

Assessment of the Effects Induced by Two Triterpenoids on Liver Mitochondria Respiratory Function Isolated from Aged Rats

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Betulin and betulinic acid are natural compounds widely distributed in the plant kingdom, known to exhibit multiple biological activities, including: antitumoral, anti-inflammatory, antiangiogenic, anti-HIV, immunomodulatory and hepatoprotective. The aim of this study was to evaluate the effects of betulin and betulinic acid formulated as complexes with cyclodextrins on mitochondrial respiration using mitochondria isolated from the liver of aged rats. The mitochondria were isolated by the differential centrifugation technique and the oxygen consumption studies were measured using a Clark-type electrode. The respiratory parameters evaluated were basal respiration (State 2), active respiration (State 3) and the respiration control ratio (RCR).

Keywords: betulin, betulinic acid, GCDG, mitochondria, aging

Pentacyclic triterpenes represent a class of natural compounds with a plethora of pharmacological activities intensely studied in the past years. Betulin (Bet - 3-lup-20(29)-ene-3 β ,28-diol) and its oxidized form, betulinic acid (BA-3 β -Hydroxy-lup-20(29)-en-28-oic acid) are the most representative compounds of this class, based on the great number of publications focused on this subject. The two natural compounds, Bet and BA possess a lupan-type chemical structure and can be obtained from natural sources such as the birch tree bark (betulin can be obtained in yields up to 30%) or by chemical synthesis, especially, BA from Bet via an oxidation reaction [1, 2].

BA attracted the interest of the researchers, especially, due to its capacity to induce apoptosis in a variety of cancer cells and the absence or its very low toxicity on normal cells, both *in vitro* and *in vivo* [3-5]. Besides its potent anticancer activity, BA was described to exhibit other pharmacological effects, including: anti-inflammatory, antiangiogenic, immunomodulatory, anti-HIV, anti-bacterial, hepatoprotective, protective against cerebral ischemia-reperfusion injuries [2, 6]. Bet in comparison with BA has a lower potency as anticancer agent, but was presented as an active inhibitory compound in processes, such as: inflammation, angiogenesis, hepatotoxicity and viral and bacterial pathologies [7].

A major handicap of these compounds is their very low solubility in aqueous solutions what limits their parental administration *in vivo*. Considerable efforts were made to find a proper formulation in order to increase their solubility and their bioavailability, respectively. On this line, there was proposed the inclusion of BA and Bet in cyclodextrins for

obtaining complexes with increased solubility in water [8, 9].

Aging is a biological process characterized by a gradual decline of physiological functions and metabolic processes generated by the cumulative oxidative damage, the repercussion of reactive oxygen species (ROS) activity. A key role in the process of aging is played by mitochondria since these organelles are on one hand the primary sources of ROS and on the other hand the target of ROS effects [10, 11].

The purpose of this study was to investigate the effects of Bet and BA formulated as complexes with gamma-cyclodextrins on liver mitochondria respiratory function, mitochondria isolated from senescent rats.

Experimental part

Material and methods

Animals

In this study were used senescent Sprague Dawley male rats (20-24 weeks old) with the body weight ranging from 400-450 g. The experimental procedures and protocols that were applied in the present study were in agreement with the European Directive 2010/63/EU regarding the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Research Ethics of “Victor Babeş” University for Medicine and Pharmacy of Timișoara, Romania. The animals received food and water *ad libitum* and were kept in the University animal facility under standard conditions (constant temperature of 22.5 \pm 2°C and relative humidity of 55% \pm 5%, 12 h (light) – 12 h (dark) cycle).

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Reagents

The reagents used for mitochondria isolation, incubation and oxygen consumption studies (KCl, mannitol, sucrose, HEPES, BSA – bovine serum albumin, EGTA, KH_2PO_4 , MgCl_2 , ADP - Adenosine 5'diphosphate potassium salt, FCCP - Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazon, L-Glutamic acid sodium salt, L-Malic acid, oligomycin, rotenone, and succinate disodium salt - hexahydrate) were purchased from Sigma-Aldrich, Germany and Merck and were of analytical purity. Betulinic acid (BA) and betulin (Bet) were purchased from Sigma Aldrich Ltd. (Taufkirchen, Germany, purity over 99%) and the octakis-[6-deoxy-6-(2-sulfanyl ethanesulfonate)]- α -CD (GCDG) was synthesized at the University of Saarland and received as a gift.

Preparation of BA and Bet complexes with GCDG

The inclusion of betulinic acid (BA) and betulin (Bet) in gamma-cyclodextrins was performed using the kneading method based on physical mixtures of BA/Bet and GCDG obtained by mixing the compounds with the same quantity of a solvent mixture consisting of ethanol and water (1:1). The protocol used was described in our previous work [9, 12, 13]. The binary products were prepared using a molar ratio of 1:1 [12, 13].

Mitochondria isolation

The isolation liver mitochondria protocol was described in one of our previous articles [14] and also in the literature [15]. In brief, the mice were sacrificed after they were anesthetized with a mixture of xylazine (5 mg/kg body weight) and ketamine (20 mg/kg body weight) that was administered intraperitoneally. The livers were immediately excised, cleansed of fat, blood and connective tissue and rinsed in ice-cold 0.9% KCl solution. The liver mitochondria were isolated in a specific media (210 mM mannitol, 70 mM sucrose, 10 mM HEPES - pH = 7.4; improved with 125 mg BSA 5 mg/mL and 0.25 ml EGTA 1 mM) using the differential centrifugation technique [15]. Protein concentration was obtained by the means of Biuret method using as standard BSA. The final concentration of proteins used for the oxygen consumption studies was 1 mg/ml [14, 16].

Oxygen consumption studies

The mitochondrial respiratory parameters were measured using a Clark-type electrode (Strathkelvin 782 Oxygen System) at 37°C. The measurements required the

addition of mitochondria suspension (1 mg mitochondrial protein /mL) into the incubation media consisting of 100 mM KCl, 2 mM KH_2PO_4 , 10 mM HEPES and 1 mM MgCl_2 , pH=7.4. The respiratory parameters evaluated in this study were: State 2 (basal respiration), State 3 (active respiration) and RCR (respiratory control ratio – calculated as the ratio between State 3 and State 2).

The protocol of oxygen consumption studies consisted of the following sequence of additions: addition of Complex I (glutamate – G, 10 mM and malate – M, 2 mM) dependent substrates and measurement of basal respiration (State 2); addition of the test compounds; active respiration was determined by subsequent ADP addition (State 3 – oxidative phosphorylation); cytochrome c was added in order to assess mitochondria integrity; ATP synthase was inhibited by oligomycin and uncoupled respiration was obtained by FCCP titration in steps of 0.1 μM . Data are expressed in $\text{pmol O}_2 \cdot \text{s}^{-1} \cdot \text{mg}^{-1}$ [14, 15].

Statistical analysis

Our results are presented as mean and standard deviation and were analyzed using one-way ANOVA and Tukey's post hoc analysis.

Results and discussions

For the evaluation of the effect of the two compounds, BA and Bet formulated as complexes with gamma-cyclodextrins on liver mitochondria respiratory function, we isolated mitochondria from the livers excised from aged Sprague Dawley male rats and we added in the electrode chamber two different concentrations of the test solutions: 1 and 3 μM . The respiratory parameters measured were: State 2 (basal respiration) – in the presence of glutamate (G) and malate (M), the substrates of complex I, State 2 – active respiration – after the addition of ADP and RCR – calculated as the ratio between of State 3 and State 2.

Our results showed that addition of 1 μM Bet solution led to slight increase of the State 2, but not statistically significant as compared to control group (fig. 1). BA (1 μM) addition had no influence on the State 2 respiration. The two compounds induced an improvement of State 3 respiration as compared to control group, an increase of this parameter being observed also after addition of the cyclodextrin itself, so we could not say at this moment if the cyclodextrin or the compounds are responsible for the effect on State 3 respiration. In the case of RCR there were no significant changes between the groups (fig. 1).

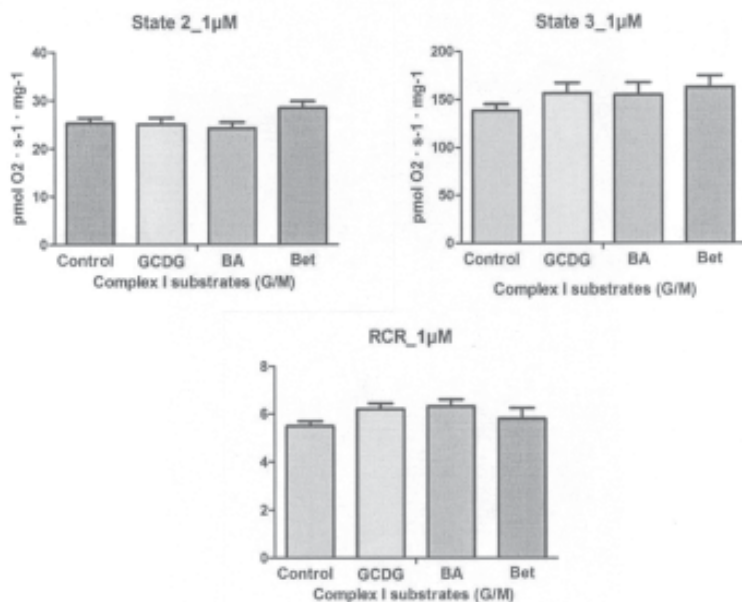


Fig. 1. Quantification of respiratory parameters in presence of Complex I dependent substrates (glutamate/malate) after addition of 1 μM of test compounds: BA, Bet and GCDG.

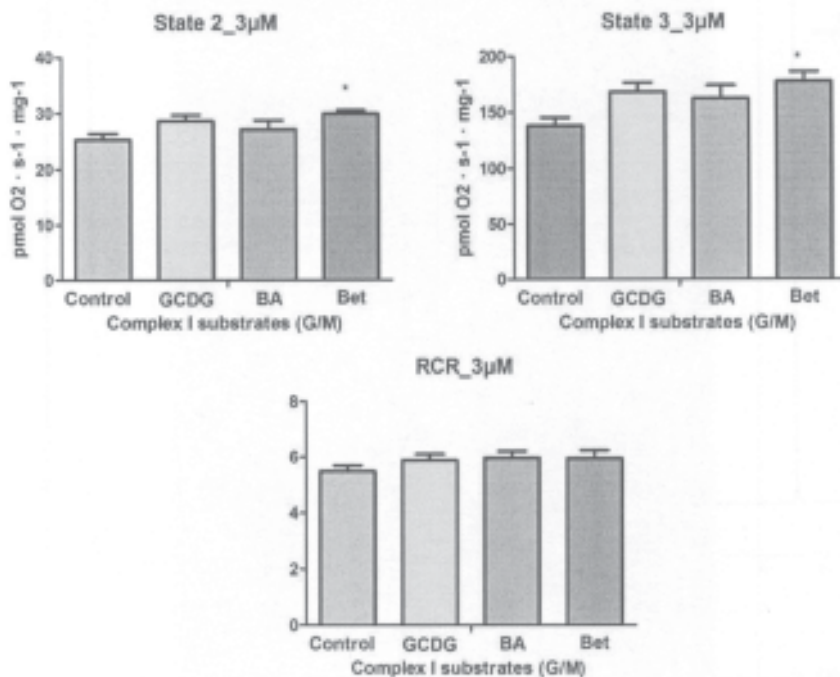


Fig. 2. Quantification of respiratory parameters in presence of Complex I dependent substrates (glutamate/malate) after addition of 3 μ M of test compounds: BA, Bet and GCDG

Since no eloquent changes were observed after addition directly into the electrode chamber of a 1 μ M solution of test compounds, we decided to verify a higher concentration, 3 μ M. In this case a significant increase of State 2 respiration was recorded after the addition of Bet solution as compared to the control (fig. 2). As mentioned for the lower concentration, BA had no effect on this parameter. Regarding the State 3 respiration, there was seen a significant augmentation of the parameter after Bet addition, an increase being also recorded in the case of BA and GCDG but not statistically significant as compared to control. RCR is directly proportional with State 2 and State 3, therefore no important changes of the two parameters are associated with no changes of the values of this parameter, too.

One of the reasons that determined us to test the effects of this compounds on liver mitochondria respiratory function was represented by the fact that mitochondria is a key player in the antitumoral mechanism of action of BA and Bet [4] and, moreover, the hepatoprotective mechanism of these compounds is not fully elucidated.

In one of our previous studies, we showed that the liver mitochondrial respiratory function of aged rats was impaired as compared to the adult rats, the impairment consisting in a significant decline of State 2 and State 3 [17]. There are also other studies which affirm that aging affects liver mitochondrial function, together with a decline in the intrinsic metabolic activity of the hepatic parenchyma, and in the gene expression of proteins involved in intermediary metabolism and drug metabolism [18, 19]. Ventura and co-workers proved that the Complex I activity was significantly decreased in liver mitochondria from aged rats (30 month-old) [20].

According to our results, we could affirm that addition of Bet solution increased the mitochondrial respiratory parameters, basal respiration (State 2) and oxidative phosphorylation (State 3) in the presence of Complex I dependent-substrates, glutamate and malate, what could be associated with a hepatoprotective effect. When it was added BA+GCDG complex solution (both concentrations 1 and 3 μ M), we detected a mild effect on the State 3 since State 2 wasn't affected by the action of BA.

Betulin proved its hepatoprotective effects against Cd-induced cytotoxicity in human hepatoma cell lines by

blocking the production of ROS induced by Cadmium [21]. Furthermore, a birch bark extract (BBE) with a high content in betulin (75%) was administered to patients with chronic hepatitis C and the results were characterized by reduced values of hepatic enzymes (ALT) [22].

In a previous study, we showed that topical application of a betulin nanoemulsion improved the liver mitochondria respiration using liver mitochondria isolated from mice with two-stage skin carcinogenesis and topically treated with betulin nanoemulsion [16].

The inclusion of betulin in a complex with gamma-cyclodextrins denoted an augmentation of betulin as anticancer agent by decreasing tumor growth to C57BL/6J mice inoculated with murine melanoma cells [8].

Besides its anticancer, anti-inflammatory and antiangiogenic effects, BA is known as a compound with protective effects on liver cells. It was demonstrated that pretreatment with BA and Bet reduced the toxic effects of acetaminophen and ethanol, Bet exhibiting a stronger effect than BA [23]. These data are in agreement with our results that show a stronger effect of Bet on mitochondrial respiration than BA. In another study, Zheng and coworkers showed that BA counteracted the hepatic toxicity associated with D-galactosamine and LPS (lipopolysaccharide) [24].

In one of our recent studies, we indicated that BA+GCDG complex administered daily intraperitoneally for 18 days at C57BL/6J injected with murine melanoma cells induced an increase of both basal and active respiration as compared to control group [12] in the presence of both complex I and complex II dependent-substrates.

Conclusions

Our data showed that addition of Bet complex with gamma-cyclodextrin in the electrode chamber on liver mitochondria isolated from aged rats induced an increase of State 2 and State 3 respiratory parameters in the presence of glutamate and malate as compared to control group. No significant changes were detected when BA complex solution was added. These results represent a possible mechanism of action for the elucidation of Bet hepatoprotective effects not only against different noxious agents, but also against aging.

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