A PHARMACO-TOXICOLOGICAL EVALUATION OF BETULINIC ACID MIXED WITH HYDROXIPROPILGAMMA CYCLODEXTRIN ON IN VITRO AND IN VIVO MODELS

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Abstract
Betulinic acid (3β, hydroxy-lup-20(29)-en-28-oic acid, BA) is a pentacyclic triterpene previously tested on several cell lines of neuroectodermal origin and on some in vivo tumor models, such as the melanoma model. The aim of the present work was to test its activity by increasing its water soluble fraction with gamma type cyclodextrins (hydroxipropilgamma cyclodextrin). The study consisted in a preliminary in vitro evaluation on A431 and MCF-7 cell lines and in vivo tests on fertilized eggs. The dissolution capacity in presence of cyclodextrin was analysed by the phase diagram. The main conclusion was that betulinic acid possessed an important antiangiogenic activity and a significant cytotoxicity, especially on the A431 cell line.

Keywords: betulinic acid, skin pathology, cyclodextrin, cancer

Introduction
Betulinic acid (3β, hydroxy-lup-20(29)-en-28-oic acid, BA) is a pentacyclic triterpene previously tested on several cell lines of neuroectodermal origin and on different tumor cells [1, 2, 3]. Recent literature data revealed that this natural compound is found in various plants and can be isolated by extraction with organic solvents from different sources such as: Quisqualis fructus, Coussarea paniculata, Caesalpinia
paraguariensis, Ilex macropoda, Chaenomeles lagenaria, Tetracentron sinense, Syncarpa glomulifera, Zizyphus joazeiro, Syzygium claviforum and others [2,4]. Betulinic acid was selected a few years ago as a compound accepted in the RAID (Rapid Access to Intervention in Development) program [4]. In some cases its efficacy is completed by the anti-inflammatory activity. Its selectivity on pathologic cells was explained by different mechanisms including the low pH of cancer cells, the proapoptotic activity etc [2, 5]. Betulinic acid has a potential antitumor activity and has no toxicity [2]. It is active on in vivo models correlated with melanoma [2, 3]. The mechanism of its antiangiogenic activity is not fully known [2, 6]. In vitro antitumor activity was observed at doses in the micromolar range while in vivo effects were reported for doses over 250 mg/kg body weight [6, 7]. A major problem for betulinic acid is its low solubility, which is more important for testing its biological activity [2, 8]. Cyclodextrins are compounds known for the capacity of forming complexes with the active compound and thus increasing its solubility [8, 9]. QSAR (Quantitative structure-activity relationship) specific studies on betulinic acid proved that gamma type cyclodextrins are more appropriate for increasing the compound’s solubility [8]. The increased incidence of skin pathologies, including melanoma and skin cancer, is another argument in favor of the present study [10, 11]. The aim of the present study was to evaluate the pharmacotoxicological activity of the mixture of betulinic acid and hydroxipropilgamma cyclodextrin (HPGCD).

Materials and methods

Betulinic acid was purchased from Sigma Aldrich (Germany) and the cyclodextrins were purchased from Cyclolab Res. & Dev. Ltd., (Hungary).

Phase solubility evaluation

The aqueous solubility of betulinic acid at various concentrations of HPGCD was studied by the method reported by Higuchi and Connors [12]. Samples of betulinic acid in quantities exceeding its aqueous solubility were shaken at room temperature with aqueous solutions of HPGCD in increasing concentrations (0-300 mmol/L), for a period of five days, until equilibrium was established. The solutions were then filtered and analysed by UV spectrophotometry at 210 nm.

The apparent stability constant can be calculated from the solubility data, using the following equation:
\[
K = \frac{\text{Slope}}{S_0(1 - \text{slope})}
\]

where \(S_0\) is the intrinsic solubility of BA and the \text{slope} is the slope of the solubility diagram.

Complex preparation
For complex preparation the kneading procedure (Kp) was applied and the chosen ratio for active compound and cyclodextrin was 1:2 as it was suggested from previous physico-chemical evaluations [8, 13]. First, it was performed a simple powder mixing using the mortar and pestle and then the kneading with 50% ethanolic solution until the bulk of solvent evaporated. The mixture was then dried at room temperature for 24 hours and after that it was heated in the oven, at 105°C for several hours. The final product was pulverized and sieved.

Electronic scanning microscopy (ESM)
Particle morphology was examined using an electronic scanning microscope Hitachi 2400S (Hitachi Scientific Ltd, Japan). A thin-layer covering device (Bio-Rad SC 502, VG Microtech, England) was used to obtain an electric conductivity to the surface of the sample. Air pressure was 1.3-13.0 mPa.

Chorioallantoic Membrane (CAM) Assay
Fertilized chicken eggs preparation was performed according to methods from the literature [14]. Fertilized chicken eggs were placed into a special incubator as soon as embryogenesis started and were kept under constant humidity at 37°C. On the first days of incubation (day 3), 3-4 mL albumen from the pointed end of the egg were removed, so that the developing chorioallantois can detach from the shell itself and the underlying CAM vessels are disclosed. The next day a window was cut into the shell with the aid of scissors. The opening was closed with a silk tape and incubation went on until the day of the experiment (day 6). One \(\mu\)L of a 20 mM betulinic acid/HPGCD solution was applied.

MTT-assay.
Antiproliferative effects of the test compound were measured \textit{in vitro} on the following human cell lines: MCF7 (breast adenocarcinoma) and A431 (skin epidermoid carcinoma), by using the MTT ([3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay [15]. Cancer cells (5000/well) were seeded onto a 96-well microplate and attached to the bottom of the well overnight. The process continued on the second day when 200 µL of new medium with the test substances was added. After incubation for 72 h, the living cells were assayed by the addition of 20 µL of 5 mg/mL MTT ([3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide]) solution. MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a period of minimum 4 h contact. The medium was then removed, and the precipitated crystals were dissolved in 100 µL of dimethyl sulfoxide during a 60 min period of shaking. Finally, the reduced MTT was assayed at 545 nm, using a specific microplate reader; wells with untreated cells were used as controls samples. All in vitro experiments were carried out on microplates with a number of parallel wells. Stock solutions of the tested substances were prepared in dimethyl sulfoxide (DMSO) solutions and the highest DMSO concentration (0.3-0.5%) of the medium didn’t affect significantly the cell proliferation. The active compound with cyclodextrin was dissolved in water and DMSO (10 mM).

Results and discussion

Phase solubility curve

Fig. 1 shows the phase solubility diagram of BA with HPGCD in water.

![Phase solubility curve](image_url)

**Figure 1.**
Phase solubility curve for the mixture of betulinic acid (BA) and gamma type cyclodextrin (HPGCD)
According to the method introduced by Higuchi and Connors, an A_L type curve was obtained for BA with HPGCD and the slope of the diagram was less than 1, which leads to the conclusion that a 1:1 inclusion complex was formed.

The following equation is obtained by linear regression:

\[ y = 0.0083x + 0.356 \]

where \( x \) = HPGCD concentration (mmol/L)
\( y \) = BA concentration (mmol/L).

The correlation coefficient was \( r = 0.9940 \) (\( r^2 = 0.9881 \)).

The stability constant, calculated according to the equation of Higuchi and Connors was \( 22.38 \text{ M}^{-1} \). The phase solubility analysis lead to the conclusion that a 1:2 complex preparation would increase the BA solubility.

ESM images present a detailed aspect for betulinic acid, the applied cyclodextrin and their 1:2 Kp (kneading procedure) complex. (Fig. 2)

![ESM images](image)

**Figure 2.**
Betulinic acid (A), the gamma type cyclodextrin (B) and their 1:2 Kp complex (C) presented as ESM images

The image (Fig. 2) shows the differences between the pure substance and its complex. The kneading procedure is an easy and efficient method for
complex preparation. Betulinic acid (sample A) has irregular, rounded shape crystals. In sample B there can be seen smaller particles. In the complex (sample C) there can be seen crystals with column or plated shape, which are formed during preparation. The changes in betulinic acid properties could improve its biological availability especially in the presence of water.

Figure 3.
Evolution of the angiogenesis: initially (A), after 2 days (B) and at the end of the experiment (C) following the application of the active compound.

Figure 4.
Detailed image of the blood vessels after the application of the betulinic acid preparation

The results and images obtained from the in vivo test confirm the antiangiogenic activity of the tested compound, in agreement with literature data. In figure 3, a strong antiangiogenic effect can be observed for the betulinic acid solution (A₂, B₂). However, the underlying mechanisms of action for these responses are unknown. It is known that BA affected and influenced the endothelial sprouting, the capillary network formation [2, 16]. Staining for both VEGF (vascular endothelial growth factor) and CD31
(cluster of differentiation) (microvessel density) was decreased in mouse tumor model treated with betulinic acid. The mechanisms of its activity, more specific of its antiangiogenic one are associated with induction of proteasome-dependent degradation of surfactant proteins (Sp) in prostate tumor cells. More detailed, Sp protein expression was decreased in SK-MEL2, indicating that the betulinic acid–dependent down-regulation of Sp proteins was correlated with both proapoptotic and antiangiogenic responses and represented an initial description of the mechanism in the betulinic acid anticarcinogenic activity [16, 17]. Our data confirmed its intense antiangiogenic potential that is sustained by the reduced period of embryo surviving (around 11 days) (Fig.3 and Fig.4).

The addition of cyclodextrin to the formulation didn’t change this compound’s property but offered the possibility of its dissolution in an adequate solvent (distilled water) for biological application. The detailed vessel image has shown the antiangiogenic activity and the possible damages at the level of blood vessels, aspect analysed for the antitumor mechanism confirmation.

![Inhibition of proliferation](image)

**Figure 5.**

*In vitro* cytotoxic activity of betulinic acid in different cell lines

It is known that BA is a selective compound on melanoma cells such as: MEL-1, MEL-2, MEL-4 and active on other cells that are related to melanoma [1, 2, 18]. An important effect of the betulinic acid is the citotoxicity on B16 (mouse melanoma) cells in concentrations of µg/mL [7]. Even though the most important reports included its antimelanoma activity it is also cited for the activity on neuroblastoma, leukemia, glioblastoma, head, neck, lung and prostate cancer etc [2]. The *in vitro* tests confirmed the literature data regarding the cytotoxic activity of betulinic acid which is significant on cells related to skin pathology. The high activity on A431
(skin carcinoma call line) indicated the compound as an important antitumor one, effect that is maintained even if the concentration in the biological environment is changed. This aspect could be related to the latest studies that confirm the inhibitory potential of betulinic acid in two-stage mouse skin carcinogenesis [2]. The dissolution with cyclodextrins didn’t change its antitumor activity that is higher and easily detectable. Compared to literature data, betulinic acid still proved its important citotoxicity on tumor cells.

Conclusions
Betulinic acid is an important antitumor compound and could be used in skin pathologies including skin cancers. Its dissolution in an aqueous environment can be improved by different methods including cyclodextrin complexation. The gamma type cylodextrins are useful for this aim. It is a strong antiangiogenic compound and implicitly an antitumor substance. All the correlations and tests confirmed once again the importance of betulinic acid therapeutic activity. An adequate formulation can be useful for the preparation of various formulations for in vivo administration.

Acknowledgements
This work was supported by CNCSIS-UEFISCSU, project number PNII-IDEI code 1257/2007

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Manuscript received: March 4th 2010