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# Final scientific report
# - 2018 – 2020 -

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**

**Project manager: Conf. univ. dr. farm. Coricovac Elena-Dorina**

**Mentor: Prof. univ. dr. farm. habil. Dehelean Cristina Adriana**

## Introduction

The present scientific report presents in a synthetic manner all the experimental results obtained during the 24-months period of the project highlighting the published/accepted results and also that are to be published. This project was aimed to elucidate the antimelanoma mechanism of action of betulinic acid (BA), a natural compound that proved to be very active from a pharmacological perspective against tumor cells and harmless against healthy cells, mechanism that is very complex and incompletely known until present.

The selection of melanoma was based on the following premises: i) it is a very aggressive disease that causes a significant number of deaths despite the small percentage of cases as compared to the other types of skin cancer and ii) the lack of a curative treatment for metastatic

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melanoma, the current therapies present a decreased efficacy due to the high capacity of melanoma cells to become resistant.

To develop and perform this project, there were proposed several objectives and related activities, as follows:

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

To accomplish this objective, we used different healthy primary human cell lines as, human fibroblasts (HDFa) and human keratinocytes (HEKa) and human (SK-MEL-28 and SK-MEL-5) and murine (B16F10 and B16F0) melanoma cell lines (see figure 1).

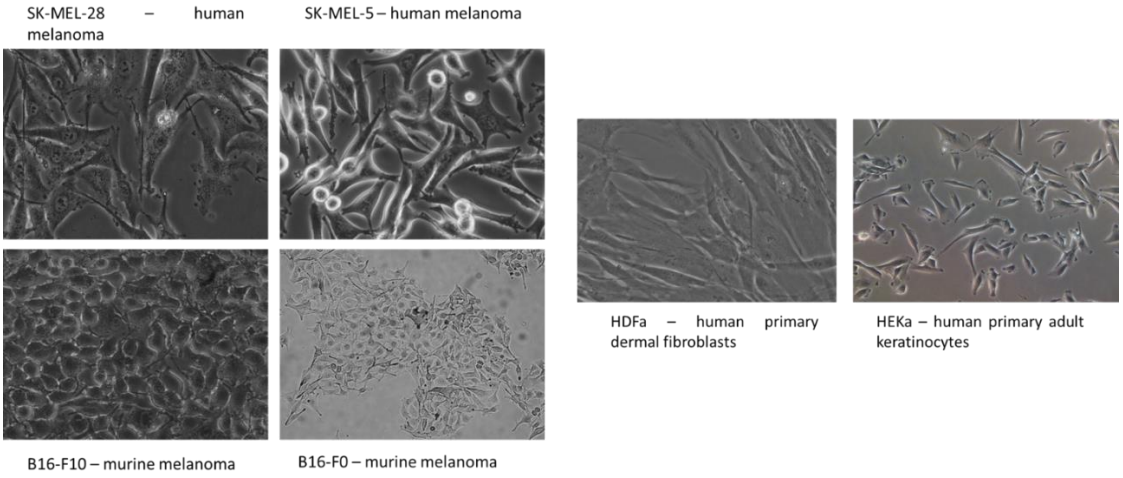


**Figure 1. The appearance in culture conditions of the healthy and tumor cell lines used in the study.**

The experimental design consisted of establishing the toxicological profile of BA by verifying different concentrations of BA solubilized in DMSO (2.5; 5; 10; 20 and 25 µg/ml) for different time points (24, 48 and 72 h) in the cell lines mentioned above.

The following results were obtained:

- BA exerts a dose- and time-dependent cytotoxic effect on tumor cell lines

- the most susceptible cell line to BA effect was SK-MEL-28, cell line selected for futher experiments

- BA exerted a slightly cytotoxic effect in healthy cells only at the highest concentration tested and at the longest time point – 72h.

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The toxic potential of BA was also tested on hepatic and renal healthy cells, and no toxicity was observed after 24 h stimulation. Moreover, BA proved to be non-toxic in a 3D model of epidermis tissue.

These data show that BA has a selective toxicity oriented towards tumor cells and will be published in an ISI article with an impact factor higher than 1.5.

By performing the experiments proposed to realize the second objective (**Melanoma gene profiling**), it was shown the impact of BA on different genes involved in apoptosis, angiogenesis and epithelial-to-mesenchymal transition (the main directions of the present research), impact that was quantified by further techniques as qRT-PCR and Western Blot. The results obtained at this objective, together with a part of the results from objectives 3, 4 and 5 will be disseminated as an article published in an ISI international journal with an impact factor higher than 4.

To complete objective 3 (**Evaluation of BA antitumoral effects on apoptosis via mitochondrial pathway**) several standardized techniques were applied, as: - immunofluorescence (DAPI and Hoechst 33342 staining), flow cytometry (Annexin V/PI), qRT-PCR, Western Blot, High Resolution Respirometry (to assess mitochondrial/cellular respiration), ELISA, and Amplex Red (for ROS determination).

The following results were obtained:

- BA induces apoptosis in human melanoma cells starting at 10 µg/mL (see figure 2)

- BA-induced apoptosis involves intrinsic pathway (mitochondrial pathway) by activating caspases 3, 8, and 9, up-regulating pro-apoptotic markers and down-regulating anti-apoptotic markers expressions

- BA interferes with mitochondrial respiration (Oroboros Oxygraph-2k) and mitochondrial bioenergetic profile (assessed by XFe24 Extracellular Flux Analyzers - Seahorse Scientific)

- BA induces changes in mitochondria localization (immunofluorescence)

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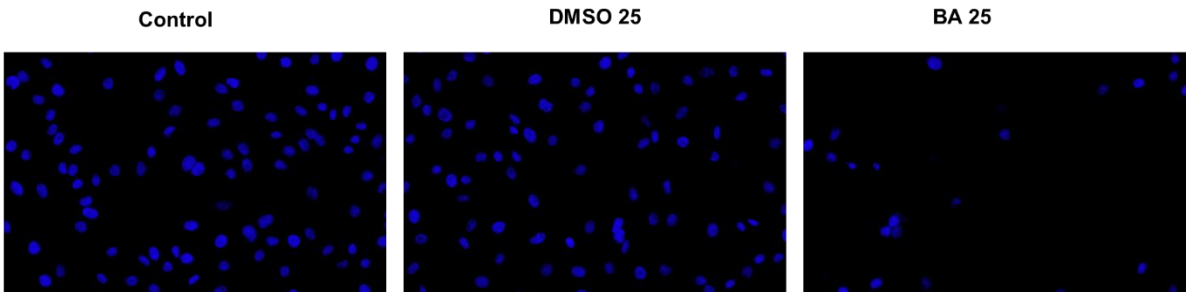


**Figure 2. SK-MEL-28 cells stained with Hoechst 33342 after BA and DMSO stimulation for 24 h.**

Objective 4 was focused on the discovery of the BA antiangiogenic mechanism of action, mechanism that is not entirely known at present. To get some insights in this matter, we evaluated the impact of BA on several proteins (Sp1, Sp3 and Sp4) and specific markers (VEGF) with key roles in angiogenesis by applying standardized techniques as: qRT-PCR, Western Blot and immunofluorescence. Our results indicated that BA antiangiogenic mechanism of action involves the tested proteins and VEGF by inhibiting their expressions. Moreover, EGFR signaling pathway is also implicated in BA antiangiogenic effect.

Another objective proposed in this project was to find the BA antimetastatic signaling pathway, metastasis being a specific feature of melanoma. To fulfill this objective, it was verified the effect of BA on specific epithelial (E-cadherin, Laminin-1) and mesenchymal (Vimentin, Fibronectin, N-cadherin) markers, melanoma cells migration and invasion and on MMPs by performing qRT-PCR, Scratch assay and Western Blot.

The following data were obtained:

- BA inhibits mesenchymal markers expression
- BA stimulates epithelial markers expression
- BA inhibits melanoma cell migration and invasion
- BA interferes with MMPs expression
- BA reverses EMT in melanoma cells.

At the end of this project, based on the data obtained, we could assert that our results will have a significant impact as scientific background for the literature due to the novelty of these

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results regarding the antimelanoma mechanism of action of BA, representing also a starting point for new research directions.

All the graphs and the images obtained during the studies will be used as parts for the following articles, and in order to avoid auto plagiarism, we decided not to include them in this report.

All the data that weren't published yet will be disseminated until the end of this year as articles/chapter in international books with high visibility and elevated impact factor.

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**Results dissemination**

**Conferences:**

1. Coricovac DE, Dehelean CA. Functionalization of betulinic acid by nanotechnology improved its in vitro antiproliferative activity. Nano Engineering & Technology, 10-11 December 2018, Rome Italy. – poster

2. Coricovac D, Macasoi I, Pinzaru I. Dehelean C. Betulinic acid exhibits a distinctive antimelanoma effect by interfering with epithelial-to-mesenchymal transition (EMT). The 5th German Pharm-Tox Summit 2020, The 86th Annual Meeting of the German Society for Experimental and Clinical Pathology and Toxicology (DGPT), 2-5 March 2020, Leipzig, Germany – poster. Abstract book: Naunyn-Schmiedeberg's Arch Pharmacol (2020) 393 (Suppl 1):S1–S97, DOI 10.1007/s00210-020-01828-y. IF = 2.058

**Webinars:**

1. Merck Romania: Introduction to Merck "Green" Nucleic Acid Purification Technology – 28.05.2020

2. Merck Romania: Restart Cell Culture with Merck – 10.06.2020.

**Published articles:**

1. Farcas, C. G., Macasoi, I., Pinzaru, I., Chirita, M., Chirita Mihaila, M. C., Dehelean, C., Avram, S., Loghin, F., Mocanu, L., Rotaru, V., Ieta, A., Ercuta, A., & **Coricovac, D.** Controlled Synthesis and Characterization of Micrometric Single Crystalline Magnetite With Superparamagnetic Behavior and Cytocompatibility/Cytotoxicity Assessments. **Frontiers in Pharmacology**, 2020, 11, 410. https://doi.org/10.3389/fphar.2020.00410. **IF = 4.225**

2. Ghiulai, R., Avram, S., Stoian, D., Pavel, I. Z., **Coricovac, D.,** Oprean, C., Vlase, L., Farcas, C., Mioc, M., Minda, D., Motoc, A., Szuhanek, C., Danciu, C., Soica, C., & Sima, L. Lemon Balm Extracts Prevent Breast Cancer Progression In Vitro and In Ovo on Chorioallantoic Membrane Assay. Evidence-based complementary and alternative medicine: eCAM, 2020, 2020, 6489159. https://doi.org/10.1155/2020/6489159. **IF = 1.984**

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3. Bratu T, Berceanu M, **Coricovac D* (corresponding author),** Crainiceanu Z, Gyori Z, Roman A, Marcovici I, Dehelean C, Pinzaru I. Melanin Quantification by Chemical Methods in Healthy and Melanoma Cell Lines Correlated with the Importance in the Development of Melanoma. Revista de Chimie 2020, 71(4), 430-437. https://doi.org/10.37358/RC.20.4.8084. **IF = 1.755.**

4. Iftode A, Draghici GA, Macasoi I, Marcovici I, **Coricovac DE* (corresponding author**), Dragoi R, Tischer A, Kovatsi L, Tsatsakis AM, Cretu O, Dehelean C. Exposure to Cadmium and Copper triggers cytotoxic effects and epigenetic changes in human colorectal carcinoma cell-line – HT-29. Experimental and Therapeutic Medicine – under revision. **IF = 1.785.**

**Diploma Thesis:**

1. Marcovici Iasmina (student), Dehelean Cristina (coordinator): Observații experimentale *in vitro* privind rolul melaninei în răspunsul celulelor melanomice la tratament

2. Brumar Roxana (student), Coricovac Dorina (coordinator): Compusi naturali cu efect supresor asupra tranzitiei epithelial-mezenchimale asociata procesului tumoral

3. Linca Alexandra (student), Coricovac Dorina (coordinator): Rolul aditivilor alimentari de tip edulcoranti in bolile cornice.

Project manager: Conf. Univ. dr. Dorina Coricovac