradiated or irradiated with increasing doses of UVA as indicated, harvested after 16h of incubation, stained with AnnexinV/PI and subjected to flow cytometry. Percentage of apoptotic cells in each treatment is shown, '*' represents $P < 0.001$ with respect to untreated control.

Figure 2. Silibinin pretreatment enhances UVA-induced apoptosis in HaCaT cells

(A) HaCaT cells were pretreated with either DMSO or 75μM silibinin for 2h and sham/ UVA irradiated. After 24h of irradiation, both floating and adherent cells were collected at different time points as indicated and subjected to Trypan blue dye exclusion assay as mentioned in 'Materials and Methods'. (B) HaCaT cells were pretreated with 75μM silibinin for 2h and sham/UVA irradiated. After 6h, 12h, 18h or 24h of irradiation, both floating and adherent cells were collected, lysates were prepared and western blotting was done for cleaved caspase-3 and cleaved PARP. Expression of β-Actin is shown as loading control.

Figure 3. Silibinin enhances UVA-induced ROS in HaCaT cells

(A) HaCaT cells cultured in 24 well plates were pretreated with DMSO or 75μM silibinin and/or 10mM NAC for 2h and sham/UVA irradiated as indicated. After 30 min, 1h or 2h of UVA exposure, the ROS generation was measured using DCFH-DA as described in 'Materials and Methods'. Data represents mean $\pm$ SE from experiments done in triplicates. '#' indicates no statistically significant difference, '*' indicates $P < 0.001$ and '\$' indicates $P < 0.05$. (B) NAC and/or silibinin pretreated HaCaT cells were sham irradiated or exposed to 5J/cm² UVA, both floating and adherent cells were collected after 24h and percentage cell viability was determined by Trypan blue exclusion assay. (C) NAC and/or silibinin pretreated HaCaT cells were sham irradiated or exposed to 5J/cm² UVA, cell lysates were prepared after 24h and western blotting was carried out for cleaved caspase-3 and cleaved PARP. β-Actin expression is shown as loading control. (D) NAC and/or silibinin pretreated HaCaT cells were sham irradiated or exposed to 5J/cm² UVA as indicated, harvested after 16h of incubation, stained with AnnexinV/PI and subjected to flow cytometry. Percentage of apoptotic cells in each treatment is shown.

Figure 4. Silibinin pretreatment enhances UVB-induced ER-stress mediated apoptosis
(A) NAC and/or silibinin pretreated cells were irradiated with 5J/cm² UVA or sham-irradiated, cell lysates were prepared after 24h and western blotting was done for GRP78 and CHOP. (B) 10mM NAC and/or 75μM silibinin pretreated cells were sham irradiated or exposed to 5J/cm² UVA, incubated with DMSO or 100nM Piericidin A for 24h, cell lysates were prepared after 24h and western blotting was done for GRP78, CHOP, cleaved caspase-3 and cleaved PARP. β-Actin expression is shown as loading control.

Figure 5. Schematic illustration of UVA-induced apoptotic pathway and its modulation by silibinin in HaCaT cells.