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Scientific report – Phase II – 2021

Project title**:** ***Delivery to the dermocosmetic market of a modern topical formulation with betulinic acid encapsulated in proniosomes***

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Introduction

The present project was aimed to obtain a novel pharmaceutical formulation based on betulinic acid (BA) encapsulated in proniosomes for topical application in different cutaneous pathologies as adjuvant treatment. The selection of BA as active compound for the topical formulation was based on the data found in the literature, but also on our scientific background on this subject. BA is a natural compound, a pentacyclic triterpene, found in the bark of birch tree, that exhibits potent anticancer activity in different types of cancer including skin cancers (both *in vitro* and *in vivo*) and a low/absent toxicity in healthy cells. The main handicap of this compound is represented by its low solubility in aqueous media, limiting its use *in vivo.* To overcome this issue, this project proposes the formulation of BA as proniosomes, formulation that improves both its solubility and bioavailability.

To accomplish the main objective of the project, the project was organized in seven different objectives that comprise two activities each, except for objective 6. The corresponding objectives for phases I and II are the first four objectives that will be further summarized.

Scientific and technical report

O.1. Synthesis and physicochemical characterization of proniosomes

A.1.1. Synthesis of the proniosomes – as nanoplatform for natural compounds with hydrophobic character

A.1.2. Physicochemical characterization: surface morphology, size distribution, zeta potential, and stability during formulation process and storage

O.2. **Synthesis and physicochemical characterization of proniosomes loaded with betulinic acid**

A.2.1. Synthesis and physicochemical characterization: surface morphology, size distribution, zeta potential, and stability during formulation process and storage

A.2.2. Assessment of the BA encapsulation efficiency of proniosomes and in vitro release profile

Applied methodology

The methods applied for the preparation and characterization (physicochemical and rheological) are standardized and internationally validated. The coacervation phase-separation

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method was performed for the preparation of the BA-based and blank proniosomal gels. The surfactants used to obtain the proniosomal gel formula are GRAS (Generally Recognized as Safe) substances, as follows: cholesterol, sorbitan monostearate (Span 60) and soy lecithin (Lipoid S 75). The method was conducted according to the protocols described in the literature and adapted to our laboratory conditions. The percentage of BA in the proniosomal gel was 0.3%.

The surface morphology of the proniosomal gels was assessed by the means of scanning electron microscopy – SEM (Hitachi SU8230 electronic microscope provided with a STEM module and EDX X-Max^N 80 detectors). The pH was measured by the means of Sension ™ 1 portable digital pH meter (Hach Company, U.S.A). The encapsulation efficiency was determined spectrophotometrically. The rheological features (flow behavior, viscosity, consistency, penetration degree and spreadability) were analyzed by a stress-controlled rheometer (RheoStress 1, HAAKE, France) equipped with a cone-plate geometry (1/60), a penetrometer (PNR 12, Petrolab, Germany) equipped with a micro-cone and suitable container, and the Pozo Ojeda-Sune Arbussa extensometer.

Results

- the obtained proniosomal gels are non-Newtonian systems with a pronounced pseudoplastic-thixotropic behavior (figure 1)

Figure 1. The visual appearance of the obtained pronisomal gels – Blank and BA (0.3%) after 6 months of storage.

- The pH value of the proniosomal gel containing betulinic acid was slightly lower (6.556±0.04) as compare to neutral pH, due to the acidic character of the drug, still the determined pH values were within the provided range for semisolid preparations (4.5-8.5) by the national pharmacopoeia suggesting that will be well tolerated by the skin

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- characterization of the rheological properties of the BA-based gel indicated a higher viscosity and thixotropy as compared to blank gel, a higher degree of penetration, but lower spreading capacity. All these properties fall within the specific parameters of semi-solid pharmaceutical preparations.

- the encapsulation efficiency of BA in the gel exceeded 50%.

- the blank gel presents slightly spherical agglomerated vesicles, being somewhat evenly distributed

- BA-based proniosomal gel showed a rather high viscosity compared to the blank, which may mean that the betulinic acid has been successfully encapsulated in the structure of the gel.

The methods used to obtain the proniosomal gel and those applied for its characterization were standardized and described in the article published by Pinzaru et al., Antioxidants (Basel) 2021 (partial funding from this research project).

O.3. In vitro toxicological profile of hollow and BA-loaded proniosomes

A.3.1. Assessment of selected test compounds impact on healthy human skin cells: keratinocytes, melanocytes, and fibroblasts.

A.3.2. Assessment of selected test compounds impact on 3D skin model: EpiDerm™ (MatTek In Vitro Science Laboratories)

Applied methodology

The toxicological profile of the proniosomal gels (blank and BA) was assessed using 3D human reconstructed skin tissues (EPI-200 - Figure 2; EPI-200-SIT and EPI-200-PHO) purchased from MatTek Corporation.

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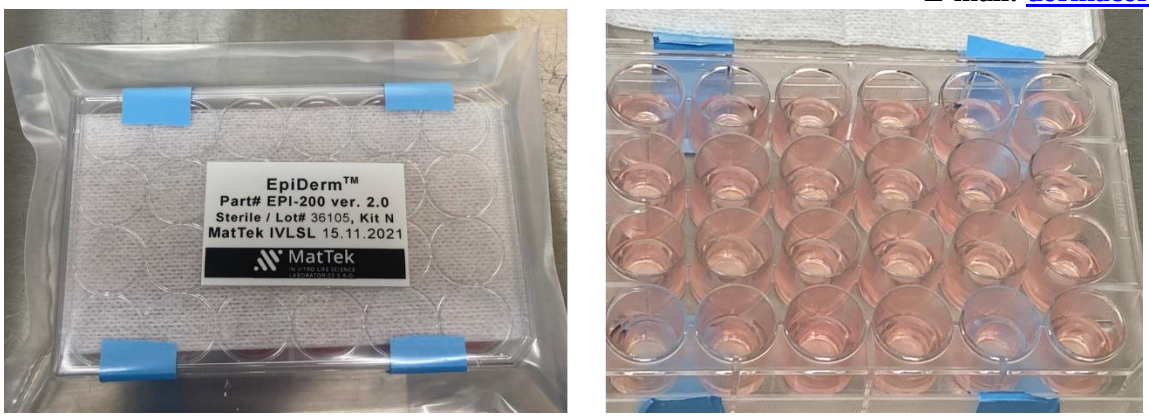

Figura 2. 3D human reconstructed skin tissues purchased from MatTek Corporation.

The *in vitro* experiments using 3D human skin tissues were performed according to the protocols provided by the manufacturer and the following parameters were analyzed: the impact of gels (blank and BA), but also of BA (powder) on tissue viability, irritant and phototoxic potential of them. Standardized techniques have been used, adapted to the specific conditions of 3D tissues, such as: MTT ((3- (4,5-Dimethyl-2-thiazolyl) -2,5-diphenyltetrazolium bromide) - measures the viability and LDH (lactate dehydrogenase) - measures cytotoxicity. The methods are detailed in the publication of Pinzaru et al., Antioxidants (Basel) 2021 (partial funding from this research project). The *in vitro* testing on healthy cell lines will be performed using proniosomes formulated as powder (an ongoing process at the present moment).

Results

- BA powder does not reduce the viability of 3D tissues and has no irritant or phototoxic potential
- blank proniosomal gel has a slight irritating effect (tissue viability approx. 80-85%), while BA gel slightly stimulates the viability of other tissues
- UVA exposure decreased the viability of tissues exposed to blank gel, while BA gel provided protection against the phototoxic effect induced by UVA.

These results together with those described in the previous objectives (preparation and characterization of proniosomal gels) will be disseminated in the form of a scientific article (in the drafting phase) which will be published in an international journal with high impact factor (over 5).

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O.4. *In vitro* pharmacological profile of hollow and BA-loaded proniosomes

A.4.1. Assessment of test compounds impact on skin cancer cell lines: A431 – human epidermoid carcinoma, TE354.T – human basal cell carcinoma and SK-MEL-31 – human primary melanoma.

A.4.2. In vitro metabolism of BA loaded niosomes using Hepa RG cells

Applied methodology

Assessment of the pharmacological profile of BA was performed using consecrated cell viability and cytotoxicity methods, as: MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and LDH (lactate dehydrogenase assay). To evaluate the changes induced at nuclear level was applied the Hoechst 33342 staining. The healthy cell lines (HDFa – human primary fibroblasts, HEKa – human primary keratinocytes, HEMa – human primary melanocytes, and HepaRG – human hepatocytes – figure 3) and the tumor ones (A431 – human epidermoid carcinoma, TE354.T – human basal cell carcinoma and SK-MEL-31 – human primary melanoma) were purchased from ATCC (American Type Culture Collection) and were cultured according to manufacturer's recommendations.

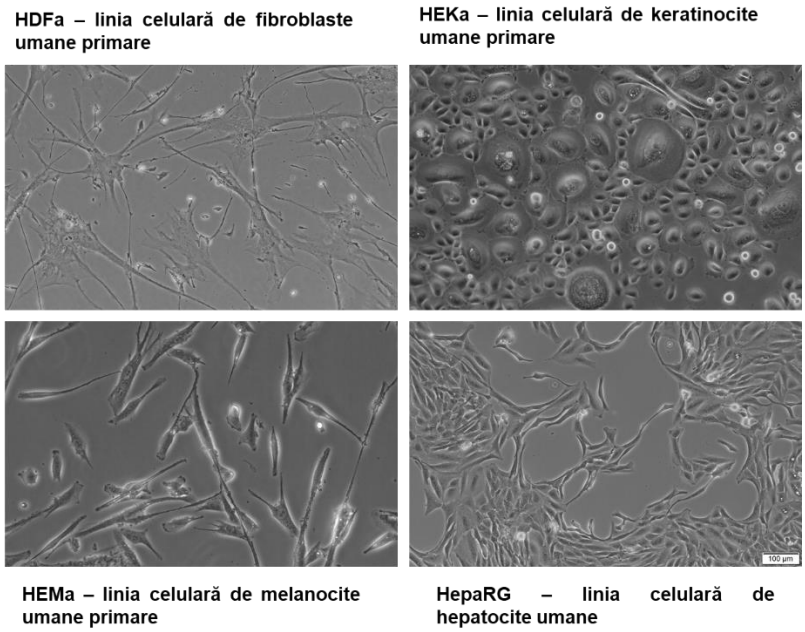

Figure 3. Human healthy cell lines studied

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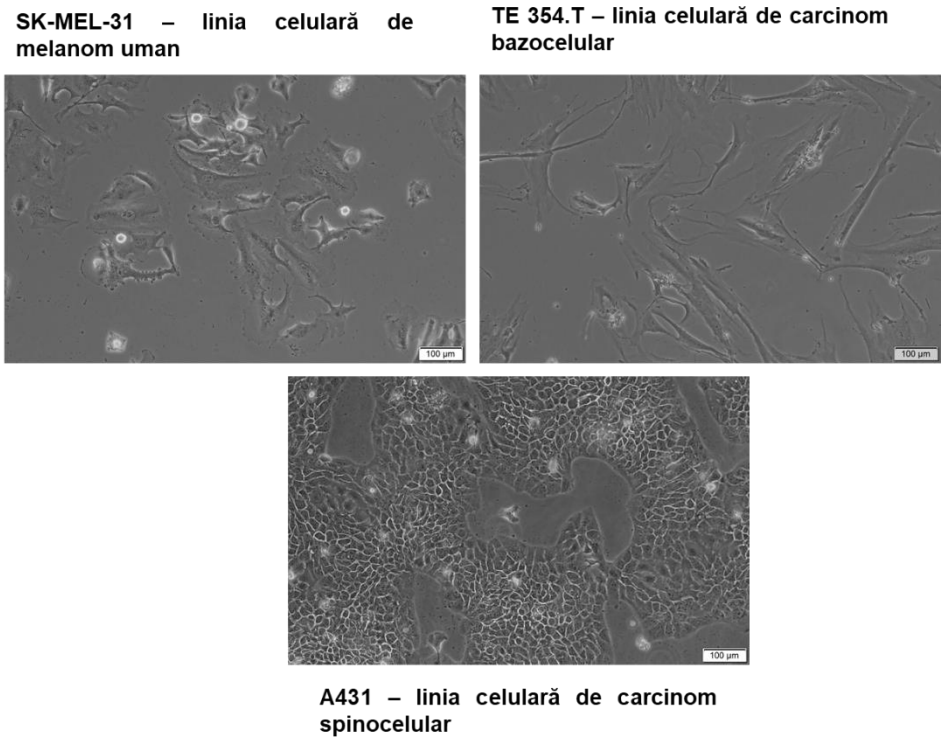

Figure 4. Human tumor cell lines studied.

The following parameters were monitored: cell viability (MTT and Alamar blue), changes in cell morphology, cytotoxicity (LDH) and changes in the nucleus (specific signs of apoptosis / necrosis) - Hoechst 33342.

Results

- BA in DMSO induces a dose-dependent toxicity in tested tumor cell lines - TE 354.T, A431 and SK-MEL-31, toxicity characterized by decreased cell viability, cytotoxicity, morphological changes (round cells floating in the environment, reduced confluence, cell debris) and apoptotic changes at the nucleus level (fragmentation of the nucleus, condensation of chromatin, etc.)
- BA in DMSO showed a selective cytotoxic action in healthy cells (HDFa, HEKa, HEMa, and HepaRG), which are significantly affected by the highest concentrations tested - 50 and 75 µM.

All the graphs and 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.

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Results dissemination

Conferences:

1. Pinzaru I, Marcovici I, Coricovac D, Macasoi I, Dehelean C. In vitro toxicological profile of rutin as antimelanoma agent. Farmacia: de la inovare la buna practica farmaceutica, Ed. Universitatii din Oradea, Congresul National de Farmacie editia a XVIII-a, 15-17 Septembrie 2021, pp.82. ISBN 78-606-10-2144-4 – oral presentation
2. Dehelean C. Coricovac D. Natural compounds a promising alternative for SARS-CoV-2 treatment. Farmacia: de la inovare la buna practica farmaceutica, Ed. Universitatii din Oradea, Congresul National de Farmacie editia a XVIII-a, 15-17 Septembrie 2021, pp.83. ISBN 78-606-10-2144-4. – oral presentation
3. Coricovac D, Marcovici I, Pinzaru I, Macasoi I, Vlaia L, Soica C, Dehelean C. Betulinic acid formulated as proniosomal gel for topical use in skin disorders. Farmacia: de la inovare la buna practica farmaceutica, Ed. Universitatii din Oradea, Congresul National de Farmacie editia a XVIII-a, 15-17 Septembrie 2021, pp.99. ISBN 78-606-10-2144-4. – oral presentation

Published articles:

1. Pinzaru I, Tanase A, Enatescu V, Coricovac D, Bociort F, Marcovici I, Watz C, Vlaia L, Soica C, Dehelean C. Proniosomal Gel for Topical Delivery of Rutin: Preparation, Physicochemical Characterization and In Vitro Toxicological Profile Using 3D Reconstructed Human Epidermis Tissue and 2D Cells. Antioxidants (Basel). 2021 Jan 10;10(1):85. doi: 10.3390/antiox10010085. **IF = 6.313**
2. Coricovac D, Dehelean CA, Pinzaru I, Mioc A, Aburel OM, Macasoi I, Draghici GA, Petean C, Soica C, Boruga M, Vlaicu B, Muntean MD. Assessment of Betulinic Acid Cytotoxicity and Mitochondrial Metabolism Impairment in a Human Melanoma Cell Line. Int J Mol Sci. 2021 May 4;22(9):4870. doi: 10.3390/ijms22094870. **IF = 5.924**
3. Pinzaru I, Sarau C, Coricovac D, Marcovici I, Utescu C, Tofan S, Popovici RA, Manea HC, Pavel IE, Soica C, Dehelean C. Silver Nanocolloids Loaded with Betulinic Acid

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