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Original article

The new esters derivatives of betulin and betulinic acid in epidermoid squamous carcinoma treatment – *In vitro* studies

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ABSTRACT

Background: Betulinic acid and betulin are triterpenes with documented cytotoxic properties toward various cell lines. Unfortunately both betulinic acid and its metabolic precursor, betulin, are very poorly soluble in aqueous buffers, thus their bioavailability and bio-distribution are insufficient in terms of medical applications.
Objective: To investigate the specific anticancer role of the newly synthesized betulin derivatives in human epidermoid carcinoma cells.
Methods: In the present study we synthesized five amino acid esters of betulin. For the synthesis we selected alanine (Boc-L-Ala-OH, negative control) and four basic amino acids – natural lysine (Boc-L-Lys(Boc)-OH) and three its unnatural derivatives (Boc-L-Dap(Boc)-OH, Boc-L-Dab(Boc)-OH, and Boc-L-Orn(Boc)-OH). Boc-protected amino acids were most convenient for the synthesis. All new esters have one (betulin-L-Ala-NH_2) or two free amino groups which significantly increase their solubility in water and facilitate their transport through the cell membrane. It is worth noting that the biological activity of new esters of betulin is positive correlated with the length of the side chain of L-amino acid. The highest biological activity displayed compound containing lysine side chain (Lys, $-CH_2-CH_2-CH_2-CH_2-NH_2$). Considering the biological activity, other derivatives can be set in the following series: Orn ($-CH_2-CH_2-CH_2-NH_2$) > Dab ($-CH_2-CH_2-NH_2$) > Dap ($-CH_2-NH_2$) > Ala (CH_3) > betulin. New betulin esters were tested in normal human keratinocytes (HaCaT) and human epidermoid carcinoma cells (A431). To assess cytotoxicity, MTT test was performed after 24, 48 and 72 h of incubation with the test compounds at a concentration range of 0.75–100 μM. In case of apoptotic activity, a TUNEL method and comet assay were performed. Additionally expression of caspase-3 and PARP1 was evaluated immunocytochemically.
Results: The highest cytotoxicity in cells induced skin cancer new compounds, particularly compound containing a lysine side chain (IC50 = 7 μM) and ornithine (IC50 = 10 μM). The highest number of apoptotic cells was observed in case incubation with compound containing Orn, Dab and Dap side chain.
Conclusions: The new betulin ester derivatives display enhanced antitumor activity compared to their non-modified precursors. It is worth emphasizing their specific toxicity against epidermoid carcinoma cells.

1. Introduction

The new anticancer agents and therapies are constantly searched. Researchers focus mainly on new applications of well-known drugs and also look for new, more efficient compounds with anti-tumor activity. Therefore, the development of novel and natural potent, non-toxic anticancer agents is worth a continuous effort. Natural substances derived mostly from plants provide an excellent platform for new drugs discovery. Herbal medicine based on drugs derived from plant in medicinal practice often becomes a kind of an alternative treatment [1,2]. The majority of anticancer factor are of natural origin. The natural environment is a rich source of such materials which are currently used in oncology either in their intact/raw form, or as chemically modified derivatives (vinblastine, vincristine, paclitaxel, etoposide, teniposide) [3]. Moreover, it is known that natural products have been widely used for fighting human

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ailments [4,5]. Recently, the researchers are interested in pentacyclic triterpens. These natural agents are formed by arrangement of squalene epoxide and are found in most plants. One of such compounds with promising anticancer activity are betulin (found mostly in the bark of the birch), betulinic acid (oxidation product of betulin) and their chemical derivatives [6,7]. Previously reported *in vitro* and *in vivo* studies have demonstrated that these compounds have anti-tumor properties, and induce apoptosis in various tumor cells (human melanoma lines Mel-1, Mel-2, squamous carcinoma line A-431, cancer of the stomach sensitive and resistant to daunorubicin) [8–12].

Betulinic acid and betulin are triterpenes with documented cytotoxic properties against various cell lines [13]. Unfortunately both betulinic acid and its metabolic precursor, betulin, are very poorly soluble in aqueous buffers, thus their bioavailability and bio-distribution are insufficient in terms of medical applications. The newly synthesized betulin derivatives have a much better solubility in water while maintaining a high biological activity [11,14]. In 2006, the action of triterpenes derived from bark of the birch on tumor squamous cell line A431 and also on normal human keratinocytes was described. The preliminary studies were performed in clinical trials in patients with a clinical diagnosis of actinic keratosis using the suspended in oil triterpenes extract [15]. Moreover, these compounds can induce apoptosis in cancer cells derived from keratinocytes. It was also demonstrated that betulin derivatives can stimulate differentiation in normal keratinocytes [16,17]. Actinic keratosis (AK) is in the skin considered as precancerous lesion. These lesions are occurred in the skin damaged by the sun's rays. From the focal lesion can develop squamous cell carcinoma. The malignant neoplasm derived from keratinocytes can metastasize. Despite the raised frequency of skin cancer, the knowledge is still very poor and requires further research. The problem is very complex due to the trends in fashion for sunbathing, continues exposition on ultraviolet radiation, ozone depletion expanding and lengthening the survival of the population. Currently there are many applied treatments of AK, however none of them is sufficiently effective. The local formulations containing cytostatic (5-fluoroacyl) and immunomodulatory substances (imiquimed, diclofenac) are associated with large inflammatory reaction in the treated skin. Moreover, laser therapy and photodynamic therapy are very expensive methods, requiring specialized equipment and proper photosensitizers. Therefore, the new method and chemotherapeutics are needed for AK treatment. Recent studies showed the high efficiency in the AK treatment of the oil extract from triterpenes, derived from the birch bark. Moreover, the obtained results indicated very good skin tolerability [15,17,18].

Our research group derivatization of betulin from birch bark with selected natural amino acids (National patent application No PL211589 of 28.12.2011), which had better water solubility than betulin. We have also found, that new compounds have proapoptotic activity in cancer cells [11] what was has been also indicated in other studies [19,20]. The purpose of our studies was to estimate the anticancer potency of new derivatives of betulin and betulinic acid in malignant cells derived from keratinocytes. The effect of those compounds will be also evaluated on model cells – normal human keratinocytes.

2. Material and methods

2.1. Cell culture

The human epidermal cancer cell line was used (A431), and normal keratinocytes (HaCaT) was use as a control. The epidermoid squamous carcinoma cell line A-431, derived from an 85-year-old female, is one of a series of cell lines established from solid tumors by D.J. Giard et al. [21]. The HaCaT cell line was a well-known immortalized human keratinocyte cell line, which was utilized for their high ability to proliferation and differentiation *in vitro* (ATCC) The cells were grown in DMEM (medium, Sigma) with the addition of 10% fetal bovine serum (Sigma–Aldrich) and supplemented by antibiotics (Antibiotic-Antimycotic Stabilized, Sigma). For further studies, cells were removed by trypsinization (Trypsin-EDTA, Sigma) and washed with PBS. The cells were grown in a humidified atmosphere at 37 °C and 5% CO_2.

2.2. Anti-cancer drug synthesis

In the present study we synthesized five amino acid esters of betulin. One mmol of the appropriate Boc protected L-amino acid was dissolved in 5–6 ml of anhydrous THF and then 1 mmol of the CDI was added. The mixture was preincubated for 30 minutes in room temperature, and after this time 1 mmol of betulin was added. The reaction was carried out under the reflux for 24 h in the inert gas atmosphere (nitrogen or argon). After the reaction was completed THF was removed under reduced pressure and remaining reaction mixture was dissolved in ethyl acetated and washed with citric acid (5%), $NaHCO_3$ (5%) and brine. The organic phase was dried using $MgSO_4$ and evaporated under reduced pressure. The remaining product was purified *via* column chromatography on silica gel. The eluents mixture was chloroform/ethyl acetate (4:1, v/v). The appropriate organic fractions were collected, and were removed under reduced pressure to obtain pure Boc protected monosubstituted betulin esters (purity was confirmed by TLC analysis; eluent chloroform/ethyl acetate 4:1, v/v). In the next step, 3 M HCL in methanol was used to remove Boc groups from obtained compounds (Table 1). The Boc deprotection reaction was monitored by TLC analysis. After reaction was completed, the mixture was suspended in diethyl ether, sonicated and filtered to obtain desired product. For the synthesis we selected alanine (Boc-L-Ala-OH, negative control) and four basic amino acids – natural lysine (Boc-L-Lys(Boc)-OH) and three its unnatural derivatives (Boc-L-Dap(Boc)-OH, Boc-L-Dab(-Boc)-OH, and Boc-L-Orn(Boc)-OH). All new esters have one (betulin-L-Ala-NH_2) or two free amino groups which significantly increase their solubility in water and facilitate their transport through the cell membrane. It is worth noting that the biological activity of new esters of betulin is positive correlated with the length of the side chain of L-amino acid. The highest biological activity displayed compound containing lysine side chain (Lys, –CH_2–CH_2–CH_2–CH_2–NH_2). Considering the biological activity, other derivatives can be set in the following series: Orn (–CH_2–CH_2–CH_2–NH_2) > Dab (–CH_2–CH_2–NH_2) > Dap (–CH_2–NH_2) > Ala (CH_3) > betulin (Figs. 1 and 2).

Table 1
The HR-MS characteristic of synthesized Boc protected and deprotected betulin esters with amino acids.

	HR-MS (calculated)	HR-MS (measured)	Rf ($CHCl_3$/EtOAc 4:1)
Betulin-Ala-Boc	614.4784	614.4688	n.d.
Betulin-Dap(Boc)-Boc	728.5340	729.5406	0.40
Betulin-Dab(Boc)-Boc	743.5574	743.5566	0.44
Betulin-Orn(Boc)-Boc	757.5731	757.5728	0.46
Betulin-Lys(Boc)-Boc	771.5887	771.5881	0.47
Betulin-Ala-NH_2	514.4260	514.4261	n.d.
Betulin-Dap-NH_2	529.4369	529.4383	n.d.
Betulin-Dab-NH_2	543.4526	543.4515	n.d.
Betulin-Orn-NH_2	557.4682	557.4671	n.d.
Betulin-Lys-NH_2	571.4839	571.4827	n.d.

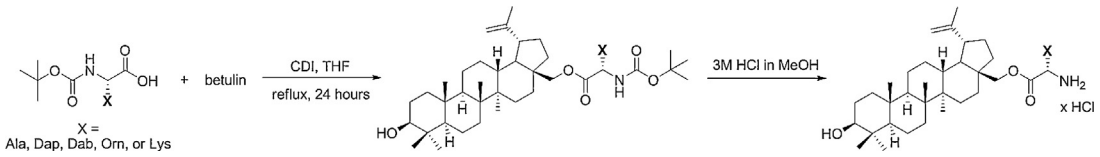

Fig. 1. The general procedure for the synthesis of monosubstituted betulin esters containing L-amino acids.

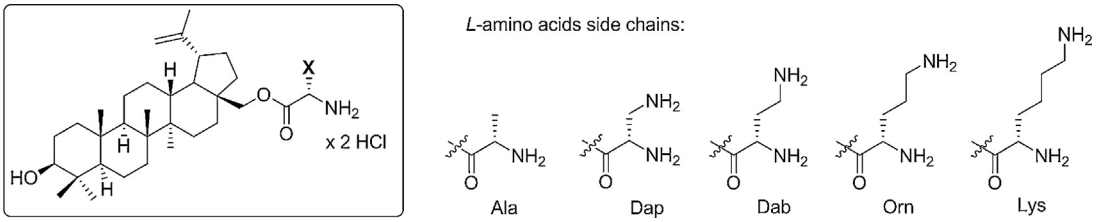

Fig. 2. The general architecture of 5-membered collection of betulin esters containing.

2.3. Survival assay

MTT assay was applied for evaluation anticancer effectiveness of betulin and its new derivatives after 24, 48 and 72 h soft incubation with the test compounds at the concentration range of 0.75–100 μM. The cytotoxicity MTT test is based on the colored reaction of the tetrazolium salt.

2.4. Alkaline comet assay

The alkaline comet assay method was used for the detection of DNA fragmentation associated with apoptosis [22,23]. After incubation with betulin derivatives cells at a concentration of 1×10^5/ml were mixed with low melting-point agarose (Sigma) at a ratio of 1:10 (v/v) and spread on a slide. The slides were submerged in precooled lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, pH 10, 10 mmol/L Tris base, and 1% Triton X-100) at 4 °C 186 for 60 min. After being lysed and rinsed, the slides were equilibrated in TBE solution (40 mmol/L Tris/boric acid, 2 mmol/L EDTA, pH 8.3), electrophoresed at 1.0 V/cm^2 for 20 min, and stained using the silver staining method. To score the comet pattern, 100–200 nuclei from each slide were counted by two independent investigators.

2.5. TUNEL assay

DNA fragmentation was visualized using an ApopTag® (Qbiogene) kit. In the TUNEL assay the enzyme terminal deoxynucleotidyltransferase labels the 3′-OH ends of DNA generated during apoptosis with biotinylated nucleotides. These fragments are detected by immunoperoxidase staining. The apoptosis detection kit distinguishes apoptosis from necrosis by specifically detecting the DNA cleavage and chromatin condensation connected with apoptosis. Human epidermal cancer cells (A431) and normal keratinocytes (HaCaT) were seeded on 8-well slides (20,000 cells per slide, Roth, Germany). Cells were left for attachment overnight and then interaction with betulin derivatives was performed. After treatment *in vitro* the cells were fixed with 4% formalin in PBS for 10 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. TUNEL assay was performed according the manufacturer's (Millipore) instructions. Cells were counterstained with hematoxylin. Then samples were mounted by DPX (Thermo Fisher Scientific, Germany) on glass slides. Cells with stained nuclei were determined by counting 100 cells in 3 randomly selected fields. The counting was achieved by two independent investigators. Samples were evaluated with an upright microscope BX51 (Olympus, Japan).

2.6. Immunocytochemical studies

The expression of caspase-3 and PARP-1 was detected immunocytochemically in case of apoptotic activity determination. Immunocytochemistry was performed using ABC method. Human epidermal cancer cells (A431) and normal keratinocytes (HaCaT) were seeded on 8-well slides (20,000 cells per slide, Roth, Germany). Cells were left for attachment overnight and then interaction with betulin derivatives was performed. After treatment *in vitro* the cell cultures were fixed in 4% paraformaldehyde. Samples were then permeabilized and blocked by incubation with 0.1% Triton X-100 (Sigma) in PBS. Expression of proteins was visualized with the mouse monoclonal antibody (1:100, CAS3 and PARP-1, Santa Cruz Biotechnology). For conventional bright-field microscopy (peroxidase-ABC labeling), the samples were incubated with diaminobenzidine–H_2O_2 mixture (DAKO) to visualize the peroxidase label, counterstained with hematoxylin (Alchem, Poland) for 30 s. Samples were examined on upright microscope BX51 microscopy (Olympus, Japan). Stained cell numbers were determined by counting 100 cells in 3 randomly selected fields. The counting was performed by two independent investigators. The result was judged to be positive if staining was observed in more than 5% of cells. The intensity of immunohistochemical staining was evaluated as (−) negative (no reaction), (+) weak, (++) moderate, and (+++) strong. All experiments were repeated three times.

3. Results

3.1. Survival assay

The highest cytotoxicity in cells skin cancer induced new compounds, particularly compound containing a lysine side chain (IC50 = 2.25 μM for 72 incubation hours and 7.28 for 48 h incubation) and ornithine (IC50 = 4.5 μM for 72 h incubation and 10 μM for 48 h incubation) in comparison to betulin and betulinic acid. For betulin-Dab-NH_2, betulin-Dap-NH_2l and betulin-Ala-NH_2 the effect was significant only after 72 h incubation (Table 2). The enhanced antitumor activity was observed in the new betulin esters compared to their non-modified precursors. In HaCaT control cells the opposite response was obtained (Table 2). The highest cytotoxicity effect was observed after 24 and 48 h

Table 2
IC50 evaluated after 24, 48 and 72 h incubation with active compounds in skin cancer A431 cells and human keratinocytes as a normal control cells.

Compound	IC_{50} for HaCat cells [μM]			IC_{50} for A431 cells [μM]		
	24 h	48 h	72 h	24 h	48 h	72 h
Betulin-Lys-NH_2	0.6	4.1	145.2	97.2	7.3	2.3
Betulin-Orn-NH_2	4.8	4.5	146.3	96.6	10.1	4.5
Betulin-Dab-NH_2	17.4	9.6	158.2	54.3	56.4	5.2
Betulin-Dap-NH_2	266.2	52.1	149.8	258.1	380.8	37.5
Betulin	13.8	43.8	150.2	76.5	80.2	45.2
Betulinic acid	14.3	16.0	160.2	210.5	40.9	35.6
Betulin-Ala-NH_2	47.8	26.9	160.2	217.1	102.3	9.3
DMSO	230.5	92.4	159.7	126.9	246.7	150.2

IC_{50} calculated according the best trend line fit (polynomial, logarithmic or linear).

incubation. However, it is worth emphasizing their specific toxicity against epidermoid carcinoma cells (Table 2).

3.2. Alkaline comet assay (ACA)

The results of ACA are shown in Table 3 for 24 h incubation and in Table 4 for 72 h incubation for both treated cell lines. The insignificant number of apoptotic and necrotic cells were observed after different compounds (betulin, betulinic acid and their derivatives in A431 cells). The higher number of undefined damage and undamaged nuclei was observed (Tables 3 and 4A). Similar results were obtained for normal human keratinocytes HaCaT (Tables 3 and 4B).

3.3. TUNEL assay

The results of TUNEL assay are presented in Table 5. The selected microphotographs are presented in Fig. 3. The highest TUNEL positivity was observed in A431 cells after incubation with betulin derivatives containing ornithine side chain betulin-Orn-NH_2 (85%) and betulin-Dab-NH_2 (98%) (Table 5 and Fig. 3). After incubation with betulin and betulinic acid the percentage of stained cell nuclei was lower 79% and 53% respectively. Only particular not significant apoptotic cells were observed in normal keratinocytes (data not shown).

3.4. Immunocytochemical staining PARP-1 and caspase 3

PARP-1 is cleaved by caspase-3 during apoptosis, which leads to its inactivation. The results from immunocytochemical assay for PARP1 and casp-3 after incubation with active compounds are presented in Table 6. The PARP-1 overexpression was observed approximately in 100% of observed cells and the low amounts of caspase-3 (<5%) was obtained for modified and non-modified compounds (Table 6).

4. Discussion

Multiple anti-precancerous lesion (actinic keratosis), basal skin cancers, derived from keratinocytes, therapies including surgical procedure, chemotherapy, photodynamic therapy have been developed. Some of them can cause side effects like baldness, immunodeficiency, organ damage and are not enough effective in skin cancer treatment [24,25]. Majority type of cancer cells is resistant to apoptosis. In many cases malignant cells are characterized by mutation in genes responsible for this death [26–28]. All anticancer strategies are mainly based on elimination of malignant cells through inhibition of cell and reactivation of apoptotic cell death. Recently, due to high incident of skin cancer the interest in medicines from plant has increased significantly. Betulin and betulinic acid derived from the birch bark meet these criteria and offer numerous pharmacological benefits [1,27,29]. Although betulin and betulinic acid affect various types of cancer cells including multidrug resistance ones, the normal cells are rather resistance to those compounds [30]. However, their biological activity is limited due to poor water solubility [12]. Antitumor activity of biological substance can be investigated on different level. In our studies we examined the viability of cells by cytotoxicity test *in vitro*. Our results demonstrate that the highest cytotoxicity in skin cancer cells was induced by new

Table 3
Cell death evaluation by alkaline comet assay after 24 h incubation with active compounds in (A) skin cancer A431 cells; (B) human keratinocytes as a normal control cells.

	Apoptosis	Necrosis	Intermediate damage	Not damaged nuclei
(A) A431 cells				
Control cells	6.94	0.58	17.34	75.14
Betulin-Lys-NH_2	0.88	0	6.14	92.98
Betulin-Orn-NH_2	2.00	0	18.00	80.00
Betulin-Dab-NH_2	6.67	0	28.89	64.44
Betulin-Dap-NH_2	5.00	0	15.00	80.00
Betulin	0.91	0	35.45	83.64
Betulinic acid	4.55	1.95	9.10	84.40
Betulin-L-Ala-NH_2	2.60	1.96	18.30	77.14
DMSO	1.94	0.97	19.42	77.67
(B) HaCaT cells				
Control cells	0.93	0	10.28	88.79
Betulin-Lys-NH_2	0.78	3.13	10.16	85.93
Betulin-Orn-NH_2	0	2.00	13.00	85.00
Betulin-Dab-NH_2	2.74	1.83	3.67	91.76
Betulin-Dap-NH_2	1.00	0	2.00	97.00
Betulin	0.96	1.90	20.95	76.19
Betulinic acid	3.00	2.00	7.00	88.00
Betulin-Ala-NH_2	2.00	0	4.00	94.00
DMSO	4.00	1.00	7.00	88.00

Table 4
Cell death evaluation by alkaline comet assay after 72 h incubation with active compounds in (A) skin cancer A431 cells; (B) human keratinocytes as a normal control cells.

	Apoptosis	Necrosis	Intermediate damage	Not damaged nuclei
(A) A431 cells				
Control cells	1.00	0	0	99.00
Betulin-Lys-NH_2	4.31	6.89	19.83	68.97
Betulin-Orn-NH_2	2.26	3.76	3.76	90.21
Betulin-Dab-NH_2	4.07	2.44	12.19	81.30
Betulin-Dap-NH_2	9.24	2.52	2.52	85.72
Betulin	21.26	5.17	12.64	60.93
Betulinic acid	8.46	2.31	12.31	76.92
Betulin-Ala-NH_2	0	0	0	100.00
(B) HaCaT cells				
Control cells	11.11	0	33.33	55.56
Betulin-Lys-NH_2	1.00	0	2.00	97.00
Betulin-Orn-NH_2	7.00	0	0	93.00
Betulin-Dab-NH_2	1.00	0	0	99.00
Betulin-Dap-NH_2	2.00	0	0	98.00
Betulin	0.85	0.85	42.74	55.56
Betulinic acid	10.22	0.73	12.41	12.41
Betulin-Ala-NH_2	13.16	3.51	13.16	70.17

Table 5
Apoptosis detected by TUNEL assay 72 h incubation with active compounds in skin cancer A431 cells.

Active compound	% of A431 cells with stained nuclei
Control cells	0%
Betulin-Lys-$NH_2 \times 2HCl$	75%
Betulin-Orn-$NH_2 \times 2HCl$	85%
Betulin-Dab-$NH_2 \times 2HCl$	98%
Betulin-Dap-$NH_2 \times 2HCl$	97%
Betulin	79%
Betulinic acid	53%
Betulin-Ala-$NH_2 \times HCl$	49%

betulin esters containing lysine or ornithine side chain. Moreover, the active doses of these betulin derivatives were significantly lower than betulin itself. The obtained data showed also that viability of human keratinocytes decreased especially after 24 h incubation with betulin, betulinic acid and their derivatives. In the other investigation it was reported, that triterpenes from birch bark with betulin as the main compound present dose-dependent cytotoxic effects in immortalized human HaCaT keratinocytes and skin cancer [15]. Huyke at al. also found that oleo-gel from birch bark containing betulin and its derivatives was effective in the current treatment of actinic keratosis [18,31]. Suresh at al. examined the inhibitory effect of betulinic acid and its four derivatives against four cancer cell lines melanoma A375, neuroblastoma SH-SY5Y, breast adenocarcinoma MCF-7 and epidermoid carcinoma. Their ionic derivatives have constantly presented much lower IC50 values against four cancer cell lines and indicated elevated cytotoxicity than betulinic acid [16]. In another study, Qian showed that a series of new imidazole

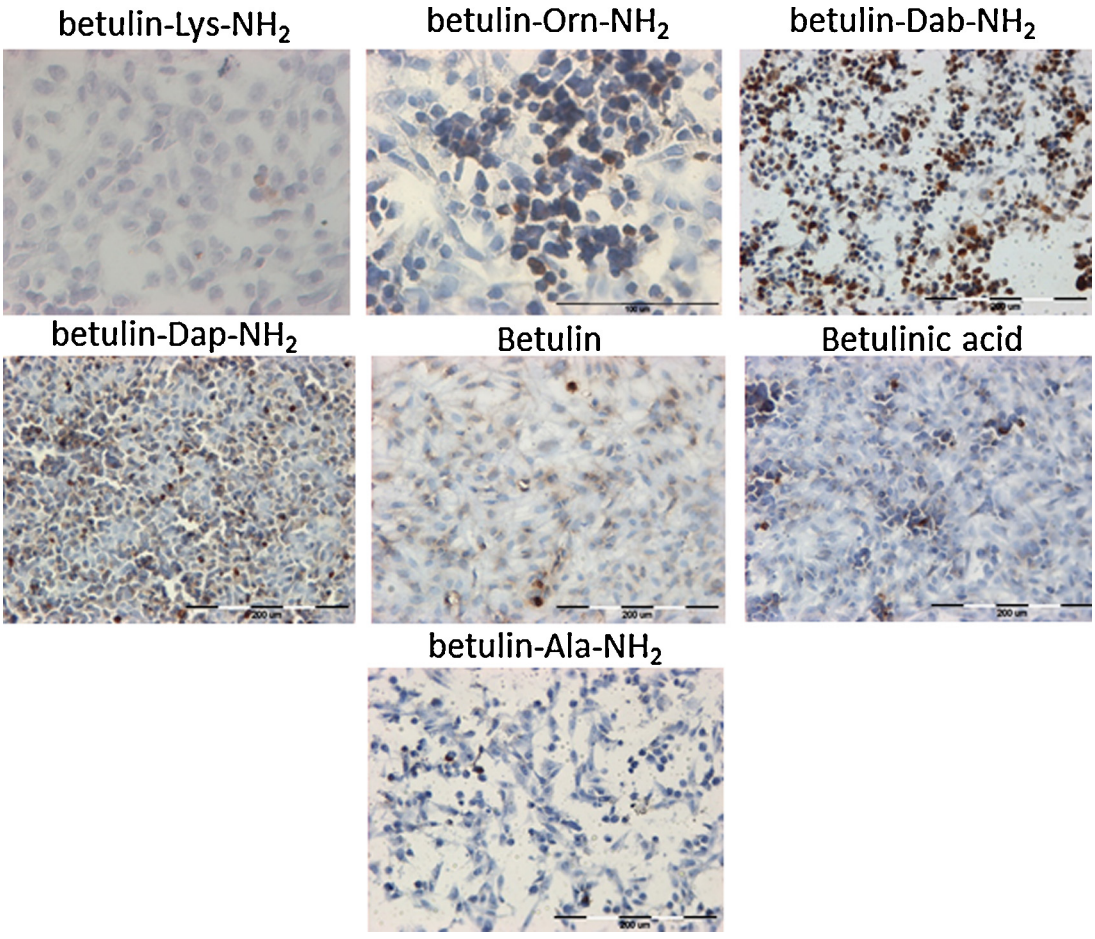

Fig. 3. TUNEL assay evaluated after 72 h incubation with active compounds in skin cancer (A431) cells.

Table 6
Immunocytochemical determination of PARP-1 and Caspase 3 in skin cancer A431 cells and human keratinocytes as a normal control cells.

Lp	A431				HaCaT			
	PARP-1 12.5 μM	PARP-1 6 μM	Casp 3 12.5 μM	Casp 3 6 μM	PARP-1 12.5 μM	PARP-1 6 μM	Casp 3 12.5 μM	Casp 3 6 μM
Control cells	<5% −/+		<5% −/+		30% +/++		0%	0%
Betulin-Lys-NH_2	Difficult to evaluate	98% ++	0%	0%	100% ++/+++	100% ++	0%	<5%
Betulin-Orn-NH_2	95% ++	95% +/++	0%	0%	100% ++/+++	100% ++	<5% disturbed morphology	0% normal cells
Betulin-Dab-NH_2	85% −/+	100% ++/+++	0%	0%	100% ++/+++	100% ++/+++	0%	0%
Betulin-Dap-NH_2	98% ++/+++	95% +/++	0%	0%	95% +/++	90% +/++	0%	0%
Betulin	100% ++/+++	100% ++/+++	0%	0%	98% +/++	90% +/++	0%	0%
Betulinic acid	100% ++	100% ++	0%	0%	98% +/++	35% +/++	<5%	0%
Betulin-Ala-NH_2	100% ++/+++	100% ++/+++	0%	0%	80% ++	80% +/++	0%	0%
DMSO	100% ++/+++	100% +/++	0%	0%	95% +/++	80% +/++	0%	0%

carboxylic esters (carbamates) and N-acylimidazole derivatives of betulinic acid have the better cytotoxicity profile than betulinic acid. The new compounds were screened for *in vitro* cytotoxicity activity against human cancer cell lines HepG2, Jurkat and HeLa [32].

The strategy of cancer cell chemotherapy is based on the reactivation of apoptotic pathways which have been silenced or modified in tumor cells. We have examined the level of PARP-1 and caspase-3 proteins immunocytochemically. PARP-1 is cleaved by caspase-3 during apoptosis, which leads to its inactivation. In all investigated cell lines normal HaCaT and skin cancer A431 the PARP-1 overexpression was observed and the low amounts of caspase-3 was obtained for modified and non-modified compounds. Therefore, it has been postulated that tested compounds can induce cell death by necrosis. However, the other our results especially TUNEL assay showed that examined cells revealed apoptosis. Additionally in our data from alkaline comet assay necrotic cells were not observed. Probably, the different apoptotic pathway was activated, excluding caspase-3 pathway. Some recent reports indicate that betulin and betulinic acid in combination with cytostatic drugs (doxorubicin, cisplatin, taxol, actinomycin D) induce apoptosis by activation of caspases in various types of tumor cell lines [26,33]. It is believed that the anti-tumor effect of betulin, betulinic acid and their derivatives is the inclusion of a mechanism of apoptosis in cancer cells [8,20,34,36,37]. It was observed that the apoptotic process is induced irrespective of the P53 gene and excluding CD95 receptor. Mitochondria are altered by betulin and betulinic acid. These triterpenes make mitochondrial membrane permeable and the apoptogenic proteins are released in the intermembrane space of mitochondria [8,19,20]. Previous studies also indicated, that apoptosis induced by betulinic acid can activate the production of reactive oxygen species (ROS), blocking of topoisomerase I, inhibition of angiogenesis and activation of MAP-kinase cascades [35,38]. In our previous studies we concluded that new derivatives of betulin and betulinic acid, which have been designed and synthesized by our team, exhibit greater solubility in aqueous media and thus a higher bioavailability than their precursors. We have also found that the novel and particularly – lysine esters of betulinic acid and betulin induce apoptosis in tumor cells of human pancreatic cancer and stomach (lines sensitive and resistant to cytotoxic drugs) more effectively than betulin and betulinic acid [11,12].

5. Conclusion

The results obtained in the current study indicate that new derivatives of betulin and betulinic acid can be effectively applied in human squamous cell lines. The most benefit is that the new compounds are safe for normal human keratinocytes. These findings demonstrate that new modified compounds derived from betulin have the high therapeutic potential in actinic keratosis.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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