ANTITUMORAL ACTIVITY OF BETULIN, A COMPOUND PRESENT IN BIRCH TREE, IN FORMULATIONS WITH CYCLODEXTRIN

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ABSTRACT. Betulin is a triterpene with an important therapeutic activity even on tumour treatment. It is very low soluble in water and biological environment. The increasing of its solubility by cyclodextrin complexation could lead to an improvement in the biological activity. Materials and methods used betulin and 6-O-maltosyl-beta-cyclodextrin and a simple kneading procedure. The compound and final product were evaluated by SEM pictures, in vitro tests on A431 cell line and on an embryonated egg model. Results and discussions indicated the important activity of betulin as antitumor agent and an antiangiogenic activity. The mixture with the ramified cyclodextrin seems to be a complex formation because of the shape of compounds changes. The complexes had a better biological activity. The main conclusion is that betulin is an important antitumor compound and increasing of its solubility could improve the biological activity.

Keywords: betulin, cyclodextrins, antitumor, angiogenesis

INTRODUCTION
Betulin is a triterpenoid related to vegetal sources such as birch tree that contains higher amounts of this compound in the outer bark (in some types over 40%) (Muceniece R. et al., 2007; Mullauer F.B. et al., 2009). It possesses important anti-inflammatory and anticancer actions. Its activity was studied correlated with a very close structure, betulinic acid c (Muceniece R. et al., 2007; Patocka J., 2003). Their intervention in skin pathology such as melanoma and skin cancer is remarkable (Muceniece R. et al., 2007; Fulda S., 2008). The last details about their mechanism of activity on melanoma cells refer to mitogen-activated protein kinase pathway (Muceniece R. et al., 2007; Mullauer F.B. et al., 2009). They are considered also phospholipase A2 inhibitors and modulators in the production of interleukin-10, interferon gamma, etc (Muceniece R. et al., 2007). Betulinic acid is a propapoptotic agent but betulin seems not to have a fully described mechanism (Muceniece R. et al., 2007; Pisha E. et al., 1995). Betulin seems to avoid the involving of death receptor pathway but is dependent on the mitochondria (Muceniece R. et al., 2007; Mullauer F.B. et al., 2009).

MATERIALS AND METHODS
Betulin was purchased from Sigma Aldrich (Bucharest, Romania-Redox) and cyclodextrins from Cyclolab Res. & Dev. Ltd., (Hungary). All materials were used as received.

1. Complex preparation
For complex preparation was applied kneading procedure. The ratio for active compound and cyclodextrin was 1:2 as was suggested from previous physico-chemical evaluations (Szejtli J., 1998; Soica C.M. et al., 2008). First we prepared a simple powder mixing on mortar with pestle and then the kneading with 50% ethanolic solution until the bulk of solvent evaporated. Then, the mixture was dried at room temperature for 24 hours and after that was put in the oven, at 105°C for several hours. The final product was pulverized and sieved.

2. Scanning electronic microscopy
Particle morphology was examined using electronic scanning microscope Hitachi S4700 (Hitachi Scientific Ltd, Japan). A thin-layer covering device (Polaron E 5100, Bio-Rad Microscience Division, England) was used to obtain an electric conductivity to the surface of the sample. Air pressure was 0.1 Torr (13332 mPa).

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3. CAM assay

Fertilized chicken eggs preparation used known methods from the literature into a special incubator as soon as embryogenesis starts and was kept under constant humidity at 37°C (Ribatti D. et al., 2003). On the first days of incubation (day 3), was removed 3-4 ml albumen at the more pointed end of the egg, so that the developing chorionallantois 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 is closed with a silk tape and incubation goes on until the day of the experiment (day 5). The applied solution was a 0.01 mg/ml betulin/6-O-maltosyl betacyclodextrin, 1μl.

4. MTT-assay

Antiproliferative effects of the test compound were measured in vitro on the A431 (skin epidermoid carcinoma) cell line, by using the MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay (Mosmann T. et al., 1983). Cancer cells (5000/well) were seeded onto a 96-well microplate and attached to the bottom of the well overnight. The process continues on the second day when 200 μL of new medium with the test substances was added. After an incubation time 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. The medium was then removed, and the precipitated crystals were dissolved in 100 μL of dimethyl sulfoxid 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 utilized as controls samples. The active compound with cyclodextrin was dissolved in water and DMSO from a stock solution 0.01 mg/ml.

RESULTS AND DISCUSSIONS

The SEM pictures for betulin, cyclodextrin and their complexes indicated important shape changes (Figures 1, 2).
Antitumoral activity of betulin, a compound present in birch tree in formulations with cyclodextrin

The SEM images show differences between the pure substances and their complex. Betulin is an acicular substance with a specific shape, different from ramified cyclodextrin who looks like dense sand. The complexes are different comparing with the initial substances aspect that suggests the possible complex formation. Any changes in the initial form indicated a physico-chemical transformation that is important in complex formation observations.

Betulin complexed with cyclodextrin is involved in antinagiogenic activity (Fig. 3), aspect mentioned in our previous studies (Feflea S. et al., 2009) and it remains on this field of activity even it is dissolved in other way. The activity of tested compound was observable even from the first days of experiment. It induced a relative shorter survival period (incubation day 14) of embryo compared to the blank specimen (incubation day 17), and it caused a decrease number of capillaries. Analyzing the immunostained (with antigen anti smooth muscle actin) chorioallantoic membrane modification of both mesenchyme and epithelium are observed. This can explain in part the indirect antiangiogenic effect, by the densification of the stroma (intense positive reaction to smooth muscle actin) correlated with the low number of blood vessels.

**CONCLUSIONS**

Betulin is an important antitumor compound that is active on skin pathology. It is a very low water soluble compound that could be mixed with cyclodextrins to form more active complexes.
The ramified cyclodextrins can be helpful in modulations of triterpenes activity for improving their physical and biological properties. The antiangiogenic activity of betulin can influence the antitumor intervention and detailed studies will be able to show all the mechanism details.

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