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Scientific report – Phase I – 2018

Project title: ***NEW INSIGHTS INTO THE ANTIMELANOMA MECHANISM OF ACTION OF BETULINIC ACID***

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Introduction

This project aims to elucidate the antimelanoma mechanism of action of a natural origin compound, betulinic acid – BA. Betulinic acid proved to be a very active anticancer agent with a high selectivity on tumor cells and no toxicity on healthy/normal cells.

Although, a considerable number of studies were focused on elucidating the antitumoral mechanism of action of BA, there still are some missing pieces to gather in order to have a complete picture of the BA antimelanoma mechanism of action.

To perform the experimental procedures proposed in the present project, it was used a solution of BA solubilized in DMSO – dimethyl sulfoxide (the solvent in which BA is soluble and proved to be non-toxic for cells *in vitro* when is used at low concentrations - < 1%).

The first phase of the project consisted of the following objectives and activities:

Objective 1. Characterization of BA *in vitro* effects in terms of cell viability

Activity 1.1. Assessment of BA cytotoxicity by using different doses (2.5; 5; 10; 20 and 25 μg/ml) on normal human cells: HDF – human primary dermal fibroblasts and HEKa – human primary adult keratinocytes, by the means of : MTT, Alamar blue, LDH and Trypan blue assays.

Activity 1.2. Assessment of BA cytotoxicity by using different doses (2.5; 5; 10; 20 and 25 μg/ml) on several human and murine melanoma: SK-MEL-28 and SK-MEL-5 (human melanoma cells derived from metastases) and B16-F10, B16-F0 (murine melanoma cell lines), by the means of : MTT, Alamar blue, LDH and Trypan blue assays.

Activity 1.3. Selection of the most sensitive melanoma cell lines and the dose associated with the induction of apoptosis for the employment in the experiments required to the other objectives.

Objective 2. Melanoma gene profiling

Activity 2.1. Evaluation of the effects induced by BA administration on melanoma specific genes expression - gene microarray assay, RT-PCR and Western Blot

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Activity 2.2. Stimulation of selected melanoma cells with the apoptotic dose for 24 h and quantification of the BA effects on apoptosis markers (pro- (Bax, Bak) and antiapoptotic markers – Bcl-2, Bcl-xL, Mcl-1)

To realize the objectives and the activities described above, we used the following materials and methods:

Materials

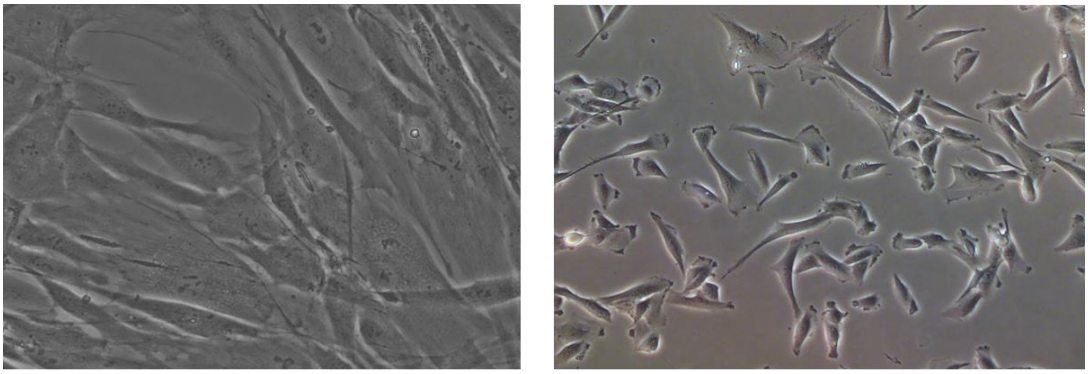
Reagents

Reagents used to perform the experiments were of analytical grade purity and were purchased from de la Sigma Aldrich (Germany), Thermo Fisher Scientific (USA) and ATCC (American Type Culture Collection). Cell culture media and the other supplements required for cells growth were bought from Sigma Aldrich and ATCC.

Cell lines

The cell lines used in the experiments were purchased from ATCC:

- Healthy/Normal cell lines: HDFa - human primary dermal fibroblasts and HEKa - human primary adult keratinocytes (Figure 1).



HDFa – human primary dermal fibroblasts

HEKa – human primary adult keratinocytes

Figure 1. The appearance of healthy cell lines in culture: HDFa and HEKa.

- Human melanoma cell lines - SK-MEL-28 and SK-MEL-5 (cell lines isolated from different metastatic tumors) and murine melanoma cell lines - B16-F10, B16-F0 (highly pigmented) (Figure 2).

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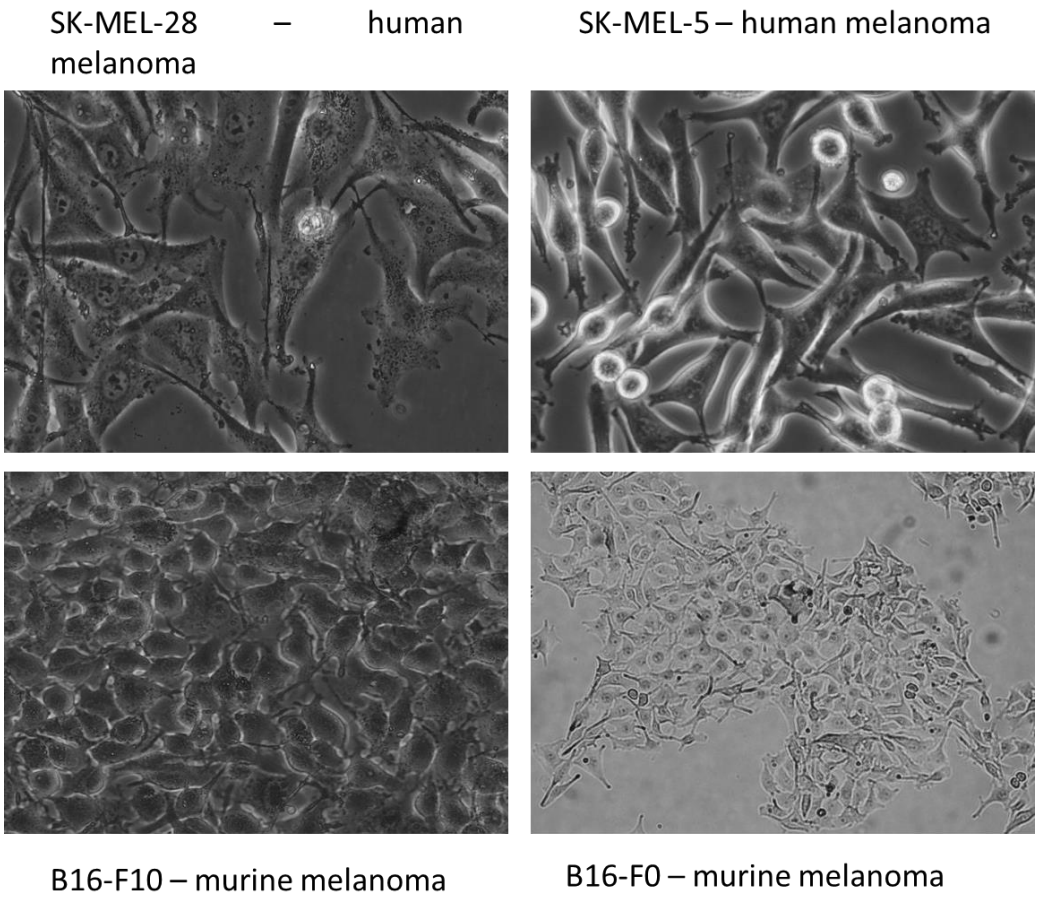

Figure 2. The appearance of melanoma cells in culture: SK-MEL-28 – human melanoma, SK-MEL-5 – human melanoma, B16-F10 – murine melanoma and B16-F0 – murine melanoma.

Methods applied

Cell culture

- the cells were grown in specific medium, as follows: HEKa - Dermal Cell Basal Medium (ATCC®-PCS-200-030™) + Keratinocyte Growth Kit (ATCC®-PCS-200-040™); HDFa - Fibroblast Basal Medium (cod: ATCC®-PCS-201-030™) + Fibroblast Growth Kit-Serum-Free (ATCC®-PCS-201-040™); SK-MEL-28 and SK-MEL-5 - EMEM (Eagle’s Minimum Essential Medium), and B16-F10 and B16-F0 - DMEM (Dulbecco’s Minimum Essential Medium).

Cell viability assessment

- Alamar blue technique – to determine viable cells

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- MTT assay – to assess cell viability
- Trypan blue – to detect necrotic cells
- LDH (lactate-dehydrogenase) assay – to evaluate cytotoxicity

Induction of apoptosis

- RT-PCR – by evaluating the impact of BA on mRNA expression of pro-apoptotic (bax, bak) and anti-apoptotic markers (Bcl-2, Bcl-xL, Mcl-1)

Experimental design

To perform the experiments proposed in the study, we established the following test groups:

- Control (C): unstimulated cells
- DMSO (D): cells stimulated with DMSO – the same doses as tested for BA
- BA+D (2.5, 5, 10, 20 și 25 µg/mL): cells stimulated with different concentrations of BA solubilized in DMSO for 24 and 72h

Preliminary Results

Cell viability assessment

BA impact on healthy cells viability

- The highest dose tested - 25 µg/mL of BA induced a slight decrease of fibroblasts and keratinocytes (5-10%) after 72h stimulation, whereas smaller concentrations had no toxic effect on healthy cells viability. These results confirm the very low/lack of toxicity induced by BA.

BA impact on melanoma cells viability

There were observed some differences between human and murine melanoma cells, as follows:

- Human melanoma cells are more susceptible to BA effect as compared to murine cells, low doses as 5 µg/mL inducing a significant decrease of viable cells percentage. The cytotoxic effect of BA was dose and time-dependent.

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- A decrease of viable cells percentage in the case of murine melanoma cells was noticed at higher concentrations as compared to human melanoma – starting at 10 ug/mL, the effect was also dose and time-dependent.
- The highest susceptibility to BA effect was exhibited by SK-MEL-28 – human melanoma cells (decreased viability percentage – 80% at 2.5 µg/mL) and this cell line was selected for melanoma gene profiling experiments.
- Changes of cells shape and morphology were observed after BA stimulation.

To determine the impact of BA stimulation on melanoma cells gene profile it was used Whole Human Genome Oligo Microarray (Agilent Technologies, Santa Clara, CA, USA) and Agilent Technologies Scanner G2505C US45102867.

The results obtained during this first phase will be included in an article that will be send for publication to an international journal ISI indexed.