Full Paper

Synthesis and Anticancer Activity of Novel Betulinic acid and Betulin Derivatives

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A series of novel betulinic acid derivatives **3–11** and betulin derivatives **12–17** were synthesized. The compounds were characterized by the means of ^{1}H- and ^{13}C-NMR spectroscopy as well as mass spectrometry. The compounds have been tested on ten tumor cell lines of different histogenic origin. The most active derivatives, containing a chloroacetyl group on C-3 in betulinic acid **9** and C-28 in betulin **15**, were up to ten times more cytotoxic and many fold more selective towards tumor cells in comparison to normal cells (fibroblasts) than betulinic acid. Furthermore, compound **15** was found to possess cell growth inhibition even when treated for a short time on anaplastic thyroid cancer cells (SW1736).

Keywords: Apoptosis / Betulin / Betulinic acid / Cytotoxicity

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Introduction

Triterpenes represent a varied and important class of natural compounds. Among these, pentacyclic lupane-type triterpenes are one of the most significant subclass which has been shown to possess several medicinal properties. The antitumor properties of lupane-derived triterpenoid plant extracts have been demonstrated for the past 25 years for their cytostatic activity on various *in-vivo* cancer model systems [1–5]. Betulinic acid **1** and its precursor betulin **2** (Fig. 1) are members of this class of pentacyclic triterpenes and are found to possess antitumor properties. Both compounds were also found to possess an anti-inflammatory activity which has been attributed to inhibition of non-neurogenic pathways [6]. Previous investigations suggested that betulinic acid inhibited HIV-1 replication [7]. Along with these properties, betulinic acid and betulin were also found to show antimalarial, antimicrobial, and spasmogenic activities [8].

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Abbreviations: pivaloxy methyl (Pom); sulforhodamine B (SRB); trichloroacetic acid (TCA)

Initially, betulinic acid was considered to be melanoma-specific [9–11], but recent studies suggested that it shows anticancer activity against a broad spectrum of cancers [12–14].

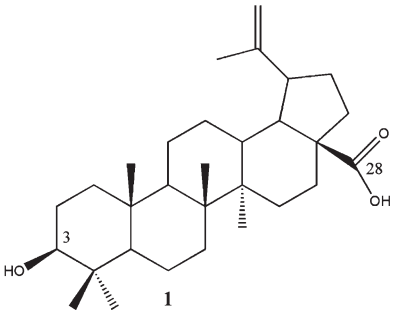
Betulinic acid was found to show anticancer activity by induction of apoptosis through the activation of caspases independent of the p53 gene status and CD95 activation [15]. Betulinic acid induced apoptosis was mediated via direct effects on mitochondria [16].

In this paper, a single-mode lab-scale microwave (CEM labs, USA) was used to synthesize some of the new derivatives of betulin and betulinic acid. Furthermore, *in-vitro* anticancer activity as well as the structure–activity relationships, selectivity, and mode of cell death are described.

Results

Chemistry

Synthesis of betulinic acid derivatives **3–11** and betulin derivatives **12–17** has been described in Schemes 1 and 2, respectively. Synthesis of 3-*O*-acetylbetulinic acid **3** was achieved in two ways. Betulinic acid **1** was reacted with acetic anhydride in ethanol for 8 h to obtain **3**. Using the other approach, betulinic acid and a small excess of acetic anhydride were suspended in dichloromethane and were treated



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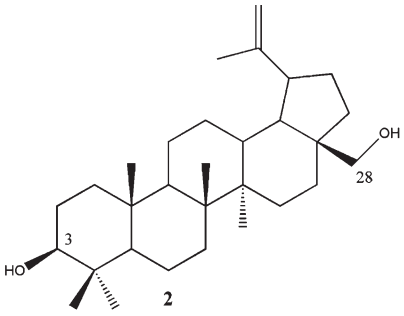

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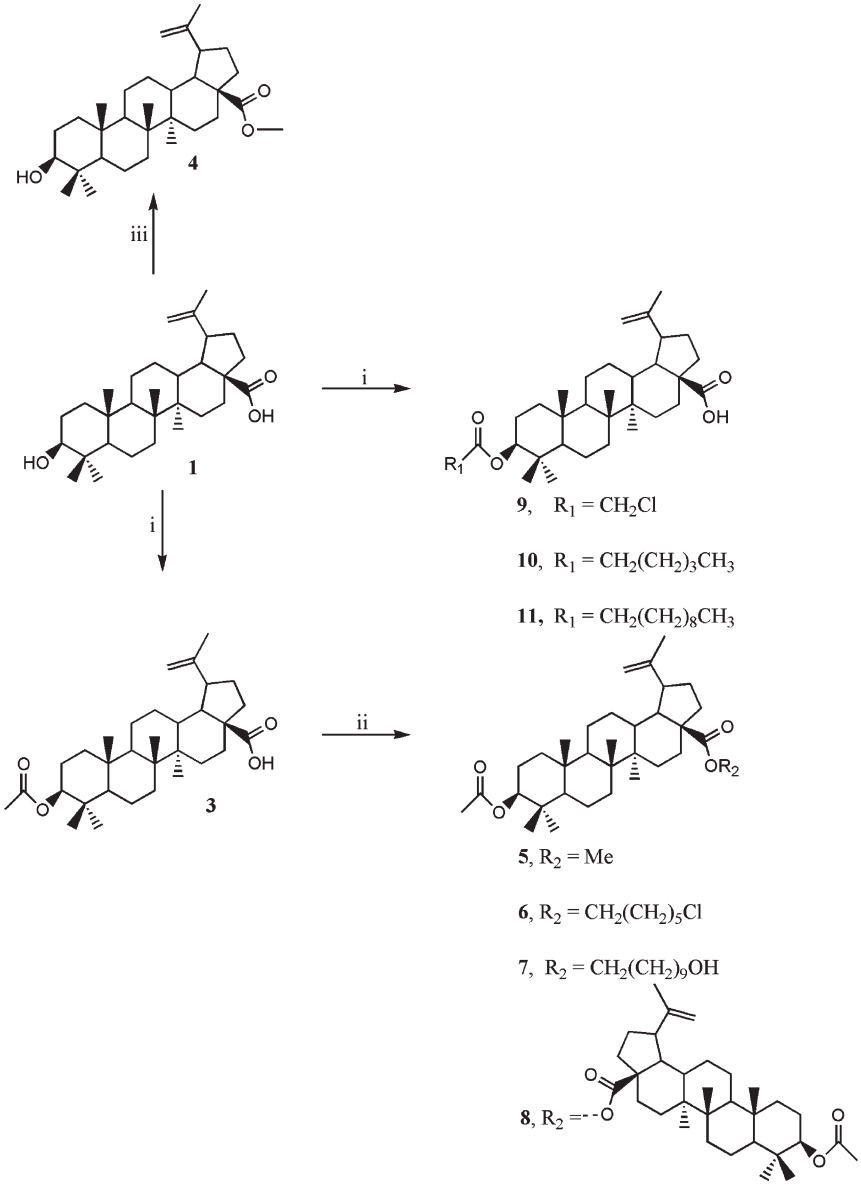
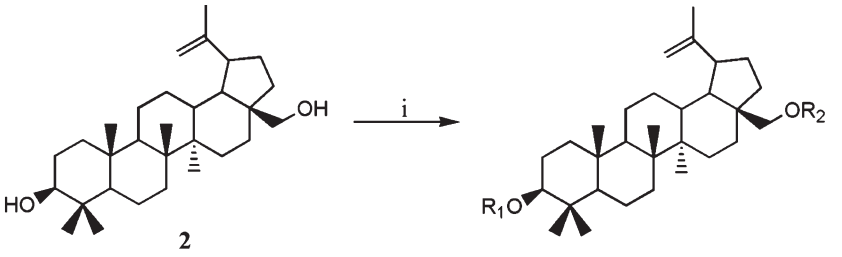
Figure 1. Structures of betulinic acid **1** and betulin **2**.



Reactions and conditions: i) Alkylanhydride, CH_2Cl_2, MW, 90°C; ii) oxalyl chloride, CH_2Cl_2, ROH; iii) CH_2N_2, ether, r. t.

Scheme 1. Synthesis of compounds **3–11**.



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12, R_1 = OAc, R_2 = OAc
13, R_1 = OAc, R_2 = H
14, R_1 = H, R_2 = OAc
15, R_1 = H, R_2 = $COCH_2Cl$
16, R_1 = H, R_2= $CO(CH_2)_9CH_3$
17, R_1 = R_2 = PoM

Scheme 2. Synthesis of compounds **12–17**.

Reactions and conditions: i) Alkylanhydride, CH_2Cl_2, MW, 90°C.

with microwave energy for 30 min at 90°C to obtain 3-*O*-acetylbetulinic acid in good yield. Compound **4**, betulinic acid methyl ester was prepared according to the method described in the literature [17]. Compound **3** was treated with oxalyl chloride in dichloromethane for 20 min; the resulting acetyl betulinic acid chloride was treated with methanol to get **5**, acetyl betulinic acid methyl ester. The acid chloride of compound **3** was reacted with 6-chlorohexanol in presence of DMAP (dimethylaminophenol) to obtain **6**. Similarly, acetyl betulinic acid chloride was treated with 1,10-decandiol to get **7**, during this reaction, compound **8** was obtained as a side product, which might have formed by a reaction with unreacted acetyl betulinic acid **3** and acetyl betulinic acid chloride. Derivatives **9**, **10**, and **11** were synthesized by microwave-assisted synthesis using the corresponding anhydrides. Chloroacetylbetulinic acid **9** was obtained by reacting **1** with an excess of chloroacetic anhydride in hexane at 94°C for 20 min. Hexanoylbetulinic acid **10** was afforded by reaction of **1** with an excess of hexanoic anhydride in dichloromethane at 90°C for 30 min. Similarly, laurylbetulinic acid **11** was prepared using lauric anhydride in dichloromethane at 90°C for 45 min.

Compound **2** was treated with an excess of acetic anhydride in dichloromethane and heated with microwave energy for 20 min to afford 3,28-bisacetylbetulin **12**. The reaction also yielded small amounts of **13** and **14** which could be separated using column chromatography. 28-*O*-acetylbetulin **14** was prepared using acetylation of the primary hydroxyl group of **2** with acetic anhydride in pyridine at 0°C for 2 h. Compounds **15**, **16**, and **17** were prepared using a lab scale microwave. Compound **15** (28-*O*-chloroacetylbetulin) was obtained by treating **2** with chloroactic anhydride and **16** (28-*O*-laurylbetulin) with lauric anhydride in 1:1 ratio. 3,28-Bispivaloxybetulin **17** was obtained using excess of pivaloxy methyl anhydride in dichloromethane with **2** in a microwave for 40 min.

The derivatives **3**, **9–11**, and **12–17** have been synthesized using a lab-scale single-mode microwave. This helped us to achieve higher yields in a shorter time in comparison to conventional heating procedures. For instance, **3**, when synthesized using conventional methods, required 8 h reflux with ethanol in the final step, whereas using a microwave, it took 30 min with a yield of 85% in a single step.

Biological studies

In-vitro antitumoral studies

The *in-vitro* cytotoxic activity of the derivatives of betulinic acid and betulin was studied on ten different tumor cell lines, 8505C and SW1736 (anaplastic thyroid tumor), A253 (head and neck tumor), A431 (cervical), A2780 (ovarian), DLD-1, HCT-8, HCT-116, SW480 (colon), and liposarcoma (connective tissue) by sulforhodamine B colorimetric assay method [18]. The compounds showed dose-dependent antitumoral activity against the investigated cell lines. The IC_{50} values of each compound tested on these cell lines are given in Table 1.

Our preliminary investigation showed that betulinic acid **1** and betulin **2** derivatives are potential lead compounds for new antitumor agents. The majority of the derivatives showed increased cytotoxic activity judged against the starting compounds. The lowest activity in the panel of derivatives made from betulinic acid was seen in case of compounds **6**, **7**, and **8**. Moderate activity was observed in **3**, **4**, and **10**. On the other hand, derivative **9** showed a substantially better activity than the parent compound. Similarly, among the derivatives synthesized from betulin, **12**, **13**, **16**, and **17** showed moderate to low activity, whereas compound **14** showed moderate activity. The betulin derivative **15** was found to exhibit a better activity than betulinic

Table 1. IC_{50} values (μM) of new derivaties of betulinic acid and betulin.

Compound	8505C	A253	A431	A2780	DLD-1	HCT-8	HCT-116	Liposarcoma	SW480	SW1736
1	7.26 ± 0.96	9.18 ± 0.04	12.60 ± 0.29	11.07 ± 0.44	11.87 ± 0.29	13.10 ± 0.50	10.80 ± 0.24	12.07 ± 0.63	6.48 ± 0.12	13.09 ± 0.48
3	15.34 ± 0.28	11.84 ± 0.45	10.72 ± 0.22	18.99 ± 0.10	12.35 ± 0.36	10.60 ± 0.20	9.62 ± 0.36	9.01 ± 3.19	8.53 ± 0.48	7.30 ± 0.66
4	26.37 ± 1.62	18.51 ± 1.72	26.99 ± 1.07	29.31 ± 2.78	45.86 ± 5.83	17.25 ± 1.72	20.47 ± 1.02	23.26 ± 3.73	17.92 ± 075	32.10 ± 1.00
5	56.97 ± 17.5	43.41 ± 7.63	63.94 ± 2.73	53.70 ± 4.22	>100	33.58 ± 1.34	58.79 ± 2.14	66 ± 15.07	17.88 ± 0.59	>100
6	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
7	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
9	8.99 ± 2.31	4.75 ± 0.10	2.78 ± 0.71	4.5 ± 1.90	3.75 ± 0.42	2.04 ± 0.92	1.90 ± 0.51	9.90 ± 1.10	4.07 ± 0.28	1.15 ± 0.33
10	27.24 ± 1.02	21.14 ± 3.68	29.57 ± 2.91	12.47 ± 1.28	27.65 ± 7.71	23.57 ± 3.06	11.43 ± 0.89	16.56 ± 1.03	23.29 ± 1.09	17.80 ± 1.50
12	>100	40.18 ± 8.70	22.26 ± 1.22	20.25 ± 1.51	87.19 ± 4.09	27.23 ± 2.03	34.94 ± 2.69	26.02 ± 0.78	45.10 ± 0.26	18.23 ± 2.65
13	60.19 ± 3.77	32.22 ± 1.02	29.64 ± 2.71	28.52 ± 1.28	33.43 ± 7.69	54.06 ± 5.93	13.66 ± 2.60	67.69 ± 6.85	47.01 ± 10.1	27.08 ± 1.88
14	14.74 ± 0.86	12.87 ± 3.06	12.19 ± 2.55	11.74 ± 2.66	13.12 ± 1.05	17.98 ± 0.44	10.71 ± 0.65	15.54 ± 1.23	13.68 ± 0.39	11.95 ± 2.39
15	4.96 ± 0.75	4.66 ± 0.52	2.17 ± 0.37	4.59 ± 0.82	4.59 ± 0.82	12.58 ± 1.93	4.07 ± 0.73	8.49 ± 0.35	7.42 ± 0.72	3.54 ± 0.66
16	>100	>100	36.08 ± 0.01	>100	>100	>100	>100	89.50	>100	>100
17	32.86 ± 1.19	52.91 ± 1.37	42.77 ± 1.98	46.23 ± 2.49	80.50 ± 5.22	63.42 ± 3.81	41.21 ± 2.02	–	42.15 ± 2.87	49.29 ± 1.40

acid on almost all the cell lines. Based on the results obtained, we can summarize that derivatization of the hydroxyl groups of triterpenes by chloro acetic anhydride increases cytotoxic activity. Modification of C-3 of betulinic acid with acetic anhydride resulted in a slight decrease of activity compared to betulinic acid. Esterification of C-28 carboxylic acid of **3**, acetyl betulinic acid, either with short- or long-chain alkyl groups led to either loss or a decreased cytotoxic activity in comparison to the parent compound. Even substitution with a chloro hexyl group has not yielded a better result. The most active derivatives **9**, 3-*O*-chloroacetylbetulinic acid, and **15**, 28-*O*-chloroacetylbetulin, were both found to show high cytotoxic activity against a broad spectrum of tumors from different histogenetic origin. Moreover, compound **9** was five to eight times more cytotoxic than betulinic acid on HCT-116, HCT-8 (both colon cancer), and SW1736 (anaplastic thyroid tumor) cell lines. Similarly, compound **15** showed a fivefold increased cytotoxicity on the A431 (cervical) cell line compared to betulinic acid at equitoxic concentrations. Compound **9** has shown a very high degree of cytotoxicity on SW1736 and compound **15** on A431 cell lines.

Selectivity

Furthermore, the most active derivatives **9** and **15** were screened for their selectivity towards tumor cells. For this the compounds were tested on human fibroblasts (WWO70327). Interestingly, it was observed that compounds **9** and **15** were less toxic on these cells than on the investigated tumor cell lines (IC_{50} = 20.83 ± 0.20 μM (**1**); 10.31 ± 1.87 μM (**9**); 15.82 ± 3.50 μM (**15**). It was observed that **15** showed a higher selectivity than betulinic acid towards all tumor cell lines except for one type of colon cancer HCT-8 cell line (Table 2). The selectivity of this compound was 4.5 times higher in the cervical A431 cancer cell line when compared to betulinic acid. Similarly, **9** was also found to be selective to tumors, it was found to be nearly sixfold more selective than betulinic acid in the anaplastic thyroid tumor SW1736 cell line. In addition, human fibroblasts showed a better tolerance to both these compounds in comparison with the cancer cells in most cases.

*Compounds **9** and **15** induce apoptosis*

To test whether **9** and **15** induced cell death mediated by apoptosis, floating cells from SW1736 and A431 after a 24-h treatment with IC_{90} concentrations of compounds **9** and **15**, respectively, were collected and analyzed by DNA laddering technique (Fig. 2). In both cell models with both compounds occurrence of the typical DNA ladders was observed.

*Compound **15** induces cell death faster than betulinic acid in SW1736 cell line*

Comparative time-dependent cytotoxic investigations were preformed for **1**, **9**, and **15** with equitoxic concentrations on anaplastic thyroid cancer SW1736 and cervical cancer A431 cell lines. Both cell lines were treated with corresponding IC_{50} concentrations of all three compounds for 0, 4, 8, 24, 36, 48, 72, and 96 h (Fig. 3). On the anaplastic thyroid cancer SW1736 cell line it was observed that for cells treated with **15**, growth inhibition starts at the end of a 4-h treatment and

Table 2. Selectivity index[§] of **1**, **9**, and **15** towards tumor cells.

Cell line	1	9	15
8505C	2.87 ± 0.38	1.15 ± 0.36	3.19 ± 0.85
A253	2.27 ± 0.01	2.17 ± 0.40	3.39 ± 0.84
A431	1.65 ± 0.04	3.71 ± 1.16	7.29 ± 2.04
A2780	1.88 ± 0.07	2.29 ± 1.05	3.45 ± 0.98
DLD-1	1.75 ± 0.04	2.75 ± 0.59	3.45 ± 0.98
HCT-8	1.59 ± 0.06	5.05 ± 2.46	1.26 ± 0.34
HCT-116	1.93 ± 0.04	5.43 ± 1.73	3.89 ± 1.11
Liposarcoma	1.72 ± 0.09	1.04 ± 0.22	1.86 ± 0.42
SW 480	3.21 ± 0.06	2.53 ± 0.49	2.13 ± 0.52
SW 1736	1.59 ± 0.06	8.97 ± 3.04	4.47 ± 1.29

[§] Selectivity index IC_{50} of human fibroblasts/IC_{50} on corresponding tumor cell line.

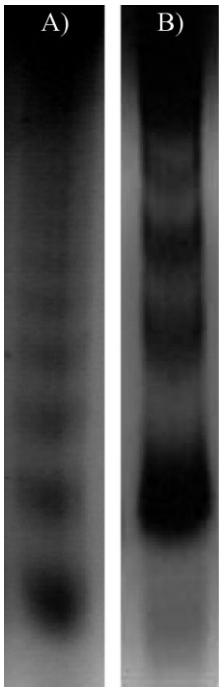

Figure 2. DNA laddering test after action of compound: A) **9** on SW1736 cell line; B) **15** on A431 cell line.

reaches 50% inbetween 72 and 96 h. On the other hand, compounds **1** and **9** show no significant cell growth inhibition until treated for 36 h; both these compounds achieve 50% inhibition of growth at the end of the 96-h treatment. In case of the cervical cancer A431 cell line, compound **15** showed significant cell inhibition at the end of 8 h, whereas compounds **1** and **9** only after a 24-h treatment.

Discussion

Our study demonstrates that modification of the C-3-hydroxyl group of betulinic acid with an acetyl moiety leads to a small change in cytotoxicity when compared to betulinic acid. The change though cannot be generalized, compound **3** is more toxic than betulinic acid, **1** is more toxic on colon cancer (HCT-116, HCT-8, SW480) and anaplastic thyroid cancer cell line (SW1736), whereas less toxic than betulinic acid for the rest of the cell lines. On the other hand, modification of the C-28 acid group into a methyl ester (**4**) has lead to a drop in cytotoxicity. The conversion of the C-28 carboxylic acid group into a methyl ester (**5**), in 3-*O*-acetylbetulinic acid, also rendered a drop in toxicity. Moreover, modification of C-28-carboxylic acid group of compound **3** into a long-chain alkyl ester (**7**) or chloro substituted medium-chain alkyl ester (**6**) yielded non-toxic derivatives. In contrast, conversion of the C-3 hydroxyl group in betulinic acid with a chloro substituted acetyl group yielded a highly toxic derivative (**9**). Compound **9** was found to be 1.5 to 10 times more cytotoxic than betulinic acid. Interestingly, **9** was ten times more cytotoxic than betulinic acid on SW1736 and less toxic than betulinic acid on 8505C, both tumor cell lines are two different types of anaplastic thyroid cancers. The chloroacetyl derivative of betulinic acid **9** was found to be highly cytotoxic on colon cancer lines (DLD-1, HCT-8, HCT-116) and the cervical cancer cell line (A431). C-3 substitution with longer-chain alkyl groups (**10** and **11**) has lead to a decrease in cytotoxicity values.

Betulin has a secondary β-hydroxyl group at C-3 and a primary hydroxyl group at C-28 which can be easily modified and substituted. Substitution of acetyl groups at C-3 and C-28 of the comparatively inactive betulin yielded a moderate to less active derivative **12**. Of the side products obtained during the reaction, **13**, C-3 hydroxyl substituted with an acetyl group, showed less to moderate activity, and **14**, modification of C-28-hydroxyl with an acetyl group, showed a moderate to good activity on the tumor cell lines used. Taking this as a lead, we modified the C-28 primary hydroxyl group with a chloro substituted acetyl group **15**, and long-chain (lauryl) alkyl group **16**. Compound **15** showed activity on similar cell lines like compound **9**, and the activity was found to be better than betulinic acid. The best activity was seen for the cervical

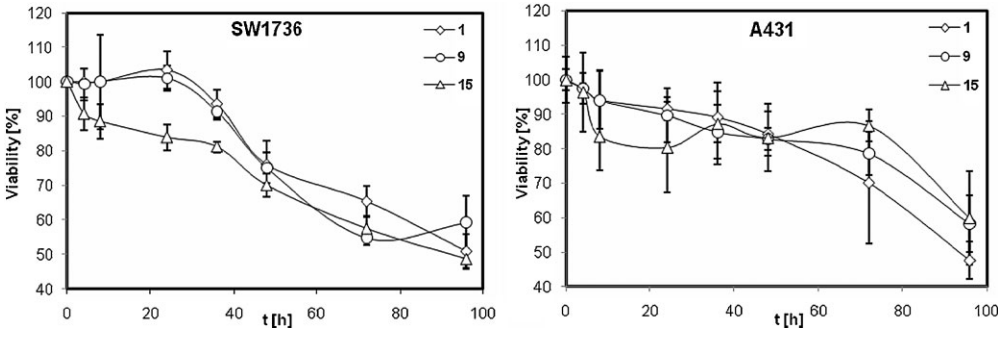

Figure 3. Kinetics of tumor cell growth inhibition of SW1736 and A431 cells treated with equitoxic concentrations of **1**, **9**, and **15** for various time periods.

cancer cell line (A431). Modification of both hydroxyl groups of betulin with the pivaloxy methyl (POM) group, **17**, leads to a moderately active derivative. Long-chain alkyl ester substitution (**16**) at C-28 in betulin caused the loss of cytotoxicity.

The most active derivatives among this panel, **9** and **15** were found to be selective towards tumor cells. Compound **9** was found to be nine times more specific to anaplastic thyroid tumor cell line (SW1736) compared to human fibroblasts (WWO70327), similarly, compound **15** was seven times more selective for cervical cancer cell line (A431).

The active derivatives **9** and **15** were found to induce apoptotic cell death on SW1736 and A431 tumor cell lines, respectively. This was proved by the DNA fragmentation assay.

Compound **15** tends to show significant growth inhibition with short time treatments on anaplastic thyroid cancer (SW1736) and cervical cancer cell line (A431) 4 and 8 h, respectively, whereas treatment with **1** and **9** is successful after 36 and 24 h, respectively.

Conclusion

Modification of the C-28-carboxylic acid group into esters with short- or long-chain alkyl groups has lead to derivatives with lower toxicity. Modification of the C-3 secondary hydroxyl group into esters containing medium-chain alkyl groups showed moderate activity whereas ester with a chloroacetyl side chain yielded a potentially toxic derivative. Modification of C-28 primary hydroxyl group in betulin furnished moderate to good toxic derivatives. Esterification of both primary and secondary hydroxyl groups of betulin gave moderately active derivatives. The active derivatives **9** and **15** were found to induce cell death in tumor cell lines (A431 and SW1736) by apoptosis. Compound **15** was found to show significant growth inhibition within a short time treatment. Compounds **9** and **15** seem to show good selectivity towards tumor cell lines compared to normal fibroblasts.

Experimental

General

Betulinic acid **1** and betulin **2** were obtained as a kind gift from BioSolutions Halle GmbH (Halle, Germany). Compounds **3**, **4**, **8**, **12-14** were synthesized by the procedures reported in the literature [17, 19–24]. The structures of the mentioned compounds were confirmed with ^{1}H- and ^{13}C-NMR spectroscopy. Solvents were obtained commercially and used without further purification. Syntheses under pressure were performed in a lab-scale single-mode microwave from CEM Corp, USA. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Unity 500 NMR spectrometer (Varian, USA) in $CDCl_3$. MS spectra were recorded on LCQ – Thermo (Germany).

Synthesis

*Methyl ester of 3-O-acetylbetulinic acid **5***

3-*O*-Acetyl betulinic acid (456.7 mg, 1 mmol) was dissolved in 5 mL of dry dichloromethane. Then, 500 μL of freshly distilled oxalyl chloride were added. The mixture was stirred until the CO_2 bubbling stopped; approximately 20 min. Dichloromethane and the excess of oxalyl chloride were removed *in vacuo*. The synthesis of acetyl betulinic acid chloride has to be carried out under exclusion of moisture. After cooling down to room temperature, the residue was dissolved in a mixture of a very high excess (10 mL) of methanol with the addition of 10 mg DMAP and stirred at 50°C for 2 h and the solvent was removed rapidly under vacuum. The crude product was dried and cleaned using column chromatography (petrol ether/ethyl acetate, 5:2). White solid; yield: 164 mg (36%); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.77 and 1.66 (C1H_2), 0.81 (C24H_3/C23H_3/C25H_3), 0.87 (C26H_3), 0.93 (C27H_3), 1.09 and 1.66 (C15H_2), 1.15 and 1.43 (C12H_2), 1.18 and 1.48 (C11H_2), 1.38 and 1.61 (C6H_2), 1.39 (C9*H*), 1.41 (C5*H*), 1.42 and 1.60 (C21H_2), 1.59 and 1.67 (C2H_2), 1.61 (C13*H*), 1.65(C7H_2), 1.83 and 2.01 (C16H_2), 1.89 and 2.17 (C22H_2), 1.97 (C30H_3), 2.11 (C2′H_2), 2.17 (C19*H*), 3.63 (C1″H_3), 2.22 (C18*H*), 4.43 (C3*H*), 4.57 and 4.70 (C29H_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.7 (*C27*), 15.9 (*C26*), 16.1 (*C25*), 16.5 (*C24*), 18.2 (*C6*), 19.4 (*C30*), 20.9 (*C11*), 21.1 (*C2′*), 23.2 (*C2*), 25.4 (*C12*), 27.1 (*C15*), 27.9 (*C23*), 29.6 (*C16*), 29.8 (*C21*), 34.1 (*C22*), 34.3 (*C7*), 37.1 (*C10*), 37.8 (*C13*), 38.2 (*C1*), 38.4 (*C4*), 40.7 (*C8*), 42.4 (*C14*), 46.7 (*C17*), 47.0 (*C18*), 49.4 (*C19*), 50.4 (*C9*), 51.2 (*C1″*), 56.5 (*C5*), 80.9 (*C3*), 109.6 (*C29*), 150.5 (*C20*), 170.9/176.6 (*C28*/*C1′*). LC/MS/MS $[M + H]^+$: 513.7.

*Chorohexyl ester of 3-O-acetylbetulinic acid **6***

Compound **6** was synthesized exactly the same way as described for compound **5** except for using 6-chloro-1-hexanol in twofold excess (273 mg, 2 mmol) instead of methanol. The crude product was dried and cleaned using column chromatography (petrol ether/ethyl acetate, 3:1) to obtain a white powder. White solid; yield: 206 mg (45%); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.77 and 1.66 (C1H_2), 0.82 (C24H_3/C23H_3), 0.83 (C25H_3), 0.91 (C26H_3), 0.95 (C27H_3), 1.09 and 1.66 (C15H_2), 1.15 and 1.43 (C12H_2), 1.18 and 1.48 (C11H_2), 1.38 and 1.61 (C6H_2), 1.39 (C9*H*), 1.41 (C5*H*), 1.42 and 1.60 (C21H_2), 1.43 (C3″H_2), 1.49 (C4″H_2), 1.59 and 1.67 (C2H_2), 1.61 (C13*H*), 1.62 (C2″H_2), 1.65 (C7H_2), 1.77 (C5″H_2), 1.80 and 2.04 (C16H_2), 1.85 and 2.20 (C22H_2), 2.03 (C30H_3), 2.11 (C2′H_2), 2.19 (C19*H*), 2.23 (C18*H*), 3.54 (C6″H_2), 4.08 (C1″H_2), 4.45 (C3*H*), 4.59 and 4.72 (C29H_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.6 (*C27*), 16.0 (*C26*), 16.1 (*C25*), 16.4 (*C24*), 18.1 (*C6*), 19.3 (*C30*), 20.9 (*C11*), 21.2 (*C2′*), 23.6 (*C2*), 25.4 (*C3″*), 25.5 (*C12*), 26.5 (*C4″*), 27.6 (*C15*), 27.9 (*C23*), 28.6 (*C2″*), 29.6 (*C16*), 30.6 (*C21*), 32.2 (*C5″*), 34.2 (*C22*), 34.3 (*C7*), 37.1 (*C10*), 37.8 (*C13*), 38.3 (*C1*), 38.4 (*C4*), 40.7 (*C8*), 42.4 (*C14*), 44.8 (*C17*), 45.0 (*C6″*), 47.0 (*C18*), 49.3 (*C19*), 50.4 (*C9*), 51.2 (*C1″*), 56.5 (*C5*), 80.9 (*C3*), 109.6 (*C29*), 150.5 (*C20*), 171.0/176.1 (*C28*/*C1′*). LC/MS/MS $[M + H]^+$: 634.3.

*1,10-Hydroxydecanete ester of 3-O-acetylbetulinic acid **7***

Compound **7** was synthesized exactly the same way as described for compound **5** except for using 1,10-decanediol in twofold excess (349 mg, 2 mmol) instead of methanol. The crude product was dried and cleaned using column chromatography (petrol ether/ethyl acetate, 4:1) to obtain a white powder. White solid; yield: 192 mg (42%); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.77 and 1.66 (C1H_2), 0.82 (C24H_3/C23H_3), 0.83 (C25H_3), 0.91 (C26H_3),

0.95 (C27H_3), 1.09 and 1.66 (C15H_2), 1.15 and 1.43 (C12H_2), 1.18 and 1.48 (C11H_2), 1.29 (C4″H_2–C8″H_2), 1.38 and 1.61 (C6H_2), 1.39 (C9*H*), 1.41 (C5*H*), 1.42 and 1.60 (C21H_2), 1.43 (C3″H_2/C8″H_2), 1.49 (C4″H_2), 3.00 (C9″H_2), 1.59 and 1.67 (C2H_2), 1.61 (C13*H*), 1.62 (C2″H_2), 1.65 (C7H_2), 1.77 (C5″H_2), 1.80 and 2.04 (C16H_2), 1.85 and 2.20 (C22H_2), 2.03 (C30H_3), 2.17 (C2′H_2), 2.19 (C19*H*), 2.23 (C18*H*), 3.00 (C10″H_2), 4.07 (C1″H_2), 4.45 (C3*H*), 4.59 and 4.72 (C29H_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.6 (*C27*), 16.0 (*C26*), 16.1 (*C25*), 16.4 (*C24*), 18.1 (*C6*), 19.3 (*C30*), 20.9 (*C11*), 21.2 (*C2′*), 23.6 (*C2*), 25.5 (*C12*), 25.7 (*C3″*/*C8″*), 27.6 (*C15*), 27.9 (*C23*), 28.7 (*C2″*), 29.4 (*C4″*), 29.5 (*C5″–C7″*), 29.6 (*C16*), 30.6 (*C21*), 32.2 (*C9″*), 34.2 (*C22*), 34.3 (*C7*), 37.1 (*C10*), 37.8 (*C13*), 38.3 (*C1*), 38.4 (*C4*), 40.7 (*C8*), 42.4 (*C14*), 44.8 (*C17*), 47.0 (*C18*), 49.3 (*C19*), 50.4 (*C9*), 63.1 (*C10″*), 63.9 (*C1″*), 56.5 (*C5*), 80.9 (*C3*), 109.5 (*C29*), 150.6 (*C20*), 171.0/176.2 (*C28*/*C1′*). LC/MS/MS $[M + H]^+$: 672.04.

3-O-Chloroacetylbetulinic acid ***9***

A mixture of betulinic acid (228 mg, 0.5 mmol) and chloroacetic anhydride (170 mg, 1 mmol) in hexane (3 mL) was stirred with full microwave power (300 W) at 94°C under a maximum pressure of 10 bar for 20 min. After separation by column chromatography with silica gel (eluent: dichloromethane/methanol, 10:0.1), it yielded a white powder. White solid; yield: 162 mg (71%); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.77 and 1.70 (C1H_2), 0.83 (C24H_3), 0.84 (C23H_3), 0.85 (C25H_3), 0.92 (C26H_3), 0.96 (C27H_3), 1.04 and 1.69 (C15H_2), 1.15 and 1.46 (C12H_2), 1.18 and 1.48 (C11H_2), 1.37 and 1.60 (C6H_2), 1.39 (C9*H*), 1.41 (C5*H*), 1.42 and 1.60 (C21H_2), 1.59 and 1.94 (C22H_2), 1.60 and 1.67 (C2H_2), 1.62 (C13*H*), 1.63 (C7H_2), 1.72 and 2.02 (C16H_2), 1.92 (C18*H*), 1.97 (C30H_3), 2.17 (C19*H*), 4.02 (C2'H_2), 4.53 (C3*H*), 4.59 and 4.72 (C29H_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.7 (*C27*), 16.1 (*C25*), 16.2 (*C26*), 16.4 (*C24*), 18.2 (*C6*), 19.4 (*C30*), 20.9 (*C11*), 23.6 (*C12*), 25.5 (*C2*), 28.0 (*C23*), 29.7 (*C21*), 30.6 (*C15*), 32.2 (*C16*), 34.3 (*C7*), 37.1 (*C10*), 37.2 (*C22*), 38.1 (*C4*), 38.4 (*C1*), 38.5 (*C13*), 40.8 (*C8*), 41.2 (*C2′*), 42.5 (*C14*), 47.0 (*C18*), 49.3 (*C19*), 50.4 (*C9*), 55.4 (*C5*), 56.4 (*C17*), 83.3 (*C3*), 109.7 (*C29*), 150.2 (*C20*), 167.0 (*C1′*), 182.1 (*C28*). LC/MS/MS $[M + H]^+$: 534.1.

3-O-Hexanoylbetulinic acid ***10***

A mixture of betulinic acid (1350 mg, 3 mmol) and hexanoic anhydride (6 mL, 25 mmol) in dichloromethane (9 mL) was stirred with full microwave power (300 W) at 90°C under a maximum pressure of 10 bar for 30 min. The reaction mixture was separated with column chromatography (dichloromethane/methanol, 10:1) to obtain a white powder. White solid; yield: 850 mg (63%); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.81 (C6′H_3), 0.83 (C24H_3), 0.87 and 1.66 (C1H_2), 0.91 (C23H_3), 0.95 (C25H_3/C26H_3/C27H_3), 1.05 and 1.67 (C15H_2), 1.15 and 1.45 (C11H_2), 1.18 and 1.48 (C12H_2), 1.30 (C4′H_2), 1.34 (C5′H_2), 1.38 and 1.59 (C6H_2), 1.39 (C9*H*), 1.41 (C5*H*), 1.42 and 1.62 (C21H_2), 1.57 (C3′H_2), 1.58 (C7H_2), 1.59 and 1.83 (C22H_2), 1.60 and 1.64 (C2H_2), 1.62 and 2.01 (C16H_2), 1.64 (C13*H*), 1.70 (C3′H_2), 1.94 (C18*H*), 1.96 (C30H_3), 2.17 (C19*H*), 2.26 (C2′H_2), 2.34 (C2′H_2), 4.43 (C3*H*), 4.59 and 4.71 (C29H_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.0 (*C6′*), 14.8 (*C27*), 16.2 (*C24*), 16.3 (*C25*), 16.6 (*C26*), 18.3 (*C6*), 19.5 (*C30*), 21.0 (*C11*), 22.4 (*C5′*), 23.9 (*C12*), 24.9 (*C4′*), 25.6 (*C2*), 28.1 (*C23*), 29.8 (*C21*), 30.7 (*C15*), 31.4 (*C3′*), 32.3 (*C16*), 34.4 (*C2′*), 34.9 (*C7*), 37.1 (*C10*/*C22*), 37.2 (*C4*), 37.9 (*C1*), 38.5 (*C13*), 40.8 (*C8*), 42.5 (*C14*), 47.0 (*C18*), 49.4 (*C19*), 50.5 (*C9*), 53.4 (*C5*), 55.5 (*C17*), 80.6 (*C3*), 109.7 (*C29*), 150.2 (*C20*), 173.5 (*C1′*), 180.5 (*C28*). LC/MS/MS $[M + H]^+$: 555.7.

3-O-Laurylbetulinic acid ***11***

A mixture of betulinic acid (228 mg, 0.5 mmol) and dodecanoic anhydride (200 mg, 0.5 mmol) in dichloromethane was stirred with full microwave power (300 W) at 90°C under a maximum pressure of 10 bar for 45 min to obtain a brownish-white crystalline powder. It was purified by column chromatograph (dichloromethane/methanol, 8:2) on silica gel. Yield: 171 mg (75%) as a white solid; ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.81 (C11′H_3), 0.85 (C24H_3), 0.87 and 1.66 (C1H_2), 0.91 (C23H_3), 1.04 and 1.67 (C15H_2), 1.17 and 1.47 (C11H_2), 1.18 and 1.48 (C12H_2), 1.24 (C25H_3/C26H_3/C27H_3), 1.33 (C5′H_2/C6′H_2/C7′H_2), 1.36 (C4′H_2/C8′H_2/C9′H_2), 1.37 and 1.59 (C6H_2), 1.38 (C10′H_2),1.39 (C9*H*), 1.40 (C5*H*), 1.41 and 1.61 (C21H_2), 1.57 (C3′H_2), 1.58 (C7H_2), 1.58 and 1.83 (C22H_2), 1.59 and 1.65 (C2H_2), 1.62 and 2.01 (C16H_2), 1.63 (C13*H*), 1.93 (C18*H*), 1.94 (C30H_3), 2.17 (C19*H*), 2.34 (C2′H_2), 4.43 (C3*H*), 4.58 and 4.70 (C29H_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.2 (*C11′*), 14.8 (*C27*), 16.2 (*C24*), 16.3 (*C25*), 16.7 (*C26*), 18.4 (*C6*), 19.5 (*C30*), 21.1 (*C11*), 22.8 (*C10′*), 23.9 (*C12*), 24.9 (*C8′*), 25.6 (*C2*), 28.1 (*C23*), 29.2 (*C4′*), 29.4 (*C5′*/*C8′*), 29.6 (*C6′*/*C7′*), 29.8 (*C21*), 30.7 (*C15*), 25.3 (*C3′*), 32.0 (*C9′*), 32.3 (*C16*), 34.3 (*C2′*), 35.0 (*C7*), 37.2 (*C22*), 37.2 (*C10*), 38.0 (*C4*), 38.5 (*C13*), 38.6 (*C1*), 40.9 (*C8*), 42.6 (*C14*), 47.0 (*C18*), 49.4 (*C19*), 50.6 (*C9*), 55.7 (*C5*), 56.5 (*C17*), 80.7 (*C3*), 109.7 (*C29*), 150.3 (*C20*), 173.6 (*C1′*), 182.7 (*C28*). LC/MS/MS $[M + H]^+$: 626.06.

28-O-Chloracetylbetulin ***15***

A mixture of betulin (442 mg, 1 mmol) and chloroacetic anhydride (170 mg, 1 mmol) in dichloromethane (3 mL) was stirred with full microwave power (250 W) at 90°C under a maximum pressure of 10 bar for 20 min. After separation by column chromatography with silica gel (eluent: dichloromethane/methanol, 10:0.1), it yielded a white powder. White solid; yield: 385 mg (87%); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.74 (C24H_3), 0.80 (C25H_3), 0.87 and 1.64 (C1H_2), 0.94 (C27H_3), 0.95 (C23H_3), 1.00 (C26H_3), 1.02 and 1.82 (C22H_2), 1.05 and 1.63 (C12H_2), 1.06 and 1.72 (C15H_2), 1.19 and 1.50 (C11H_2), 1.21 and 1.94 (C16H_2), 1.27 (C9*H*), 1.39 (C7H_2), 1.51 and 1.97 (C21H_2), 1.59 (C2H_2/C18*H*), 3.15 (C3*H*), 0.64 (C5*H*), 1.37 and 1.55 (C6H_2), 1.63 (C13*H*), 1.66 (C30H_3), 2.42 (C19*H*), 3.93 and 3.96 (C28H_2), 4.34 (C2″H_2), 4.57 and 4.67 (C29H_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.9 (*C27*), 15.4 (*C24*), 16.1 (*C25*/*C26*), 18.4 (*C6*), 19.2 (*C30*), 20.9 (*C11*), 25.3 (*C12*), 27.1 (*C15*), 27.5 (*C2*), 28.1 (*C23*), 29.6 (*C21*), 29.8 (*C16*), 34.3 (*C22*), 34.5 (*C7*), 37.2 (*C10*), 37.7 (*C13*), 38.8 (*C1*), 38.9 (*C4*), 40.9 (*C8*/*C2″*), 42.7 (*C14*), 46.6 (*C17*), 47.1 (*C18*), 48.9 (*C19*), 50.4 (*C9*), 55.4 (*C5*), 64.8 (*C28*), 78.9 (*C3*), 109.8 (*C29*), 149.7 (*C20*), 167.4 (*C1″*). LC/MS/MS $[M - H]^-$: 518.2.

28-O-Laurylbetulin ***16***

A mixture of betulin (442 mg, 1 mmol) and lauric anhydride (383 mg, 2 mmol) in dichloromethane (3 mL) was stirred with full microwave power (300 W) at 90°C under a maximum pressure of 10 bar for 20 min. After separation by column chromatography with silica gel (eluent: dichloromethane/methanol, 10:0.05), it yielded a white powder. White solid; yield: 287 mg (65%); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.65 (C5*H*), 0.73 (C24H_3), 0.83 (C25H_3), 0.89 and 1.64 (C1H_2), 0.93 (C27H_3), 0.94 (C23H_3), 1.00 (C26H_3), 1.02 and 1.81 (C22H_2), 1.05 and 1.63 (C12H_2), 1.06 and 1.71 (C15H_2), 1.19 and 1.48 (C11H_2), 1.22 and 1.93 (C16H_2), 1.25 (C5″H_2/C6″H_2/C7″H_2), 1.26 (C9*H*), 1.36 (C4″H_2/C8″H_2/C9″H_2), 1.38 and 1.53 (C6H_2), 1.39 (C7H_2), 1.49 and 1.96 (C21H_2), 1.55 (C3″H_2), 1.57 (C2H_2), 1.58 (C18*H*), 1.63 (C13*H*), 1.67

(C30H_3), 2.28 (C2″H_2), 2.41 (C19*H*), 3.15 (C3*H*), 3.80 and 3.82 (C28H_2), 4.55 and 4.65 (C29H_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.1 (*C11″*), 14.8 (*C27*), 15.4 (*C24*), 16.1 (*C25*/*C26*), 18.3 (*C6*), 19.2 (*C30*), 20.8 (*C11*), 22.7 (*C10″*), 25.1 (*C9″*), 25.3 (*C12*), 27.1 (*C15*), 27.4 (*C2*), 28.0 (*C23*), 29.2 (*C8″*), 29.3 (*C16*/*C7″*), 29.5 (*C6″*), 29.6 (*C5″*), 29.7 (*C4″*), 29.9 (*C21*), 31.9 (*C3″*), 34.2 (*C2″*), 34.5 (*C22*), 34.3 (*C7*), 37.2 (*C10*), 37.6 (*C13*), 38.8 (*C1*), 38.9 (*C4*), 40.9 (*C8*), 42.5 (*C14*), 46.5 (*C17*), 47.9 (*C18*), 48.9 (*C19*), 50.7 (*C9*), 55.5 (*C5*), 62.5 (*C28*), 78.8 (*C3*), 109.6 (*C29*), 149.8 (*C20*), 173.9 (*C1″*). LC/MS/MS $[M + H]^+$: 611.5.

Bispivaloxymethylbetulin ***17***

Betulin (442 mg, 1 mmol) was reacted with pivalic anhydride (372.5 mg, 2 mmol) in chloroform (3 mL) and was heated to 110°C under a maximum pressure limit of 10 bar for 40 min. The obtained reaction mixture was separated with silica gel column chromatography using dichloromethane/methanol (10:1) as eluent. White solid; yield: 305 mg (69%); ^{1}H-NMR (500 MHz, $CDCl_3$) δ (in ppm): 0.66 (C5*H*), 0.75 (C24H_3), 0.82 (C25H_3), 0.89 and 1.65 (C1H_2), 0.97 (C27H_3/C23H_3), 1.03 (C26H_3), 1.03 and 1.83 (C22H_2), 1.04 and 1.63 (C12H_2), 1.05 and 1.71 (C15H_2), 1.18 and 1.41 (C11H_2), 1.19 (3C3′H_3/3C3″H_3), 1.20 and 1.94 (C16H_2), 1.28 (C9*H*), 1.38 and 1.53 (C6H_2), 1.40 (C7H_2), 1.41 and 1.96 (C21H_2), 1.58 (C2H_2/C18*H*), 1.64 (C13*H*), 1.68 (C30H_3), 2.41 (C19*H*), 3.18 (C3*H*), 3.31 and 3.79 (C28H_2), 4.58 and 4.68 (C29H_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ (in ppm): 14.7 (*C27*), 15.3 (*C24*), 16.0 (*C25*), 16.1 (*C26*), 18.2 (*C6*), 19.1 (*C30*), 20.7 (*C11*), 25.2 (*C12*), 27.0 (*C15*), 27.0/27.2 (*C2′*/*C2″*), 27.2 (*C2*), 27.9 (*C23*), 29.6 (*C16*), 29.8 (*C21*), 34.1 (*C22*), 34.5 (*C7*), 37.1 (*C10*), 37.5 (*C13*), 38.5/39.0 (*C1′*/*C1″*), 38.7 (*C1*), 38.8 (*C4*), 40.8 (*C8*), 42.7 (*C14*), 46.6 (*C17*), 47.8 (*C18*), 48.8 (*C19*), 50.3 (*C9*), 55.2 (*C5*), 62.5 (*C28*), 79.0 (*C3*), 109.8 (*C29*), 150.1 (*C20*), 178.9 (*C28*), 184.5 (*C1′*). LC/MS/MS $[M + Na]^+$: 633.5.

In-vitro **antitumoral studies**

Cell lines and culture conditions

The cell lines 8505C, A253, A2780, A431, HCT-8, HCT-116, DLD-1, SW480, SW1736, and liposarcoma were included in this study. All these cell lines were kindly provided by Dr. Thomas Mueller, Department of Hematology/Oncology, Martin Luther University of Halle-Wittenberg, Halle (Saale), Germany. Cultures were maintained as monolayers in RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with 5–10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) and penicillin/streptomycin (PAA Laboratories) at 37°C in a humidified atmosphere with 5% CO_2.

Cytotoxicity assay

The cytotoxic activities of all the compounds were evaluated using the sulforhodamine-B (SRB) (Sigma Aldrich, Germany) microculture colorimetric assay. In short, exponentially growing cells were seeded into 96-well plates on day 0 at the appropriate cell densities to prevent confluence of the cells during the period of the experiment. After 24 h, the cells were treated with serial dilutions of the compounds (0 to 100 μM) for 96 h. The final concentration of DMSO solvent never exceeded 0.5%, at which it was non-toxic to the cells. The percentages of surviving cells relative to untreated controls were determined 96 h after the beginning of drug exposure. After a 96-h treatment, the supernatant medium from the 96-well plates was thrown away and the cells were fixed with 10% TCA. For a thorough fixation, plates were now allowed to stand at 4°C. After fixation, the cells were washed in a strip washer. The washing was done four times with water using alternate dispensing and aspiration procedures. The plates were then dyed with 100 mL of 0.4% SRB for about 45 min. After dyeing, the plates were washed again with 1% acetic acid to remove the dye; then, they were allowed to air-dry overnight. 100 mL of 10 mM Tris base solution was added to each well of the plate and absorbance was measured at 570 nm using a 96-well plate reader (Tecan Spectra, Crailsheim, Germany). The IC_{50} and IC_{90} values, defined as the concentrations of the compound at which 50 and 90% cell inhibition is observed, were estimated from the semi-logarithmic dose-response curves.

Apoptosis test - DNA fragmentation assay

The determination of apoptotic cell death was performed by DNA gel electrophoresis. Briefly, SW1736 and A431 were treated for 24 h with IC_{90} doses of **9** and **15**, respectively. Floating cells induced by drug exposure were collected, washed with phosphate buffered saline (PBS), and lysed with lysis buffer (100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 0.8% SDS; all from Sigma Aldrich). Then, cells were treated with RNAse A at 37°C for 2 h, and proteinase K at 50°C (both from Roche Diagnostics Chemical Company, Mannheim, Germany). DNA laddering was observed by running the samples on 2% agarose gel followed by ethidium bromide (Sigma Aldrich) staining.

Kinetic studies

The activity of compounds **9** and **15** along with betulinic acid on A431 and SW1736 cells was analyzed by treating them for 4, 8, 24, 36, 48, 72, and 96 h at equitoxic (IC_{50}) concentrations of each compound, respectively. The cytotoxic activity was performed exactly in accordance with the SRB microculture colorimetric assay except, that after each scheduled time point the cells were washed with PBS and further grown in drug-free media for the remaining time to complete 96 h. After working up the 96-well plates according to the published SRB assay protocol, absorbance was measured at 570 nm in a 96-well plate reader.

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References

[1] M. Ogura, G. A. Cordell, N. R. Farnsworth, *Lloydia* **1977**, *40*, 157–168.

[2] K. Sheth, S. Jolad, R. Wiedhopf, J. R. Cole, *J. Pharm. Sci.* **1972**, *61*, 1819.

[3] D. H. Miles, U. Kokpol, L. H. Zalkow, S. J. Steindel, J. B. Nabors, *J. Pharm. Sci.* **1974**, *63*, 613–615.

[4] E. R. Trumbull, E. Bianchi, D. J. Eckert, R. M. Wiedhopf, J. R. Cole, *J. Pharm. Sci.* **1976**, *65*, 1407–1408.

[5] F. Sandberg, K. Duchevska, V. Khristov, S. Spasov, *Acta Pharm. Suec.* **1987**, *24*, 253–256.

[6] M. C. Recio, R. M. Giner, S. Manez, J. Gueho, *et al*., *Planta Med.* **1995**, *61*, 9–12.

[7] T. Fujioka, Y. Kashiwada, R. E. Kilkuskie, L. M. Cosentino, *et al*., *J. Nat. Prod.* **1994**, *57*, 243–247.

[8] R. H. Cichewicz, S. A. Kouzi, *Med. Res. Rev.* **2004**, *24*, 90–114.
[9] J. Patocka, *J. Appl. Biomed.* **2003**, *1*, 7–12.
[10] S. Ramadoss, M. Jaggi, M. J. A. Siddiqui, (Dabur Ltd.), USP 6,048,847 **2000**.
[11] E. Pisha, H. Chai, I. S. Lee, T. E. Chagwedera, *et al.*, *Nat. Med.* **1995**, *1*, 1046–1051.
[12] S. Fulda, I. Jeremias, H. H. Steiner, T. Pietsch, K. M. Debatin, *Int. J. Cancer* **1999**, *82*, 435–441.
[13] M. L. Schmidt, K. L. Kuzmanoff, L. Ling-Indeck, J. M. Pezzuto, *Eur. J. Cancer* **1997**, *33*, 2007–2010.
[14] V. Zuco, R. Supino, S. C. Righetti, K. Cleris, *et al.*, *Cancer Lett.* **2002**, *175*, 17–25.
[15] S. Fulda, C. Friesen, M. Los, C. Scaffidi, *et al.*, *Cancer Res.* **1997**, *57*, 4956–4964.
[16] S. Fulda, C. Scaffidi, S. A. Susin, P. H. Krammer, *et al.*, *J. Biol. Chem.* **1998**, *273*, 33942–33948.
[17] M. Urban, J. Sarek, I. Tislerova, P. Dzubak, M. Hajduch, *Bioorg. Med. Chem.* **2005**, *13*, 5527–5535.
[18] M. Kvasnica, J. Sarek, E. Klinotova, P. Dzubak, M. Hajduch, *J. Bioorg. Med. Chem.* **2005**, *13*, 3447–3454.
[19] P. Skehan, R. Storeng, D. Scudiero, A. Monks, *et al.*, *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.
[20] S. Ramadoss, M. Jaggi, M. J. A. Siddiqui, (Dabur Ltd.), USP 6,670,345 **2003**.
[21] D. Thibeault, C. Gauthier, J. Legault, J. Bouchard, *et al.*, *J. Bioorg. Med. Chem.* **2007**, *15*, 6144–6157.
[22] M. Urban, J. Sarek, J. Klinot, G. Korinkova, M. Hajduch, *J. Nat. Prod.* **2004**, *67*, 1100–1105.
[23] G. Zhao, W. Yan, *J. Carbohydr. Chem.* **2009**, *28*, 234–243.
[24] A. V. Symon, N. N. Veselova, A. P. Kaplun, N. K. Vlasenkova, *et al.*, *Russ. J. Bioorg. Chem.* **2005**, *31*, 286–291.

