

Effects of ursolic and oleanolic on SK-MEL-2 melanoma cells: *In vitro* and *in vivo* assays

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Abstract. Among the triterpenoids, oleanolic acid (OA) and its isomer, ursolic acid (UA) are promising therapeutic candidates, with potential benefits in the management of melanoma. In this study, we aimed to examine the *in vitro* and *in vivo* anti-invasive and anti-metastatic activity of OA and UA to determine their possible usefulness as chemopreventive or chemotherapeutic agents in melanoma. For the *in vitro* experiments, the anti-proliferative activity of the triterpenic compounds on SK-MEL-2 melanoma cells was examined. The anti-invasive potential was assessed by testing the effects of the active compound on vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) adhesion to melanoma cells. Normal and tumor angiogenesis were evaluated *in vivo* by chicken embryo chorioallantoic membrane (CAM) assay. The two test triterpenoid acids, UA and OA, exerted differential effects *in vitro* and *in vivo* on the SK-MEL-2 melanoma cells. UA exerted a significant and dose-dependent anti-proliferative effect *in vitro*, compared to OA. The cytotoxic effects *in vitro* on the melanoma cells were determined by the examining alterations in the cell cycle phases induced

by UA that lead to cell arrest in the S phase. Moreover, UA was found to affect SK-MEL-2 melanoma cell invasiveness by limiting the cell adhesion capacity to ICAM molecules, but not influencing their adhesion to VCAM molecules. On the whole, in this study, by assessing the effects of the two triterpenoids *in vivo*, our results revealed that OA had a greater potential to impair the invasive capacity and tumor angiogenesis compared with UA.

Introduction

A recent article (2017) of the American Cancer Society presenting key statistics for cutaneous melanoma reported that among all types of malignancies, skin cancer is the most common (1). Although squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are more frequent, melanoma, the so-called cancer of the Western world, accounts for approximately 1% among all types of skin cancers. The aggressiveness of this particular skin pathology is due to its highly metastatic potential and increased resistance to chemotherapeutic agents, events associated with a low survival of affected patients (2,3). When localized to the primary site, the chances of cure are high; however, after it develops and reaches the lymph nodes, the prognosis becomes poor, with the 5-year survival rate being around 29%. The marked decrease in survival presents once the major organs are invaded by malignant cells, with the 5-year survival rate reaching only about 7% (4). As regards therapeutic strategies, the treatment approach is straightforward in the early stages of the disease when the tumor is localized, and involves the wide excision of the primary tumor site. The challenges in treatment strategies present once metastasis occurs. Chemotherapy, radiation therapy, immunotherapy and targeted therapy represent current standard approaches for the management of this highly aggressive disease, with varying results (5-7).

The high mortality rates associated with the disease are due to its heterogenous molecular pattern based on multiple progressive mutations that can occur. The most frequent

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subtype of malignant melanoma, found in 40-50% of cases, harbors the oncogene mutation, B-RAF V600, which is related to sun exposure. It is followed by a mutation of the oncogene N-RAS, detected in 15-20% of patients. However, although this mutation exhibits significant frequency, its effect has not yet been widely studied and the response to the few treatment options is relatively poor (8-10). Based on these data, in this study, in order to investigate possible targeted new therapeutics, we decided to investigate the SK-MEL-2 melanoma cell line that harbors two mutant genes, N-RAS and TP53.

Currently, for the development of treatment options, natural compounds are intensively investigated for their chemopreventive and anti-invasive potential, in addition to lower secondary effects (11,12). Angiogenesis, the process through which new blood vessels are formed from pre-existing ones, is one of the key steps in tumor growth and metastasis. This process is being highly investigated as a possible therapeutic approach in cancer, after Folkman *et al* (13) introduced the concept of tumor angiogenesis.

The discovery of novel drugs from natural sources targeting cancer and angiogenesis was first based on the traditional practices of treating vascular-dependent pathologies (14). As an unbalanced type of diet is increasingly associated with cancer pathologies, a growing number of natural compounds found in healthy diet foods is being evaluated as anticancer agents (15). An important class of phytochemicals with demonstrated anticancer potential is represented by the triterpenoids. Triterpenoid compounds are secondary metabolites widely distributed in plants. Structurally, they are formed by 30 atoms of carbon, organized in isoprene units. Important triterpenoids are derivatives of the pentacyclic carbon skeleton, including lupane (e.g., betulinic acid), oleanane [e.g., oleanolic acid (OA)] and ursane [e.g., ursolic acid (UA)]. Several representatives are known for their potential therapeutic benefits as antioxidant, anti-inflammatory, anti-bacterial, anti-malaria and anti-viral agents. Importantly, anticancer properties have been attributed to these compounds in various types of cancer cell lines, in which they have been shown to exert anti-proliferative, pro-apoptotic and tumor anti-invasive effects (16-19).

Among the triterpenoids OA (3- β -3-hydroxy-olean-12-ene-28-oic-acid) and its isomer, UA (3- β -3-hydroxy-urs-12-ene-28-oic-acid) (Fig. 1), are promising therapeutic candidates. They are highly abundant in edible plant foods, such as apples, pears, olives or aromatic plants from the Lamiaceae family, such as oregano, basil, rosemary or lemon balm (20-23). The pharmacological value of the two compounds is demonstrated both by the multiple pharmacological targets, but also by their low toxicity (24,25). To date, the various pharmacological effects of UA and OA exerted via multiple mechanisms are not yet fully completely understood. Thus, they are the subject of current research.

Despite structural similarities, the effectiveness of their anticancer activity differs. Some studies have reported an increased antitumor activity both *in vitro* and *in vivo* for UA, but OA has also been assigned with anticancer properties (26,27). Hence, the selection of one of the two triterpenic compounds for cancer chemoprevention should be carried out taking into account the involved cancerous cell line and/or target molecules engaged in each type of cancer (28). Some available data suggest the potential benefits of OA and UA

in the management of melanoma. *In vitro*, various IC₅₀/EC₅₀ values for OA and UA have been obtained for several melanoma cell lines (29-31).

MAP/extracellular signal-regulated kinase (MEK) protein kinases form a family of proteins that next to mammalian target of rapamycin (mTOR), are involved in cell proliferation, survival, differentiation and angiogenesis, and are considered interesting synergistic targets for N-RAS mutated cancers (8). Modern approaches for the discovery of active compounds involve the virtual screening of large natural compound databases against druggable targets in cancer (32-34). In this study, for the selection of the two triterpenic compounds, we considered the availability from natural sources and their promising anticancer activities. Moreover, recent studies have indicated the possible role of UA and OA in modulating some of the deregulated kinases involved in the progression of various types of cancer (35,36). However, the mechanisms of action and the potential effects *in vivo* in N-RAS-mutated melanoma of the two compounds are not yet fully understood.

The tumor microenvironment, as regards tumor-associated inflammation and angiogenesis, as well as specific targets, has been intensively studied (18,19,37-39) in order to elucidate the mechanism(s) of action of triterpenic compounds. In the present study, we aimed to test the *in vitro* and *in vivo* anti-invasive and anti-metastatic activity of OA and UA to determine their possible use as chemopreventive or therapeutic agents in melanoma. For the *in vitro* experiments, the anti-proliferative activity of the triterpenic compounds on SK-MEL-2 melanoma cells was examined. The anti-invasive potential was assessed by examining the effects of the active compounds on vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) adhesion to melanoma cells. Normal and tumor angiogenesis was evaluated *in vivo* by chicken embryo chorioallantoic membrane (CAM) assay.

Materials and methods

In vitro analysis

Cell culture. The SK-MEL-2 human melanoma cells (ATCC, Manassas, VA, USA) were cultured in Eagle's minimum essential medium (EMEM), containing 10% fetal bovine serum (FBS) (both from ATCC) and 1% penicillin-streptomycin (Pen/Strep, 10,000 IU/ml; PromoCell, Heidelberg, Germany). The cells were maintained in an atmosphere of 5% CO₂ at 37°C.

***In vitro* cell proliferation assay: AlamarBlue assay.** The SK-MEL-2 cells were seeded in 96-well microplates (5,000 cells/plate) and incubated overnight in order to allow attachment of the cells to the bottom of the plate. Subsequently, 150 μ l of fresh EMEM medium containing UA or OA was added and the cells were incubated for 48 h. UA or OA was added at concentrations between 25 and 100 μ M. After 48 h, 15 μ l of AlamarBlue solution were added and the cells were incubated for 4-10 h at 37°C. The samples were spectrophotometrically analyzed at 570 and 600 nm using an xMark™ Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA). Wells with untreated cells were used as controls. The test substances were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Ayrshire, UK) and stock solutions were stored at 2-8°C. The final concentrations were prepared by diluting the

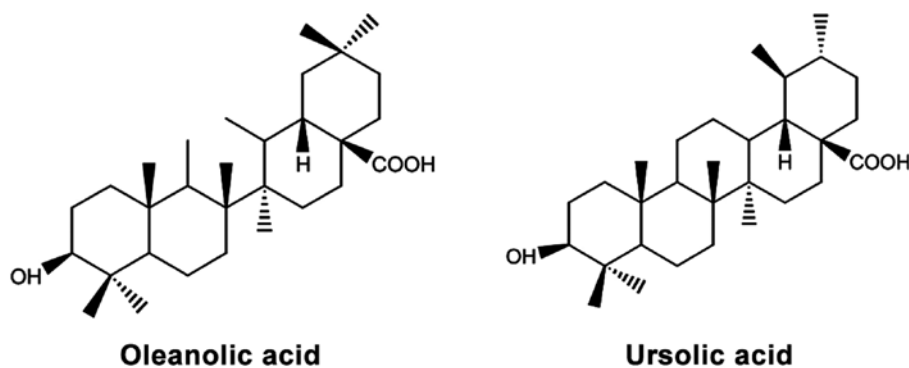


Figure 1. Chemical structures of oleanolic acid and ursolic acid.

stock solution with the EMEM growth medium. The highest DMSO concentration (0.1%) of the medium did not exert any significant effect on cell proliferation. The experiments were performed using microplates with at least 4 parallel wells. The results are presented as the mean value \pm standard deviation. One-way ANOVA was used to determine statistically significant differences between various experimental groups.

Cell cycle analysis. The SK-MEL-2 human melanoma cells were seeded in 25 cm² plates (10⁶ cells/plate) and treated with UA and OA (30, 50, or 75 μ M). After 48 h of treatment, the cells were collected, fixed with cold 70% ethanol and stored for 30 min at 4°C. Following centrifugation (1,500 rpm, 22°C), cold PBS was used to wash the cells. Subsequently, 50 μ l of propidium iodide (concentration, 50 μ M) (BD Pharmingen; BD Biosciences, San Diego, CA, USA) were added to the cells and the cells were incubated for 10 min in the dark, in order to stain the DNA. A FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) was used to perform the DNA content analysis. The percentage of cells present in the different cell cycle phases was determined using Modfit software.

Flowchamber assay. The SK-MEL-2 tumor cell lines were cultured according to the protocol described above. The cells were treated with 30 and 50 μ M of UA. After 48 h of treatment, the cells were harvested by trypsinization and the adhesive capacity of the cells exposed to mechanical stress (shearstress) on the VCAM and ICAM substrates (R&D Systems, Abingdon, UK) was evaluated using the flow chamber method.

Flowchamber analysis follows several consecutive steps beginning with the coating of the flowchamber channels with adhesion molecules (VCAM and ICAM), used at a concentration of 2 μ l/ml, 30 μ l/channel 15 min prior to the beginning of the experiment (3 channels for each adhesion molecule). The cells were trypsinized and incubated while the peristaltic pump (Ismatec - IPC High Precision Multichannel Dispenser (IDEX Corporation, Glattbrugg, Switzerland) was prepared. The preparation of the peristaltic pump consists of assembling the tubulature, passing the HBSS medium (100 ml PBS with Ca²⁺ and Mg²⁺, 50 ml DMEM high glucose medium, 0.75 ml BSA 20%) through the tubes and fixating the flowchamber to the microscope connected to the camera. The tube is fixated in the 'OUT' position and to the 'waste' recipient. The supernatant in the 'IN' position is discarded.

In continuation, 100 μ l well omogenized cellular suspension (10⁵ cells) was inserted at the 'IN' end of the channel, after

which the tube connecting to the pump was inserted. The cells were allowed 3 min to adhere to the substrate, after which, using a Leica ICC50HD camera (Leica, Bucharest, Romania) an image was acquired representing the START moment. After that moment, shearstress was introduced following this schedule: 0.35 dyne/cm² - 1 min; 2 dyne/cm² - 30 sec; 5 dyne/cm² - 30 sec; 8 dyne/cm² - 30 sec; 15 dyne/cm² - 30 sec. Following each time-speed period, an image was acquired using a Leica ICC50HD camera and Leica DMD108 microscope (Leica). At the end of the experiment, all images were analyzed in order to count the adhered cells at the initial moment and the remaining cells throughout the experiment. The cell number was correlated to the increase of shear stress. Variations of at least 15% from the total number of cells were considered significant in comparison to the control cells for the same flux values.

In vivo analysis

CAM assay. Fertilized eggs (*Gallus gallus domesticus*), obtained from a local poultry farm, were disinfected with 70° ethanol, dated and subsequently incubated in a horizontal position, at constant humidity and 37°C. On the third day of incubation, 3-4 ml of albumen was removed, so that the developing chorioallantoic membrane could be detached from the eggshell, and the blood vessels from the extra-embryonic vascular plexus could be easily observed. On the 4th day of incubation, a window was cut, resealed with adhesive tape, and set to incubate until the beginning of the experimental procedures, as previously described (40).

Evaluation of angiogenesis and tumor angiogenesis on the chorioallantoic membrane. The present study was conducted in ovo, beginning on the 7th day of incubation of the chick embryos. The effect of UA and OA on the process of angiogenesis of the chick embryo CAM was firstly evaluated in the absence of tumor cells between the 7th and the 11th embryonic days. During this interval, the vascular network is in a rapid growing phase, and the endothelial cells exhibit a high mitotic rate, similar to that of tumor-associated angiogenesis (41). The samples were tested at a concentration of 30 mM, using 3% DMSO. Three doses of the test samples and blank solutions containing only 3% DMSO were applied in triplicate daily for 5 days. Volumes of 5 ml were added inside a plastic ring with a diameter of 5 mm previously placed on the CAM surface in vascularized areas and the specimens were returned for incubation. Evaluation was performed daily by means of

a stereomicroscope and relevant images were captured for further analysis. Finally, on day 12 of incubation, the specimens were sacrificed and the fine CAMs were harvested.

The SK-MEL-2 cells were cultured according to the protocol described above. After harvesting the cells from the culture plate through trypsinization, the cells were resuspended in the culture medium until reaching the final concentration of $10^5/3 \mu\text{l}$. On the 10th day of incubation, $3 \mu\text{l}$ of the SK-MEL-2 melanoma cell suspension were inoculated inside a sterile ring previously placed on the CAM. The control samples were only inoculated with $3 \mu\text{l}$ of cell culture medium.

UA, OA and blank (DMSO) solutions were added in volumes of $5 \mu\text{l}$ inside the rings 1 day after the inoculation of the cells. Samples were applied daily for 5 days. The process was dynamically examined and relevant captures were saved. On the final day of the experiment, all the specimens were sacrificed and prepared for further histological analysis.

Zeiss Axio V16 stereomicroscope was used for the in ovo examination of the specimens. Images were registered by means of the Zeiss Axio Cam equipment and image analysis was done using Zeiss ZEN and Image J softwares. Morphometric analysis was applied on the stereomicroscopic photographs of the tested CAMs, using an arbitrary 0-5 scale that scores the intensity of vascular density on the area of application. Low values are correlated with a reduced angiogenic process, while the higher scores indicate an activated process. The results are expressed as mean values \pm standard deviation. Statistical analysis was performed using SPSS software (IBM SPSS Statistics for Windows, version 20.0; IBM Corp., Armonk, NY, USA).

Results

Effects of UA and OA on the proliferation of SK-MEL-2 melanoma cells. Cell proliferation experiments demonstrated that UA exerted an inhibitory and dose-dependent effect on SK-MEL-2 human melanoma cell growth (Fig. 2), with an IC_{50} value of $58.43 \mu\text{M}$. Specifically, the lowest concentration presented an insignificant inhibitory index of only $2.68 \pm 8.35\%$, with a gradual increase until reaching a value of $87.34 \pm 5.50\%$ for the highest tested concentration ($100 \mu\text{M}$) ($p < 0.05$). As regards OA, a tendency of the inhibition of cell proliferation was observed (Fig. 2), which did not however, reach statistical significance ($p = \text{n.s.}$).

Effects of UA on SK-MEL-2 cell cycle progression. The aneuploid character of the SK-MEL-2 cells was demonstrated utilizing flow cytometric analysis, which indicated that the diploid cells represented 65% of the total number of cells and the aneuploid cells represented 35% of the total cell population. Therefore, the cell cycle passage of both the aneuploid and diploid cell populations is separately presented and demonstrated in Table I and Fig. 3 (dip, diploid cells; ane, aneuploidy cells). There was a significant shift in the cell cycle in both the diploid and the aneuploid cells as compared with the controls. Of note, UA exerted effects on the cell cycle passage of aneuploid and diploid cells in a distinct manner. Thus, at $30 \mu\text{M}$, there was an even distribution of diploid cells among the G0/G1, S and G2/M phase, whereas there was a massive distribution of aneuploid cells in the G2/M phase.

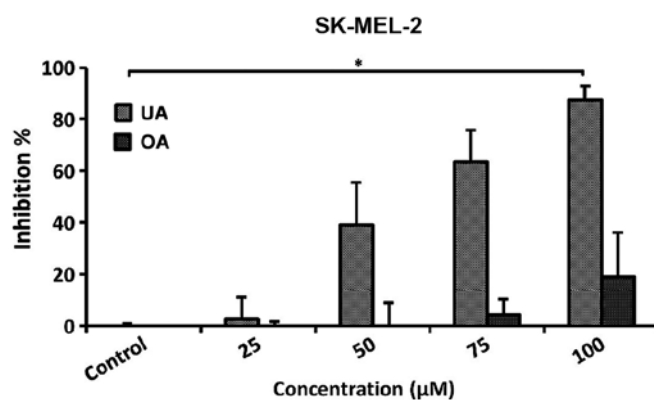


Figure 2. Effects of ursolic and oleanolic acid on SK-MEL-2 human melanoma cell proliferation. * $p < 0.05$. UA, ursolic acid; OA, oleanolic acid.

An interesting fact is that for both the diploid and aneuploid populations, the exposure to $50 \mu\text{M}$ UA resulted in S phase cell cycle arrest ($p < 0.05$) (Table I). Exposure of the SK-MEL-2 cells to the concentration of $75 \mu\text{M}$ led to the destruction of the cells and their detachment from the culture plate, not allowing for cell cycle analysis.

Effects of UA on SK-MEL-2 melanoma cell adhesion in the flow chamber assay. The flow chamber method represents an *in vitro* model which simulates the exposure of cells to the dynamical flux of fluids in a physiological environment (42). During the assay, the cells are subjected to mechanical stress (shear stress) of fluids. The method allows for the evaluation of the adhesive capacity to key molecules correlated with cancer progression, such as ICAM-1 and V-CAM 1, and enables the quantification of the cancer metastasis patterns of tumor cells (43,44).

As shown in Fig. 4A, flow chamber analysis of the SK-MEL-2 cells demonstrated that the untreated cells remained attached to the VCAM substrate at the end of progressive exposure to shear stress at a percentage of 95.90%. Data collected for the cells exposed to UA demonstrated slight alterations in the cell adhesive capacity (Fig. 4A). These alterations were not considered significant and, upon completing the experiment, a difference of only 4.70% compared with the adhesive ability of the controls was shown for a concentration of $30 \mu\text{M}$ and only 5.30% for a concentration of $50 \mu\text{M}$ UA.

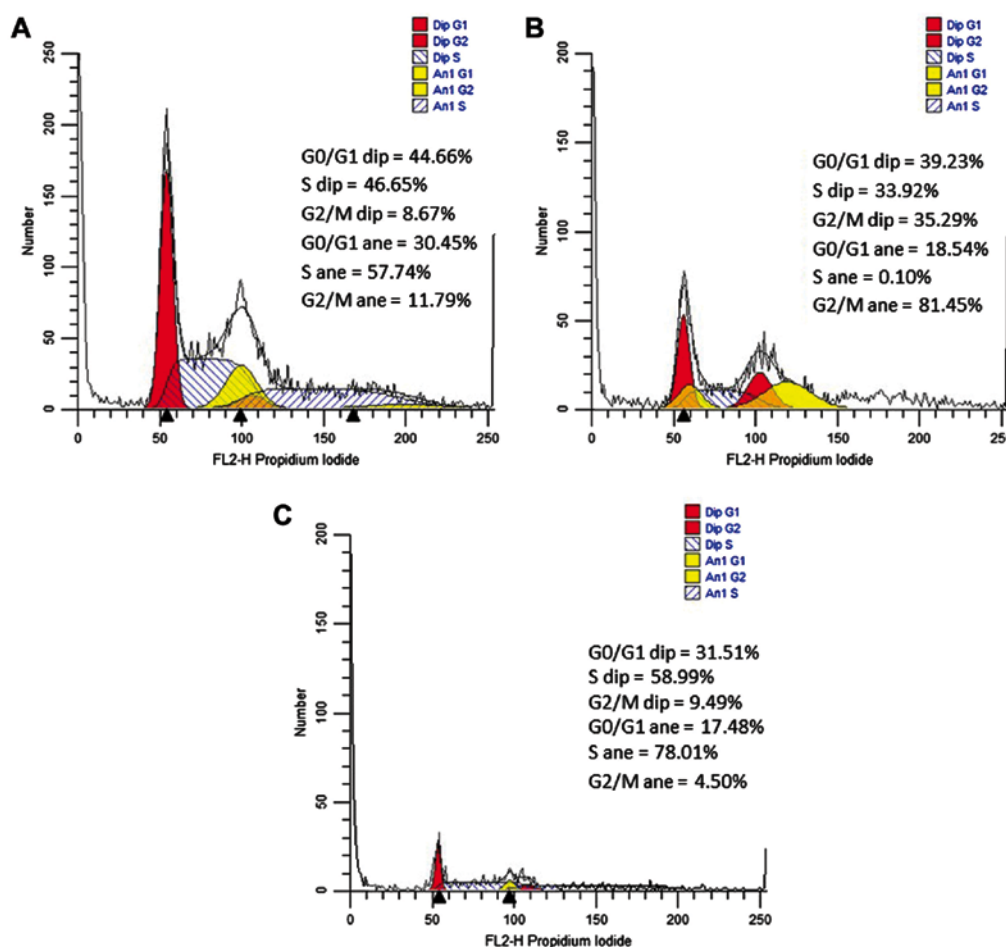
Likewise, treatment with $30 \mu\text{M}$ UA did not affect the ability of the cells to attach to the ICAM substrate ($p = \text{n.s.}$) (Fig. 4B). Significant changes in cell adhesive ability were noted for the cells treated with $50 \mu\text{M}$ UA. Indeed, upon the increase in shear stress (from 0.35 dyne/cm^2 to 15 dyne/cm^2) there was a proportional decrease in the number of attached cells with a difference of 24% lower cell number as compared with the controls ($p < 0.05$).

Effects of UA and OA on angiogenesis utilizing CAM assay. The anti-angiogenic potential of UA and OA was assessed in an *in vivo* protocol by CAM assay. Normal angiogenesis during the high mitotic phase of endothelial cells and tumor-associated angiogenesis were investigated using the SK-MEL-2 melanoma cells. The evaluation was performed in ovo by means of stereomicroscopy.

Table I. Effect of ursolic acid on cell cycle phases in the SK-MEL-2 melanoma cells.

Cell cycle distribution (%)						
SK-MEL-2 cells						
Treatment	G0/G1 dip	S dip	G2/M dip	G0/G1 ane	S ane	G2/M ane
0	44.66	46.65	8.67	30.45	57.74	11.79
UA 30 μ M	39.23	33.92	35.29	18.54	0.10	81.45
UA 50 μ M	31.51	58.99 ^a	9.49	17.48	78.01 ^a	4.50

^ap<0.05, significant differences vs. control (no treatment). UA, ursolic acid; dip, diploid cells. ane, aneuploid cells.

Figure 3. Cell cycle distribution in the SK-MEL-2 cells: (A) control, (B) cells treated with 30 μ M UA, and (C) cells treated with 50 μ M UA. UA, ursolic acid; dip, diploid cells; ane, aneuploidy cells.

The chicken embryo specimens (inoculated or not with tumor cells) exhibited good viability and survival rates with the test compounds, which were similar to those of the controls, e.g., around ED13 for all specimens. For the assessment of normal angiogenesis, modifications of the vascular plexus were observed throughout the experiment, and a gradual increase in the effects was noted, as shown in Fig. 5A-F. After 5 days of treatment, relevant images indicating the affected vessel architecture were registered and analyzed. Both OA and UA induced changes in the vascular network inducing a decrease

in vessel number inside the application area, as compared with the blank specimens. A more prominent effect was noted with OA, which induced a reduction in vascular density and several areas with very low number of fine capillaries were noted inside the ring (Fig. 5E and F).

An investigation of the angiogenic modulatory effects of the two triterpenoid compounds was also conducted in a melanoma model upon SK-MEL-2 cell inoculation to the CAM. The process was stereomicroscopically examined from the first day of the triterpene application onto previously inoculated

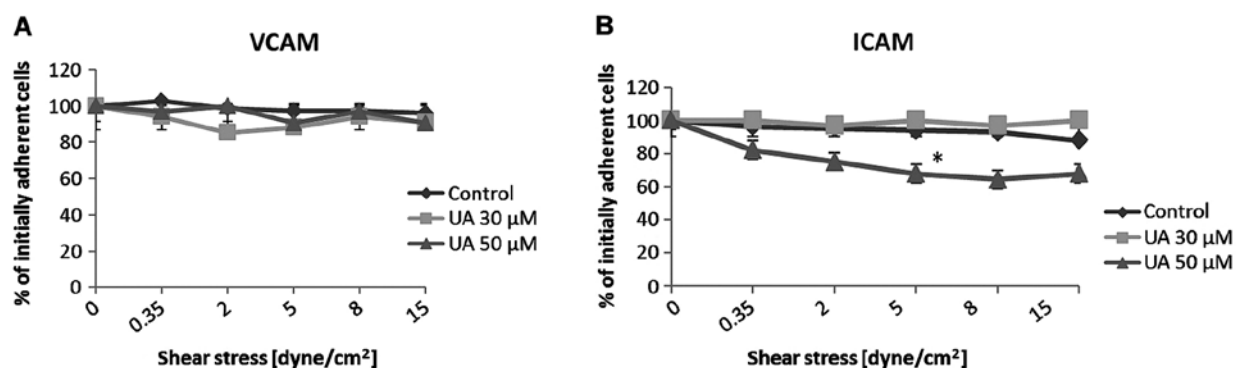


Figure 4. SK-MEL-2 cell adhesion to (A) VCAM and (B) ICAM substrates following treatment with UA. UA, ursolic acid; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule. * $p < 0.05$

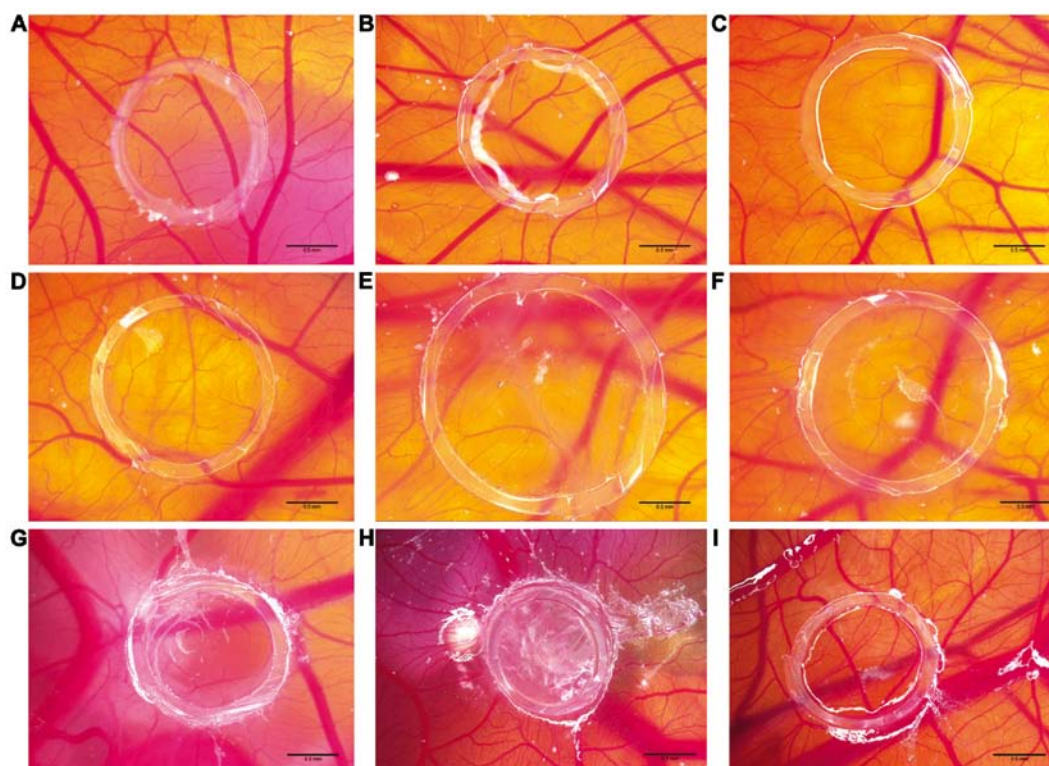


Figure 5. Effects of UA and OA on CAM assay. (A-C) Normal angiogenesis, with two doses of the test samples on ED9. (A) Blank (DMSO, 3%), (B) UA, (C) OA. (D-F) Normal angiogenesis, with four doses of the test samples on ED11. (D) Blank (DMSO, 3%), (E) UA, (F) OA. (G-I) SK-MEL-2 melanoma cells, with four doses of the test samples on ED13. (G) Control (cell medium), (H) SK-MEL-2 + UA, (I) SK-MEL-2 + OA. UA, ursolic acid; OA, oleanolic acid; CAM, chorioallantoic membrane; DMSO, dimethyl sulfoxide.

cells to the CAM. After 4 doses of triterpene solutions, relevant changes in the vascular arrangement surrounding the tumor cell implants were noted, accompanied by some alterations in the process of tumor growth. In correlation with the effects on the normal developing vascular plexus, OA exerted a significant effect on melanoma-associated angiogenesis, which seemed to have contributed to the growth of the tumor area inside the ring (Fig. 5I). The angiogenic reaction in the OA-treated CAMs was clearly reduced compared to both the control and the UA samples (Fig. 5G-I). The 'spokes wheel' type of vascular reaction can be observed converging towards the tumor site for the control and UA samples, but not for cells treated with OA (Fig. 5I). Conversely, UA did not severely impair the tumor-associated capillary density, the growth of melanoma

cells was not inhibited (Fig. 5H), and the invasiveness of the melanoma cells was higher. Although the anti-angiogenic effects were more potent with OA than with UA, the metastatic potential was not inhibited with either agent. For the applied experimental conditions, the two triterpenes influenced, but did not inhibit the invasiveness of the SK-MEL-2 melanoma cells.

The morphometric evaluation of the angiogenic process by applying a 0-5 scale in correlation with the degree of vascular density also indicated that vessel growth was inhibited to a greater extent by OA during the rapid growing stage of the embryo, as well during the tumor growth process and related angiogenesis, using the SK-MEL-2 cell line. Treatment with OA led to a lower vascular density for the assessment

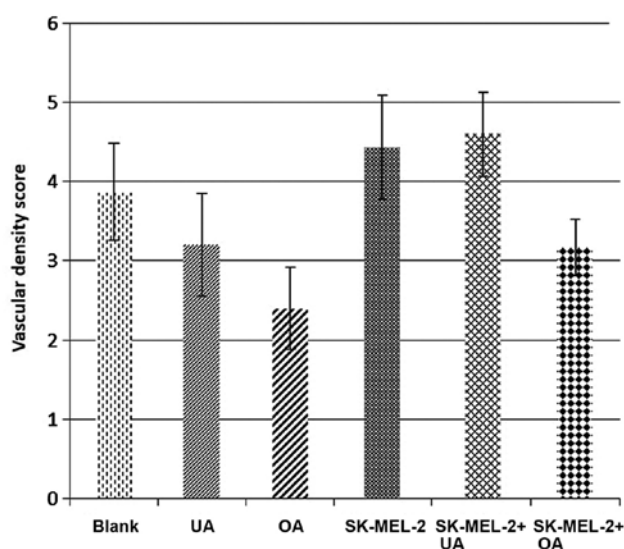


Figure 6. Vascular density scores induced by the test and control samples on CAM. Normal conditions and incubation with SK-MEL-2 melanoma cells. Data were assessed after four doses of UA and OA, using a 0-5 scale, by means of stereomicroscopy. CAM, chorioallantoic membrane; UA, ursolic acid; OA, oleanolic acid.

of melanoma-induced angiogenesis, while UA induced very similar or slightly higher values than those of the control specimens with only melanoma cells (Fig. 6).

Discussion

Proliferation and apoptosis constitute a very precise equilibrium in the healthy human body. Solid tumors represent a cluster of cells originating from a transformed founder cell with an increased capacity of proliferation, a decreased rate of apoptosis, an augmented metabolic rate, a high invasive capacity and an altered morphology (45,46). The proliferative ability of cancer cells at a distant site is essential for metastasis, and difficulties in establishing secondary growth may explain why <0.01% of circulating tumor cells (CTCs) actually form metastases (47).

In this study, we evaluated the potential use of two widely distributed natural triterpenoids as chemopreventive agents in the management of N-RAS-mutated melanoma, obtaining new data on the possible effects of UA and OA on the deregulated pathways of this type of melanoma not related to sun exposure. UA is known to induce apoptosis through the inhibition of the MEK and phosphoinositide 3-kinase (PI3K)/mTOR pathways, involved in a synergistic manner in N-RAS-mutated cancers (8), as demonstrated in prostate cancer (48) and leukemia cells (49), but not in melanoma cells. Less data are available on the modulation of these involved tyrosine kinase receptors by OA. Our results are indicative of the potential benefits of both compounds possibly in combination, for targeting the affected signaling keys of the N-RAS type of melanoma.

Our results revealed an intense anti-proliferative activity of UA *in vitro* on the human melanoma cell line, SK-MEL-2, as compared to the effects of OA, whose anti-proliferative activity was lower. This difference in potency was also highlighted by other research groups. Meng *et al* (48) reported a strong

anti-proliferative potential for UA compared to OA in the case of Caco2 colon cancer cells with IC₅₀ values of 200 μ M for OA and 70 μ M for UA. Fuliás *et al* (50) demonstrated that UA exerted a significant apoptotic activity against A2058 human melanoma cells, an effect that was not observed in the case of OA. In a separate study, our research group reported a superior activity of UA on several breast cancer tumor cell lines, as opposed to OA to which cancer cells were resistant (51).

The cytotoxic activity of triterpenoid compounds on *in vitro* melanoma assays shown in other studies has exhibited variability in potency when tested on different cell lines. Both OA and UA were shown to exert more potent effects on B164A5 cells as compared to the A431 and A375 melanoma cells (52). Due to their very low solubility in water, detrimental to their bioavailability, triterpenic acids were also investigated in different pharmaceutical formulations utilized to improve these parameters. Gamma-cyclodextrin complexes with UA and OA were also tested for their biological activities, indicating better results than for the pure compounds on three melanoma cell lines (52). Some other triterpenoids have been tested under the form of myristoyl ester derivatives, which exhibited increased cytotoxicity values as compared to the compounds alone, on both A431 and A375 melanoma cells (53).

In normal cells, the cell cycle is tightly regulated. In cancer cells, due to genetic alterations, this process becomes disrupted, resulting in uncontrolled growth. Thus, the cell cycle represents the basis of tumor pathology and compounds proven to be active on cell cycle regulation may be candidates in anticancer therapy (54,55).

Taking into account reports that show an anti-proliferative effect of UA on melanoma cells, which may be caused by alterations in cell cycle phases, we performed cell cycle analysis of SK-MEL-2 melanoma cells treated with UA, as previously described (56). In the present study, OA did not exert a significant decrease in cell proliferation; therefore, the activity on cell cycle events was investigated only for UA. Our study confirmed that the anti-proliferative effects of UA were mediated by alterations in the cell cycle, with exposure to 50 μ M of UA inducing arrest in the S phase. It has been previously demonstrated that UA induces cell cycles arrest at the G1 phase in MCF-7 and PC-3 cells (57,58). Others have reported the involvement of OA in the G2/M phase of the cell cycle by decreasing Cyclin B1/cdc2 activity in HepG2 cells (18).

Although metastasis is widely regarded as an inefficient process, the majority of cancer patients succumb to the disease due to metastases rather than from their primary tumors. In the metastasis cascade, the adhesion of cancer cells to vascular endothelial cells through adhesion molecules is a crucial step (47). This intercellular interaction takes place in the context of a permanent mechanical shear stress of the bloodstream, where a balance between the hydrodynamic friction forces and the intercellular liaisons (cancer cells, endothelium) is established (59). Due to these pressures, only a small percentage of CTCs survives in order to metastasize (60). This is why the inhibition of intercellular interaction has become an important therapeutic target in the attenuation of metastases (61). Thus, tests which measure cell adhesion to surfaces/substrates are useful in the characterization of cellular superficial interactions (62). Investigations into tumor

cell-endothelial contact formations have been based on similarities to the leukocyte-endothelial cell interactions during inflammation (61).

Since cell adhesion and cell interaction are key steps in the metastatic process, in this study, we aimed to examine the influence of UA, the most active compound, on cell adhesive capacity. Using the flow chamber protocol, we observed that incubating the SK-MEL-2 cells with high concentrations of UA (50 μ M) led to a decreased adhesion capacity to the ICAM substrate, without however, influencing the adherence capacity of the cells to VCAM.

VCAM 1 (also known as CD106) is an adhesion molecule which mediates the adhesion of leukocytes to the endothelium. It is expressed by endothelial cells following stimulation by cytokines. It has been reported that colorectal cancer cells adhere to the vascular endothelium by binding to VCAM-1 (63). In addition, some types of melanoma cells are known to adhere to the endothelium through VCAM-1 (64). ICAM-1 (also known as CD54) is a transmembrane glycoprotein, part of the immunoglobulin family, and is widely expressed by hematopoietic and non-hematopoietic cells, including endothelial cells, leukocytes, fibroblasts or cancer cells; however, under cytokine stimulation it may be expressed on every human cell type (65). It is known that the inhibition of ICAM-1 expression on melanoma cells may reduce their metastatic potential (66).

In order to obtain more data regarding the possible implication of the two selected triterpenoid compounds on the aggressive metastatic process induced by melanoma cells with an N-RAS mutation, in conjunction to the *in vitro* studies, we chose to apply an *in vivo* method, CAM assay. The choice of the protocol was based on multiple advantages, such as low costs, consumed time and number of sacrificed animals that render this assay effective for pre-screening model studies (67).

Following stereomicroscopic evaluation, we observed that the two analyzed compounds functioned differently on the *in vivo* developing vascular system and the tumor microenvironment. OA exerted more potent effects as compared to UA, on the normal process of angiogenesis, but without influencing the viability of the embryos. Inside the application ring, OA induced a decrease in the number of newly formed capillaries during a highly angiogenic interval (i.e., EDD7-EDD11) (68). The vascular branching pattern was also influenced outside the application site, but to a lower extent compared to UA.

The two triterpenoids also differently influenced the development of *in vivo* SK-MEL-2 melanoma on the chick embryo CAM. UA induced a lower impairment of the tumor progress, without important limitations in tumor growth, which was in contrast with the *in vitro* results of the SK-MEL-2 cytotoxicity assay. Moreover, tumor angiogenesis was not inhibited. Therefore, the invasiveness of the tumor was not restricted, showing extended areas of secondary tumors outside the application spot. At a concentration of 30 μ M, it seemed that UA had stimulatory implications on tumor progression, compared with the untreated control CAMs. Conversely, OA, the compound considered to exert less potent effects on tumor growth *in vitro* on the SK-MEL-2 cells, limited to a greater extent the growth of tumor cells inside the ring, *in vivo*.

Other studies have reported the anti-angiogenic effects of the two compounds. Cárdenas *et al* (69) reported the *in vitro*

effects of UA on angiogenesis, proving that it inhibited certain stages of angiogenesis (proliferation, migration and endothelial cell differentiation), while stimulating other stages (extracellular matrix degradation by MMP-2 and urokinases). Still, both compounds were shown to inhibit the production of vascular endothelial growth factor (VEGF), one of the factors responsible for angiogenesis.

Lin *et al* (70) reported the anti-angiogenic effects of UA *in vitro* and *in vivo* in colorectal cancer. It reduced intratumoral microvascular density in mice with colorectal cancer, as well as the number of blood vessels in the CAM pattern, and it also inhibited the expression of certain angiogenic factors. Both UA and OA exerted anti-angiogenic effects on liver cancer cells (71).

It has been reported that VEGF-induced angiogenesis can be modulated by ICAM (72), a cellular adhesion molecule with high impact in developing recurrence, invasion and metastatic process (73), its production being inhibited by the two studied acids (28). As we only tested UA for this effect, and it showed an anti-adhesive potential, particularly towards ICAM molecules *in vitro*, we considered that the invasive pattern of the SK-MEL2 cells incubated with UA on the CAM assay was rather determined by a different pathway.

As regards the anti-angiogenic effects of 30 μ M UA and OA on normal CAM, our results obtained for UA were contrary to those reported by Cárdenas *et al* (69), who observed a reduction in angiogenesis in 50% of the eggs treated with 20 μ mol UA. In addition, Sohn *et al* (74) reported an increased anti-angiogenic potential for UA, compared to OA, with IC₅₀ values of 5 and 20 μ M for UA and OA, respectively. However, both studies were performed using a bovine aortic endothelial cell CAM model. On the other hand, Kiran *et al* (75) reported an impairment of angiogenic modulators after the administration of UA at concentrations >10 μ M, while lower concentrations did not affect the angiogenesis of human umbilical vein endothelial cells (HUVECs). No data were found regarding the effects of OA or UA in a SK-MEL-2 melanoma model on the CAM assay.

In conclusion, the two tested triterpenoid acids, UA and OA, exerted differential effects *in vitro* and *in vivo* on SK-MEL-2 melanoma cells. UA exerted a significant dose-dependent anti-proliferative effect *in vitro*, compared to OA. The cytotoxic effects *in vitro* on the melanoma cells were determined by the alterations in the cell cycle phases by UA that induced cell arrest in the S phase. Moreover, UA may contribute to the capacity of SK-MEL-2 melanoma cell invasiveness by limiting the cell adhesion capacity to ICAM molecules, but not influencing the adhesion to VCAM.

Assessing the effects of the two triterpenoids *in vivo* using SK-MEL-2 melanoma cells on a CAM model, our results revealed the potential impairment of the invasive and angiogenic tumor process to a greater extent for OA compared to UA. These elements are suggestive of testing the two compounds as a mixture, with possible synergic or additive effects on blood vessels and tumor cells. Even though further studies are warranted to confirm our effects, the easily available compounds from accessible natural sources, UA and OA, may be considered effective chemopreventive agents for possible use in the management of N-RAS-mutated melanoma.

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