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# The effect of delphinidin on the mechanical properties of keratinocytes exposed to UVB radiation

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ABSTRACT

The usage of active compounds of dietary phytochemicals in prevention of UV-induced skin diseases is increasingly gaining attention in the development of skin care products. The purpose of this study was to measure the influence of delphinidin (as a botanical agent) on the cell mechanical properties evaluated by the atomic force microscopy (AFM) technique in the immortalized human keratinocyte cell line (HaCaT) exposed to UVB radiation.
The cells were treated with various doses of UVB radiation with and without pre and post-treatment with selected concentrations of delphinidin. The measurements of the elastic properties revealed that the exposure of HaCaT cells to high dose of the UVB radiation (100 mJ/cm$^2$) caused a decrease in the cell elastic modulus. It was accompanied by the decrease of metabolic activity, rearrangement of actin cytoskeleton and disappearance of the cell repair marker 53BP1. Both pre-treatment and post-treatment with delphinidin at non-cytotoxic concentrations (5 or 10 μM), restored the elastic modulus of irradiated keratinocytes. A direct AFM analysis showed that the UVB-mediated decrease of the cell stiffness was restored more effectively when cells were treated with delphinidin after the UVB irradiation. The results demonstrate the regenerative effect of delphinidin on the mechanical properties of cells exposed to UVB radiation (100 mJ/cm$^2$), which may be due to antioxidant and inhibitory effect on matrix metalloproteinases activation.



## 1. Introduction

The use of the botanical agents in photochemoprevention of skin diseases is of current interest [1,2]. Among them, the anthocyanins, natural pigments present in fruits and vegetables are believed to prevent many serious health conditions like cardiovascular diseases, diabetes, arthritis, inflammations and they also show significant inhibitory effects on the growth of certain cancer cells [3–6]. Particularly, delphinidin - the aglycon form of anthocyanin, was reported to exert a potent inhibitory effect on UVB–dependent skin damage, as compared to other anthocyanidin compounds [5]. Delphinidin protection against the UVB radiation and oxidative stress in normal and immortalized HaCaT keratinocytes was shown by Noda et al. [7] and Afaq et al. [5]. However, its effect on the mechanical properties of cells has not been yet investigated.

Particular attention is given to investigations of molecular mechanisms of the effects of UVB radiation, as the one of the major risk factors of human skin diseases, and an early recognition as well as protection of skin barriers against damage on the level of epidermis. Majority of data concern various cancer cell lines while less information is available about normal or immortalized cell lines that are not cancerous. Immortalized HaCaT keratinocytes are often used models and the effects of UVB radiation on that cell line are already well recognized including the oxidative stress and pro-inflammatory cytokine and chemokine release [8,9]. However, many data reveal the different responses of HaCaT cells and natural human keratinocytes [10–12].

Damaging effects on the skin of both UVA and UVB radiation have been also the subjects of many investigations concerning DNA chromosomal aberrations, induction of the single and double DNA strand breaks (SSB and DSB) and effects due to the generation of the reactive oxygen species (ROS) (for review see [13,14]). It is well recognized that DNA double strand breaks (DSB) generated by external and/or internal factors, belong to the most deleterious forms of DNA damage. The p53 binding protein 1 (53BP1) belongs to the DNA damage response (DDR) pathway and is recognized as the DNA-damage checkpoint activator and DNA repair marker of DSB created by various genotoxic factors including UV radiation [15–18]. The HaCaT cells demonstrate UV-type specific p53 mutation [19] and remains non–tumorigenic until induced by specific factors, including irradiation with 20 J/cm$^2$ UVA for 5 weeks [20].

The important point is that anthocyanidins, including delphinidin, act differently in normal as compared with cancer cells, particularly with highly malignant, metastatic and drug-resistant. In cancer cells delphinidin showed prooxidant and proapoptotic activity, whereas the cancer preventing properties of that compound are connected with

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their free-radical scavenger and detoxifying properties (for review see [21]).

The pleiotropic functions of matrix metalloproteinases (MMPs), the zinc-dependent endopeptidases responsible for the degradation of proteins in the extracellular matrix required for cell translocation, development and remodeling as well as in diseases progression in response to environmental conditions are well characterized [22]. The role of MMPs in skin pathology has been recognized particularly in skin cancer initiation and development [23]. However, the induction of MMP-9 in primary human keratinocytes by UVB irradiation in dose – dependent manner was documented by Onoue et al. [24]. Induction of the other MMPs including MMP-1, −2, −7 and −9 by UVB irradiation in HaCaT cells was confirmed by many investigators [25,26]. Jiang et al. [27] described stimulation of MMP-1 in HaCaT cells at low doses of UVB irradiation without effect on cell viability. The effect of MMPs activity on the cytoskeletal organization in HaCaT cells was shown [28,29].

The deleterious effects of UV radiation on the actin cytoskeleton and focal adhesion dynamics in keratinocytes were described by Reich et al. [30] and Liu et al. [31], respectively. The increasing evidences confirm that cytoskeleton organization and the changes in cell morphology are accompanied by alterations in their mechanical properties that can be quantitatively investigated by their deformability [32]. To study the mechanical properties of cells, the atomic force microscopy (AFM) technique has been applied [33]. This technique allows the detection of even the smallest changes in cell elasticity, provides information about the living cells cytoskeleton rearrangement and overall cell condition (for review see [34] and [35]). The use of the fluorescent microscopy as a complementary method enables observation of the cytoskeleton filaments rearrangement [36].

Only a few studies have examined the effect of UV irradiation on the mechanical properties of skin fibroblasts [37,38]. It has been demonstrated that UVB irradiation can dose-dependently decrease the elastic modulus of the studied cells [38].

The protective effect of various flavonoids on the UVB-dependent MMPs induction in normal keratinocytes and HaCaT cells is already well recognized [25,26,28]. Therefore, the present study was conducted to determine the effect of delphinidin on the modulation of the mechanical properties of UVB-irradiated HaCaT cells.

## 2. Materials and Methods

### 2.1. Cell Culture

The spontaneously immortalized human keratinocyte cell line (HaCaT) was obtained from Deutsches Krebsforschungszentrum Stabsstelle Technologietransfer Heidelberg, Germany. All materials used for the cell culture were obtained from Life Technologies (USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with phenol red, 4.5 g/l glucose and supplemented with 10% FBS, 2 mM L-glutamine and an antibiotic mixture (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown at 37 °C in a humidified atmosphere of 5% $CO_2$. Approximately 80% confluent cultures were washed with phosphate-buffered saline (PBS) and harvested with 0.25% trypsin/EDTA solution. Subsequently, the cells were counted in the Neubauer hemocytometer. For the experiments they were seeded at $8 \times 10^3$ cells/ml onto 10 mm glass coverslips (Menzel Gläser, Germany), placed into 24-well plates and cultured at 37 °C in a humidified atmosphere of 5% $CO_2$ for 24 h prior to further treatment (UVB and/or delphinidin).

### 2.2. UVB Irradiation

As the UVB light source a sunlamp TL 20W/12 RS SLV (Philips, UK), with the UV spectrum from 290 to 315 nm was used. The dose of UVB irradiation was measured with a VLX 312 radiometer equipped with a UVB sensor (Vilber Lourmat, Marne-la-Vallée Cedex, France). In order to receive an energy density of 25, 50 and 100 mJ/cm$^2$, the cells were exposed to the UV for 45 s, 89 s and 179 s, respectively. Before the radiation in order to prevent UV quenching, the medium was removed, the cells were washed with PBS buffer, and replaced with PBS. Then the cells were exposed to different doses of UVB radiation (25, 50 and 100 mJ/cm$^2$). After the irradiation, PBS was changed to a fresh DMEM medium and the cells were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$ for 24 h, prior to further measurements or delphinidin treatment. Non irradiated and non-treated cells were used as a control.

### 2.3. Delphinidin Treatment

The delphinidin stock solutions were prepared in DMSO (Carl Roth, Germany). Three concentrations of delphinidin (Sigma-Aldrich, USA) were examined (5, 10, and 20 μM), and amounted to 0.25% medium volume per well. Incubations were carried out for 24 h in a DMEM medium at 37 °C in a humidified atmosphere of 5% $CO_2$. In the separate experiment we verified that DMSO at the concentration of 0.25% did not change the keratinocyte metabolic activity nor elasticity (*data not shown*).

### 2.4. AFM Analysis of Cell Stiffness

The HaCaT cell stiffness measurements were performed using a commercial microscope (XE120 model, Park Scientific Instruments, South Korea). The gold-coated silicon nitride cantilevers (MLCT, Bruker, USA) with a spring constant of 0.01 N/m were applied. Coverslips with cells were washed 3 times with a DMEM medium before each measurement. All measurements were performed in a DMEM medium at room temperature. In order to locate a cell and control the AFM cantilever position, an optical microscope was used. Force curves were collected from randomly chosen keratinocytes, localized by a top-view optical camera integrated with the AFM and imaged before the elasticity measurements. Regions around the cell center were selected for the measurement. At each selected region, a grid of $4 \times 4$ points was created. Force curves were recorded at the scan velocity equal to 9 μm/s. The determination of the elastic modulus was based on a subtraction of the two force curves: the calibration curve recorded on the glass coverslip without the cells and the other on a given cell [36] at an indentation depth of 300 nm. The choice of indentation depth equaled to 300 nm is slightly arbitrary, however, it reflects the elasticity of the layer when we could expect the mechanical response originating from actin cytoskeleton. The obtained *force versus indentation curve* can be evaluated by means of the Sneddon extension of the Hertz model [39] assuming that the AFM tip is an infinitely stiff indenter of a selected geometry (usually conical or parabolic). In our studies the best match was achieved when the force-indentation curves were analyzed assuming that the shape of the AFM tip was a parabola. In this case the elastic modulus can be calculated according to the equation:

$$F(\delta) = \frac{4}{3} \cdot \sqrt{R} \cdot E_{eff} \cdot \delta^{\frac{3}{2}} \quad (1)$$

where $R$ is the curvature of the AFM tip, $\delta$ is the indentation depth, and $E_{eff}$ is the reduced elastic modulus which considers both the cell and the AFM cantilever stiffness linked in the series. Since the Young's modulus of the AFM cantilever is much larger than that of the cells, the $E_{eff}$ can be re-written as follows:

$$E_{eff} = \frac{E_{cell}}{1 - \mu_{cell}^2} \quad (2)$$

where $\mu_{cell}$ is the Poisson coefficient set to 0.5 since cells can be treated as an incompressible material [40].