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Esters of betulin and betulinic acid with amino acids have improved water solubility and are selectively cytotoxic toward cancer cells

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ABSTRACT

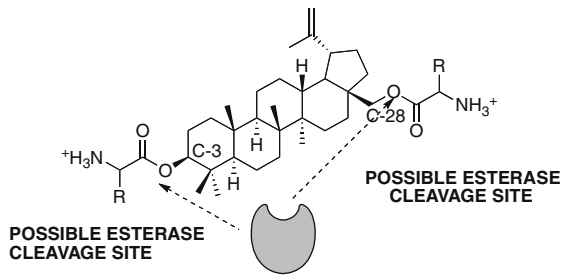
Betulin and betulinic acid are naturally occurring pentacyclic triterpenes showing cytotoxicity towards a number of cancer cell lines. Unfortunately they are practically insoluble in aqueous media and therefore their overall absorption index is not satisfactory. We have modified structures of both compounds by simple transformation to mono- and disubstituted esters of L-amino acids. This allowed us to achieve better water solubility without loss of the observed earlier anticancer properties. Comet assay on various cancer cell lines demonstrate that these compounds act via an apoptotic mechanism.

Natural compounds to treat various types of cancer have recently attracted considerable interest due to their versatile biological properties and usually broad safety window during administration. One such group of compounds are triterpenes with betulinic acid being the best documented example demonstrating cytotoxicity toward several cancer cell lines.[1] This feature of betulinic acid originally demonstrated by Pisha et al. on melanoma cells makes this compound an interesting drug candidate. Betulinic acid, for example, is currently undergoing phase II clinical trials for the treatment of melanoma.[2]

Unfortunately, betulinic acid and its metabolic precursor betulin, that also harbors anticancer properties, are not very much soluble in aqueous media and therefore their overall absorption index is not satisfactory enough. However, their versatile molecular structure makes them ideal candidates for modifications using medicinal chemistry approaches. To date several reports have been published demonstrating that either simple or advanced modifications may be performed without loss of the desired biological properties.

There are two types of possible modifications of betulin and betulinic acid.[3] First, the hydroxy groups could be transformed into more soluble derivatives.[4,5] While, the second approach involves changing the triterpenes core rings, mostly by various substitutions or other types of chemical modifications.[6,7]

In the approach presented in this manuscript, we decided to transform betulin and betulinic acid into their respective esters of amino acids. In the patent literature there is one example of a glycine ester of betulinic acid showing some cytotoxicity toward melanoma cells, but no further studies were performed within this group of compounds.[8] We assumed that modifications of betulin into diester derivatives should especially benefit the solubility index of these compounds and even if esterases cleave the ester



R = side chain of the amino acid

Figure 1. Esters of betulin with amino acids and possible cleavage sites by esterases.

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prodrug in vivo it will still fall apart into the desired triterpene (drug) and the non-hazardous amino acids (Fig. 1).

Our preliminary attempts in the synthesis of esters of betulin and betulinic acid with L-Boc protected amino acids using DCC (*N*,*N*′-dicyclohexylcarbodiimide)/DMAP (4-dimethylaminopyridine) in THF were only partially successful (the usual yield after 5–10 days of coupling was only in the range of 10–15%). Therefore, we decided to use the much stronger coupling reagent CDI (1,1′-carbonyldiimidazole) (Scheme 1). In the patent literature there is one example of coupling Boc-Gly to betulinic acid using this method.[8] We found that this protocol in every case yields the desired esters in high yields even after only 24–48 h of coupling (Table 1).[9] In the case of betulinic acid, esters were obtained at carbon C-3, while in the case of betulin only at carbon C-28 (Fig. 1) as determined using ^{1}H NMR by shift of the CH-OH proton at carbon C-3 (the very characteristic resonance signal from an unsubstituted derivatives is usually at around 3.0–3.2 ppm, while substitution of carbon C-3 shifts this signal downfield by at least 1.0 ppm). In the case of the disubstituted derivatives, the first esterification takes place at carbon C-28 and than at carbon C-3 as determined by ^{1}H NMR, as well. Interestingly, we have never observed monosubstituted betulin derivatives at carbon C-3. Deprotection of the Boc group was achieved using 3 M solution of HCl in dioxane for 30 min.[10] The yield of this step was between 64% and 82%. All the compounds were in the form of white crystalline solids and were characterized using ^{1}H NMR and HR-MS.

Water solubility was evaluated by dissolving 1 mg of the compound in 100 μl of DMSO as described earlier by Jeong for other betulinic acid derivatives.[11] 10 μl aliquots were treated with distilled water to achieve dilutions equal to 5×, 10×, 20×, 50×, 100×. Subsequent analysis of the presence of the precipitate or gel-like suspension was performed in order to classify all the compounds synthesized. The lysine disubstituted derivative of betulin (**2g**) had excellent solubility in aqueous media. Disubstituted derivatives of betulin and monosubstituted esters of betulinic acid with lysine or alanine also improved solubility to a certain extent (Table 2). While derivatives with bulky and hydrophobic side chains (**2e**, **2f** and **4d**) were even less soluble, however still significantly better when compared to betulin or betulinic acid. Alternative assay conditions (at pH 7.4 in 0.05 M Tris buffer and fixed 5% DMSO concentration) confirmed our water solubility results obtained in previous experiment as well.

In order to determine the chemical stability of the compounds at physiological pH (7.4) we have performed RP-HPLC assays for compound **2g**. We have found that **2g** is stable for at least 6 days of incubation in phosphate buffer (pH 7.4, 0.02 M) at room temperature. This suggests that the triterpene ester is stable throughout the cell culture in vitro.

Proliferation profiles of human gastric carcinoma parental (EPG85-257P) and its daunorubicin resistant (EPG85-257RDB) cell lines, and of human pancreatic carcinoma parental (EPP85-181P) and its daunorubicin resistant (EPP85-181RDB) cell lines were evaluated in multiple experiments after 24 h of treatment with the compounds using SRB tests for all the compounds tested.[12] Usually, most experiments aiming to determine cytotoxicity of betulinic acid or betulin in the literature are performed for at least 72 h.[5,13] In our case, this time of incubation was much too long for both tested parental cell lines. The most active compounds (**2c**, **2d**, **2f** and **2g**) completely destroyed the cells (none of the cell types could be detected using comet assay performed after 72 h), making final data not very reliable (not shown here). Additionally, the response of daunorubicin resistant cell lines after 24 h was not as good as of parental cell lines.

On the other hand, this observation demonstrates that besides achieving better water solubility, the mode of action of the tested compounds is much faster than for betulinic acid and betulin. The choice of the tested cell lines was based on two major criteria. Tumor models in this study correspond to human organs (stomach and pancreas), which potentially could be easily 'reached' by the tested compounds after oral administration. Keeping in mind the known problems with the delivery of betulinic acid to the target cells this argument should not be neglected. The second criteria corresponds to the multidrug resistance of several known cancer cells, therefore we have tested sensitive and daunorubicin resistant cell lines as well, to additionally check the selectivity of our compounds.[14]

For both pancreatic cell lines we have found that the most active compound is the lysine diester of betulin **2g** (IC_{50} = 3.8 μM), and only slightly weaker is alanine diester of betulin **2d** (IC_{50} = 4.6 μM). However, for both compounds after 24 h of treatment, the activity towards the sensitive cell line was only around four times better than compared to the resistant one. Proliferation profiles of the other tested betulin esters were much less impressive. Considering derivatives of betulinic acid, substitutions with amino acids we have found that lysine ester **4a** was about twice better (IC_{50} = 14 μM) from L-α-aminobutanoic acid ester **4b** (IC_{50} = 26 μM), while molecules with bulky, hydrophobic side chains had practically no effect on the cells. Control compounds, betulinic acid and betulin were around 10 times weaker than the best compounds **2d** and **2g**.

In case of the human pancreatic carcinoma cell lines after 24 h of incubation we have observed significant effects only for the parental (EPG85-257P) cell line. Also, for this cell line the most active compound was the lysine diester of betulin **2g** (IC_{50} = 4.1 μM). Around twice weaker was the lysine ester of betulinic acid **4a** (IC_{50} = 9.7 μM), while around four time weaker was the alanine diester of betulin **2d** (IC_{50} = 17 μM). Betulin and betulinic acid were

Table 1
Numbering, structure and yield of the betulin and betulinic acid protected and deprotected esters with amino acids

Compds	R^1	R^2	Yield (%)	Compds	Yield (%)	HR-MS (calcd)	HR-MS (exp.)
1a	H	Gly	85	**2a**	68	500.4104	500.4109
1b	Gly	Gly	91	**2b**	81	557.4318	557.4297
1c	H	Ala	83	**2c**	65	514.4260	514.4241
1d	Ala	Ala	72	**2d**	68	585.4631	585.4628
1e	Phe	Phe	71/4[a]	**2e**	80	737.5258	737.5261
1f	Met	Met	66/7[a]	**2f**	71	705.4698	705.4673
1g	Lys	Lys	64/5[a]	**2g**	68	699.5789	699.5792
3a	Lys	—	62	**4a**	64	585.4631	585.4628
3b	Abu	—	78	**4b**	77	542.4210	542.4531
3c	Ala	—	73	**4c**	69	528.4053	528.4059
3d	Phe	—	65	**4d**	82	604.4366	604.4304

Additionally, in the case of deprotected derivatives (**2**, **4**) mass of the compound obtained using high-resolution mass spectroscopy in ESI mode (HR-MS ESI) is presented.
[a] Values showing yield of the disubstituted and monosubstituted derivative, respectively.

Table 2
Cytotoxicity of the tested compounds, betulin (BE) and betulinic acid (BA) towards human gastric carcinoma parental (EPG85-257P) and daunorubicin resistant (EPG85-257RDB) cell lines and towards human pancreatic carcinoma parental (EPP85-181P) and daunorubicin resistant (EPP85-181RDB) cell lines evaluated after 24 h of the treatment using SRB test

Compds	Cytotoxicity toward cancer cell lines IC_{50} (μM)				Water solubility
	EPP85-181P	EPP85-181RDB	EPG85-257P	EPG85-257RDB	
BE	>50[a]	46	26	>50	Weak
BA	32	>50	42	>50	Middle
2a	>50	>50	>50	>50	Good
2b	>50	>50	>50	>50	Very good
2c	>50	>50	38	>50	Good
2d	4.6	21	17	>50[a]	Very good
2e	>50	>50	>50	>50	Good
2f	22	>50	31	>50	Good
2g	3.8	18	4.1	>50[a]	*Excellent*
4a	14	>50	9.7	>50	Very good
4b	26	>50	33	>50	Very good
4c	>50	>50	48	>50	Very good
4d	>50	>50	>50	>50	Good

Water solubility of the tested compounds was evaluated as described in the text.
[a] Estimated value between 50 and 100 μM.

Table 3
Apoptosis detection using comet assay of human gastric carcinoma parental (EPG85-257P) and human pancreatic carcinoma parental (EPP85-181P) cell lines after 24 h of incubation with the tested compounds at 25 μM

Compds	Apoptosis detection			
	EPP85-181P		EPG85-257P	
	%Apoptosis	%Normal	%Apoptosis	%Normal
BE	2	98	0.5	99.5
BA	16	84	4	96
2a	1.4	98.6	9.8	90.2
2b	0.5	99.5	—[a]	—[a]
2c	5.2	94	42	58
2d	100	0	100	0
2e	1	99	9	91
2f	24.7	75.3	15.1	84.9
2g	99.5	0.5	100	0
4a	99	1	—[a]	—[a]
4b	69.6	30.4	1.7	98.3
4c	25.9	74.1	1.4	98.6
4d	3.1	96.9	2.5	97.5

[a] Cells in the transient phase between apoptosis and normal stage with multiple cells already in apoptosis phase.

less active than the best compounds and their IC_{50} values were 26 μM and 42 μM, respectively. As for the human gastric carcinoma daunorubicin resistant (EPG85-257RDB) cell line, much longer incubation time was required to observe significant cytotoxicity (data not shown).

Cell proliferation experiments for all the tested cell lines showed that the most active compounds are the ones that are the most soluble in water. The best of all the compounds tested, the lysine diester of betulin **4g** was the most active in every case. Besides the solubility effect, we think that additional advantage of this compound is the presence of basic side chains, which facilitates penetration through the plasma-membrane, a well-known feature of compounds with this type of modification. Generally, we have found that derivatives of betulin in the form of diesters were usually among the most active compounds and that presence of non-hydrophobic side chains further improved their ability to maintain their normal mode of action on the cells tested. Similar effect was observed for the esters of betulinic acid. Compounds bearing hydrophobic side chains, while much more soluble in aqueous media as compared to betulinic acid, were not as effective as the lysine ester **4a**.

Betulin and betulinic acid are compounds known to usually kill the cells via apoptosis. One suggested mechanisms of action is binding to the mitochondrial membrane and the subsequent release of cytochrome *c*, which ignites caspase activation and the cell death cascade.[15] The ability to kill the cells via the apoptotic pathway is a well-desired feature for anticancer compounds.

In order to test if compounds described here are acting via this mechanism we have performed comet assays as described by Collins.[16,17] This type of experiment allows telling-apart the apoptotic cells form normal cells as well as determining the amount of each type of cells, being apoptotic or normal (Table 3). Apoptotic cells in this assay are comet shaped due to the DNA damage as a result of the cells destruction in this late phase of apoptosis (Fig. 2).

We have performed comet assay for two cell lines that responded best to the compounds, namely human gastric carcinoma parental (EPG85-257P) and human pancreatic carcinoma parental (EPP85-181P) cell lines (Table 3). Analysis of the results for both cell lines clearly demonstrates presence of apoptotic cells, as visualized by the comet shape. The ratio of apoptotic versus normal

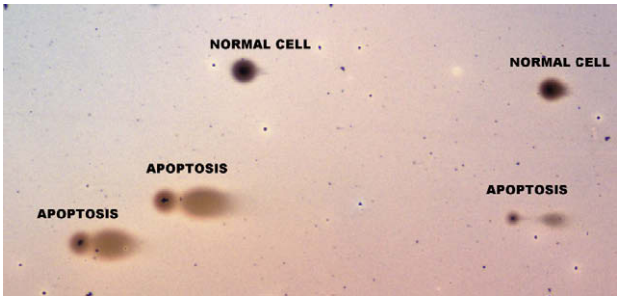
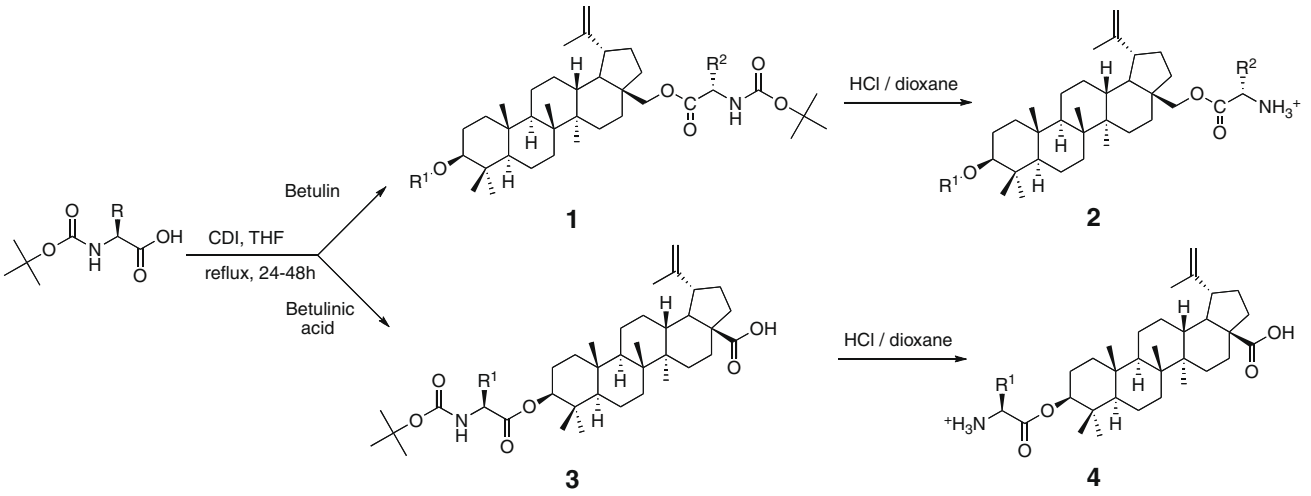


Figure 2. Criteria for differentiation of normal from apoptotic cells in the comet assay for human gastric carcinoma parental (EPG85-257P) cell line as example.

cells strongly correlates with the ability of the compounds to inhibit cell proliferation.

Generally, the more active the compound was the higher the amount of apoptotic cells were detected after 24 h of treatment. In the case of lysine and alanine diesters of betulin **2d** and **2g** all of the cells in both tested cell lines were in the apoptotic phase. Similar results were observed for the lysine ester of betulinic acid **4a**. However, in case of the human gastric carcinoma parental (EPG85-257P) cells we have observed that most of the cells were in a transient phase between apoptosis and normal stage, with additional cells already in the apoptotic phase. In case of the betulin and betulinic acid only between 0.5% and 16% of the cells were already in the apoptotic phase.

In summary, we have demonstrated synthesis of a series of esters of betulin and betulinic acid, that reveal much better solubility in aqueous media compared to parental compounds. The compounds tested here demonstrate towards multiple tested cell lines and kill these cells via an apoptotic mechanism. We can see strong correlation between the type of ester with the type of the side chain of the amino acid used and their biological activity. Currently, we are in the process of synthesizing and testing a much broader group of this type of molecules. Full analysis of several new compounds and their activity towards a broader spectrum of cancer and normal cell lines will be published in a separate report in due course.



Scheme 1. Synthesis of the esters of betulin and betulinic acid with amino acids and their subsequent deprotection. R and R^2— side chain of amino acid, R^1—H or amino acid for compounds **1** and **2** and side chain of amino acid for compounds **3** and **4**.

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