IN VITRO INSIGHTS REGARDING THE ROLE OF MELANIN IN MELANOMA CELLS’ RESPONSE TO BETULINIC ACID TREATMENT

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Abstract
Malignant melanoma (MM), the cancer arising from the normal-to-malignant switch of melanocytes, remains the most aggressive type of skin cancer with an annual increasing incidence worldwide. Based on the presence or absence of melanin, MM is divided in melanotic (pigmented) or amelanotic (non-pigmented) melanoma. Melanin is the natural pigment providing a protective role against harmful ultraviolet (UV) radiations and oxidative stress to epidermal melanocytes and surrounding keratinocytes. Recent studies reveal the ability of melanin to bind drugs via covalent and non-covalent bonds, leading to their accumulation within pigmented tissues which might influence the response of MM to chemotherapy. Betulinic acid (BA) is a pentacyclic triterpenoid possessing a multitude of therapeutic properties, including antimelanoma activity. The present study aimed to investigate the response of two MM cell lines to the antitumour effect of BA, considering their melanin content and to evaluate the cytotoxic potential of BA in healthy primary fibroblasts. The results reveal a dose-dependent decrease in the viability and confluence of all cell lines. However, SK-MEL-5 cells (IC₅₀ = 13.67 µM for 24 h, and 7.48 µM for 72 h) exerted a higher sensitivity to BA when compared to B16-F10 cells (IC₅₀ = 52.12 µM for 24 h, and 25.54 for 72 h). BA induces a differential cytotoxicity in healthy, MM non-pigmented SK-MEL-5 and pigmented B16-F10 cells. Therefore, further studies are required to confirm the interaction between BA and melanin.

Rezumat
Melanomul malign (MM), cancerul care rezultă din transformarea malignă a melanocitelor, rămâne cel mai agresiv tip de cancer cutanat, având o incidență anuală aflată în continuă creștere. În funcție de prezența sau absența melaninei, MM se clasifică în melanom melanotic (pigmentat) și amelanotic (nepigmentat). Melanina reprezintă pigmentul natural care oferă protecție melanocitelor și keratinocitelor epidermice împotriva radiațiilor ultraviolete (UV) și a stresului oxidativ. Studii recente au evidențiat abilitatea melanin a lega medicamente prin legături covalente și non-covalente, și influența acestuia asupra unor celule sănătoase – fibroblaste primare. Rezultatele au arătat o scădere doză-dependentă în viabilitatea și confluența tuturor liniilor celulare. Totuși, linia celulară SK-MEL-5 (IC₅₀ = 13.67 µM pentru 24 h și 7.48 µM pentru 72 h) a arătat o sensibilitate mai ridicată la acțiunea BA comparativ cu linia celulară B16-F10 (IC₅₀ = 52.12 µM pentru 24 h și 25.54 pentru 72 h). BA induce o citotoxicitate dependată de tipul de celule, celule sănătoase, nepigmentate SK-MEL-5 și respectiv pigmentate B16-F10. Sunt necesare studii suplimentare pentru a confirma interacțiunea dintre BA și melanină.

Keywords: betulinic acid, melanoma, viability, morphology, primary fibroblasts

Introduction
Despite the recent progress made in diagnosing and treating cancer, this pathology still represents a major health problem, being the second leading cause of death worldwide [1]. In terms of incidence, skin cancer which covers both melanoma and non-melanoma neoplasms, is the most common type of malignancy in Caucasians. Malignant melanoma (MM) is ranked as the most aggressive subtype [2], with an annual incidence increase of 0.6% [3]. The starting point for
the development of cutaneous melanoma is represented by melanocytes, as a result of the interaction between the host and the environmental factors. One of the most recognized risk factors for the development of MM is represented by excessive exposure to ultraviolet radiation (UVR). However, genetic predisposition also has a major impact on the occurrence of this type of neoplasia [4]. The leading cause of death among patients diagnosed with melanoma is represented by its ability to metastasize rapidly, especially in the lungs, liver, and neurons [5]. The behaviour of MM is driven by several factors as hormones, growth factors, cytokines, peptides and neurotransmitters. Both normal and malignant melanocytes are equipped with a unique metabolic pathway, leading to the biosynthesis of melanin through the oxidation of tyrosine [6]. The term “melanin” refers to the most enigmatic pigments of natural origin [7] which protect normal and malignant melanocytes, as well as the surrounding keratinocytes against physical and chemical insults [6]. Cutaneous melanin is a mixture of eumelanin - a black-to-brown pigment, and pheomelanin which is yellow reddish in colour [8]. Pigmentation is highly deregulated within melanoma cells which can easily shift from pigmented to non-pigmented states [6]. Furthermore, depending on the presence or absence of melanin, MM has been divided in two subcategories, namely: melanotic or pigmented melanoma and amelanotic or unpigmented melanoma [9]. Melanin is known as a photoprotector against UVR-induced damage by reducing the skin penetration of UV light and counteracting harmful ROS [10, 11]. It also possesses a potent chelating property, being able to bind pharmacologically active compounds via both covalent and non-covalent bonding, due to its abundance in functional groups such as o-quinone, amine, catechol and imine [12], ability which might influence the efficiency of MM treatment.

Current MM treatment strategies resort to surgery, chemotherapy, targeted therapy and immunotherapy. In recent years, there has been an improvement in the treatment of melanoma with the development of new therapeutic agents such as BRAF, CTLA4 and PD1 inhibitors [13, 27]. Although major advances have been made in the treatment of melanoma, these types of new therapeutic approaches face many challenges. First, therapeutic doses are often associated with systemic toxic reactions. In addition, due to the heterogeneity of patients and tumours, clinical outcomes in the treatment of melanoma are negatively influenced by the emergence of resistance to therapy [14]. Since ancient times, plants have been used for the prevention and treatment of various diseases [15]. Currently, over 60% of drugs used in the treatment of cancer have been obtained from herbal sources. Among the most widely used compounds of plant origin in the treatment of cancer are triterpenes. They have many therapeutic activities such as antiangiogenic, antioxidant, but also a targeted action on various receptors involved in cancer pathogenesis such as androgen receptor and vascular endothelial growth factor receptor [16]. Among these compounds, betulinic acid (BA) has been noted for its biological and therapeutic properties such as anti-inflammatory, anti-infective and antitumour. Due to its selective cytotoxic action (highly cytotoxic against tumour cells and very low cytotoxic in healthy cells), BA has become a promising therapeutic agent in various cancers [17]. Since the late 1990s, BA has been noted for its beneficial effects in the treatment of MM [18]. Its antitumour effect is based on the ability of BA to induce CD95- and p53-mediated cellular apoptosis. In addition, BA also possesses other molecular mechanisms such as the formation of reactive oxygen species (ROS), the activation of mitogen-activated protein kinase, but also the inhibition of angiogenesis [19]. Another molecular mechanism described for BA is the anti-melanogenic activity by inhibiting the tyrosine kinase activity [20].

The present study aims to investigate the selective in vitro cytotoxic effect of BA on healthy cells (human primary dermal fibroblasts - HDFa) and on both melanotic and amelanotic MM cell lines, as well as to verify whether melanoma cells’ response to treatment is influenced by the pigmentation grade.

Materials and Methods

Reagents
The reagents used in the present study, as: betulinic acid (BA), dimethyl sulfoxide (DMSO – BA solvent), specific culture media - Fibroblast Basal Medium (ATCC PCS-201-030), Eagle’s Minimum Essential Medium (EMEM) and Dulbecco’s Modified Eagle’s Medium (DMEM), cell culture supplements – Fibroblast Growth Kit–Low Serum (ATCC PCS-201-041), foetal bovine serum (FBS), penicillin, streptomycin, phosphate saline buffer (PBS), trypsin-EDTA solution, MTT viability kit were acquired from ATCC (American Type Culture Collection, Virginia, USA), Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and Sigma Aldrich, Merck KGa Group (Darmstadt, Germany), respectively. The reagents were of analytical purity and approved for cell culture use.

Cell lines
The experiments described in the present study were conducted using immortalized melanoma cells of human – SK-MEL-5 (human melanoma cell line – code HTB-70™) and murine origin – B16-F10 (murine melanoma cell line – code CRL-6475™) and primary human dermal fibroblasts (HDFa - PCS-201-012™) (Figure 1). All the cell lines were classified as BSL 1 (Biosafety Level 1) and were purchased from ATCC (American Type Culture Collection, Virginia, USA) as frozen vials being kept in liquid nitrogen until the start of the experiment.
Figure 1.

Microscopical aspect of human (SK-MEL-5) and murine (B16-F10) melanoma cells and of human primary dermal fibroblasts (HDFa) in culture. The scale bar was 50 µm.

Cell culture
The melanoma cells (SK-MEL-5 and B16-F10) were cultured according to the manufacturer’s recommendations in specific culture media: EMEM (ATCC catalogue No. 30-2003, for SK-MEL-5) and DMEM (ATCC catalogue No. 30-2002, for B16-F10) that were supplemented with FBS (final concentration 10%) and a mixture of antibiotics (penicillin/streptomycin – final concentration 1%). In the case of primary fibroblasts, HDFa, the cells were cultured in specific medium Fibroblast Basal Medium (ATCC PCS-201-030) that was completed with a specific growth kit - Fibroblast Growth Kit–Low Serum (ATCC PCS-201-041) and a mixture of antibiotics (Penicillin-Streptomycin-Amphotericin B Solution - ATCC PCS-999-002). During the experiment, the cells were kept in a humidified CO₂ incubator in standard conditions: 37°C and 5% CO₂. To establish the number of cells/well we used an automatic cell counter – Countess II Automated Cell Counter (Thermo Fisher Scientific, Inc., Waltham, MA, USA), after staining with Trypan blue.

Assessment of cell viability by MTT (3-(4,5-dimethylthiazol-2-yl) assay
In order to verify the impact of BA solution on both healthy (HDFa) and melanoma cells’ (SK-MEL-5 and B16-F10) viability it was applied the MTT technique, a standardized assay to determine the percentage of viability cells following exposure to a specific treatment. The protocol applied was previously described [21, 32] and was adapted to our laboratory facilities. In brief, the following steps were performed: (i) seeding of 10⁴ cells/well/200 µL culture medium in 96-well plates; (ii) addition of fresh medium (100 µL/well final volume) and stimulation with different concentrations of BA in DMSO (in the range of 1 - 75 µM) for two time points: 24 and 72 h; (iii) addition of 10 µL MTT reagent/well and incubation for 3 h at 37°C; (iv) after the 3 hours of incubation it was added a volume of 100 µL/well solubilization buffer followed by 45 minutes incubation at room temperature and dark; and (v) absorbance reading at 570 nm using a microplate spectrophotometer (xMark™ Microplate Spectrophotometer, Biorad). There were also tested the same concentrations of DMSO (BA’s solvent) as for BA. As control, the unstimulated cells were used.

Cellular morphology
Changes in cells’ morphology represent an important parameter in the assessment of toxic potential of a test compound. In this line, it was also verified the impact of BA treatment on healthy and human and murine melanoma cells in terms of cells’ morphology,
microscopically using an inverted microscope the Olympus IX73 (Olympus, Tokyo, Japan). The pictures were taken at 24 and 72 h post-stimulation, for each of the concentrations tested and were analysed using the cellSens Dimensions v.1.8. Software (Olympus, Tokyo, Japan).

**Statistical analysis**

The cell viability data obtained were statistically analysed using one-way ANOVA test followed by Dunnett’s multiple comparison post hoc test (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com). The results were presented as means ± SD. The differences between the groups were considered statistically significant if p < 0.05.

**Results and Discussion**

BA, the pentacyclic triterpene also known as “mitocan” due to its capacity to target mitochondria in tumour cells, was highly investigated as an antitumour agent [21]. This natural compound was initially described by Pisha et al. as an antimelanoma agent and further studies proved its antitumour properties against multiple types of cancer [22]. Several antitumour mechanisms of action were elucidated for BA (as a pro-apoptotic agent by targeting mitochondria, as antiangiogenic agent, a prooxidant effect by generating ROS, and others) [23], but the complete mechanism is far from being discovered.

Melanin is a natural polymer produced within the human epidermis by specialized cells known as melanocytes. Melanin plays a protective role against UV radiation and oxidative stress. Recent studies denote the drug- and metallic-binding ability of melanin, leading to their accumulation within pigmented tissues [24]. On the other hand, due to this property, melanin acts as scavenger of toxics, protecting pigmented cells by retaining harmful substances, which are subsequently released in non-toxic concentrations [25]. The role of melanin in the melanoma therapeutic response is not clearly defined.

Several studies presented the antimelanogenic effect of BA by exerting an inhibitory effect on tyrosinase activity [26] and by regulating MEK/ERK and PI3K/Akt pathways [20]. This capacity of BA to inhibit melanin synthesis could be considered a novel potential antimelanoma mechanism of action, but also highlights its benefice use in cosmetics as an anti-hyperpigmentation agent.

Based on the current data from the literature, the present study was oriented toward the investigation of BA antimelanoma effect both in non-pigmented and melanin-pigmented melanoma cells and to establish a potential correlation between BA’s therapeutic effect and the pigmentation grade of the cells. In addition, it was also tested the effect of BA’s treatment in healthy cells, primary fibroblasts of human origin, to highlight the selective cytotoxicity of BA, a feature that is highly desired for all the anticancer agents. Thereupon, an *in vitro* investigation has been conducted using two MM cell lines of different origin, B16-F10 (murine melanoma cells) and SK-MEL-5 (human melanoma cells) and a healthy primary human cell line, HDFa (primary human dermal fibroblasts) in terms of cell viability evaluation and morphological changes monitorization following BA treatment. The healthy human cell line, HDFa (primary fibroblasts) was chosen for this study based on the similarity cell-type morphology with the melanoma cells investigated (a fibroblastic-like morphology) and on the key role played by fibroblasts in melanoma development [28].

The two melanoma cell lines were selected based on the results of one of our previous studies indicating that B16-F10 cells contain the highest amount of melanin (56.452 µg/mL), while SK-MEL-5 cells lack pigmentation [29].

**Figure 2.**

*In vitro* assessment of BA’s (1, 5, 10, 50 and 75 µM) impact on HDFa cells’ viability at 24 h post-stimulation by MTT assay

The results are expressed as cell viability percentage (%) normalized to control (unstimulated) cells. The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was performed to check the statistical differences in rapport with control followed by Dunnett’s multiple comparisons post-test (**** p < 0.0001).

The evaluation of BA’s impact on human fibroblasts, HDFa viability percentage after a 24 h treatment, indicated that BA significantly reduced cells’ viability only at the highest concentrations tested (50 and 75 µM), whereas the lowest ones did not affect HDFa viability (Figure 2). The calculated IC50 after a 24 h treatment was 64.26 µM. Similar results were obtained after a 72 h stimulation (data not shown). It was also tested the impact of DMSO, the solvent used for BA solubilization, and no significant effects were recorded in cells’ viability percentages, this representing the reason for the normalization of the results to control cells (unstimulated cells).

Analysis of the morphological changes induced by BA in HDFa showed changes in cells’ shapes and the
presence of cellular debris only in the cells groups treated with the highest concentrations of BA (50 and 75 µM), whereas in the case of the lowest concentrations the HDFa cells presented a morphology similar to the control cells (Figure 3).

**Figure 3.**
Representative photos of the morphological features of HDFa cells after the treatment for 24 h with BA in DMSO (1, 5, 10, 50 and 75 µM). The scale bar was 50 µm.

**Figure 4.**
*In vitro* assessment of BA’s (1, 5, 10, 50 and 75 µM) impact on SK-MEL-5 cells’ viability at 24 and 72 h post-stimulation by MTT assay

The results are expressed as cell viability percentage (%) normalized to control (unstimulated) cells. The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was performed to check the statistical differences in rapport with control followed by Dunnett’s multiple comparisons post-test (**** p < 0.0001).

Our main findings in terms of cell viability in human melanoma cells, SK-MEL-5, reveal that BA treatment for 24 and 72 h induced a significant decrease of human melanoma cells’ viability in a dose- and time-dependent manner (Figure 4). The IC₅₀ values calculated for 24 and 72 h were 13.67 and 7.48 µM, respectively. Together with the decrease of SK-MEL-5 cells viability there were also observed significant changes in cells morphological aspect (Figure 5). BA treatment induced
a decreased confluence of the human melanoma cells in a dose-dependent manner, leading to cell rounding and floating in the medium at both time intervals. The most significant changes in cells’ shape were detected at the highest concentrations (50 and 75 µM, cell debris) after 24 h, whereas a longer incubation period (72 h) with BA determined a significant cytotoxic effect even at the concentration of 10 µM (Figure 5).

![Figure 5](image)

Representative photos of the morphological features of SK-MEL-5 cells after the treatment for 24 and 72 h with BA in DMSO (1, 5, 10, 50 and 75 µM). The scale bar was 20 µm.

In the case of murine melanoma cells, B16-F10, that are highly pigmented, the therapeutic response was lower as compared to non-pigmented melanoma cells, SK-MEL-5, but still it was also observed a dose-dependent decrease of cells viability percentage (Figure 6). The 72 h treatment with BA reduced the percentages of B16-F10 cells as compared to the ones calculated for 24 h, but not as significant as seen for SK-MEL-5 cells. The IC_{50} values calculated were: 52.12 µM for 24 h and 25.54 for 72 h. These values were calculated using GraphPad Prism software.

![Figure 6](image)

In *vitro* assessment of BA’s (1, 5, 10, 50 and 75 µM) impact on B16-F10 cells’ viability at 24 and 72 h post-stimulation by MTT assay. The results are expressed as cell viability percentage (%) normalized to control (unstimulated) cells. The data represent the mean values ± SD of three independent experiments performed in triplicate. One-way ANOVA analysis was performed to check the statistical differences in rapport with control followed by Dunnett’s multiple comparisons post-test (**** p < 0.0001).

BA treatment also affected the morphology of B16-F10, but at a lower extent as compared to SK-MEL-5 cells (Figure 7). Round and floating cells were seen at the highest concentrations tested (50 and 75 µM). Moreover, it can be clearly seen the presence of melanin within the B16-F10 cells (Figure 7).

![Figure 7](image)

The DMSO (BA’s solvent) cytotoxic potential was also tested, but there were not significant differences between control (unstimulated cells) and the ones stimulated with DMSO. A decrease in both cells’ viability percentages was observed only at the highest concentrations (50 and 75 µM), but at a much lower extent as compared to BA treatment (data not shown).
In recent years, BA was placed into the spotlight due to its abundance of biological properties such as antitumour, antimicrobial, anti-inflammatory, and antiangiogenic [22]. Previous in vitro studies directed the utilization of BA towards the treatment of MM [23]. Our previous work established several new potential antimelanoma mechanisms of action for BA as antitumoral agent by interfering with the epithelial-to-mesenchymal transition [30] and by impairing mitochondrial metabolism of melanoma cells [21]. The current results indicate the following: (i) BA at low concentrations (< 50 µM) exerts a selective cytotoxicity (HDFa cells’ viability was not affected, while in melanoma cells was significantly decreased – Figures 2, 4 and 6), still by increasing the concentration the selectivity is lost; and (ii) BA treatment proved to be highly cytotoxic on both pigmented (B16-F10) and non-pigmented (SK-MEL-5) cells (with a higher susceptibility recorded in the case of SK-MEL-5) by decreasing cells’ viability percentages and by inducing morphological changes in melanoma cells shape, changes that indicate an apoptosis-like effect. The selectivity of BA towards cancer cells and a lack/a very low toxicity against healthy cells was also described in previous studies [21, 31].

The cytotoxic effect of BA in melanoma cells is supported by other results from the literature. Soica et al. showed that BA inhibited the growth of both non-metastatic and metastatic B164A5 cells following a 72 h of treatment [33]. In another study developed by Farcas et al., it has been revealed that BA decreases the viability of B164A5 and A375 MM cells at the concentrations of 5 and 25 µM following 24 and 48 h [34]. In a recent study by Coricovac et al., BA is shown to induce a dose-dependent cytotoxicity in A375 MM cells after 24 h, accompanied by apoptotic nuclear features (shrinkage, chromatin condensation), up-regulation of pro-apoptotic markers (Bax, Bad, and Bak), inhibition of mitochondrial respiration and glycolysis, and decrease in the mitochondrial membrane potential [21]. A very recent study showed that BA proved to be very active against equine melanoma cells [35].

Conclusions

The preliminary results that were presented in the current study indicate a dose-, time- and cell line-dependent cytotoxic effect induced by BA treatment in healthy, non-pigmented and pigmented melanoma cells characterized by a reduction of cells viability and induction of apoptotic-like morphological changes. An interesting finding consisted in the different response of non-pigmented (SK-MEL-5) and pigmented (B16-F10) melanoma cells following BA treatment, the non-pigmented cells being more susceptible as compared to pigmented ones, what led us to the hypothesis that melanin might interfere with BA antimelanoma effect. Further studies are required to elucidate the connection between BA and melanin what might represent a novel potential antimelanoma mechanism of action.

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Conflict of interest

The authors declare no conflict of interest.

References


