Project description

Title: New insights into the antimelanoma mechanism of action of betulinic acid

Cancer still represents one of the leading causes of death worldwide [1]. Taking into account the increased incidence and the high mortality rate of cutaneous malignant melanoma, this disease is still considered to be a great matter of concern. Albeit, melanoma represents only 5% of all skin cancers, its aggressiveness and its increased potential to metastasis, makes it the deadliest type of skin cancer [2, 3]. According to the EUCAN reports from 2012, in Romania, the number of the cutaneous malignant melanoma cases in terms of incidence (I), mortality (M) and 5-year prevalence (P) were as follows: for women - I number = 593; M number = 175 and 5-year P = 2034 and for men – I number =528; M number = 189 and 5-year P = 1857 [4].

Melanoma originates from the malignant transformation of the melanocytes and the prognosis and survival of melanoma patients is established based on several items, such as: early detection, diagnosis and treatment; an early diagnosis (stages I and II) being associated with a 5-year relative survival rate of about 98.2%, whereas for stages III and stages IV the survival rate decreases to 61.7% and 15.2%, respectively [3, 5]. It is well known that melanoma is resistant to conventional therapies, especially, in advanced stages; the response rate to the action of single therapy was described to be of only 15-30% and the median duration of response was of few months [6]. Moreover, the side effects associated with the administration of the chemotherapy and radiotherapy are severe and decline the compliance of the patients to these types of treatment.

A considerable amount of research papers were published in order to elucidate the molecular mechanisms involved in the development and progression/metastasis of melanoma and to find an effective curative treatment, but there are still some gaps that need to be filled concerning this subject. As alternative therapy for melanoma were proposed compounds of natural origin that proved to be active and their side effects were minimized as compared to the conventional therapy.

Betulinic acid (BA), a compound of natural origin, member of the pentacyclic triterpenes family, became an actor into the spotlight of melanoma research field starting with 1995, when Pisha and collaborators, proved that this compound, selected from 2500 plant extracts during a drug screening program conducted by the American Cancer Institute, exhibited selective antimelanoma effects both *in vitro* (against several human melanoma cell lines) and *in vivo* [7, 8]. Further studies (at present there were obtained 1064 results after a search on PubMed of the keyword “betulinic acid”) were developed in order to explore the pharmacological effects of BA and the data obtained showed that this compound, not only exhibited a potent and broad antitumor activity against multiple tumors of different origin (human and murine melanoma, neuroblastoma, medulloblastoma, breast cancer, pancreatic cancer, leukemia, lung cancer, colon cancer, etc.) [8], but also exerted a panel of other biological effects, including: antiangiogenic [9], anticoagulant and anti-inflammatory [10], immunomodulatory, antiviral, hepatoprotective [8], protective effects against the diabetic neuropathy induced by streptozocin [11] and some others. Moreover, it was demonstrated that BA has no toxic effects neither *in vitro* on normal cells, nor *in vivo* even at doses of 500 mg/body weight [7, 8].

A great interest was assigned to the discovery of the antitumoral mechanism of action of BA and some hypotheses were proposed and proved, but the mechanism is far to be fully elucidated: (i) induction of apoptosis via mitochondrial pathway independent of cell death - CD-95 ligand/receptor and wild-type p53 protein in tumor cells of neuroectodermal origin (neuroblastoma, medulloblastoma, Ewing's sarcoma and glioma), by triggering mitochondrial permeability transition, activation of caspases cascade, new protein synthesis, reactive oxygen species (ROS) formation and nuclear fragmentation [12-14]; (ii) cell death via release of cytochrome c from the mitochondria, formation of the apoptosome, activation of caspases, PARP cleavage and DNA fragmentation in Jurkat cells [15] and (iii) inhibition of different tumor cells growth (lung, breast, colon, pancreatic and prostate cancers) by downregulating the expressions of Sp-specificity protein factors (Sp1, Sp3 and Sp4) and that of Sp-regulated genes (survivin, vascular endothelial growth factor – VEGF, p65 - subunit of NF-κB, epidermal growth factor – EGF and cyclin D1) involved in key processes such as: cell survival, proliferation and angiogenesis [16-20].

Regarding the BA's antimelanoma mechanism of action, there were discovered the following effects: (i) induction of apoptosis by producing cytoplasmic shrinking, surface blebbing and DNA fragmentation [7]; (ii) involvement of MAPK (Mitogen-activated Protein Kinase) proteins in BA-induced apoptosis, characterized by activation of p38 and stress activated protein kinase/c-Jun NH2-terminal kinase in response to ROS generation, a gradual depolarization of mitochondrial membrane potential without the activation of caspases [21]; (iii) apoptosis via intrinsic pathway by releasing cytochrome c from the mitochondria and DNA fragmentation in CD95-resistant and CD95-sensitive melanoma cells independent of Bax/Bcl-2 ratio (proapoptotic/ antiapoptotic proteins from Bcl-2 family) [22] and (iv) apoptosis by targeting cdk4 protein with role in cell cycle, a player in ERK signaling pathway [23]. The aforementioned data concerning the BA's antimelanoma mechanism of action are not considered enough to offer a clear and complete overview of the signaling pathway involved in BA's activity, therefore are required further studies to gather new insights into the pathway in order to elucidate it.

Among the drawbacks of BA it could be cited its low solubility in aqueous solutions, fact that led to a limitation of BA administration in vivo and to numerous studies focused on finding a proper formulation for this compound. Our group contributed to the progress of the research in this direction by offering important data of the BA's novel formulations behavior both *in vitro* and *in vivo*: (i) BA complexed with a gamma-cyclodextrin decreased the proliferation and *in vivo* tumor development of murine melanoma cells – B164A5 and improved the respiratory function of liver mitochondria isolated from the mice with murine melanoma in the presence of both complex I and complex II dependent substrates [24, 25] and (ii) BA formulated as nanoemulsion exhibited an antiangiogenic effect *in vivo* on embryonated eggs by using chorioallantoic membrane (CAM) assay [9].

Based on the data existent in the literature and our own background, the present project aims to provide new insights into the BA's antimelanoma mechanism of action in order to fill the existent gaps by trying to find a connection between the pro-apoptotic, antiangiogenic and antimetastatic effects exerted by this compound. The present project proposes three different approaches in order to gain insights into the molecular antimelanoma mechanism of action of BA:

(i) antitumoral approach via induction of apoptosis; (ii) antiangiogenic approach via VEGF, specificity proteins and EGFR signaling pathway and (iii) antimetastatic approach via epithelial to mesenchymal transition (EMT) and matrix metalloproteases (MMPs).

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