

Effects of Glycyrrhizin on UVB-irradiated Melanoma Cells

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Abstract. It is known that liquorice root is rich in compounds which exert several pharmacological actions. In the present study, we evaluated the effect of glycyrrhizin (the main constituent of liquorice root) and of its metabolite aglycone, 18 β -glycyrrhetic acid, on UVB-irradiated human melanoma cells: SKMEL-2 from metastatic tissue and SKMEL-28 from primary malignant melanoma. Tests performed (Trypan blue exclusion test, MTT and Western blot) showed that glycyrrhizin is not toxic for both types of cells. In SKMEL-28 cells, Bcl-2 expression was low after UVB irradiation, but it was increased when treated with glycyrrhizin. On the contrary, in the SKMEL-2 cell culture, Bcl-2 expression was not modified by the substances under study. The results show that glycyrrhizin treatment might offer protection from the damage induced in humans by UVB radiation, while it seems to be ineffective on metastatic cells. Further studies must be performed to understand the mechanism of the protective effect.

Different antioxidant compounds are present in liquorice root and the literature supports that glycyrrhizin (GL) and its aglycone, glycyrrhetic acid (GA), have a wide range of pharmacological actions including anti-allergic, anti-carcinogenic and anti-immune-mediated cytotoxicity (1). It has been demonstrated that GL is able to stimulate melanogenesis and to inhibit the growth of B16 melanoma cells, inducing a cell cycle arrest in G1-phase (2). More recently, Jung *et al.* investigated the mechanism of GL effects on melanogenesis in B16 murine melanoma cells. The study showed that the stimulation of GL-induced melanogenesis is likely to occur through transcriptional activation (3). Ultraviolet radiation is a dangerous environmental agent known to be responsible for

severe effects in the skin and in the eye of both animals and humans. Sun-exposure without protection and the depletion of the stratospheric ozone have resulted in an increasing incidence of malignant melanoma in humans (4, 5). Recent important findings have correlated the exposure to excessive ultraviolet radiations with the presence of reactive oxygen species (ROS), which are involved in all stages of multi-step cancer development (6). Cutaneous carcinogenesis is effectively reduced by antioxidant agents (7) and, in the present study, we investigated if Bcl-2 expression in UVB-irradiated (300 mJ/cm²) melanoma cells is modified by treatment or pre-treatment with antioxidants such as GL and its aglycone, 18 β -glycyrrhetic acid (18 β -GA).

Materials and Methods.

Compounds. Glycyrrhizin (Sigma Chemical Co, USA) and 18 β -glycyrrhetic acid (Sigma) were tested at 30 μ M (final concentration). All the substances were solubilised in sterile dimethylsulphoxide (final concentration 0.01%) and the solutions of each compound sterilised by filtration.

Cells. SKMEL-2 from metastatic tissue (ATCC HTB-68) and SKMEL-28 from primary malignant melanoma cells (ATCC HTB-72) were chosen for the present study.

Culture medium. Eagle's Minimum Essential Medium (CAMBREX Bio Science, Belgium) enriched with 1% non essential aminoacids, sodium pyruvate 1 mM, bovine insulin 10 μ g/ml, 5% foetal bovine serum (FBS), 1% antibiotic solution (penicillin 50 U/mL and streptomycin 0.5mg/mL) and 1% L-glutamine.

Tests. The Trypan blue exclusion test, MTT test, TUNEL test and Western blot analysis were performed.

Statistical analysis. Statistical comparison was carried out using a Student's *t*-test. A *p* value < 0.05 was considered significant.

Cell culture. Flasks containing the different cell lines were maintained at + 37°C in a fully humidified atmosphere of 5% CO₂. Once cell growth was well established, the cells were transferred under sterile conditions into chamber slides (Nunc, Naperville, USA) for the TUNEL technique, into 60-mm petri dishes (Falcon, Becton

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Key Words: Glycyrrhizin, UVB radiation, Bcl-2, SKMEL-28 cells, SKMEL-2 cells.

Dickinson, NJ, USA) for Western blot analysis and into 96-multiwell plates (Falcon, Becton Dickinson) for the MTT test.

Cytotoxicity assays. Cells were plated at $1 \times 10^4/\text{cm}^2$ in 96-multiwell until near confluence. After 48-hour incubation at 37°C in 5% CO_2 in the presence of increasing micromolar concentrations of the compounds, the cytotoxicity tests were performed.

Trypan blue exclusion test. In order to define the exact concentration to use for apoptotic studies, equimolecular increasing concentrations of the compounds dissolved in the culture medium were first added to the melanoma cells maintenance medium: 1mL of each dilution of GL and of $18\beta\text{-GA}$ was placed into the wells. A well containing 1mL of cells and 1mL of medium served as a control. The plates were again incubated at 37°C for 48 hours and, after this period, a count of both live and dead cells was carried out in a Bürker cell-count chamber under the optical microscope (magnification 400x) using a solution of Trypan blue 0.5% in PBS (phosphate buffer solution).

MTT test. In the MTT assay, a yellow tetrazolium salt is reduced to a blue formazan product by mitochondrial succinic dehydrogenase which is present only in metabolically active cells (8). The crystals formed are solubilized and the resulting coloured solution is quantified. Twenty μL of MTT (5 mg/ml) were added to each well for 4 hours. After removing the supernatant, the formed crystals were solubilized with 200 μL of dimethylsulphoxide (DMSO, J.T. Baker Deventer, Holland) and shaken for 10 minutes. The resulting coloured solution was quantified at 540 nm using a Bio-Rad 550 microplate reader.

All tests were carried out in triplicate and compared with control wells.

Treatment with GL, $18\beta\text{-GA}$ and UVB radiation. Melanoma cells were treated with GL and $18\beta\text{-GA}$ with two different modalities: in the first case, cells were pretreated with the compounds over 12 hours. After this period, the medium was removed and the cells were washed twice with PBS with $\text{Ca}^{++}\text{-Mg}^{++}$ (PBS+, CAMBREX, Belgium). GL and $18\beta\text{-GA}$ were added to PBS also during irradiation.

The cells were exposed to ultraviolet B light ($300 \text{ mJ}/\text{cm}^2$) using a UVB TL-20Watt/ 12 RS lamp (Philips, Netherlands). After UVB irradiation, the cells were provided with fresh medium added with GL and $18\beta\text{-GA}$.

In the second case, the cells were irradiated without pretreatment. Only after UVB irradiation were the cells provided with fresh medium enriched with GL and $18\beta\text{-GA}$.

TUNEL staining. The TUNEL (TdT-mediated dUTP nick end labelling) technique is based on the direct and specific labelling *in situ* of DNA breaks in nuclei, and allows the detection of apoptosis in individual cells. Melanocytes were directly stained on chamber slides by the "In situ cell death detection kit" (Roche Diagnostics, Mannheim, Germany) following the manufacturer's recommendation. After incubation with the compounds and after irradiation, the melanoma cells were fixed with 4% buffered paraformaldehyde solution before permeabilization with Triton X (0.1%) and sodium citrate. Subsequently, the cells were incubated with fluorescein-labelled nucleotides and terminal deoxynucleotidyl transferase (TdT) for 1 hour at 37°C . Each experiment was carried out in triplicate. In negative controls, the primary incubation was replaced with a nucleotide mixture without TdT. Fluorescent specimens were analyzed by a Confocal Scanning Laser Microscopy (Leica TCS4D) in conjunction with a conventional optical microscope (Leica DM IRBE).

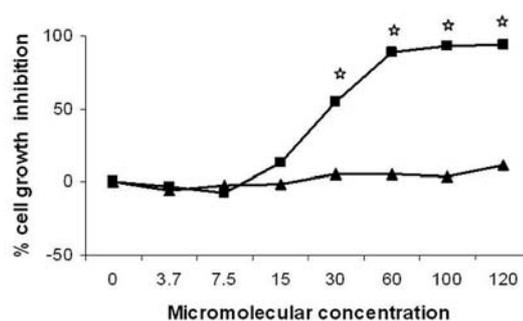


Figure 1. Antiproliferative activity of glycyrrhizin (▲) and $18\beta\text{-glycyrrhetic acid}$ (■) on SKMEL-28 cell growth. MTT test was performed with equimolecular concentration of the compounds under study. Data obtained are expressed as percentage of growth inhibition. [☆ significantly different (p value < 0.05) by t -test with respect to control (0% inhibition)].

Western blot test. Monolayer cultures in 60-mm dishes were washed with PBS and extracted by scraping with 100 μL of extraction buffer consisting of 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1% Na deossicolate, 1% Triton X-100, 0.1% SDS, 0.2% NaN_3 with the proteases inhibitors aprotinin (0.2 TIU/mL, Sigma), leupeptin (0.01 mg/mL, Sigma) and PMSF (4 mM, Sigma). Protein concentration was measured by means of the "Protein Assay Reagent" (Protein Assay, Bio-Rad, Hercules, CA, USA) in a total volume of 1 mL with bovine serum albumin as standard.

Fifty μg of protein per lane were loaded onto a 13% polyacrylamide gel and transferred to nitrocellulose. To verify equal loading of total protein in all lanes, the membrane was stained with Red Ponceau. The blot was incubated with 1:40 anti-human Bcl-2 mouse monoclonal antibody (Dako, A/S, Denmark) in 1% non-fat dry milk overnight at 4°C . The blots were washed three times in PBS/Tween and incubated with peroxidase-conjugate goat anti-mouse IgG (Bio-Rad) at 1:2000 dilution for 45 minutes at room temperature. Detection was performed using the ECL chemiluminescent system (Amersham, IL, USA) and autoradiographic film (Hyperfilm-ECL, Amersham).

Results

The Trypan blue exclusion test and MTT test confirmed that GL and $18\beta\text{-GA}$ are not toxic at the concentrations ($30\mu\text{M}$) added to the melanoma cells for apoptotic studies (Figures 1, 2). Therefore, the apoptotic studies in melanoma cell lines were carried out using GL and $18\beta\text{-GA}$ $30\mu\text{M}$. Results from the TUNEL test demonstrated that the presence of GL in the medium did not induce apoptosis on both cell lines (Figures 3, 4); while 24-hour incubation with $18\beta\text{-GA}$ $30\mu\text{M}$ stimulated apoptosis in SKMEL-2 cells (Figure 4). As expected, UVB irradiation induced apoptosis, but if the cells were treated with GL prior to, during or after the irradiation, the percentage of apoptotic cells significantly decreased. The protein concentration (Figure 5), determined by the Bradford analysis (9), confirms the protective activity of the compounds against UVB damage on cells. Finally, results from the Western blot

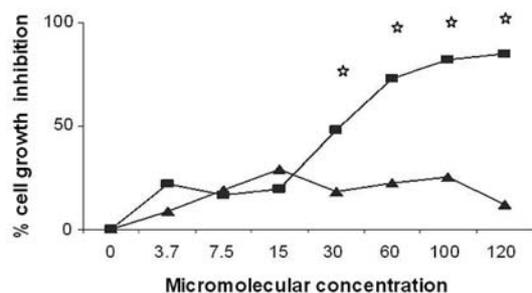


Figure 2. Antiproliferative activity of glycyrrhizin (▲) and 18β-glycyrrhetic acid (■) on SKMEL-2 cell growth. MTT test was performed with equimolecular concentrations of the compounds under study. Data obtained are expressed as percentage of growth inhibition. [☆ significantly different (p value < 0.05) by t -test with respect to control (0% inhibition)].

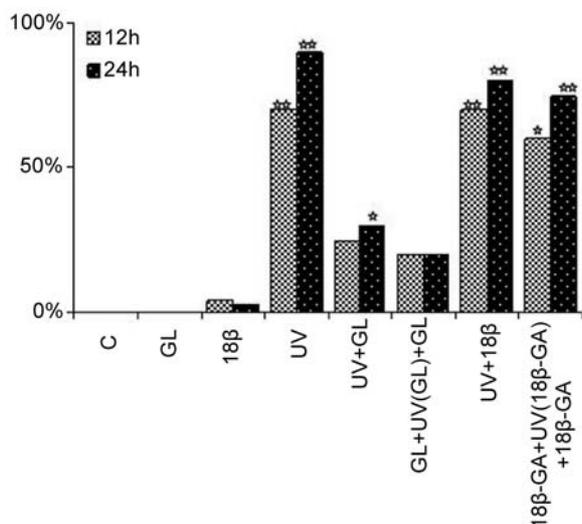


Figure 3. SKMEL-28 TUNEL staining. After UVB irradiation and the addition of the compounds, around 100 cells were counted in randomly selected fields for each point and percentages of labelled cells are shown. As expected, the presence of GL during the UVB radiation decreased the number of apoptotic cells. [significantly different (☆ = p value < 0.05, ☆☆ = p value < 0.01) by t -test with respect to control (0% inhibition)].

test clearly show (Figure 6 and Table I) that Bcl-2 expression was modified in cultures treated with GL and 18β-GA 30μM. The UVB radiation (300mJ/cm²) reduced the Bcl-2 content both in SKMEL-2 and in SKMEL-28 cells, but if GL or 18β-GA 30 μM were added to the culture medium, Bcl-2 expression was improved, and this was particularly evident in SKMEL-28 cells after 24-hour GL treatment.

Discussion

Our results confirm that UV irradiation-induced cell damage can be reduced by the application of protective agents; this depends on the characteristics of the cell lines used. These

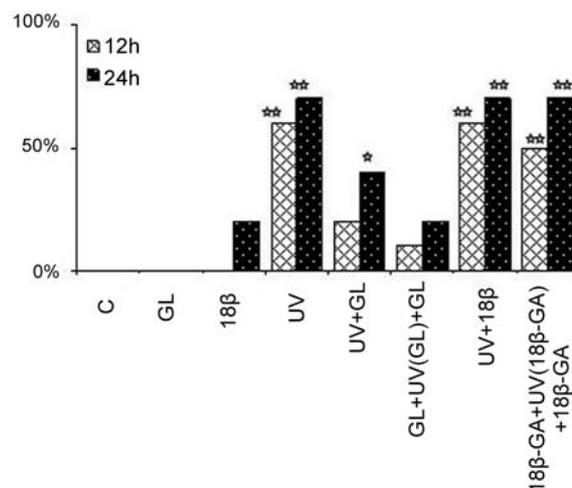


Figure 4. SKMEL-2 TUNEL staining. After UVB irradiation and the addition of the compounds, around 100 cells were counted in randomly selected fields for each point and percentages of labelled cells are shown. As expected, the presence of GL during the UVB radiation decreased the number of apoptotic cells. [significantly different (☆ = p value < 0.05, ☆☆ = p value < 0.01) by t -test with respect to control (0% inhibition)].

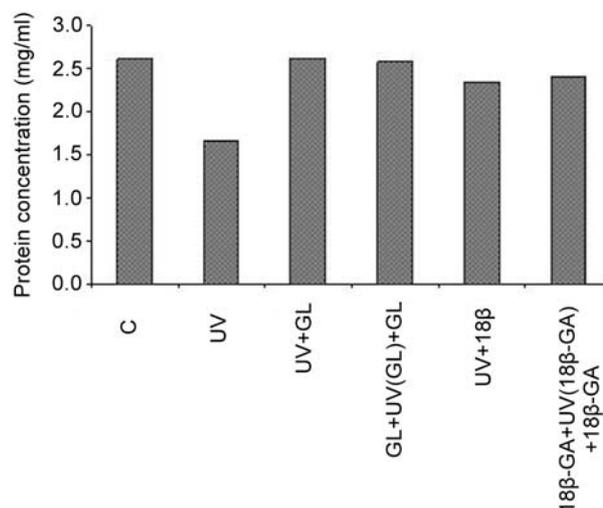


Figure 5. Protein concentration performed by Bradford analysis. SKMEL-28 showed an evident increase in protein content after UVB irradiation when added to GL and GL18β-GA.

preliminary results suggested that GL, and in some cases 18β-GA, could be added to the list of protective agents since SKMEL-28 cells were really protected from radiation damage, which was proved by the results of the Western blot test. With regard to SKMEL-2 cells, the line was very sensitive to the UVB action and it may be that the irradiation caused such severe cellular damage as to make it impossible for GL to extend the same protection we observed with SKMEL-28 cells.

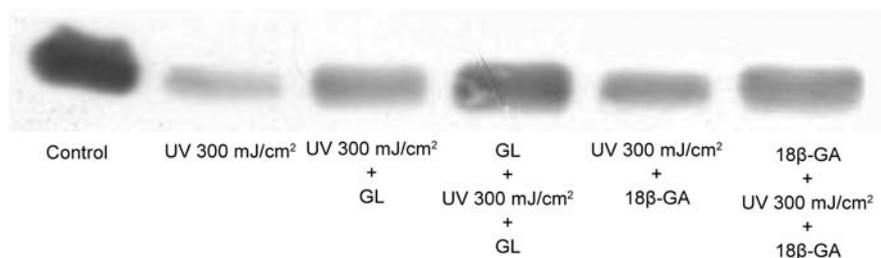


Figure 6. Expression of Bcl-2 in SKMEL-28 cells. Cellular lysates from subconfluent cultures of SKMEL-28 cells were irradiated with UVB 300 mJ/cm² and analysed by Western blot test after 24 hours. The protective action of both GL and 18β-GA is evident. Bcl-2 expression was improved with GL and this is particularly evident in lane 4, where the cells are treated before, after and during irradiation.

Epidemiological data confirm that the high incidence of skin tumours is related to human exposure to solar ultraviolet radiation, which is known to generate ROS (10-12). Another important assumption is that the natural antioxidant defence is impaired upon UVA and UVB exposure (13), therefore supplementation with antioxidant should be recommended before intensive sun-exposure. GL has been recognised to possess anti-oxidant properties (14). Moreover, it has been demonstrated that the presence of GL in topical formulations improves the penetration of anti-inflammatory agents such as diclofenac (15), while recent data have expanded the concept that inflammation is a very critical component of tumour progression (11, 16).

We conclude that GL should be considered as an important and promising agent to be added to topical formulations in the development of new strategies for the prevention of skin cancer.

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Table I. Densitometry measurements (pixel units) from Western blot analysis.

24 hours	SKMEL-28
Control	1014.86
UV 300 mJ/cm ²	209.40
UV 300 mJ/cm ² + GL	393.14
GL + UV 300 mJ/cm ² + GL	711.09
UV 300 mJ/cm ² + 18β-GA	318.42
18β-GA + UV 300 mJ/cm ² + 18β-GA	473.23

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Received November 18, 2004
Accepted December 29, 2004