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Antiproliferative and proapoptotic activity of ursolic acid in human skin malignant melanoma cells*

Antyproliferacyjna i proapoptotyczna aktywność kwasu ursolowego w ludzkich komórkach czerniaka złośliwego skóry

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Aim: Pentacyclic triterpenoid – ursolic acid is one of the most promising anticancer agents of biological origin. Especially modulation of cellular signalling pathways (STAT3, TRAIL, IRE1-TRAF-2-ASK1 signalling pathways) and enzymes inhibition (MMP-7, u-PA) may lead to apoptosis induction as well as inhibition of the following: tumorigenesis, tumor promotion, metastasis and angiogenesis. Melanoma malignum is one of the most malignant invasive cancers. It is characterized by a fast growth rate, multiple metastases, and late diagnosis. Substances of natural origin, including ursolic acid, have attracted broad attention recently, as potential antimelanoma agents. The aim of the study was to evaluate the influence of ursolic acid on the proliferation and apoptosis in human G361 malignant melanoma cell line.

Material/Methods: The effect of ursolic acid on the number of G361 cells was measured using In Vitro Toxicology Assay Kit Sulforhodamine B. DNA synthesis of G361 cells was evaluated by means of BrdU colorimetric immunoassay. Detection of caspase-3 activity was performed using Caspase 3 Assay Kit, Colorimetric.

Results: Ursolic acid had a strong effect on the number and proliferation of G361 cells. The most remarkable effect was observed at a concentration of 20 μM. Our results suggest that in some concentrations ursolic acid can induce apoptosis via activation of caspase-3 in melanoma G361 cells.

Conclusions: The presented results suggest that ursolic acid can have an influence in a dose and time dependent manner on skin *melanoma malignant* cells. Ursolic acid has antiproliferative and cytotoxic activity and it can induce apoptosis in human melanoma malignum G361 cell line.

Keywords: **Ursolic acid • melanoma malignum • anticancer agent • G361 cell line • apoptosis • cytotoxicity**

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Wykaz skrótów: Abbreviations: **Ac-DEVD-CHO** – N-Ac-Asp-Glu-Val-Asp-CHO; **Ac-DEVD-pNA** – acetyl-Asp-Glu-Val-Asp-p-nitroanilide; **AMPK** – 5′ AMP-activated protein kinase; **ANOVA** – Analysis of variance; **Bcl-2** – B-cell lymphoma 2; **bFGF** – basic Fibroblast Growth Factor; **BrDU** – Bromodeoxyuridine; **COX-2** – Cyclooxygenase-2; **DMSO** – Dimethyl sulfoxide; **ERK** – Extracellular Signal–Regulated Kinase; **EthD-1** – ethidium homodimer-1; **FOX-M1** – Forkhead box M1; **HIF-1** α – Hypoxia-Inducible Factor-1α; **ICAM-1** – Intercellular Adhesion Molecule 1; **IL-8** – Interleukin-8; **IRE1-TRAF-2-ASK1** – IRE1 (Inositol-requiring enzyme-1), TRAF-2 (TNF receptor-associated factor 2) and ASK1 (Apoptosis signal-regulating kinase 1); **JAK 1/2** – Janus kinase 1/2; **JNK** – c-Jun N-terminal Kinase; **MMP-7** – Metalloproteinase 7; **NF-κB** – Nuclear Factor kappa-light-chain-enhancer of activated B cells; **PARP** – Poly (ADP-ribose) polymerase; **PBS** – Phosphate-Buffered Saline; **pNA** – p-Nitroaniline; **STAT3** – Signal Transducer and Activator of Transcription 3; **TMB** – 3,3′,5,5′-Tetramethylbenzidine; **TNF** – Tumor Necrosis Factor; **TRAIL** – TNF-Related Apoptosis-Inducing Ligand; **Tris** – Tris(hydroxymethyl)aminomethane; **UA** – Ursolic acid; u-PA – Urokinase-type plasminogen activator; **VEGF** – Vascular Endothelial Growth Factor.

INTRODUCTION

Pentacyclic triterpenoid – ursolic acid (3β-hydroxy-urs-12-en-28-oicacid, UA, Fig. 1) is one of the most promising anticancer agents of biological origin. UA consists of six C_5 active isoprene units. In plants it occurs as either a free acidic form or aglycones of triterpene saponins [10, 24, 26]. UA like other pentacyclic triterpenes (oleanolic acid, betulinic acid, uvaol, α and β-amyrin) is widely distributed in the plant kingdom, especially in leaves and berries of medicinal plants such as: *Arctostaphylos uva-ursi* (L.) Spreng (bearberry), *Vaccinium macrocarpon* Ait. (cranberry), *Rhododendron hymenanthes* Makino, *Rosemarinus officinalis* (L.), *Eriobotrya japonica* (Thunb.) Lindl., *Calluna vulgaris* (L.) Hull, *Ocimum sanctum* (L.), *Eugenia jambolana* Lam. and in the protective wax-like coatings of apples, pears, prunes [20]. From a medical point of view, UA has several attractive biological and pharmacological activities: antimetastatic, antiangiogenic, antioxidant, antiinflammatory, antimicrobial, antiviral and hepatoprotective [8, 22]. Especially modulation of cellular signalling pathways and enzymes inhibition may lead to apoptosis induction as well as inhibition of the following: tumorigenesis, tumor promotion, metastasis and angiogenesis [16, 28]. Some authors reported synergistic cytotoxic effects of UA combined with P-gp inhibitors or DNA alkylating agents, against cell lines derived from very aggressive and difficult to treat cancers [2, 31]. This is why its application in the combination therapy to enhance tumor cell sensitivity and efficacy of treatment is considered.

Melanoma malignum is one of the most malignant invasive cancers. It is characterized by a fast growth rate, multiple metastases, and late diagnosis. Treatment of *melanoma malignum* is mainly carried out with surgery, chemotherapy and radiotherapy [6, 7, 29]. Resection of such a tumor is successful solely in its early stage of development. New treatment options for the *melanoma malignum* are needed [18]. Substances of natural origin, including UA, have attracted broad attention recently, as potential antimelanoma agents [19].

The aim of the study was to evaluate the influence of ursolic acid on the proliferation and apoptosis in the human G361 malignant melanoma cell line.

MATERIALS AND METHODS

Cell culture and reagents

The human skin malignant melanoma G361 cell line was derived from a 31-year-old Caucasian male patient was purchased from American Type Culture Collection (ATCC® CRL-1424™; LGC Stamdards). Cells were cultured in a growth medium containing McCoy's 5A medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum (PAA), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich), and 10 mM HEPES (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2.

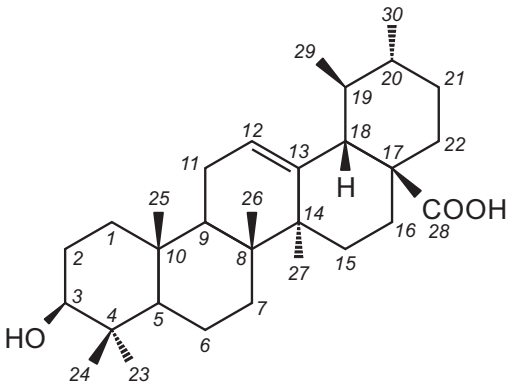

Fig. 1. Chemical structure of ursolic acid

Ursolic acid – UA (Sigma-Aldrich) was dissolved in DMSO (Sigma-Aldrich) as a 20 mM stock solution and stored at -20˚C. Further dilution was done in a cell culture medium.

Monitoring in vitro cytotoxicity of ursolic acid

To determine the cytotoxic effect of UA, G361 cells were plated at a density of 2×10^3/well in 96-well culture plates and cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 24h, media with UA at concentration range of 1 µM to 20 µM were added to the wells. Control cultures were treated with vehicle alone (0.2% DMSO). The effect of UA on number of G361 cells was measured using In Vitro Toxicology Assay Kit Sulforhodamine B based (Sigma-Aldrich) after 24 h, 48 h and 72 h of incubation time. After appropriate incubation periods cells were washed with phosphate-buffered saline (PBS, Sigma-Aldrich), fixed in 10% trichloroacetic acid and stained with 0.4% sulforhodamine B dye solution. Unincorporated dye was removed by washing wells with 1% acetic acid solution. Incorporated dye was liberated from the cells with 10 mM Tris base solution and absorbance was measured at 570 nm and 690 nm (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies).

DNA replication assay

DNA synthesis, a process restricted to the S phase of the cell cycle, was evaluated by means of BrdU colorimetric immunoassay (Roche). The assay is based on the immunoenzymatic measurement of BrdU incorporation, occurring during the replication of cellular DNA. G361 cells were plated at a density of 5×10^3/well in 96-well culture plates and cultured at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h, media were changed and media with UA (concentrations from 1 µM to 20 µM) were added. Control cultures were treated with vehicle alone (0.2% DMSO). After each incubation time (24 h and 48 h) BrdU was added to the G361 cells and cells were reincubated for 4 h in normal culture conditions. Subsequently, cells were fixed and DNA was denatured by FixDenat solution, anti-BrdU-POD was added to the wells. After the washing steps, detection of immune complexes by the subsequent substrate (TMB) reaction was carried out. The absorbance of reaction product was measured at 450 nm and 690 nm (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies).

Effect of ursolic acid on caspase-3 activity

Detection of caspase-3 activity was performed using Caspase 3 Assay Kit, Colorimetric (Sigma Aldrich). Cells were seeded at a density of 10^6 per 100-mm diameter culture dish and cultivated at 37°C in a humidified atmosphere containing 5% CO_2. After 24 h, media were changed and media with selected concentrations of UA (1 µM, 10 µM, and 20 µM) were added for the next 24 h. After incubation, cells were scrapped, lysed and centrifuged (16000 g; 10 min). This method is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val--Asp-p-nitroanilide (Ac-DEVD-pNA) by caspase-3 resulting in the release of the p-nitroaniline (pNA) moiety. Specificity of the assay was confirmed by preincubation of some samples with caspase-3 inhibitor: Ac-DEVD--CHO. Absorbance of pNA was measured at 405 nm using the MRX Revelation plate reader (Dynex Technologies). The obtained results were normalized relative to cellular protein content determined spectrophotometrically by the Bradford method [1].

Live/Dead assay

LIVE/DEAD™ Viability/Cytotoxicity Kit (Thermo Fisher Scientific Inc) was used to detect dead cells, as well as to analyze their morphology and identify changes typical for apoptosis. This method relies on the simultaneous fluorescence staining of cells with calcein AM (intracellular esterase substrate, labeling of live cells) and ethidium homodimer-1 (EthD-1; DNA bindig substance, labeling of dead cells). G361 cells were cultured in 24-well glass bottom plate (2×10^5 cell/well) and incubated with UA (1 µM, 10 µM and 20 µM) for 72 h. Control cultures were treated with vehicle alone (0.2% DMSO). Then, the plate was centrifuged (250 g, 5 min), medium was removed, and cells were incubated with fluorochromes (30 min). The observations were performed using an inverted fluorescence microscope Nikon Eclipse TS100F. Apoptotic cells were identified based on nuclear morphology (chromatin condensation, fragmentation of the nucleus).

Statistical analysis

The data obtained from 3 independent series of experiments were presented as mean values ± standard deviations. The results were checked for normality with the Shapiro-Wilk test. Statistically significant differences between groups were calculated using one-way analysis of variance (ANOVA) followed by post-hoc Tukey test for

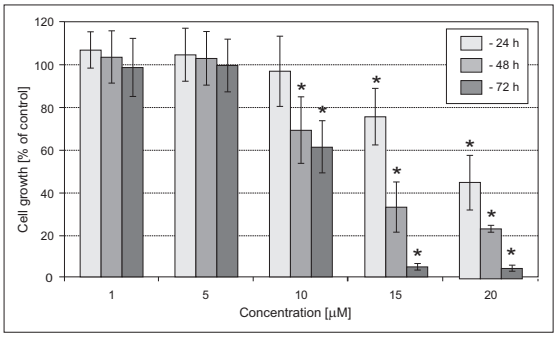

Fig. 2. Number of G-361 cells cultured in the presence of various concentrations of ursolic acid. Each bar represents the mean ± SD; *p < 0.05 compared to control

multiple comparisons. To determine the homogeneity of variance, the Brown-Forsyth test was used. The data was processed using Statistica version 10 software (StatSoft, Inc. 2011). Statistical significance was declared at p < 0.05. IC50 values were calculated using ED50plus v.1.0.

RESULTS

In vitro cytotoxicity of ursolic acid

In order to estimate the cytotoxic activity of ursolic acid, various concentrations of the compound (1 μM, 5 μM, 10 μM, 15 μM and 20 μM) were added to culture medium for 24 h, 48 h and 72 h (Fig. 2). Regardless of the incubation time, the presence of 1 and 5 μM UA did not cause significant differences in number of cells, compared to the culture without UA (p>0.05, cell percentage between 98.4% and 106.6% of control). Antiproliferative action of 10 μM UA was dependent on the incubation time. Significant inhibition of cellular growth was visible after 48h and 72h of treatment but not after 24h. The highest concentrations of UA (15 and 20 μM) significantly decreased cellular density at all the incubation periods (p<0.05). However, cytotoxic response was considerably greater after longer incubation times. Prolongation of the incubation time from 24 h to 48 h with 15 μM UA led to a sharp drop in the number of cells from 75.4% to 33.2% of control. Cellular density decreased further down to 5.2% after 72h of incubation. Similarly, incubation of cells with 20 μM UA resulted in a reduction of the cell number down to 44.6%, 23.0% and 4.6% of control, after 24, 48 and 72h of treatment respectively. IC50 values, after cell exposure for 24, 48 and 72h, were 21.2, 13.1 and 10.6 μM, respectively.

Effect of ursolic acid on DNA synthesis in melanoma cells

DNA synthesis rate was evaluated after 24 and 48h of cell treatment with UA (1 μM, 5 μM, 10 μM, 15 μM, 20 μM) using BrdU incorporation immunoassay. After 24h of incubation time (Fig. 3A) in a culture treated with 20 μM UA significant inhibition of DNA synthesis rate was observed at a level of 53.8% of control.

After 48 h of incubation time (Fig. 3B) with UA significant influence on inhibition of DNA synthesis rate had concentrations 15 μM and 20 μM. UA in concentration 15 μM significantly inhibits DNA synthesis rate at a level of 73.6% of control (p<0.05). An even stronger effect of UA was notified in G361 cells cultures treated with concentration 20 μM. Inhibition of DNA synthesis rate in this case was at the level 41% of control (p>0.05). IC50 values, after cell exposure for 24 and 48h were 25.2 and 21.5 μM, respectively.

Effect of ursolic acid on caspase-3 activity

The data shown in Fig. 4 evidenced that apoptosis activation followed treatment of G361 cells with UA, as active caspase-3 is a crucial element of the effector phase of apoptosis. Cells were treated with selected concentrations of UA: 1 μM, 10 μM, and 20 μM (Fig. 4). UA at concentration of 1 μM did not affect caspase-3 activity in G361 cells. Significant apoptosis induction was seen in cells incubated with 10 and 20 μM UA which resulted in an increase of enzyme activity 1.27-fold and 1.29-fold respectively in comparison to control (p>0.05).

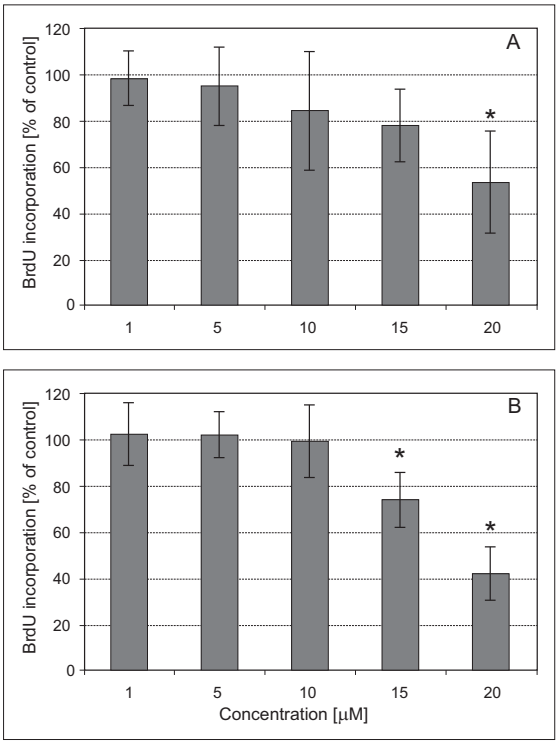

Fig. 3. DNA synthesis rate in G361 cells treated with various concentrations of UA: a) after 24 h of incubation time with UA, b) after 48 h of incubation time with UA. Each bar represents the mean ± SD; *p < 0.05 compared to control

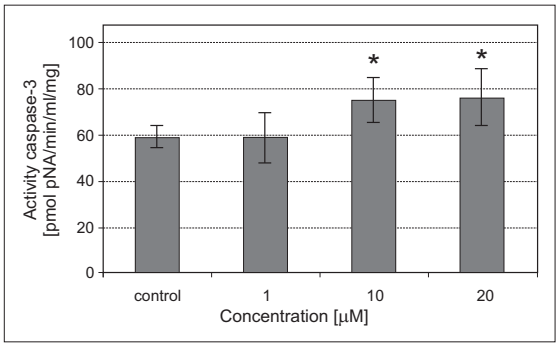

Fig. 4. The effect of 24 h exposure to 1 μM, 10 μM, and 20 μM of UA on caspase-3 activity in G361 cells. Each bar represents the mean ± SD; *$p < 0.05$ compared to control

Evaluation of cellular death

The LIVE/DEAD Viability/Cytotoxicity Kit was used to visualize cellular death, especially apoptosis, after treatment with UA. This method is based on differential staining of viable and dead cells together with an analysis of cell morphology. After 72 h of incubation, in control cultures and in the cultures treated with 1 μM UA, almost all cells were stained with calcein, which was indicative of their good viability. Only few cells were stained with ethidium homodimer-1. The majority of cells incubated with 10 μM and 20 μM UA accumulated calcein, but it was also observed that a remarkable increase took place in the number of cells emitting orange fluorescence from ethidium homodimer-1. Analysis of the EthD-1 stained cells morphology revealed that some of them have undergone apoptosis. An apoptotic nucleus is characterized by chromatin condensation with aggregation along the nuclear envelope or nuclei fragmentation to small round parts (Fig. 5).

DISCUSSION

Melanoma malignum pharmacotherapy often fails, especially in later stages of the illness, which results in a relatively high mortality rate. Therefore, a lot of research work is conducted to find new compounds, mostly from plants, which can be used in melanoma chemoprevention or in therapy. In the last years, great attention has been paid to triterpenoids, a large group of compounds, represented by, for example – ursolic acid.

UA had a strong effect on the number and proliferation of G361 cells, which was confirmed by two methods – Sulforhodamine B dye assay and BrdU colorimetric immunoassay. The most remarkable effect was observed at a concentration of 20 μM. The presented results strongly suggest that UA can have an influence, in a dose and time dependent manner, on skin melanoma malignant cells.

The evaluation of the antiproliferative effect of UA was carried out by Harmand et al. on M4Beu melanoma cell line and by Mahmoudi et al. on MM200, Mel-RM, Me4405, A375 melanoma cell lines using MTT test [4, 14]. Harmand et al. observed only a small increase in proliferation after 24 h and 48 h of incubation of M4Beu cells with UA at concentrations from 5 μM to 12.5 μM. Higher UA concentrations (15 μM and 20 μM) caused the inhi-

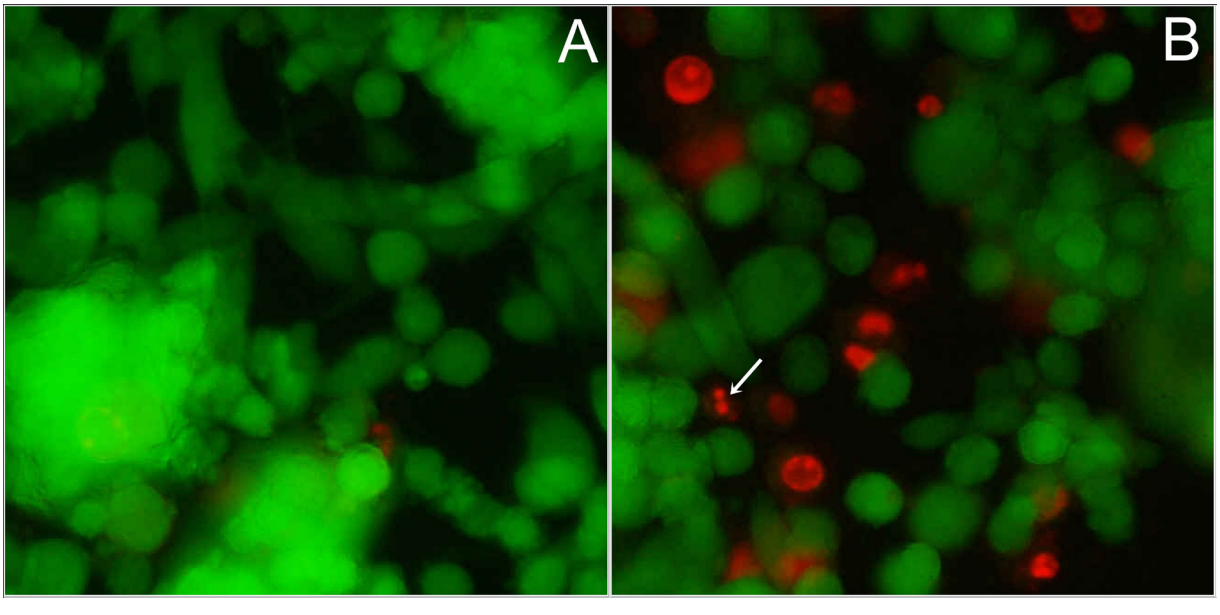

Fig. 5. Visualization of G361 cells after 72 h incubation period by double calcein (green fluorescence)/ethidium homodimer-1 (orange fluorescence) staining in: a) control culture and b) 20 μM UA treated culture; white arrow indicates example of a typical apoptotic cell (inverted fluorescence microscope Nikon Eclipse TS100F, magnification x200)

bition of M4Beu cell proliferation [4]. Mahmoudi et al. noticed the inhibition of melanoma cell proliferation by UA at concentrations starting at 20 μM. Significant cytotoxicity was observed in all the cell lines incubated with higher UA concentration, either after 24 h and 48 h of incubation. The strongest effect of UA was seen in Me4405 cell line. In this case, UA caused 100% inhibition of proliferation after 24 h and 48 h at 50 μM and 40 μM compound concentration, respectively [14]. Our results suggest higher sensitivity of G361 cells compared to Me4405, A375, Mel-RM, MM200 cell lines. Mahmoudi et al. confirmed the selectivity of UA action against melanoma cells, as normal human fibroblasts (HFF2) were considerably resistant to that substance. It has been shown that UA inhibits proliferation and decreasing amount of cancer cells, inducing apoptosis, inhibiting tumor growth, metastasis and angiogenesis. UA acts by various mechanisms, mainly by modulating signalling pathways of NF-κB and STAT3 [14].

Our results suggest that in some concentrations UA can induce apoptosis via activation of caspase-3 in melanoma G361 cells. Treatment of that cell line with UA at concentrations of 10 μM and 20 μM resulted in the significant activation of caspase-3. Occurrence of apoptosis in G361 cells treated with UA was also confirmed by calcein AM/ethidium homodimer-1 staining. Similar observations but on Me4405 melanoma cells were presented by Mahmoudi et al, where UA in concentration 30 μM induced proteolytic processing of caspase-3, which was confirmed by Western Blotting analysis [14]. Similarly, using Western blotting technique, Wang et al. detected the activation of caspase-3 in SW480 cells after treatment with UA at concentrations of 20 μM and 40 μM for 48 hours [25]. UA had an effect on several melanoma cell lines: A375, B16-F10 and M4Beu [26]. UA induced apoptosis via caspase-3 activation and mitochondrial pathway [4, 15]. Duval et al. presented UA activation of p53 and caspase-3 genes expression and inhibition of NF-κB mediated Bcl-2 activation. Duval et al. investigated the effect of UA on activity of key caspases utilizing fluorimetric assay (CaspACE Assay System Flurometric). M4Beu melanoma cell line was treated with 10 μM, 12,5 μM and 15 μM UA. Caspase-3 activity increased both after 24 h and 48 h of incubation time in cells treated with 12.5 μM and 15 μM UA, while caspase-9 activity increased in cells treated with 15 μM UA [3].

Caspase cascade activation is the primary apoptosis signal transduction pathway in human melanoma cells. Caspase-3 and -8 play a crucial role in this process [14]. It has been suggested that the main mechanism of anticancer activity of UA in breast cancer cells is the activation of the intrinsic mitochondrial pathway of apoptosis [24]. In MDA-MB-231, breast cancer cells treatment with UA resulted in the dissipation of mitochondrial membrane potential, increased pro-apoptotic Bax level and decreased antiapoptotic Bcl-2 level and translocation cytochrome C to cytoplasm and activation of caspase-9 [9]. UA also inhibits metalloproteinase 7 (MMP-7) and urokinase-type plasminogen activator (u-PA), decreasing the level of protein: NF-κB, cellular proto-oncogens (c-Jun and c-Fos), Protein kinase B (serine/threonine kinase). UA stimulates expression of p53 protein, which is responsible for cell cycle regulation, apoptosis and DNA repair. Moreover, UA inhibits the transcription and activity of cyclooxygenase-2 (COX-2). In breast cancer cell line – MCF-7 UA inhibits the expression of FoxM1 transcription factor [20, 24].

UA is also active against multiple myeloma cells. The mechanism of action is based on he modulation of STAT3 signalling pathway via the inhibition of the activity of this protein and the inhibition of the activity of tyrosine kinases (JAK 1/2 and cSRC). Th effect of UA was examined on androgen independent (DU145) and androgen-dependent (LNCaP) cell lines of prostate cancer. In LNCaP cells, UA not only blocked STAT-3 protein induced by interleukin-6 (IL-6) but also inhibited tumor necrosis factor (TNF) dependent NF-κB. In DU145 cell line UA also led to NF-κB inactivation [20, 21]. UA showing inhibitory effect on growth of hepatocellular carcinoma cells stops angiogenesis via inhibiting of hypoxia-inducible factor-1α (HIF-1 α), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), u-PA, reactive oxygen species and nitric oxide [13]. UA induces apoptosis via caspase-3 activation [22]. Activation of signalling pathway IRE1-TRAF-2-ASK1 and apoptotic pathway ASK1-JNK occurred in bladder cancer cells treated by this triterpenoid. Additionally, stimulation of 5' AMP-activated protein kinase (AMPK) and inhibition of cellular proliferation is observed [30]. UA activates also apoptosis in colorectal cancer cells via TRAIL signalling pathway. Cell death and autophagy modulation occurs through the JNK pathway. Inhibition of NF-κB, cyclin D1, metalloproteinase 9 activity, ICAM-1, VEGF and antiapoptotic proteins (c-FLIP, survivin, Bcl-2 and Bcl-xL) also is observed [17]. Apoptosis is activated by the stimulation of signalling pathway P2Y(2)/Src/p38/Cox2 [12]. Phosphorylation of extracellular signal–regulated kinase (ERK), activation of caspase-9, caspase-3 and PARP and blocking survivin by UA result in inhibition of ovarian cancer cells proliferation [23]. The inhibitory and lethal effect of UA on gemcitabine resistant pancreatic cancer cell lines (MIA PaCa-2, Capan-1 and PANC-1) following modulation signalling pathway JNK andPI3K/Akt/NF-κB has also been documented [11]. In human chronic leukaemia K562 cell line UA induces apoptosis by stimulating expression of PTEN gene, blocking AKT kinase activity and activates mitochondrial pathway [27]. UA suppresses invasive and migratory features of human non-small-cell lung carcinoma cells (A549, H3255, Calu-6) and already in time and dose dependent manner induces apoptosis [5].

CONCLUSIONS

It can be concluded that UA has antiproliferative and cytotoxic activity and it can induce apoptosis in human *melanoma malignum* G361 cell line. Effect of UA is incubation time or concentration dependent. Further investigation is needed to determine its safety and efficacy in humans. UA may be new, promising and advantageous agent in treatment of *melanoma malignum*.

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1155