POLYURETHANE NANOSTRUCTURES INCORPORATING URSOLIC AND OLEANOLIC ACIDS: *IN VITRO* ANTIPROLIFERATIVE EVALUATION

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ABSTRACT

In the last years, the frequency of melanoma is increasing while melanoma death-related causes currently represent more than 80% of all skin cancers. Oleanolic and ursolic acids are pentacyclic triterpenes that were proven as efficient antitumor agents in several cancers such as colon, liver, breast and prostate. The aim of this study was synthesis of a new delivery system for ursolic and oleanolic acids, as a solution for their poor water solubility that presumably causes low bioavailability. Using the interfacial polycondensation technique combined with spontaneous emulsification, nanoparticles with diameter between 30 and 68 nm were obtained.*In vitro*antriproliferative assay was conducted on several melanoma cell lines using the free pure compounds as blank A significant antiproliferative effect of ursolic acid on human and murine melanoma cells was observed, while the same doses of oleanolic acid exhibited only low potency on all tested cell lines. When incorporated into polyurethane nanoparticles, the active compounds lack their antiproliferative effect, probably due to a weak release from the final nanostructures.

Keywords: antiproliferative, melanoma, oleanolic acid, polyurethane nanoparticles, ursolic acid

INTRODUCTION

In the last years, the frequency of melanoma is increasing [1] while melanoma death-related causes currently represent more than 80% of all skin cancers [2]. The highest incidence occurs between age 20 and 45, with a mortality rate higher in men than in women; however, the incidence is higher among women, due to the fact that,usually, melanoma occurs on areas hard to be noticed on men, while on women it occurs on lower legs [3, reviewed in 4].

New treatment strategies are generated byincreasedresearch in the field, natural compounds being a significant part of the currently studied compounds. Oleanolic and ursolic acids are pentacyclic triterpenes that were proven as efficient antitumor agents in several cancers such as colon, liver, breast and prostate [5-7]. Also, several studies have reported their efficacy against melanoma [8, 9].

The aim of this study was synthesis of a new delivery system for ursolic and oleanolic acids, as a solution for their poor water solubility thatpresumably causes low bioavailability. The delivery system is based on polyurethane nanoparticles which were physicochemically analyzed in terms of particle size; in order to evaluate their efficacy, an *in vitro* antriproliferative assay was conducted on several melanoma cell lines using the free pure compounds as blank.

MATERIALS AND METHODS

Substances

Ursolic and oleanolic acids of analytical purity were purchased from Fluka (Sigma Aldrich, Steinheim, Germany). The reagents were purchased as follows: Merck (Germany) provided isophoronediisocyanate (IPDI), Polyethylene glycol M = 200 (PEG), acetone, Span®85 and Tween®20. 1,4-butanediol (BD) was purchased from Carl Roth GmbH (Germany) and ethylene glycol (EG) from Lach-Ners.r.o. (Czech R.).

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Synthesis of polyurethane drug delivery system
The method described in literature by Bouchemal*et al*. (10) was used for the polyurethane particles synthesis. The method consists in a multi-step procedure: interfacial polycondensation technique and spontaneous emulsification. An organic phase (mixture of 1.5 ml IPDI, 1.5 ml Span®85, and 15 ml acetone heated at 30°C) was injected into an aqueous phase (0.8 ml EG, 0.8 ml BD, 0.3 ml PEG, and 1.5 ml Tween®20 mixed with 15 ml distilled water and heated at 30° C) under magnetic stirring (500 rpm). The final mixture was heated at 40°C for four hours in order to ensure complete chemical reactions. The nanoparticles obtained from the procedure described above were maintained as layers of 3 mm thickness in Petri dishes at 80° C for 12 hours, for water and acetone removal, and then the products were washed with a mixture (water-acetone 1:1 v/v). The two triterpenic acids were added in concentration of 57 μM in two experiments and empty polymeric nanoparticles were used as a control.

Nanoparticles' size measurements
A particle size analyzer (Vasco from Cordouan Tech., France) was used in order to measure the size of the obtained polyurethane nanoparticles. Solutions of 1:100 (w/w) in ethanol were prepared. The parameters used were: 190 channels, 25° C temperature, 80% laser power, 18 μs time interval, continuous acquisition mode and Log-normal dispersion. The average value of three consecutive measurements was considered.

Alamar Blue in Vitro Analysis
A375 human melanoma cells (Sigma-Aldrich, Bucharest, Romania) were cultured in DMEM (Sigma-Aldrich, Bucharest, Romania) containing 15% FCS (fetal calf serum, PromoCell, Heidelberg, Germany) and 1% penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell). SK-mel 2 human melanoma cells (ATCC, Germany) were cultured in EMEM (ATCC, Germany) containing 10 % FBS (fetal bovine serum, ATCC, Germany) and 1% penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell). B16 4A5 murine melanoma cells were cultured in DMEM (Sigma-Aldrich, Bucharest, Romania) containing 10% FCS (fetal calf serum, PromoCell, Heidelberg, Germany),1% penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell) and 1% glutamine (PromoCell). Cells were maintained at an atmosphere of 5% CO_2 at 37°C.
The cell lines were seeded onto a 96-well microplate (5,000 cells/plate) and attached to the bottom of the well for 24 h. The next day, 150μL of new medium containing the tested substances were added and cells were incubated for 48 h.ursolic acid (UA), polyurethane nanoparticles incorporating ursolic acids (UA_nano), oleanolic acid (OA) and polyurethane nanoparticles incorporating oleanolic acid (OA_nano) were added in the concentrations of 25, 50, 75 and 100 μM. The two highest concentrations (75 and 100 μM) of theempty polyurethane nanoparticles were tested. After the exposure time, 15 μL of the Alamar Blue solution was added and the cells were incubated for at least 4 h at 37 °C. A microplate reader was used to spectrophotometrically analyze the samples at 570 nm and 600 nm respectively. Untreated cells were used as controls. Since the tested substances were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Ayrshire, UK) and stored as stock solutions, the highest concentration of DMSO in the final medium (0.1%) was tested and did not have any significant effect on cell proliferation. For all experiments, final concentrations of the tested compounds were prepared by serial dilutions of the stock solution (10 mM) with the DMEM and EMEM medium.
Cell viability was calculated using the formula:

$$\{[(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2 \text{ of test agent dilution}]/[(\varepsilon_{OX})\lambda_2 A°\lambda_1 - (\varepsilon_{OX})\lambda_1 A°\lambda_2 \text{ of untreated positive growth control}]\} \times 100$$

Where,
ε_{OX}= molar extinction coefficient of alamar Blue oxidized form (BLUE);
A = absorbance of test wells;
A° = absorbance of positive growth control well (cells without tested compounds);
λ_1= 570 nm and λ_2 = 600 nm

All *in vitro* experiments were performed on microplates with at least four parallel wells. The results are are presented as mean ± standard deviation. One way Anova test was used to determine the statistical difference between various experimental groups;*, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

Nanoparticle's diameter
Polyurethane nanoparticles with the two tested acids were prepared. Figure 1 exhibits the results of particle measurements for the polyurethane nanostructures with oleanolic acid (a), polyurethane nanoparticles with ursolic acid (b) and empty polyurethane nanoparticles (c). One can notice that nanoparticles with diameter between 30 and 68 nm were obtained, as follows: 30.91 nm for polyurethane nanoparticles with oleanolic acid, 58.90 nm for polyurethane nanoparticles containing ursolic acid and 67.63 nm for empty polyurethane samples. Only one population was obtained for all the samples, thus indicating their homogeneous nature.

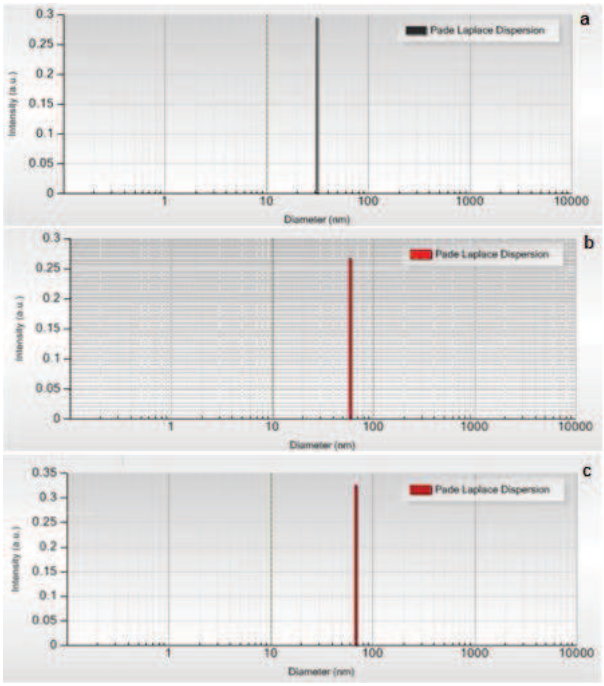

Fig. 1. Diameter of polyurethane structures for: (a) polyurethane nanoparticles with oleanolic acid, (b) polyurethane nanoparticles with ursolic acid and (c) empty polyurethane nanoparticles. Parameters used were: temperature (25° C), 190 channels, laser power (80%), time interval (18 μs), acquisition mode (continuous) and analysis mode (Pade-Laplace).

Cell proliferation assay

Figure 2 present the *in vitro*antiproliferative effects of ursolic and oleanolic acids compared to the encapsulated compounds on A375 human melanoma cell line. Exposure to ursolic acid for 48 h caused thedecrease of A375 cells' viability in a dose-dependent manner. The viability values were as follows: 96.60 ± 1.63 %, 76.25 ± 4.60 %, 52.61 ± 9.74 % and 33.23 ± 3.20 % for 25, 50, 75 and 100 μM, respectively. Encapsulation of ursolic acid inside polyurethane nanoparticles led to the following results: 97.98 ± 2.46 %, 85.74 ± 8.15 %, 100.44 ± 7.37 %, and 79.49 ± 4.54 %, for the same concentrations, respectively. In case of oleanolic acid, the viability values were: 77.94 ± 7.92 %, 75.70 ± 2.88 %, 75.15 ± 6.90 %, 82.54 ± 0.20 % for 25, 50, 75 and 100 μM, respectively. The same concentrations of the polyurethane nanoparticles incorporating ursolic acid led to the results: 86.68 ± 2.48 %, 88.03 ± 7.83 %, 88.59 ± 6.37 %, 85.58 ± 5.15 %, respectively. Also, the highest concentrations (75 and 100 μM) of empty polyurethane nanoparticles were tested and the viability values after 48 h of exposure were: 94.72 ± 6.69 % and 86.33 ± 2.20 %, respectively.

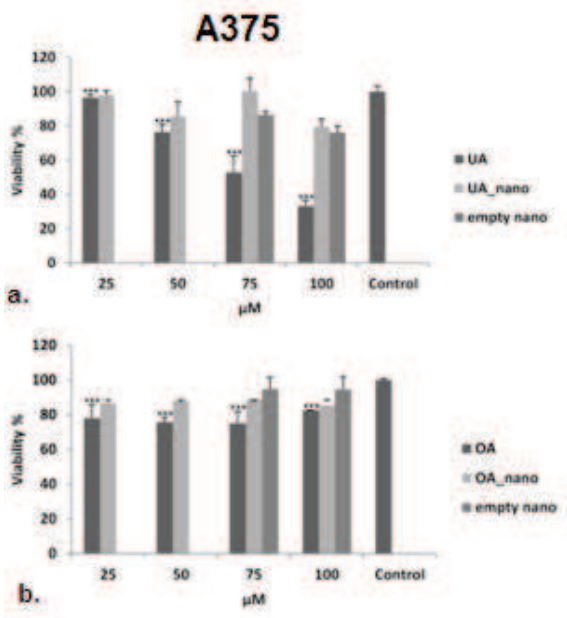

Fig. 2. *In vitro* viability of A375 human melanoma cells after 48 h of exposure to: (a) ursolic acid (UA), polyurethane nanoparticles incorporating ursolic acid (UA_nano), empty polyurethane nanoparticle (empty_nano) and (b) oleanolic acid (OA), polyurethane nanoparticles incorporating oleanolic acid (OA_nano), empty polyurethane nanoparticle (empty_nano) as compared to the untreated cells (control).

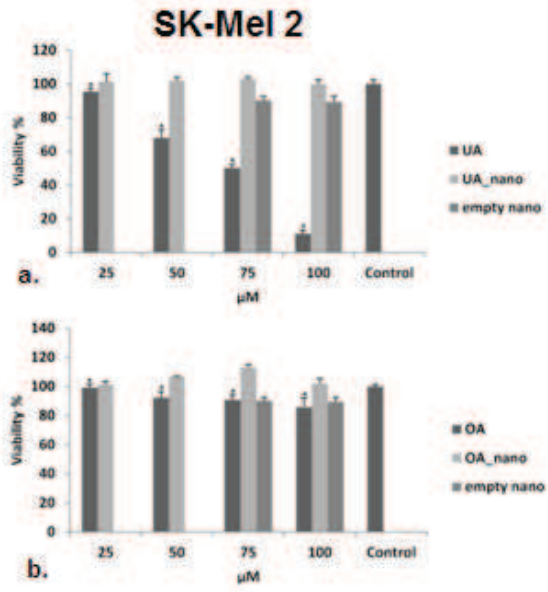

Fig. 3. *In vitro* viability of SK-Mel 2 human melanoma cells after 48 h of exposure to: (a) ursolic acid (UA), polyurethane nanoparticles incorporating ursolic acid (UA_nano), empty polyurethane nanoparticle (empty_nano) and (b) oleanolic acid (OA), polyurethane nanoparticles incorporating oleanolic acid (OA_nano), empty polyurethane nanoparticle (empty_nano) as compared to the untreated cells (control).

As figure 3 shows, the viability values for SK-Mel 2 cells treated with ursolic and oleanolic acids as well as their nanoparticles are: 95.40 ± 1.89 %, 67.90 ± 4.77 %, 50.12 ± 2.16 %, 11.24 ± 2.19 % for 25, 50, 75 and 100 µM of ursolic acid, respectively. Treatment with nanoparticles incorporating ursolic acid resulted in the following values: 101.34 ± 4.77 %, 101.76 ± 2.22 %, 102.91 ± 1.35 % and 100.00 ± 2.41 % for 25, 50, 75 and 100 µM, respectively. Oleanolic acid's treatment led to the following results: 99.25 ± 2.19 %, 92.30 ± 4.23 %, 90.83 ± 3.16 % and 85.73 ± 4.38 % for 25, 50, 75 and 100 µM, respectively. For the same concentration of polyurethane nanoparticles incorporationgoleanolic acid the viability values were: 101.23 ± 2.31 %, 106.51 ± 0.48 %, 113.07 ± 2.00 % and 102.21 ± 3.28 %, respectively. The empty nanoparticles led to 90.20± 2.37 % for 75 µMand 89.34 ± 3.26 % for 100 µM.

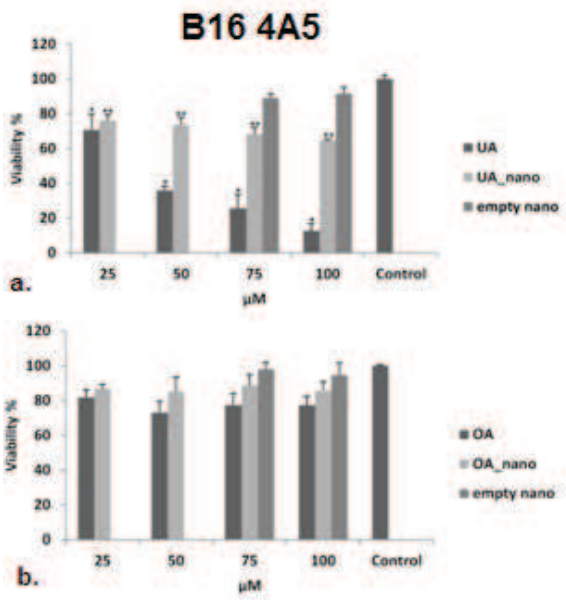

Fig. 4. *In vitro* viability of B16 4A5 murine melanoma cells after 48 h of exposure to: (a) ursolic acid (UA), polyurethane nanoparticles incorporating ursolic acid (UA_nano), empty polyurethane nanoparticle (empty_nano) and (b) oleanolic acid (OA), polyurethane nanoparticles incorporating oleanolic acid (OA_nano), empty polyurethane nanoparticle (empty_nano) as compared to the untreated cells (control).

B16 4A6 murine melanoma cells viability values after treatment with the tested compounds are presented in Figure 4. Ursolic acid's treatment produced decrease in cell viability as follows: 70.35 ± 9.23 %, 36.04 ± 2.32 %, 25.85 ± 7.80 % and 12.70 ± 4.11 % for 25, 50, 75 and 100 µM, respectively. For the same concentrations of nanoparticles with ursolic acid the results were: 76.08 ± 3.31 %, 73.68 ± 4.10 %, 68.43 ± 3.53 % and 64.77 ± 0.68 %, respectively. Treatment with oleanolic acid resulted in the following values: 81.23 ± 4.23 %, 73.00 ± 6.49 %, 77.41 ± 6.57 % and 77.31 ± 5.18 % for 25, 50, 75 and 100 µM, respectively. At the same concentrations, the results for nanoparticles with oleanolic acid were: 86.68 ± 2.48 %, 85.04 ± 8.53 %, 88.59 ± 6.37 % and 85.58 ± 5.15 %, respectively.

DISCUSSION

The role of pentacyclictritepenes in cancer treatment is the subject of many reports. Ursolic and oleanolic acid extracted from various plants were proven to act as antiproliferative agents in many cancer types. Meng*et al*. [11] reported the ursolic acid's activity on DU 145, PC-3 and LNCap prostate cancer lines with IC_{50}values ranging between 22 and 36 µM. Also, they reported ursolic acid's antitumor effect on HeLa (cervix) and RPMI 8226 (myeloma) cells [11]. Shan *et al*. [12] reported the *in vitro* antitumor activity of ursolic acid on HL60 and K562 (leukemia) cells as well on HL60 and H562 – Adriamycin resistant leukemia cells. Other studies also reported the*in vivo* anticancer activity of ursolic acid in HCT11 colon cancer xenograft [13] as well as in DU145 prostate cancer cells implanted in athymicBalb/c nude male mice [14].

In terms of oleanolic acid's anticancer activity, *in vitro* effects were reported for BGC-823 (gastric) and A549 (lung) cancer cells [15]. Kartini*et al*. reported positive results of oleanolic acid against SiHa (cervix) cells [16]. Cheng *et al*. reported the oleanolic acid antitumor activity on MCF-7 (breast) cancer cells; Cheng's results are contradictory to Hsu *et al*. that reported a weak anticancer activity of oleanolic acid on MCF-7 and MDA-MB-231 cells [17].

Our data revealed the *in vitro*antiproliferative activity of ursolic acid on all tested melanoma cell lines. The viability decrease manifested in a dose-dependent manner for all three cell lines, however with different potencies. The IC_{50}values ranged between 75 and 100 µM for A375 (human) melanoma cells, between 25 and 50 µM for B16 4A5 (murine) melanoma cells and 75 µM for SK-Mel 2 (human) melanoma cells. The same concentration of oleanolic acid seems to produce a weak response as antiproliferativeagent on the tested cells. For all cell lines, the IC_{50} values were not found in the tested range of concentration, the lower viability being recorded for B16 4A5 (73 %). SK-Mel 2 proved to be the most resistant to oleanolic acid. Our results are consistent with previous data, suggesting that ursolic acid is more effective than the oleanolic acid [18, 19] in terms of antiproliferative activity.

In recent years, nano- and micro-particles have raised medical interest, being used as drug delivery systems [20]. Some of the new strategies of drug delivery in nanotechnology are polymeric nanoparticles, microemulsions, liquid cristals systems, solid lipid nanoparticles and liposomes [21]. Along with other advantages, it was reported that nanostructures could improve the solubility and stability of the active substances

and alsoassociate compounds with different hydrophilicity/lipophilicity degrees [22, 23]. These advantages could lead to the reduction of the therapeutic dose and the improvement of pharmacological activity [21].
The present synthesis of polyurethane nanoparticles was based on the method previously described by Bouchemal*et al*. [10], who obtained nanocapsules as drug carriers for α-tocopherol, a good antioxidant with a high sensitivity on light, heat and oxygen. Based on this procedure, Borcan*et al*. also obtained microparticles using isophorondiisocyanate, with nontoxic *in vitro* activity on mesenchymal stem cells and reduced*in vivo* noxiousness on CD1Nu/Nu mice [20].
As the present study revealed, the encapsulation of ursolic and oleanolic acid into poly(ether) urethane nanoparticles did not improve the *in vitro* activity of the active compounds, leading to a high viability of the three cancer cell lines, as compared to the antiproliferative activity of the free substances. A possible explanation may consist in the strong entrapment of the active agent inside the polymeric nanostructures which presumably causes an impossibility to exert its pharmacological activity. Further research should be focused on the *in vivo* study of the polyurethane nanoparticles loaded with the active compounds.

CONCLUSIONS

The present study reportsa significantantiproliferative effect of ursolic acid on human and murine melanoma cells while the same doses of oleanolic acid exhibited only low potency on all tested cell lines. When incorporated into polyurethane nanoparticles, the active compounds lack their antiproliferative effect, probably due to a weak release from the final nanostructures.

ACKNOWLEDGEMENTS

This paper was published under the frame of European Social Found, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/136893.

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NANOSTRUCTURI POLIURETANICE INCORPORÂND ACIZII URSOLIC ȘI OLEANOLIC: EVALUAREA EFECTELOR ANTIPROLIFERATIVE *IN VITRO*

REZUMAT
În ultimii ani, frecvența melanomului este în creștere, iar mortalitatea produsă de melanom reprezintă mai mult de 80% din cancerele pielii. Acizii oleanolic și ursolic sunt triterpene pentaciclice care s-au dovedit a fi agenți antitumorali eficienți în câteva tipuri de cancere precum colon, ficat, sân și prostată. Scopul studiului a fost sinteza unui noi sistem de livrare a acidului ursolic și oleanolic, ca o soluție pentru slaba solubilitate în apă, care posibil să fie cauza unei biodisponibilități scăzute. Utilizând tehnica de policondensare interfacială combinată cu emulsificarea spontană, au fost obținute nanoparticule cu diametru intre 30 și 68 nm. S-au realizat teste antiproliferative *in vitro*, pe câteva linii celulare de melanom, utilizând compuși puri liberi ca martori. A fost observat un efect antiproliferativ semnificativ al acidul ursolic pe liniile celulare melanomice umane și murinice, în timp ce aceleași concentrații de acid oleanolic au avut o potență redusă pe toate liniile celulare testare. După incorporarea în nanoparticule poliuretanice, compușii activi și-au pierdut efectul antiproliferativ, probabil datorită unei eliberări scăzute din nanostructurile finale.
Cuvinte cheie: antiproliferativ, melanom, acid oleanolic, nanoparticule poliuretanice, acid ursolic