Volume 46, issue 1, April 2007 ISSN 0923-1811

The official journal of
The Japanese Society for
Investigative Dermatology

Available online at
ScienceDirect
www.sciencedirect.com

Journal of Dermatological Science (2007) **46**, 21—30

ELSEVIER

Journal of Dermatological SCIENCE

www.intl.elsevierhealth.com/journals/jods

Attenuation of UVA-induced damage to human keratinocytes by silymarin

Alena Svobodová*, **Adéla Zdařilová, Jana Mališková, Hana Mikulková, Daniela Walterová, Jitka Vostalová**

Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, 775 15 Olomouc, Czech Republic

Received 9 August 2006; received in revised form 19 December 2006; accepted 25 December 2006

KEYWORDS
UVA radiation;
Human keratinocytes;
Silymarin;
Oxidative stress;
Free radicals;
DNA damage;
Apoptosis;
Protection

Summary

Background: UV radiation from sunlight is a potent environmental risk factor in skin cancer pathogenesis. UVA is the major portion of UV light reaching the earth surface (~95%) and it is reported to lead to benign and malignant tumor formation. UVA-mediated cellular damage occurs primarily through the release of reactive oxygen species (ROS) and it is responsible for inflammation, immunosuppression, photoaging and photocarcinogenesis.
Objective: The aim of our study was to investigate the potency of silymarin, the polyphenol fraction from the seeds of *Silybum marianum*, to modulate UVA-induced oxidative damage to human keratinocytes.
Methods: Skin epidermal cell line HaCaT, extensively used for studying the influence of UV radiation, was chosen as an experimental model. Silymarin's effect on UVA-disrupted cell viability, proliferation, mitochondrial function, and intracellular ATP and GSH level was measured. Furthermore, silymarin's potency to reduce UVA-induced ROS generation, membrane lipid peroxidation, caspase-3 activation and DNA damage was monitored.
Results: Treatment of irradiated HaCaT (20 J/cm^2) with silymarin (0.7—34 mg/l; 4 h) resulted in concentration-dependent diminution of UVA-caused oxidative stress on all studied parameters. Silymarin application extensively reduced GSH depletion and ROS production as well as lipid peroxidation in irradiated cells. Formation of UVA-induced DNA single strand breaks and caspase-3 activity was also significantly decreased by silymarin.
Conclusion: The results suggest that silymarin may be beneficial in the treatment of UVA-induced skin oxidative injury and inflammation. However, further studies

* Corresponding author. Tel.: +420 585632324; fax: +420 585632302.
E-mail address: alf.svoboda@seznam.cz (A. Svobodová).

doi:10.1016/j.jdermsci.2006.12.009

especially whose using human systems are needed to determine efficacy of silymarin *in vivo*.

1. Introduction

UV radiation forms a part of the electromagnetic spectrum with wavelengths between 100 and 400 nm. It is divided into three categories depending on wavelength, long wave UVA (315—400 nm), medium wave UVB (280—315 nm), and short wave UVC (100—280 nm) [1]. The major source of human exposure to UV radiation is the sunlight. Although solar radiation contains all three forms of UV light, wavelengths below 295 nm (all UVC and partially UVB) are completely used up in the stratosphere to form ozone from molecular oxygen. The UV light that reaches the earth's surface comprises primarily UVA wavelengths and the remainder (approximately 5%) contains the (295—320 nm) UVB radiation [2,3]. Both UVA and UVB cause wavelength-dependent damage to human skin including skin cancer, whose incidence is dramatically increasing [4]. UVB, the minor constituent of solar UV light, acts mainly in the epidermal basal cell layer of the skin. It directly interacts with DNA bases and causes DNA lesions mostly by formation of dimeric photoproducts between adjacent pyrimidine bases on the same strand, particularly cyclobutane-pyrimidine dimers and pyrimidine—pyrimidone (6—4) photoproducts. Defective repair of these lesions leads to mutations in the epidermal cells that can cause the development of cancer cells [5]. UVA penetrates through epidermis deep into the dermis. UVA-induced responses in cells happen mainly because of oxidative processes initiated by endogenous photosensitization. After UVA exposure, singlet oxygen, H_2O_2, superoxide and hydroxyl free radicals are generated. These interact and can cause damage to cellular proteins, lipids and saccharides. UVA can also indirectly produce structural damage to the DNA (8-oxoguanine is the most common lesion) and inhibit DNA repair as well as it affects numerous signal transduction pathways and impairs the immune system [6].

Apropos the skin photoprotection, to date most of studies have involved UVB protection. Little research has involved prevention against UVA skin damage. As UVA-caused injury happens mainly through ROS overproduction exogenous supplementation of antioxidants may be an effective strategy for counteracting the deleterious effects of the ROS generated from extensive sun exposure. In the past decade, the protective activity of various herbal phenolics, namely phenolic acids and flavonoids, have been investigated. Few plant phenolic compounds and extracts have demonstrated the ability to suppress UVA-induced skin cells/skin tissue injury, e.g. carnosic acid [7] and epicatechin [8] in human skin fibroblasts, epigallocatechin-3-gallate in HaCaT keratinocytes [9,10], *Polypodium leucotomos* extract in human keratinocytes and fibroblasts [11] and quercetin in rats [12].

Silymarin (SM) is the standardized polyphenol fraction from the seeds of the milk thistle (*Silybum marianum* (L.) Gaertn., family of Asteraceae) [13]. It contains approximately 70—80% flavonolignans and 20—30% non-identified oxidized polyphenolic compounds. The mixture of flavonolignans consists mainly of silybin (silibinin, SB; Fig. 1), the major bioactive component of the extract, and isomeric isosilybin, silychristin and silydianin. Milk thistle was widely used in traditional European medicine for 2000 years especially for the treatment of liver disorders. SM is primarily known for its hepatoprotective and antioxidant activity [14]. Several studies have shown that SM and SB have chemopreventive effects against oxidative stress induced by the free radical-generating skin tumor promoting agent 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [15—17] and benzoyl peroxide (BPO) [18], as well as UVB-induced photocarcinogenesis in various animal tumor models [19—22] and it may suppress UVB damage to human keratinocytes as well [23]. However, the effects of SM in the treatment of UVA-caused damage have not been reported yet. For this reason in the present work, we investigated the potential ability of SM to ameliorate UVA-induced oxidative stress in human keratinocytes.

2. Materials and methods

2.1. Materials

Human keratinocytes (spontaneously immortalized cell line HaCaT) were a gift from the Institute of

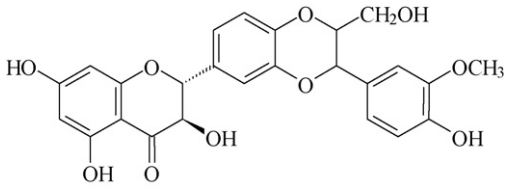

Fig. 1 Structure of silybin.

Biophysics, Academy of Science of the Czech Republic, Brno. Cell proliferation ELISA BrdU (colorimetric) kit, protease inhibitor cocktail tablet (complete™) were purchased from Roche, Germany. Western blotting luminol reagent, actin (I-19) goat polyclonal antibody, horseradish peroxidase conjugated goat anti-rabbit and rabbit anti-goat antibodies were received from Santa Cruz Biotechnology, USA. Immun-Blot™ PVDF (polyvinylidenefluoride) membrane was from Bio-Rad Laboratories, Hercules, Canada and XAR-5 film from Eastman Kodak, USA. Caspase-3 fluorescent substrate Ac-DEVD-AMC and inhibitor Ac-DEVD-CHO were purchased from Bachem AG, Switzerland. 2,2′-Dinitro-5,5′-dithiobenzoic acid was purchased from Serva, Germany. Rabbit anti-caspase-3, Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal calf serum (FCS) and all other chemicals were purchased from Sigma—Aldrich, USA.

2.2. Silymarin

SM (batch 17306S/089) containing 71% of flavonolignans was kindly provided by IVAX Pharmaceuticals (Opava, Czech Republic). Stock solutions of SM (0.14—6.83 g/l) were dissolved in DMSO.

2.3. Cell culture

HaCaT were grown in DMEM supplemented with heat-inactivated FCS (7%, v/v), streptomycin (100 U/ml), penicillin (0.1 mg/ml) and glutamine (4 mmol/l) in a humidified atmosphere with 5% (v/v) CO_2 at 37 °C. Culture medium was changed twice a week. The cells were subcultured following trypsinization. For all experiments HaCaT were seeded in plates at a density 1×10^5 cells/cm^2 and grown near to confluence.

2.4. Cytotoxicity of silymarin

Culture medium was removed and cells were treated with serum-free medium containing SM (0.7—34 mg/l) at 37 °C for 4 h. Control cells were treated with serum-free medium containing DMSO (0.5%, v/v) at the same conditions. Cell viability was evaluated using: neutral red (NR) retention, activity of cellular dehydrogenases (MTT assay), activity of lactate dehydrogenase (LDH) released into the medium, and level of intracellular ATP and GSH, lipid peroxidation (LPx) and ROS generation.

2.5. UVA irradiation

Prior to UV irradiation, cells were washed with phosphate buffered saline (PBS) and covered with a thin layer of PBS. In parallel, non-irradiated cells were treated similarly and kept in the dark in an incubator. For irradiation, a SOL-500 solar simulator equipped with a UVB-absorbing H1 filter transmitting wavelengths 315—380 nm (Dr. Hönle, UV Technology, Germany) was used. The UVA output, measured by a UVA-meter (Dr. Hönle, UV Technology, Germany) in direct contact with the cell culture dish was 6.2 mW/cm^2. For all experiments keratinocytes were irradiated on ice-cold plates with the UVA dose of 20 J/cm^2; only in the case of TBARS assay the dose of 30 J/cm^2 was used.

2.6. Effect of silymarin on UVA damage

After UVA irradiation PBS was removed, serum-free medium with SM (0.7—34 mg/l) was applied and the cells were incubated at 37 °C for 4 h. Irradiated and non-irradiated control cells were treated with serum-free medium containing DMSO (0.5%, v/v) at the same conditions. Cell viability (NR retention and activity of cellular dehydrogenases), activity released LDH, cell proliferation, level of intracellular ATP and GSH, lipid peroxidation (LPx), ROS generation, DNA breakage and caspase-3 activation were parameters used for evaluation of SM effect on UVA-induced injury.

2.7. Neutral red assay

NR uptake reveals the ability of living cells to incorporate water-soluble dye (NR) into lysosomes in the process requiring energy. NR (0.03%, w/v) in PBS was added to the cells. The cells were incubated for an additional 2 h at 37 °C, washed with a mixture of formaldehyde (0.125%, v/v) and $CaCl_2$ (0.25%, w/v) and the retained NR was dissolved using acetic acid (1%, v/v) in methanol (50%, v/v). The absorbance was read at 540 nm with a microplate reader (Sunrise, Schoeller Instruments, Austria) [24].

2.8. MTT assay

Tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), is reduced by intracellular dehydrogenases of viable living cells that leads to formation of purple formazan crystals, insoluble in aqueous solutions. After dissolution in organic solvent the absorbance is monitored. Serum-free medium supplemented with MTT (5 mg/ml) was applied to the cells for 2 h (37 °C). The medium was removed and the crystals were dissolved in DMSO with NH_3 (1%, v/v). The absorbance was measured at 540 nm [24].

2.9. LDH release assay

LDH leakage into the culture medium is commonly used for evaluation of a loss of cell membrane integrity. LDH activity was spectrophotometrically measured by the disappearance of NADH during LDH-catalysed conversion of pyruvate to lactate as a decrease in absorbance at 340 nm [24].

2.10. BrdU incorporation

Cell proliferation was evaluated using colorimetric immunoassay, based on measurement of 5-bromo-2′-deoxyuridine (BrdU), the thymidine analogue, incorporated in place of thymidine into the DNA during DNA synthesis. HaCaT proliferation was monitored using a Cell proliferation (colorimetric) kit according to the manufacturer's protocol.

2.11. Intracellular ATP level

The cells were rinsed with cooled PBS and scraped into cooled perchloric acid (6%, v/v). The suspension was neutralised with cooled KOH (3 mol/l) and centrifuged (10 min; 13 000 rpm; 4 °C). The supernatant was used for quantification of ATP by ion-pair chromatography [25].

2.12. GSH level

The cells rinsed with cooled PBS were scraped into cooled perchloric acid (1%, v/v), and sonicated. The aliquots were frozen for protein determination by Lowry assay [26]. The suspension was centrifuged (10 min; 13 000 rpm; 4 °C) and the supernatant was used for estimation of GSH in reaction with 2,2′-dinitro-5,5′dithiobenzoic acid [27]. The absorbance was read on a microplate reader at 412 nm.

2.13. Lipid peroxidation

The cells were washed with cooled PBS, scraped into trichloroacetic acid (2.8%, w/v), sonicated and aliquots were used for protein determination by Lowry assay [26]. The suspension was mixed with thiobarbituric acid (1%, w/v) in a ratio 2:1, heated (30 min; 95 °C) and centrifuged (10 min; 13 000 rpm; 4 °C). The amount of thiobarbituric acid reactive substances (TBARS) was determined spectrophotometrically at 535 nm [28].

2.14. Determination of ROS generation

To measure the rate of UVA-induced ROS production and the radical scavenging effect of SM in living cells, dichlorodihydrofluorescein assay was performed. The polar, non-fluorescent dichlorodihydrofluorescein diacetate (H_2DCFDA) undergoes deacetylation by cytosolic esterases to form dichlorodihydrofluorescein, which reacts with ROS and gives rise to the fluorescent derivative dichlorofluorescein. The fluorescence is monitored at specific excitation/emission wavelengths 488/525 nm.

After irradiation and treatment with DMSO/SM (1 h) the cells were incubated with H_2DCFDA (15 μl; 0.5 mmol/l) 15 min in the incubator. Then cells were washed with PBS and scraped into 2 ml of PBS and sonicated [29]. The protein concentration was determined by Bradford assay [30]. The fluorescence was measured using a spectrophotometer (LS 50 B, Perkin-Elmer, USA).

2.15. Comet assay

DNA single strand breaks were monitored using the single cell gel electrophoresis (Comet assay). Standard melting-point agarose (1%, w/v) was applied on an agarose-coated slide, covered with a cover slip and placed on ice for 15 min. The cells were trypsinised, pelleted, resuspended in PBS and mixed with low-melting-point agarose (1%, w/v), then transferred to the layer of agarose, covered with a cover slip and the slides placed on ice. The cover slips were then removed and the samples were incubated in cold lysis buffer (2.5 mol/l NaCl, 100 mmol/l EDTA, 10 mmol/l Tris and 1% (v/v) Triton X; 1 h; 4 °C). Electrophoresis was done (16 V; 20 min; 4 °C) in electrophoresis buffer (300 mmol/l NaOH and 1 mmol/l EDTA). The slides were washed three times with cold neutralising buffer (0.4 mol/l Tris), dried, stained with ethidium bromide and the nuclei were evaluated using inversion fluorescent microscope [31]. DNA damage was assessed in 100 nuclei per slide area by visual scoring from 0 (undamaged, no discernible tail) to 4 (almost all DNA in tail, insignificant head). Each comet was given a value according to its classification to produce an overall score for each slide ranging from 0 to 400 arbitrary units. Scores were calculated using the following formula in which N_0, N_1, N_2, N_3, and N_4 represent the number of cells in each group from 0 to 4:

$$\text{total damage } (\%) = \frac{N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4}{N_0 + N_1 + N_2 + N_3 + N_4} \times 100$$

2.16. Caspase-3 activity (Ac-DEVD-AMC cleavage assay)

Caspase-3 activity was measured using fluorogenic substrate Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) [32]. After experi-

ment the cells were washed with PBS and lysed in ice-cold lysis buffer (50 mmol/l HEPES, pH 7.4, Triton X-100 (0.5%, v/v), protease inhibitor cocktail tablet, 5 mmol/l dithiothreitol (DDT); 15 min; 4 °C). The lysate was cleared by centrifugation (14 000 rpm; 15 min; 4 °C). The protein concentration in supernatant was determined by Bradford assay [30].

The cell lysate was mixed with assay buffer (20 mmol/l HEPES, pH 7.1, 2 mmol/l EDTA, protease inhibitor cocktail tablet, 5 mmol/l DDT) containing Ac-DEVD-AMC or Ac-DEVD-AMC + inhibitor Acetyl-Tyr-Val-Ala-Asp aldehyde (Ac-DEVD-CHO) and incubated (37 °C; 60 min). Fluorescence was measured at 380/450 nm using a spectrophotometer (LS 50 B, Perkin-Elmer, USA).

2.17. Caspase-3 activation (Western immunoblot)

The cells were washed with PBS and ice-cold lysis buffer (20 mmol/l Tris, 5 mmol/l EGTA, 150 mmol/l NaCl, 20 mmol/l glycerol phosphate, 1 mmol/l NaF, Triton X-100 (1%, v/v), 1 mmol/l Na_3VO_4, Tween 20 (0.1%, v/v), protease inhibitor cocktail tablet) was added. After incubation (15 min; 4 °C) the cells were scraped and the lysate was cleared by centrifugation (14 000 rpm; 15 min; 4 °C). The supernatant protein concentration was determined by Lowry assay [26].

Proteins were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Residual binding sites on the membrane were blocked using blocking buffer (5% non-fat dry milk (w/v) in 100 mmol/l Tris—buffered saline (pH 7.5) with Tween 20 (0.05%, v/v)) for 1 h at room temperature. The membrane was then incubated with a primary antibody (rabbit anti-caspase-3 primary antibody/actin (I-19) goat polyclonal antibody; in blocking buffer) overnight at 4 °C and then with a secondary horseradish peroxidase conjugated antibody (goat anti-rabbit antibody/rabbit anti-goat antibody; in blocking buffer) for 2 h at room temperature. Caspase-3/actin expression was detected by chemiluminescence using Western Blotting Luminol Reagent and autoradiography with XAR-5 film [33].

2.18. Statistical analysis

Data are expressed as means ± S.D. Three separate experiments were performed with three replicates for each sample. Statistical comparison analysis was performed using Student's *t*-test.

Cytoxicity of silymarin is expressed as % of control:

$$\text{cytotoxicity } (\%) = \frac{|A_S|}{|A_C|} \times 100$$

A_S is the absorbance of a sample (cells treated with SM) and A_C is the absorbance of a control (cells treated with DMSO).

3. Results

3.1. Cytotoxicity of silymarin

Possible harmful effects of SM were monitored in a concentration range of 0.7—34 mg/l after 4 h treatment. SM itself caused no changes in NR retention, cellular dehydrogenases and extracellular LDH activity as well as no alteration to intracellular ATP level at the concentrations used. SM treatment also did not increase ROS generation and did not induce peroxidation of membrane

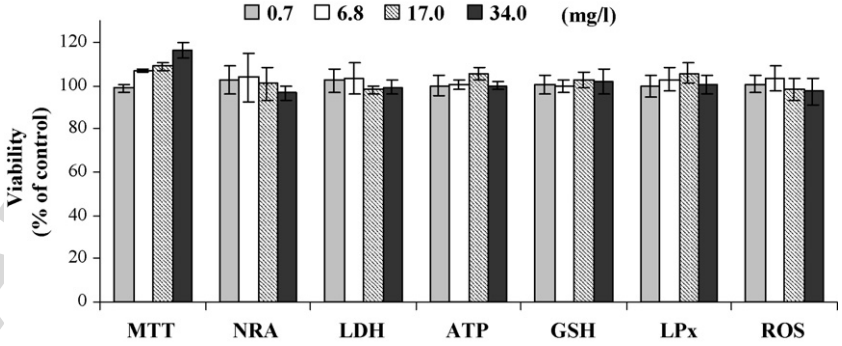

Fig. 2 Effect of SM treatment on HaCaT. Keratinocytes were treated with SM (0.7—34 mg/l) for 4 h; control cells were incubated with DMSO (0.5%, v/v) at the same conditions. Cell viability/damage was evaluated using the following parameters: cellular dehydrogenases activity (MTT); NR retention (NRA); activity of released LDH (LDH); intracellular ATP level (ATP); intracellular GSH content (GSH); peroxidation of membrane lipids (LPx); ROS generation (ROS). Data are expressed as % of control and plotted as mean ± S.D. of three independent experiments performed in triplicate.

lipids, as well as depletion of intracellular GSH content (Fig. 2).

3.2. Modulation of cell viability, membrane integrity and proliferation in UVA irradiated cells by silymarin

To evaluate the potential UVA-protective action of SM on cell viability, keratinocytes were irradiated (20 J/cm^2) and treated with SM (0.7—34 mg/l) for 4 h. As shown in Fig. 3A and B, SM provided significant protection observed as an increase in activity of cellular dehydrogenases and NR retention compared to UVA-irradiated un-treated cells. The preventive effect was concentration-dependent and maximal protection was 50% (MTT assay) or 25% (NR retention).

SM application to UVA treated cells (20 J/cm^2) reduced disruption of keratinocyte membrane integrity, which was monitored as the decrease in activity of released LDH. Protection was most potent at the highest concentration tested (34 mg/l; 50%).

SM slightly enhanced cell proliferation in UVA-exposed cells (20 J/cm^2). The effect of SM on BrdU incorporation (Fig. 3D) proved to be non-significant (10%) in comparison to the other parameters. However, the influence of UVA light on BrdU incorporation was the most extensive of all markers.

3.3. Modulation of UVA-induced impairment of cell energetic status

It is known that the amount of intracellular ATP is an important indicator of mitochondrial function and effectiveness of cellular oxidative phosphorylation. In our experimental conditions UVA exposure (20 J/cm^2; 4 h) induced strong ATP depletion (approximately 60% reduction in comparison to non-irradiated keratinocytes). Post-treatment with SM resulted in protection against the ATP decrease (Fig. 4A). The extract was most efficient at concentration of 17 mg/l (55% protection).

3.4. Modulation of UVA-induced oxidative stress by silymarin

UVA induced damage happens mainly through generation of ROS. As shown in Fig. 4B, treatment with SM led to concentration-dependent reduction of dichlorofluorescein fluorescence in irradiated keratinocytes (20 J/cm^2) corresponding to diminution of ROS level. SM concentrations of 17—34 mg/l were found very potent.

SM application to UVA-irradiated cells (20 J/cm^2) led also to dose-dependent alleviation of depletion of GSH, the most important cellular non-enzymatic antioxidant (Fig. 4C). The effect was most powerful at concentration of 34 mg/l (45% protection).

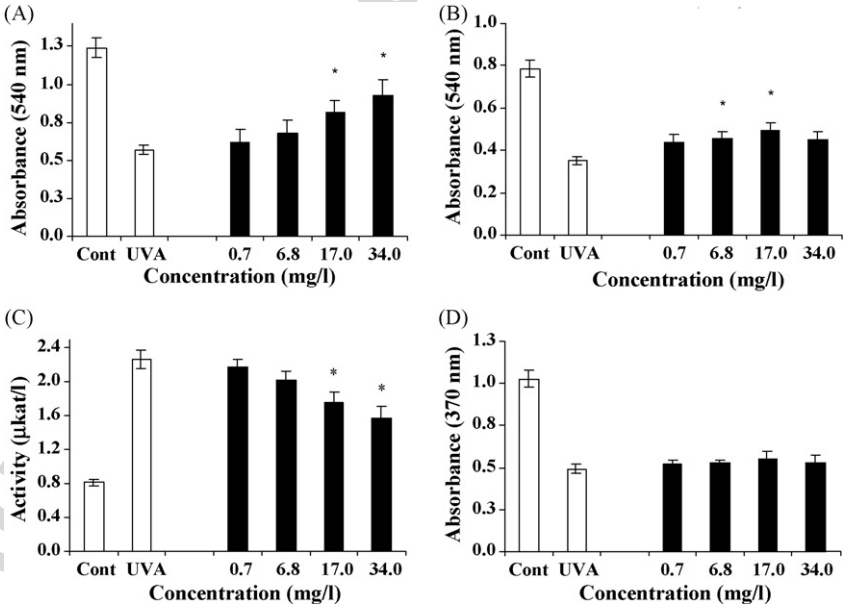

Fig. 3 Modulation of UVA-disturbed cell viability and proliferation. Keratinocytes were irradiated (20 J/cm^2) and treated with SM (0.7—34 mg/l) for 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%, v/v) at the same conditions. Effect of SM on UVA-induced alteration to (A) cellular dehydrogenases activity, (B) NR retention, (C) released LDH activity and (D) BrdU incorporation was evaluated. Data are expressed as mean ± S.D. of three independent experiments performed in triplicate. Statistical comparison analysis was performed using Student's *t*-test. $^*p < 0.05$ statistically different from irradiated cells.

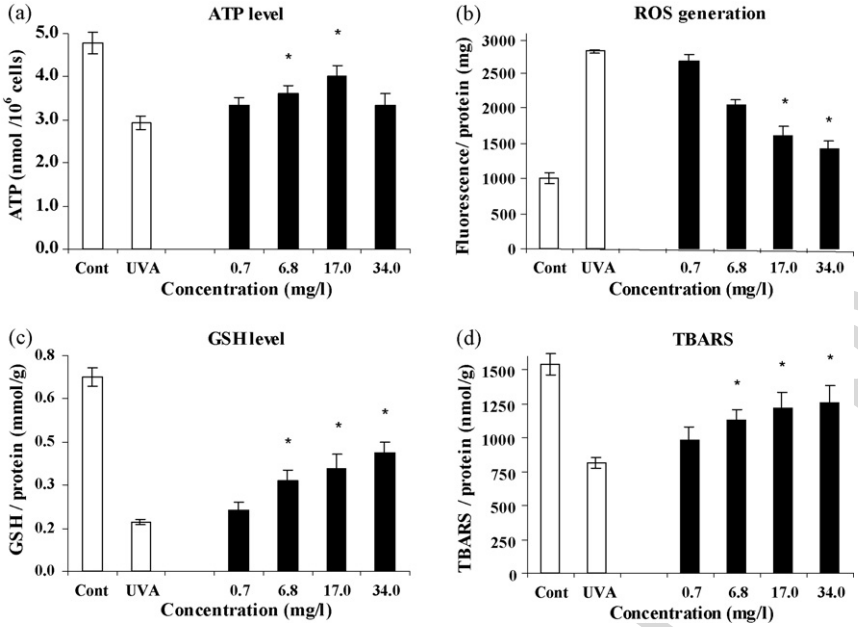

Fig. 4 Modulation of UVA-induced oxidative stress. Keratinocytes were irradiated (20 J/cm^2; in the case of lipid peroxidation 30 J/cm^2) and treated with SM (0.7—34 mg/l) for 1 or 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%, v/v) at the same conditions. Effect of SM on UVA-induced alteration to (A) intracellular ATP level (4 h), (B) ROS production (1 h), (C) intracellular GSH level (4 h) and (D) lipid peroxidation (4 h) were evaluated. Data are expressed as mean ± S.D. of three independent experiments performed in triplicate. Statistical comparison analysis was performed using Student's *t*-test. $^*p < 0.05$ statistically different from irradiated cells.

Increased ROS production and disruption of GSH functions in cells is also tightly bound up with susceptibility to UVA-caused (30 J/cm^2) membrane lipids peroxidation. As demonstrated in Fig. 4D, SM provided a dose-dependent reduction of LPx in irradiated HaCaT. An obvious decrease in TBARS (40—60%) was found in the concentration range of 6.8—34 mg/l.

3.5. Modulation of UVA-induced DNA damage and apoptosis by silymarin

DNA single strand breaks are one of the most frequent DNA lesions generated by oxidative stress. SM at the concentration range used did not itself induce the formation of single-strand breaks after 4 h incubation (data not shown). As shown in Table 1, UVA exposure (20 J/cm^2) led to extensive enhancement of DNA oxidative injury in HaCaT. In UVA-exposed cells treated with SM, the powerful ability of the extract to attenuate the appearance of the comet was evident (Table 1). The SM protection at 6.8 mg/l was more efficient than at higher concentration of 17 mg/l and was nearly 97%.

Furthermore, damage to cellular DNA was connected with activation of repairs mechanism or apoptosis. Activation of caspase-3 is a well-known apoptotic marker. SM itself did not initiate activation

Table 1 Modulation of UVA-induced DNA single-strand breaks by SM

Sample	Number of nuclei in class					Total damage (arbitrary units)
	Class number 0	Class number 1	Class number 2	Class number 3	Class number 4	
Non-irradiated cells	98	2	0	0	0	2.0
Irradiated cells	43	5	11	19	22	172.0
SM (6.8 mg/l)	90	8	2	0	0	12.0[a]
SM (17 mg/l)	72	16	12	7	3	73.0[a]

Keratinocytes were irradiated (20 J/cm^2) and treated with SM (6.8 and 34 mg/l) for 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%, v/v) at the same conditions. DNA damage was assessed by the Comet assay. The number of damaged nuclei in individual nuclei class was determined in 100 cells (nuclei) per slide. Total damage is expressed in arbitrary units, which range from 0 (undamaged cells) to 400 (totally damaged cells). The data are representatives of three independent experiments. Statistical comparison analysis was performed using Student's *t*-test.

[a] $p < 0.001$ statistically different from irradiated cells.

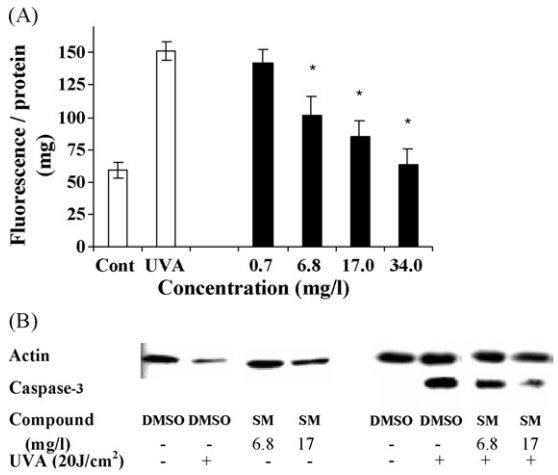

Fig. 5 Effect of SM on basal and UVA-induced caspase-3 activation in HaCaT. (A) Keratinocytes were irradiated (20 J/cm^2) and treated with SM (0.7—34 mg/l) for 4 h. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%, v/v) at the same conditions. Caspase-3 activity was evaluated by fluorimetric assay. Data are expressed as mean ± S.D. of three independent experiments performed in triplicate. Statistical comparison analysis was performed using Student's *t*-test. $^*p < 0.05$ statistically different from irradiated cells. (B) Keratinocytes were treated with SM (6.8 and 17 mg/l; 4 h), or were irradiated (20 J/cm^2) and treated with SM (6.8 and 17 mg/l; 4 h). Irradiated and non-irradiated control cells were incubated with DMSO (0.5%, v/v) at the same conditions. Basal and UVA-induced caspase-3 activation was evaluated by Western blot analysis. Data are representatives of three independent experiments.

of caspase-3 at used concentrations (Fig. 5B). SM post-treatment of UVA-irradiated (20 J/cm^2) HaCaT reduced caspase-3 activation, evaluated by fluorescent assay (Fig. 5A) and Western blot (Fig. 5B). In Ac-DEVD-AMC cleavage assay, SM protection was 50—90% and was the most powerful at the highest concentration.

4. Discussion

The ultraviolet component of solar radiation, particularly in the wavelength range of 320—400 nm, has been shown to induce ROS generation. If ROS are not challenged by the antioxidant defence systems of living organisms it results in oxidative stress in the skin. Therefore, prevention with antioxidant agents is often regarded as a possible strategy for the management of such oxidative damage. In last 20 years, naturally occurred compounds have gained considerable attention as potential (photo)protectives [1].

Recently we demonstrated that pre-treatment with SM and its main flavonolignan component silybin protected oxidative damage to HaCaT induced by hydrogen peroxide that is extensively generated during UVA exposure [34]. In this report we deal with influence of SM treatment to UVA-irradiated keratinocytes. It was published [9,35,36] and we also proved it in our experimental conditions that UVA irradiation causes depletion of intracellular GSH, extensive ROS production, peroxidation of biomembrane lipids, depletion of ATP, formation of single-strand DNA breaks, disruption of DNA synthesis, and induction of apoptotic machinery in HaCaT. We have shown that SM reduces damage to UVA-irradiated skin cells. Its protective effects may be explained by its ability to eliminate/alleviate UVA-induced radical generation. Protective activity of SM and its flavonolignans, mainly silybin, against various ROS and ROS generating drugs was previously shown *in vitro* [37—39] and *in vivo* [13,40].

It has been demonstrated that even low UVA doses (2—10 J/cm^2) caused reduction of GSH concentrations in HaCaT [41]. Addition of SM to irradiated HaCaT prevented depletion of cellular GSH the most important skin antioxidant, involved in maintaining of intracellular redox balance. We further demonstrated that in UVA-irradiated HaCaT SM also decreased TBARS levels reflecting the extent of lipid peroxidation. These beneficial effect of SM may be connected with its ability to attenuate GSH depletion, which is directly associated with degree of lipid peroxidation in cell membrane [42]. Reduction of membrane lipid peroxidation may be also associated with SM ability to decrease ROS level in UVA-irradiated keratinocytes. Another explanation of SM profitable action is its potential to enhance activities of endogenous antioxidant enzymes including glutathione peroxidase, which was previously observed *in vivo* in TPA/BPO-treated mouse epidermis [15,18].

Intracellular GSH depletion is connected with oxidative damage to mitochondrial membrane. It can trigger both abrupt opening of a high conductance of permeability transition pore in the mitochondrial inner membrane and cytochrome *c* release from these organelles, both of which can lead to interruption of electron chain transport causing another generation of ROS, loss of electrochemical gradient across the inner membrane and ATP depletion [43]. In UVA-irradiated cells, SM application led to significant protection of ATP depletion that is essential for cell energetic metabolism. Besides, ATP decrease, cytochrome *c* release [44] and/or ROS overproduction [45] are connected with first events in the apoptosis. It is well known that caspase-3 has a primordial role in triggering the cascade of events leading to apoptosis. Its activation participates in the cleavage or degradation of

various important proteins, which are involved in apoptosis, and eventually in the activation of other caspases [46]. UVA-induced increase of caspase-3 activation was reduced by SM treatment, as was shown in both fluorescent and Western blot assay.

Our observations also demonstrate that post-treatment with SM was effective in elimination of UVA-induced DNA single-strand breaks close to the level in non-irradiated cells. Cells respond to UV-induced DNA damage by activating both cellular growth arrest and DNA repair processes. Delay before replication is required for efficient repair of DNA lesions [47]. This may explain our finding that 4 h application of SM led to minimal improvement of DNA synthesis in UVA treated HaCaT.

Flavonolignan SB, which constitutes approximately 48% of all flavonolignans (71%) in our extract, is generally considered to be the main bioactive component of SM. At this moment we can only speculate that SB is responsible for remedial effect of SM. In our previous study using hydrogen peroxide as a model substance simulating UVA damage the potency SM was comparable with the pure SB [34]. However, SM, the standardized polyphenols fraction from the *S. marianum* seeds consists of several flavonolignans (71%) and polymeric flavonolignan derivatives (29%). All these compounds may contribute to reduction of UVA-caused damage.

In conclusion, human skin is constantly exposed to the UV irradiation present in sunlight. This can induce a number of pathobiological cellular changes. The development of novel preventive and therapeutic strategies depends on our understanding of the molecular mechanisms of UV toxicity. Although we have confirmed that SM has powerful antioxidant and UVA-photoprotective abilities *in vitro*, its overall effectiveness in skin protection *in vivo* have yet to be elucidated. Since, earlier studies have shown the potency of SM against UVB damage it may be a promising full-UV-spectrum protective agent. Skin care preparations supplemented with SM are likely to lead to further improvement in their photoprotective efficacy in humans. However, further studies especially those using human systems are needed to determine cellular uptake, distribution and long-term effects of SM on the skin for optimal protection.

Acknowledgements

This work was supported by the grant of Ministry of Education of the Czech Republic (MSM 6198959216). We thank to Ladislav Cvak (IVAX Pharmaceuticals, Opava, Czech Republic) for providing silymarin and Hana Kolářová for providing the fluorescent microscope.

References

[1] Svobodova A, Psotova J, Walterova D. Natural phenolics in the prevention of UV-induced skin damage. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2003;147:137—45.
[2] Van der Leun JC. The ozone layer. Photodermatol Photoimmunol Photomed 2004;20:159—62.
[3] Pattison DI, Davies MJ. Actions of ultraviolet light on cellular structures. EXS 2006;96:131—57.
[4] Pinnel SR. Cutaneous photodamage, oxidative stress, and topical antioxidant protection. J Am Acad Dermatol 2003;48:1—22.
[5] Ischihashi M, Ueda M, Budiyanto A, Bito T, Oka M, Fukunaga M, et al. UV-induced skin damage. Toxicology 2003;189:21—39.
[6] Svobodova A, Walterova D, Vostalova J. Ultraviolet light induced alteration to the skin. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2006;150:25—38.
[7] Offord EA, Gautier JC, Avanti O, Scaletta C, Runge F, Krämer K, et al. Photoprotective potential of lycopene, β-carotene, vitamin E, vitamin C, and carnosic acid in UVA-irradiated human skin fibroblast. Free Radic Biol Med 2002; 32:1293—303.
[8] Basu-Modak S, Gordon MJ, Dobson LH, Spencer JP, Rice-Evans C, Tyrrell RM. Epicatechin and its methylated metabolite attenuate UVA-induced oxidative damage to human skin fibroblasts. Free Radic Biol Med 2003;35:910—21.
[9] Tobi SE, Gilbert M, Paul N, McMillan TJ. The green tea polyphenol, epigallocatechin-3-gallate, protects against the oxidative cellular and genotoxic damage of UVA radiation. Int J Cancer 2002;102:439—44.
[10] Huang CC, Fang JY, Wu WB, Chiang HS, Wei YJ, Hung CF. Protective effects of (−)-epicatechin-3-gallate on UVA-induced damage in HaCaT keratinocytes. Arch Dermatol Res 2005;296:473—81.
[11] Philips N, Smith J, Keller T, Gonzales S. Predominant effects of *Polypodium leucotomos* on membrane integrity, lipid peroxidation, and expression of elastin and matrixmetalloproteinase-1 in ultraviolet radiation exposed fibroblasts, and keratinocytes. J Dermatol Sci 2003;32:1—9.
[12] Erden IM, Kahramant A, Kökent T. Beneficial effects of quercetin on oxidative stress induced by ultraviolet A. Clin Exp Dermatol 2001;26:536—9.
[13] Morazzoni P, Bombardelli E. *Silybum marianum* (*Carduus marianus*). Fitoterapia 1995;66:3—42.
[14] Kren V, Walterova D. Silybin and silymarin—new effects and applications. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2005;149:29—41.
[15] Zhao J, Sharma Y, Agarwal R. Significant inhibition by the flavonoid antioxidant silymarin against 12-*O*-tetradecanoylphorbol 13-acetate-caused modulation of antioxidant and inflammatory enzymes, and cyclooxygenase 2 and interleukin-1alpha expression in SENCAR mouse epidermis: implications in the prevention of stage I tumor promotion. Mol Carcinog 1999;26:321—33.
[16] Lahiri-Chatterjee M, Katiyar SK, Mohan RR, Agarwal R. Flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in SENCAR mouse skin tumorgenesis model. Cancer Res 1999;59:622—32.
[17] Singh RP, Tyagi AK, Zhao J, Agarwal R. Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein

kinases and induction of apoptosis. Carcinogenesis 2002;23: 499—510.
[18] Zhao J, Lahiri-Chatterjee M, Sharma Y, Agarwal R. Inhibitory effect of a flavonoid antioxidant silymarin on benzoyl peroxide-induced tumor promotion, oxidative stress and inflammatory responses in SENCAR mouse skin. Carcinogenesis 2000;21:811—6.
[19] Katiyar SK, Korman NJ, Mukhtar H, Agarwal R. Protective effects of silymarin against photocarcinogenesis in a mouse skin model. J Natl Cancer Inst 1997;89:556—66.
[20] Dhanalakshmi S, Mallikarjuna GU, Singh RP, Agarwal R. Silibinin prevents ultraviolet radiation-caused skin damages in SKH-1 hairless mice via a decrease in thymine dimmer positive cells and an up-regulation of p53—p21/Cip1 in epidermis. Carcinogenesis 2004;64:6349—56.
[21] Katiyar SK. Treatment of silymarin, a plant flavonoid, prevents ultraviolet light-induced immune suppression and oxidative stress in mouse skin. Int J Oncol 2002;21:1213—22.
[22] Mallikarjuna G, Dhanalakshymi S, Singh RP, Agarwal C, Agarwal R. Silibinin protects against photocarcinogenesis via modulation of cell cycle regulators, mitogen-activated protein kinases, and Akt signalling. Cancer Res 2004;64: 6349—56.
[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;25:784—92.
[24] Maines MD, Costa LG, Reed DJ, Sassa S, Sipes IG. Current protocols in toxicology. New York: John Wiley & Sons; 1998.
[25] Chlopcikova S, Psotova J, Miketova P, Sousek J, Lichnovsky V, Simanek V. Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part II. Caffeic, chlorogenic and rosmarinic acids. Phytoter Res 2004;18:408—13.
[26] Lowry OH, Rosebough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265—75.
[27] Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968;25:192—205.
[28] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302—10.
[29] Angelo SD, Ingrosso D, Migliardi V, Sorrentino A, Donnarumma G, Baroni A, et al. Hydroxytyrosol, a natural antioxidant from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells. Free Radic Biol Med 2005;38:908—19.
[30] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein—dye binding. Anal Biochem 1976;72:248—54.
[31] Tice RR, Andrews PW, Singh NP. The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. Basic Life Sci 1990;53:291—301.
[32] Ochu EE, Rothwell NJ, Waters CM. Caspase mediate 6-hydroxydopamine-induced apoptosis but not necrosis in PC12 cells. J Neurochem 1998;70:2637—40.
[33] Adhami VM, Aziz MH, Mukhtar H, Ahmad N. Activation of prodeath Bcl-2 family proteins and mitochondrial apoptosis pathway by sanguinarine in immortalized human HaCaT keratinocytes. Clin Cancer Res 2003;9:3176—82.
[34] Svobodova A, Walterova D, Psotova J. Influence of silymarin and its flavonolignans on H_2O_2-induced oxidative stress in human keratinocytes and mice fibroblasts. Burns 2006;32: 973—9.
[35] Tobi SE, Paul N, McMillan TJ. Glutathione modulates the level of free radicals produced in UVA-irradiated cells. J Photochem Photobiol B 2000;57:102—12.
[36] Soo Lee Y, Jin DQ, Beak SM, Lee ES, Kim JA. Inhibition of ultraviolet-A-modulated signaling pathways by asiatic acid and ursolic acid in HaCaT human keratinocytes. Eur J Pharmacol 2003;476:173—8.
[37] Flora K, Hahn M, Rosen H, Bernner K. Milk thistle (*Silybum marianum*) for the therapy of liver disease. Am J Gastroenterol 1998;93:139—43.
[38] Psotova J, Chlopcikova S, Grambal F, Simanek V, Ulrichova J. Influence of silymarin and its flavonolignans on doxorubicin-iron induced lipid peroxidation in rat heart microsomes and mitochondria in comparison with quercetin. Phytother Res 2002;16:S63—7.
[39] Chlopcikova S, Psotova J, Miketova P, Simanek V. Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part I. Silymarin and its flavonolignans. Phytother Res 2004;18:107—10.
[40] Pietrangelo A, Borella F, Casalgrandi G, Montosi G, Ceccarelli D, Gallesi D, et al. Antioxidant activity of silybin *in vivo* during long-term iron overload in rats. Gastroenterology 1995;109:1941—9.
[41] Larsson P, Andersson E, Johansson U, Ollinger K, Rosdahl I. Ultraviolet A and B affect human melanocytes and keratinocytes differently. A study of oxidative alterations and apoptosis. Exp Dermatol 2005;14:117—23.
[42] Schneide LA, Dissemond J, Brenneisen P, Hainzl A, Briviba K, Wlaschek M, et al. Adaptive cellular protection against UVA-1-induced lipid peroxidation in human dermal fibroblasts shows donor-to-donor variability and is glutathione dependent. Arch Dermatol Res 2006;297:324—8.
[43] Zhong JL, Yiakouvaki A, Holley P, Tyrrell MR, Pourzand C. Susceptibility of skin cells to UVA-induced necrotic cell death reflect the intracellular level of labile iron. J Invest Dermatol 2004;123:771—80.
[44] Orrenius S. Mitochondrial regulation of apoptotic cell death. Toxicol Lett 2004;149:19—23.
[45] Valencia A, Kochevar IE, Ultraviolet. A induces apoptosis via reactive oxygen species in a model for Smith-Lemli-Opitz syndrome. Free Radic Biol Med 2006;40:641—50.
[46] Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. Pharmacol Ther 2001;92:57—70.
[47] Syed DN, Malik A, Hadi N, Sarfaraz S, Afaq F, Mukhtar H. Photochemopreventive effect of pomegranate fruit extract on UVA-mediated activation of cellular pathways in normal human epidermal keratinocytes. Photochem Photobiol 2005; 82:398—405.