Scientific report – Phase II – 2019

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

Project code: **PN-III-P1-1.1-PD-2016-1982**

Contract no.: **132/07/05/2018**

Project value: **249.187, 00 Lei**

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Introduction

This intermediate report represents a synthesis of the results obtained during the second phase of the project (only a small part of these), the results that are exploited as scientific articles as mentioned in the indicators' list, and also as articles that are draft manuscripts at this moment.

The central objective of the present project is represented by the elucidation of BA (betulinic acid) antimelanoma mechanism of action, mechanism that proved to be very complex and is incompletely known at present. In order to get some insights into BA antimelanoma mechanism of action, were proposed the following objectives and activities for the second phase:

Objective 3. Evaluation of BA antitumoral effect by induced apoptosis via mitochondrial (intrinsic) pathway

A.3.1. 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) – Annexin V/PI, caspase kit assay, RT-PCR, Western Blot, ELISA.

A.3.2. Effects of BA on cellular mitochondrial respiratory function in melanoma cells – High Resolution Respirometry technique

A.3.3. Effects of BA on ROS production in melanoma cells - Amplex Red assay

Objective 4. Discovery of the BA antiangiogenic mechanism

A.4.1. Impact of BA on specificity proteins: Sp1, Sp3 and Sp4 in melanoma cells – RT-PCR and Western Blot

A.4.2. Effects of BA on VEGF and VEGF receptors expression in melanoma cells – RT-PCR, Western Blot and immunohistochemistry

A.4.3. BA activity on EGRF signalling pathway in melanoma cells – RT-PCR, Western Blot and immunohistochemistry

Objective 5. Finding the BA antimetastatic signalling pathway

A.5.1. Effects of BA on epithelial to mesenchymal transition specific markers (E-cadherin, Laminin-1, Vimentin) as antimetastatic agent – RT-PCR, Western Blot

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), Cell Signaling, Abcam 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 line that was used in the experiments of phase II of the project was SK-MEL-28 - human melanoma cell line, which is the cell line that proved to be the most sensitive to BA effect.

Methods applied

Cell culture

- the cells were grown in specific medium, as follows: EMEM (Eagle's Minimum Essential Medium) supplemented with 10% FCS and 1% mixture of antibiotics

Cell viability assessment

- Alamar blue technique – to determine viable cells
- MTT assay – to assess cell viability
- Trypan blue – to detect necrotic cells
- LDH (lactate-dehydrogenase) assay – to evaluate cytotoxicity
- Annexiv V/PI

Induction of apoptosis

- Annexin V/PI
- qRT-PCR – by evaluating the impact of BA on mRNA expression of caspases 3, 8, 9 using the specific markers
- caspase specific kit

Effect of BA on mitochondrial respiration

- High Resolution Respirometry - Oroboros Oxygraph-2k
- Amplex Red - determination of reactive oxygen species (ROS) production

Antiangiogenic effect of BA

- qRT-PCR - BA effect on mRNA expression of specificity proteins : Sp1, Sp3 and Sp4, and VEGF factor
- Western blot
- immunofluorescence

Antimetastatic effect of BA

- qRT-PCR - BA effect on mRNA expression of epithelial (E-cadherin and Laminin-1) and mesenchymal (vimentin) markers
- Western blot
- immunofluorescence

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 and 10 µg/mL) stimulated for 24h

Preliminary Results

Cell viability assessment

BA impact on healthy cells viability

- Besides the tests that were performed on healthy keratinocytes and fibroblasts, BA effect was also verified on human healthy hepatocytes - HepaRG and human renal cells - HK-2, and our results indicated a lack of toxicity even at 25µg/mL after 24h (Figure 1)

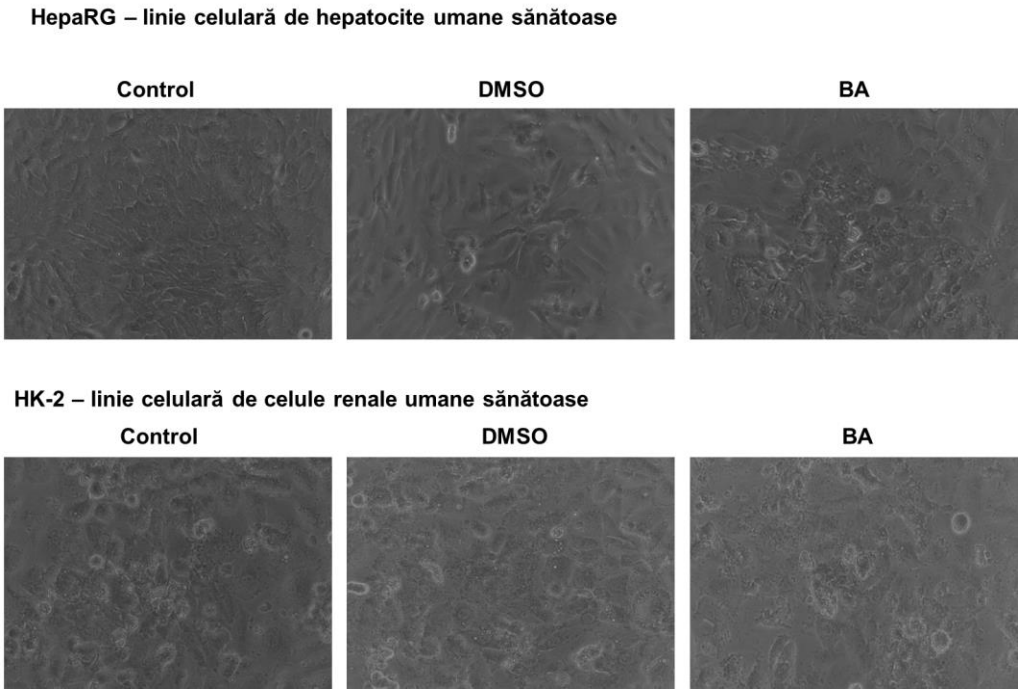

Figure 1. Morphological aspect of HepaRG and HK-2 in culture after a 24h stimulation with BA 25 µg/mL

- In addition, BA effect was also tested on a 3D epiderm model and BA exerted no irritant effect.

BA impact on apoptosis

- Annexin V/PI test confirmed the capacity of BA to induce apoptosis even at doses as 10 µg/mL.

- qRT-PCR results confirmed the BA impact on pro-apoptotic (Bax, Bak - up-regulation) and anti-apoptotic (Bcl-2 and Bcl-xL - down-regulation) markers and further experiments were perform to verifiy the effect of BA on caspases (caspases 3, 8 and 9) activation.
- BA stimulation led to the up-regulation of caspases mRNA expression, caspases 8 and 9 acting as activators of caspase 3 in mitochondrial induced-apoptosis. The caspase specific kit assay confirmed these results.
- an interesting finding was obtained by applying high resolution respirometry technique: BA modulates bioenergetics of human melanoma cells by acting directly on oxidative phosphorylation, phenomenon that is described for the first time in cancer cells.
- immunofluorescence staining of mitochondria indicated changes of mitochondria localization in human melanoma cells, changes that are correlated with the novel mechanism of action of BA (Figure 2).

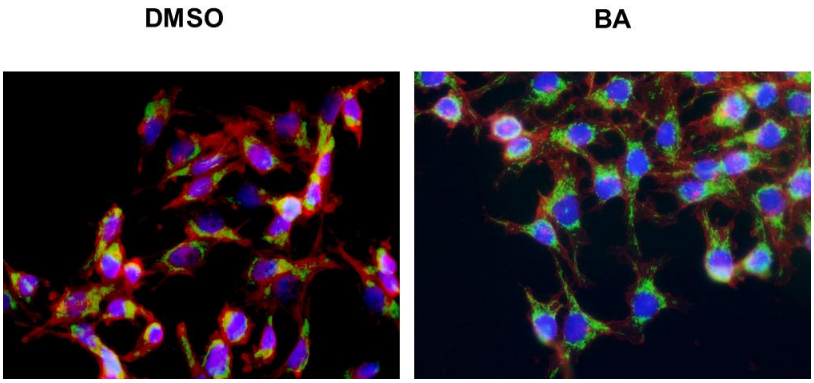

Figure 2. Staining of actin fibers (red), mitochondria localization (green) and nucleus (blue) to human melanoma cells after 24h srimulation with BA - 10 µg/mL

- BA exerted an inhibitory effect on specificity protein (Sp1, Sp3 and Sp4) and VEGF expressions, what suggests an antiangiogenic effects. Moreover, BA effects on angiogenesis was also verified on embrionated eggs by CAM assay.
- BA interferes with epithelial-to-mesenchymal transition by up-regulating epithelial markers (E-cadherin and Laminin-1) and down-regulating mesenchymal markers expressions (vimentin).

The results obtained during the second phase will be included in two articles that will be send for publication to international journals ISI indexed.