European Journal of Medicinal Chemistry 75 (2014) 460–466

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

Synthesis and studies of anticancer properties of lupane-type triterpenoid derivatives containing a cisplatin fragment

Daniel Emmerich [a], Kranthi Vanchanagiri [a], Leopoldo C. Baratto [b], Harry Schmidt [c], Reinhard Paschke [a,*]

[a] Biozentrum, Martin-Luther-Universität Halle-Wittenberg, Weinbergweg 22, 06120 Halle (Saale), Germany
[b] Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Paraná, Centro Politécnico, 81531-970 Curitiba, PR, Brazil
[c] Institut für Chemie, Martin-Luther-Universität Halle-Wittenberg, Kurt-Mothes-Straße 2, 06120 Halle (Saale), Germany

ARTICLE INFO

Article history:
Received 5 September 2013
Received in revised form
14 January 2014
Accepted 20 January 2014
Available online 4 February 2014

Keywords:
Betulinic acid derivatives
Antitumor agent
Cisplatin conjugates
Cytotoxicity
SRB assay

ABSTRACT

Both betulinic acid 1 and cisplatin are promising antitumor agents, which induce apoptotic cell death of cancer cells. In the present investigation a new series of betulinic acid–cisplatin conjugates were synthesized and cytotoxicity and selectivity were assessed against five different tumor cell lines. The aim was to combine two structural units, both related with apoptosis induction. The derivatives exerted a dose-dependent antiproliferative action at micromolar concentrations and the effect of these structural variations on anticancer activity was studied and discussed. Several compounds revealed significant antitumor activity, as the most active substance 3-*O*-acetylbetulinic (2-(2-aminoethyl)aminoethyl)amide ($IC_{50} = 1.30–2.24\ \mu M$). Interestingly, Betulinic acid–cisplatin conjugates were less cytotoxic than the precursors.

1. Introduction

Betulinic acid (1) and its derivatives are pluripotent compounds with numerous biological activities. Therefore they have been investigated widely over the last few years [1–5], focusing in the field of antitumor properties [6–10]. Since we have also successfully prepared highly active anticancer platinum complexes [11–13], we have developed a concept to combine both bioactive fragments into one molecule. The aim was to find out if a combination of two different apoptotic structures could lead to an increased cytotoxicity. An insufficient process of apoptosis is not only an important factor in the genesis of tumors, but also the main reason for malignant tumors getting resistant against chemo- and radiotherapies [14] (Fig. 1).

Combinational therapy is common in the field of chemotherapy [15–18]. The efficiency of this therapy depends strongly on the nature of the single components: how they can be delivered, how they are metabolized, and how and to which extent they can enter the cell. Therefore it could be advantageous when the components are covalently linked to each other. There are several examples for this approach. As a result of the combination of Wortmannin and Cetuximab in a "double drug" concept, the antiproliferative activity of both compounds could be improved [19]. Similarly the cytotoxic and phototoxic properties of a Ruthenium–Porphyrine conjugate are combined [20].

2. Results and discussion

2.1. Chemistry

The substances described in this work were prepared according to known methods which were modified appropriately (Schemes 1–3). Compound **3** – an alkyl amide (polyamine) – was prepared by reaction of 3-*O*-acetyl-betulinic acid (2) with diethylene triamine in dichloromethane (DCM) [21]. Platinum complexes **3(PtClS)**, **5($PtCl_2$)** and **6(PtClS)** were formed by having the respective ligand molecules react with dichlorobis(dimethyl sulfoxide)platinum(II) in CH_3OH [22]. The complexes **3($PtCl_2$)** and **6($PtCl_2$)** were prepared by a reaction of the appropriate DMSO platinum complexes with an aqueous LiCl solution [23]; **3($PtCl_2$)** was also obtained by reaction with $K_2[PtCl_4]$ and KCl. **6($PtCl_2$)** and **6(PtClS)** are platinum precursors used for the synthesis of **3($PtCl_2$)** and **3(PtClS)** from compound **3**, and **5($PtCl_2$)** and **5(PtClS)** from compound **5**. The diaminopropanol derivatives **4** and **5** (esters of

* Corresponding author. Tel.: +49 3455521600.
E-mail address: reinhard.paschke@biozentrum.uni-halle.de (R. Paschke).

0223-5234/$ – see front matter © 2014 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2014.01.031

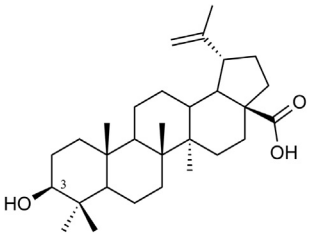
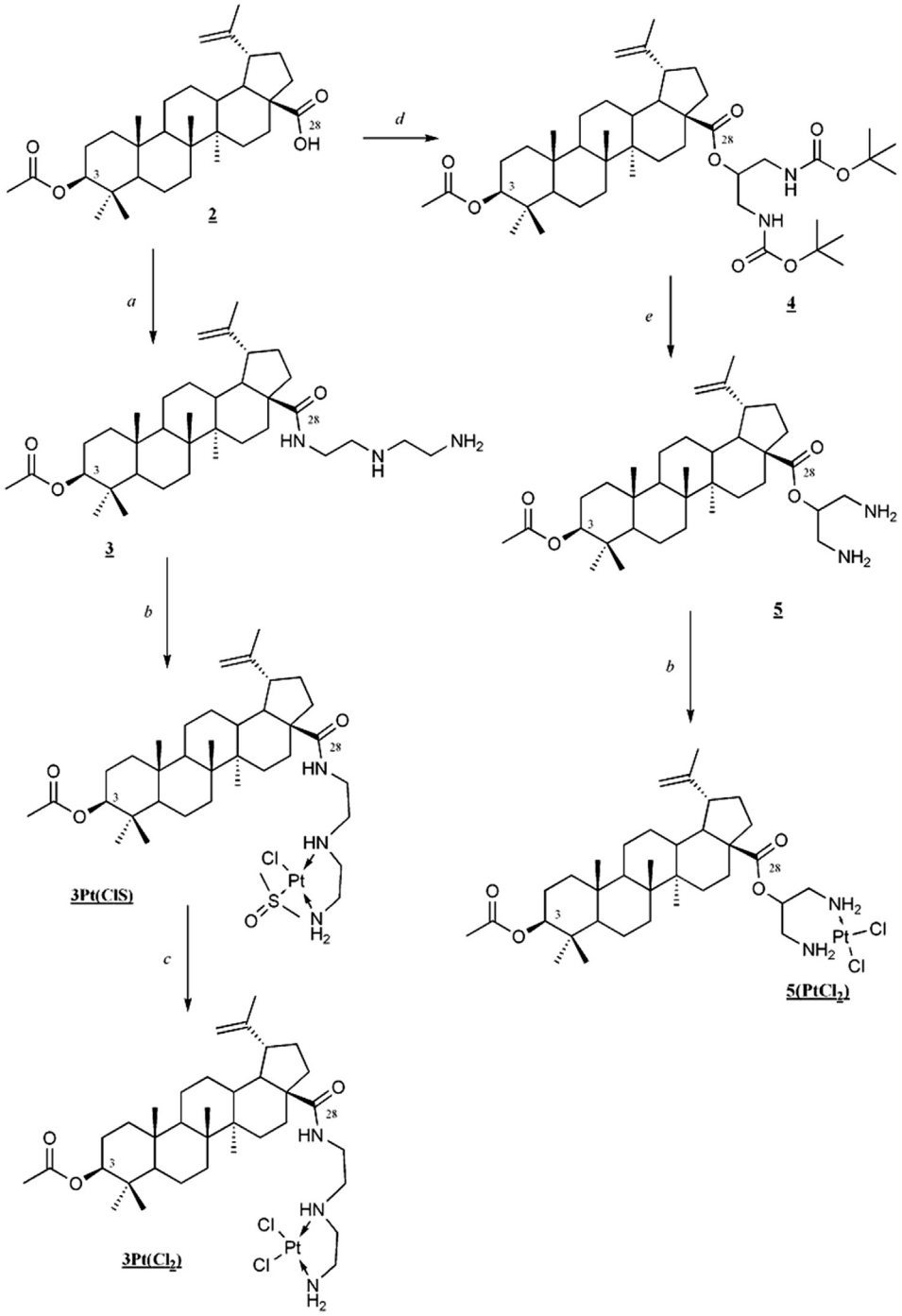


Fig. 1. Betulinic acid **1**.

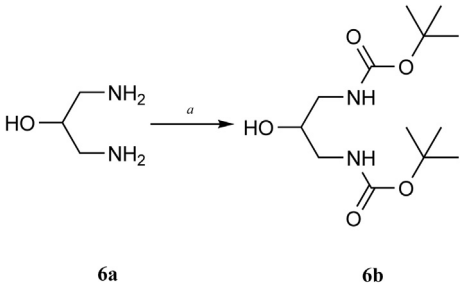
compound **2**) and **6b** have been prepared according to literature [24], as well as dichlorobis(dimethyl sulfoxide)platinum(II) [25].

2.2. Cytotoxicity

In the present study *in vitro* cytotoxic activity of betulinic acid **1** and its derivatives containing cisplatin similar ligands were studied on five different cancer cell lines: 518A2 (melanoma), A2780 (ovarian), A549 (lung carcinoma), MCF-7 (breast) and 8505C (anaplastic thyroid) as well as on one non-tumorous cell line (WWO70327) by SRB colorimetric assay method [26]. The



Scheme 1. Synthesis of 3-*O*-acetyl-betulinic acid derivatives. (a) Oxalyl chloride, diethylene triamine/DCM, 16 h rt, 6 h reflux; (b) dichlorobis(dimethyl sulfoxide)platinum(II)/CH_3OH, 2 h, rt; (c) $LiCl/H_2O$, 2 h, 80 °C; (d) oxalyl chloride, **6b**/trimethylamine/DCM, 20 h rt, 30 min reflux; and (e) TFA/DCM, 1.5 h rt.



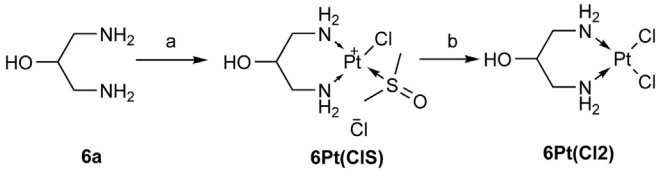
6a 6b

Scheme 2. Preparation of **6b**: (a) Boc_2O/CH_3CN, 1 h rt.

compounds showed dose-dependent antitumoral activity against the investigated cell lines. The IC_{50}, defined as the concentration of the compound at which 50 % cell inhibition is observed, was estimated from the semi-logarithmic dose–response curves (Fig. 2.) by GraphPad Prism5. The IC_{50} values were summarized in Table 1. The compounds tested here were synthesized by derivatization at C-3 (hydroxyl) and C-28 (carboxylic group) positions of Betulinic acid **1** and the structure activity relationship was developed in relation to modifications at corresponding positions.

From the IC_{50} values it was observed that compounds **1**–**5** showed moderate to strong cytotoxicity against the investigated cell lines. The lead compound **1** ($IC_{50} = 8–14$ μM) shared similar range of IC_{50} values observed for its derivatives without platinum ligands, showing higher cytotoxicity against A2780 cells. Compound **2** was slightly more cytotoxic than **1**; the presence of an acetyl group at C3 seemed to influence the activity. Compound **3** was the most active derivative ($IC_{50} = 1.3–2.24$ μM) against all tested cancer types; the presence of both acetyl and (2-(2-aminoethyl)aminoethyl) amide moieties at C3 and C28, respectively, could be responsible for the high cytotoxicity. Even though compounds **3** and **5** have symmetrical groups, it was found that **3** was 5 to 6 times more cytotoxic than **5**. The reason could be the linear alkyl amide (polyamine) group present at compound **3** that strongly influences the ability of the substance to enter the cell through the membrane as well as the capability to interact with cell components. The C28 substitutions in compounds **4** and **5** retained the cytotoxicity, but were not significant to produce more potent derivatives.

Compound **6(PtClS)** was moderately active to inactive ($IC_{50} = 19.05$ to >100 μM), while **6($PtCl_2$)** was more cytotoxic, except for the A549 cell line. Probably the presence of chloride groups in **6($PtCl_2$)** contributes more favorably to the activity than the sulfoxide groups in **6(PtClS)**. Compounds **3($PtCl_2$)** and **3(PtClS)** were moderately active with IC_{50} values between 11.04 and 28.60 μM; A549 cell line seemed to be more resistant to both compounds than other cancer types. Even though compounds **3($PtCl_2$)** and **3(PtClS)** were platinum derivatives of **3**; they exhibited cytotoxic properties similar to **1**, except for the A549 cell line. On the other hand, when compared to their precursor **3**, those compounds significantly lost activity. It was found that **3** was 5 to 14 times more cytotoxic than its platinum conjugates. In turn, the



6a 6Pt(ClS) 6Pt(Cl2)

Scheme 3. Synthesis of the diaminopropanol platinum complexes. (a) Dichlorobis(dimethyl sulfoxide) platinum(II)/CH_3OH, 3 h rt; and (b) LiCl/water, 2 h, 80 °C.

platinum conjugates **5($PtCl_2$)** and **5(PtClS)** were less active than **5**, with IC_{50} of 13.13–35.66 μM. It was observed that **5** was 1 to 3 times more cytotoxic than its platinum derivatives.

The introduction of platinum ligands into Betulinic acid (**1**), i.e. compound **3**, led to no significant loss of activity in most cancer cell lines. However, the presence of platinum groups did not have an influence in such a way as to increase the cytotoxicity of the compounds. In case of 5-platinum conjugates, loss of activity was observed when compared to **1**. Even though all compounds showed broad spectrum activity, in most substances the antiproliferative effect was pronounced against the A2780 cell line.

2.3. Selectivity

Selectivity of the compounds was assessed on human skin fibroblasts (WWO70327). The selectivity index was calculated by IC_{50} value in fibroblast divided by IC_{50} value in cancer cell lines. The results were summarized in Table 2. It was observed that compound **1** was 3 to 5 times more selective towards cancer cells than to fibroblasts, and similar selectivity was observed for **2**. The most active compound **3** and also compound **5** were the least selective substances towards all types of tumors. The selectivity of **3($PtCl_2$)** was between 2 and 3.5 times, and **5($PtCl_2$)** was around 3 to 4 times more selective towards cancer cells. Different selectivity patterns were observed for **6($PtCl_2$)**. The dose–response curves for the test compounds towards tumor (A549) and non-tumor (WWO70327) cells are given in Fig. 2.

3. Conclusions

In general when two cytotoxic groups covalently link in one molecule, the more pronounced cytotoxic activity can be expected. But our results revealed that Betulinic acid–Cisplatin conjugates more or less showed similar cytotoxicity to that of betulinic acid (1) and in all the cases they were found to be less cytotoxic than Cisplatin against all tumors. The property of double loading of two cytotoxic groups in conjugates does not contribute to exhibit improved cytotoxicity. On the other hand among the panel of derivatives compound 3 was found to be highly cytotoxic with IC_{50} of 1.3–2.24 μM on all cancers and less selective towards tumors than to normal cells. The order of cytotoxicity of all compounds on investigated cancers from highest to lowest can be given below.

3 > **2** > **1** > **5** > **4** > **3($PtCl_2$)** > **3(PtClS)** > **5($PtCl_2$)** > **5(PtClS)** > **6($PtCl_2$)** > **6(PtClS)**

In summary, the reason that the combination of the apoptotic units doesn't lead to an increase of cytotoxicity is not clear yet. More extensive investigations for instance the exact mechanism of anticancer action of the conjugates are necessary and this work is currently in progress in our laboratory and the results will be reported in due course. By this means, it should be possible to get a clear idea about our findings.

4. Experimental

4.1. Chemistry

Chemicals and solvents were used as received; DCM was dried over 4 Å molar sieves. The progress and result of every reaction were monitored by TLC on neutral aluminum oxide 60 F_{254} plates, made visible with 2% ceric ammonium sulfate solution. NMR spectra were recorded on VNMRS 400 and INOVA 500 instruments; the samples were dissolved in $CDCl_3$, CD_3OD or DMSO-d_6 with TMS (for ^1H and ^{13}C) or $Na_2[PtCl_6]$ (for ^{195}Pt) as internal standard,

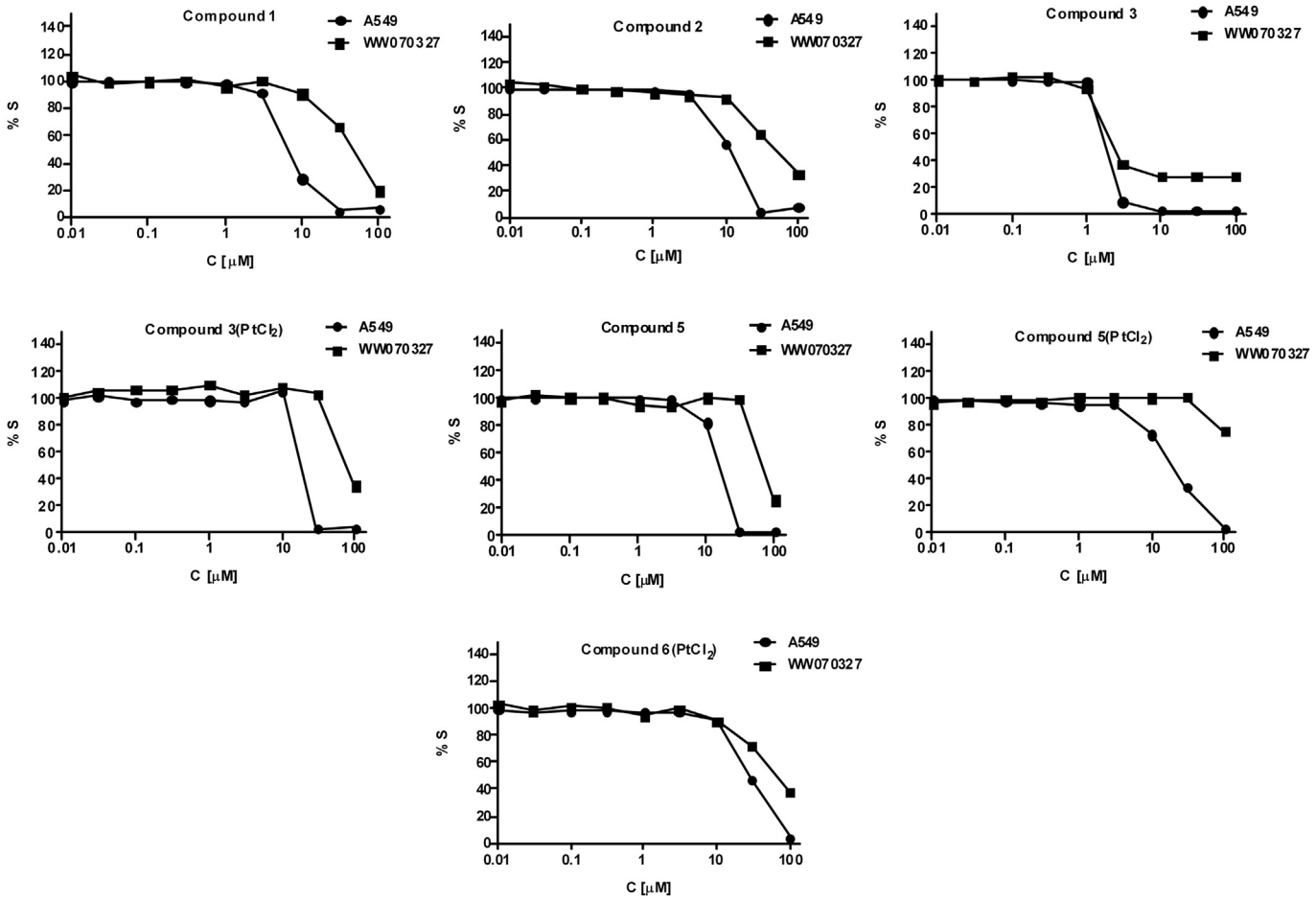


Fig. 2. Survival of tumor (A549) and non-tumor (WW070327) cells determined by SRB test after 96 h of treatment with seven selected compounds.

respectively. Chemical shifts (δ) are expressed in ppm; coupling constants in Hertz. ESI mass spectra were measured with a Thermo Finnigan LCQ-Classic, in methanolic solution. Infrared spectra were recorded on a Bruker Tensor 27 with ATR method. Betulinic acid **1** was gently supplied by BioSolutions Halle GmbH and 3-*O*-acetylbetulinic acid **2** was prepared by an esterification of **1** with acetic anhydride.

Table 1
$IC_{50} \pm$ S.E for the 96 h of activity of investigated compounds on tumor cell lines determined by SRB colorimetric assay.

Compound	A549	A2780	8505C	518A2	MCF7
1	13.3 ± 0.82	8.75 ± 0.96	12.63 ± 1.67	14.8 ± 0.6	14.03 ± 1.21
2	10.52 ± 0.01	5.95 ± 0.42	10.40 ± 0.40	10.95 ± 0.12	10.72 ± 0.40
3	2.05 ± 0.34	1.52 ± 0.29	2.24 ± 0.22	1.30 ± 0.03	1.59 ± 0.20
3($PtCl_2$)	17.32 ± 0.00	12.66 ± 4.66	11.57 ± 0.00	12.12 ± 1.63	12.41 ± 0.58
3(PtClS)	28.69 ± 0.12	12.63 ± 3.73	11.04 ± 3.77	12.45 ± 2.13	14.23 ± 1.83
4	16.91 ± 0.19	8.32 ± 0.86	15.50 ± 0.37	12.72 ± 0.25	15.06 ± 0.30
5	12.58 ± 0.24	6.60 ± 0.49	12.12 ± 0.25	11.5 ± 0.16	11.79 ± 0.27
5($PtCl_2$)	17.85 ± 1.87	13.13 ± 0.42	22.64 ± 0.44	29.83 ± 1.80	15.98 ± 0.23
5(PtClS)	14.57 ± 0.94	19.05 ± 0.43	20.13 ± 1.89	35.66 ± 0.16	19.31 ± 0.59
6($PtCl_2$)	27.12 ± 0.00	6.55 ± 1.10	9.69 ± 0.00	11.89 ± 1.66	10.47 ± 0.00
6(PtClS)	28.50 ± 4.12	23.97 ± 2.01	>100	23.87 ± 2.54	19.05 ± 1.76
Cisplatin	1.15 ± 0.05	0.33 ± 0.02	1.34 ± 0.04	0.64 ± 0.01	0.41 ± 0.01

Values are derived from dose–response curves obtained by measuring the percentage of viable cells relative to untreated controls after 96 h exposure of the test compounds to A549 (lung carcinoma), A2780 (ovarian cancer), 8505c (anaplastic thyroid cancer), 518A2 (melanoma) and MCF-7 (breast cancer) cell lines using SRB assay. Values are the average from at least three independent experiments.

*4.1.1. Preparation of the acid chloride of 3-O-acetylbetulinic acid [**2**]*

A solution of **2** (Scheme 2) ((1.0 g, 2.1 mmol) in dichloromethane (DCM) reacted with an oxalyl chloride solution (2 M in DCM, 2.4 mL, 4.8 mmol), 30 min, rt, then the solvent was removed under reduced pressure. The acid chloride of **2** – a crude white powder obtained in quantitative yield – had been directly used for the following reactions.

*4.1.2. Preparation of 3-O-acetylbetulinic (2-(2-aminoethyl) aminoethyl)amide [**3**]*

Diethylenetriamine (0.8 mL, 7.4 mmol) was dissolved in DCM (50 mL), and under vigorous stirring a solution of the acid chloride of **2** (1.0 g, 2.1 mmol) in DCM (10 mL) was added dropwise. The mixture was stirred for 16 h and refluxed for 6 h, washed twice with 10% aqueous K_2CO_3 solution and brine. The organic phases were dried over Na_2SO_4 and evaporated under reduced pressure.

Table 2
Selectivity index of the compounds towards tumor cells.

Compound	A549	A2780	8505c	518A2	MCF7
1	3.27	4.98	3.45	2.94	3.10
2	2.76	4.89	2.78	2.65	2.71
3	1.36	1.76	1.19	2.06	1.68
3($PtCl_2$)	2.30	3.15	3.44	3.29	3.21
5	1.27	2.33	1.27	1.34	1.3
5($PtCl_2$)	3.05	4.15	2.40	3.0	3.41
6($PtCl_2$)	1.06	4.39	2.96	1.22	2.74

The crude product was dissolved in CH_3OH, filtered and evaporated again to give a white solid (1.17 g, 97% yield), ^{1}H NMR (500 MHz, $CDCl_3$): 0.84 (3H, s, *H*-25), 0.85 (3H, s, *H*-24), 0.88 (3H, s, *H*-26), 0.96 (3H, s, *H*-27), 1.00 (3H, s, *H*-23), 1.68 (3H, s, *H*-30), 1.82 (2H, m, *H*-2), 2.04 (3H, s, −OAc), 2.11 (1H, m, *H*-13), 2.55 and 2.68 (1H each, m, *H*-2′), 2.68 (2H, m, *H*-3′), 2.74 (2H, m, *H*-4′), 3.08 (1H, dt, *H*-19, $^3J_{H-H}^{d} = 4.4$ Hz, $^3J_{H-H}^{t} = 10.8$ Hz), 3.23 and 3.36 (1H each, m, *H*-1′), 4.43 (1H, dd, *H*-3, $^3J_{H-H} = 11.3$ Hz, 5.2 Hz), 4.57 and 4.69 (1H each, s, *H*-29), 5.47 (1H, s, -CON*H*). ^{13}C NMR (125 MHz, $CDCl_3$): 15.1 (*C*27), 16.8 (*C*25), 16.8 (*C*26), 17.0 (*C*24), 19.3 (*C*6), 19.7 (*C*30), 21.2 (*C*2″), 22.1 (*C*12), 24.6 (*C*21), 26.9 (*C*15), 28.5 (*C*23), 30.6 (*C*16), 31.9 (*C*4), 34.1 (*C*7), 35.5 (*C*10), 38.3 (*C*1), 38.8 (*C*22), 38.9 (*C*13), 39.3 (*C*1′), 39.6 (*C*4′), 39.7 (*C*8), 41.8 (*C*14), 42.0 (*C*2′), 43.5 (*C*3′), 48.1 (*C*19), 51.4 (*C*18), 51.9 (*C*9), 56.8 (*C*5), 57.0 (*C*17), 82.5 (*C*3), 110.0 (*C*29), 152.2 (*C*20), 172.9 (*C*1″), 179.3 (*C*28). IR γ_{max} (neat): 2937, 2868, 1732, 1639, 1452, 1370, 1316, 1244, 1195, 1108, 1024, 978, 881, 543, 512. MS (ESI): 585 (100, $[M+H]^+$). Analysis for $C_{36}H_{61}N_3O_3$ (583.90): C, 74.05; H, 10.53; N, 7.20; found: C, 68.69; H, 9.59; N, 6.12.

4.1.3. Preparation of dichlorobis(dimethyl sulfoxide)platinum(II)

Dichlorobis(dimethyl sulfoxide)platinum(II) [25] was prepared as described as follows: $K_2[PtCl_4]$ (0.5 g, 1.2 mmol) was dissolved in water (4 mL), and DMSO (0.3 mL, 4.2 mmol) was added dropwise. The solution was stirred for 5 min and then allowed to stand at room temperature for 2 h. The forming solid was filtered off, washed with water, ethanol, and ether and then dried to give pale yellow crystals (448 mg, 88% yield).

4.1.4. Preparation of κN′,N″-(3-O-acetylbetulinic (2-(2-aminoethyl) aminoethyl)amide) chloro κS-dimethyl sulfoxide platinum(II) chloride [3(PtClS)]

Dichlorobis(dimethyl sulfoxide)platinum(II) (150 mg, 0.36 mmol) was suspended in CH_3OH (10 mL) and a solution of **3** (207 mg, 0.36 mmol) in CH_3OH (4 mL) was added dropwise. The mixture was stirred for 2 h at rt, then concentrated under reduced pressure to 3 mL and stored in the refrigerator overnight. The precipitate was filtered off and the solution was concentrated to 0.5 mL under reduced pressure. The precipitate was filtered off, washed with water and dried to yield a yellow solid (299 mg, 91% yield). ^{1}H NMR (400 MHz, CD_3OD): 0.84 (3H, s, *H*-25), 0.85 (3H, s, *H*-24), 0.89 (3H, s, *H*-26), 0.97 (3H, s, *H*-27), 1.00 (3H, s, *H*-23), 1.68 (3H, s, *H*-30), 2.02 (3H, s, −OAc), 2.15 (1H, m, *H*-22), 2.54 and 2.90 (1H each, m, *H*-2′), 2.66 (6H, s, DMSO) 2.90 and 3.07 (2H, m, *H*-3′), 3.07 (1H, m, *H*-19), 3.30 and 3.46 (1H each, m, *H*-1′), 3.46 (2H, m, *H*-4′), 4.43 (1H, dd, *H*-3, $^3J_{H-H} = 10.8$ Hz, 5.4 Hz), 4.58 and 4.70 (1H each, s, *H*-29). ^{13}C NMR (100 MHz, CD_3OD): 15.2 (*C*27), 16.7 (*C*25), 16.8 (*C*26), 17.0 (*C*24), 19.3 (*C*6), 19.7 (*C*30), 21.2 (*C*2″), 22.2 (*C*12), 24.7 (*C*21), 26.9 (*C*15), 28.5 (*C*23), 30.7 (*C*16), 31.9 (*C*4), 34.0 (*C*7), 35.5 (*C*10), 38.3 (*C*1), 38.8 (*C*22), 38.9 (*C*13), 39.3 (*C*1′), 39.6 (*C*8), 39.9 (*C*4′), 42.0 (*C*14), 42.1 (*C*2′), 43.5 (*C*3′), 48.1 (*C*19), 51.3 (*C*18), 51.9 (*C*9), 56.8 (*C*5), 57.0 (*C*17), 82.4 (*C*3), 110.1 (*C*29), 152.2 (*C*20), 172.9 (*C*1″), 180.1 (*C*28). ^{195}Pt NMR (86 MHz, CD_3OD): −3309 (*SP*-4-4), −3220 (*SP*-4-3). IR γ_{max} (neat): 2940, 2869, 1635, 1449, 1371, 1253, 1196, 1131, 1024, 979, 948, 879, 733, 695, 441. MS (ESI): 892.4 (100, $[M-Cl]^+$). Analysis for $C_{38}H_{67}Cl_2N_3O_4PtS$ (928.03): C; 49.18, H; 7.28, N; 4.53, S; 3.45, found: C; 51.58, H; 7.83, N; 3.29, S; 4.80.

4.1.5. Preparation of κN′,N″-(3-O-acetylbetulinic (2-(2-aminoethyl) aminoethyl)amide) dichloro platinum(II) [3(PtCl$_2$)]

4.1.5.1. Method A. Compound **3** (140 mg, 0.24 mmol) was dissolved in CH_3OH (10 mL), and a solution of $K_2[PtCl_4]$ (100 mg, 0.24 mmol) in water (2 mL) was added. The mixture has been stirred under light exclusion for 24 h, and then a 5% aqueous KCl solution (10 mL) was added and stirred for another hour. The suspension was extracted twice with DCM (30 mL), the organic phases were dried over Na_2SO_4, and then concentrated under reduced pressure to give a yellow powder (86 mg, 42% yield).

4.1.5.2. Method B. Compound **3(PtClS)** (130 mg; 0.14 mmol) was suspended in water (8 mL). LiCl (30 mg; 0.7 mmol) was added and the mixture was stirred for 2 h at 80 °C. The resulting precipitate was filtered off, washed with water, and dried to yield a yellow powder (97 mg, 81% yield).

^{1}H NMR (400 MHz, DMSO-d_6): 0.76 (3H, s, *H*-25), 0.76 (3H, s, *H*-24), 0.78 (3H, s, *H*-26), 0.83 (3H, s, *H*-27), 0.90 (3H, s, *H*-23), 1.61 (3H, s, *H*-30), 1.97 (3H, s, −OAc), 2.13 (1H, m, *H*-2), 2.50–3.40 (9H, m, br, *H*-19 + *H*-1′ + *H*-2′ + *H*-3′ + *H*-4′) 4.33 (1H, dd, *H*-3, $^3J_{H-H} = 11.1$ Hz, 4.8 Hz), 4.51 and 4.63 (1H each, s, *H*-29). ^{13}C NMR (100 MHz, DMSO-d_6): 14.3 (*C*27), 15.9 (*C*24), 15.9 (*C*25), 16.4 (*C*26), 17.7 (*C*6), 19.0 (*C*30), 20.6 (*C*11), 20.9 (*C*2″;), 23.3 (*C*2), 25.2 (*C*12), 27.7 (*C*23), 28.9 (*C*21), 30.3 (*C*15), 32.3 (*C*16), 33.8 (*C*4), 35.6 (*C*7), 36.6 (*C*10), 37.3 (*C*1), 37.6 (*C*22), 40.3 (*C*8), 41.9 (*C*14), 46.2 (*C*19), 49.6 (*C*18), 49.8 (*C*9), 54.7 (*C*5), 54.9 (*C*17), 79.9 (*C*3), 109.3 (*C*29), 150.8 (*C*20), 170.1 (*C*1″), 176.0 (*C*28). IR γ_{max} (neat): 2943, 1636, 1520. 1425, 1374, 1318, 1255, 1197, 1028, 981, 883, 443. MS (ESI): 892.4 (100, $[M+H_2O+Na]^+$), 814.4 (21, $[M-Cl]^+$). Analysis for $C_{38}H_{61}Cl_2N_3O_3Pt$ (873.92): C; 50.88, H; 7.23, N; 4.94, found: C; 51.64, H; 7.55, N; 4.54.

4.1.6. Preparation of 1,3-bis(tert-butylcarboxyamino)2-propanol (6b)

Boc_2O (2.9 g, 13.4 mmol) and 1,3-diamino-2-propanol (1.5 g, 16.6 mmol) were stirred in CH_3CN (250 mL) for 1 h. The forming precipitate was filtered off and the solution concentrated under reduced pressure to yield a white solid (2.06 g, 53% yield).

^{1}H NMR (500 MHz, $CDCl_3$): 1.34 (18H, s, −C*H*$_3$), 3.10 (4H, m, −C*H*$_2$−), 3.65 (1H, m, HOC*H*), 4.18 (1H, s, −O*H*), 5.41 (2H, s, −N*H*−). ^{13}C NMR (125 MHz, $CDCl_3$): 28.2 (−CH_3), 43.3 (−CH_2−), 70.2 (HOCH), 79.4 (C_{quart}), 157.0 (*C*=O). IR γ_{max} (neat): 3350, 2980, 2934, 2917, 1664, 1526, 1443, 1392, 1365, 1158, 1116, 853, 574. MS (ESI): 313.1 (100, $[M+Na]^+$).

4.1.7. Preparation of 3-O-acetylbetulinic (1,3-bis(tert-butylcarboxy amino) 2-propyl)ester [4]

Compound **6b** (350 mg, 1.20 mmol) and NEt_3 (0.5 mL, 3.60 mmol) were dissolved in DCM (10 mL). A solution of **2a** (520 mg, 1.00 mmol) in DCM (10 mL) was added dropwise, the mixture was stirred for 20 h, then refluxed for 30 min. The solution was washed twice with 0.12 M hydrochloric acid (50 mL) and twice with brine (50 mL), and then dried with Na_2SO_4. The solution was concentrated under reduced pressure to give a pale yellowish solid (840 mg, 99% yield).

^{1}H NMR (500 MHz, $CDCl_3$): 0.79 (3H, s, *H*-25), 0.80 (3H, s, *H*-24), 0.81 (3H, s, *H*-26), 0.91 (3H, s, *H*-27), 0.93 (3H, s, *H*-23), 1.64 (3H, s, *H*-30), 1.87 (2H, m, *H*-12), 2.00 (3H, s, −OAc), 2.17 (1H, m, *H*-2), 2.42 (1H, m, H13), 2.75 (1H, m, *H*-19), 3.13 and 3.20 (2H each, m, *H*-1′ + *H*-3′), 3.71 (1H, m, *H*-2′), 4.42 (1H, dd, *H*-3, $^3J_{H-H} = 14.3$ Hz, 4.8 Hz), 4.58 and 4.68 (1H each, s, *H*-29), 5.26 (2H, s, -N*H*). ^{13}C NMR (125 MHz, $CDCl_3$): 14.7 (*C*27), 15.9 (*C*25), 16.2 (*C*26), 16.5 (*C*24), 18.1 (*C*6), 19.3 (*C*30), 20,8 (*C*11), 21.3 (*C*2″), 25.3 (*C*12), 27.9 (*C*23), 28.4 (−C(CH_3)$_3$), 29.6 (*C*21), 29.8 (*C*15), 32.2 (*C*16), 34.2 (*C*4), 36.2 (*C*7), 37.1 (*C*10), 37.7 (*C*13), 37.8 (*C*1), 38.4 (*C*22), 40.7 (*C*8), 42.4 (*C*14), 43.7 (*C*1′ + *C*3′), 46.0 (*C*19), 49.7 (*C*18), 50.5 (*C*9), 55.5 (*C*5), 70.9 (*C*2′), 79.8 (−O*C*Me$_3$), 80.9 (*C*3), 110.3 (*C*29), 149.3 (*C*20), 157.1 (−NHCOO−), 171.0 (*C*1″), 177.3 (*C*28). IR γ_{max} (neat): 2946, 2874, 1736, 1685, 1524, 1454, 1368, 1244, 1164, 1027, 978, 889, 856, 440. MS (ESI): 793.4 (100, $[M+Na]^+$), 693.4 (15, $[M-OCOCMe_3+Na]^+$). Analysis for $C_{45}H_{74}N_2O_8$ (771.093): C, 70.09; H, 9.67; N, 3.63; found: C, 65.87; H, 9.58; N, 3.65.

*4.1.8. Preparation of 3-O-acetylbetulinic (1,3-diamino 2-propyl) ester [**5**]*

Compound **6b** (883 mg, 1.05 mmol) was dissolved in DCM (5 mL). It was stirred and TFA (0.5 mL, 6.49 mmol) was added dropwise. After about 1.5 h, the mixture was diluted with DCM (50 mL), then washed with cold concentrated K_2CO_3 solution (50 mL) and twice with brine (50 mL). The organic phase was dried over Na_2SO_4, then concentrated under reduced pressure. The precipitate was dissolved in CH_3OH, filtered, and the solution evaporated again to yield a white solid (443 mg, 75% yield).

^{1}H NMR (500 MHz, $CDCl_3$): 0.84 (3H, s, *H*-25), 0.85 (3H, s, *H*-24), 0.86 (3H, s, *H*-26), 0.94 (3H, s, *H*-27), 0.98 (3H, s, *H*-23), 1.70 (3H, s, *H*-30), 1.96 (2H, m, *H*-12), 2.05 (3H, s, −OAc), 2.25 (2H, m, *H*-2 + *H*-13), 3.01 (1H, m, *H*-19), 3.22 (4H, m, *H*-1′ + *H*-3′), 3.75 (1H, m, *H*-2′), 4.48 (1H, dd, *H*-3, $^3J_{H-H}$ = 10.5 Hz, 5.6 Hz), 4.61 and 4.74 (1H each, s, *H*-29), 5.31 (4H, s, $-NH_2$). ^{13}C NMR (125 MHz, $CDCl_3$): 14.6 (*C*27), 16.0 (*C*25), 16.2 (*C*26), 16.5 (*C*24), 18.2 (*C*6), 19.3 (*C*30), 20,9 (*C*11), 21.3 (*C*2″), 23.7 (C2), 25.5 (*C*12), 28.0 (*C*23), 29.7 (*C*21), 30.7 (*C*15), 32.4 (*C*16), 34.3 (*C*4), 37.1 (*C*7), 37.2 (*C*10), 37.8 (*C*1), 38.3 (*C*22), 38.4 (*C*13), 40.7 (*C*8), 42.5 (*C*14), 43.5 (*C*1′ + *C*3′), 47.0 (*C*19), 49.3 (*C*18), 50.4 (*C*9), 55.4 (*C*5), 56.6 (*C*17), 70.9 (*C*2′), 81.0 (*C*3), 109.6 (*C*29), 150.6 (*C*20), 171.1 (*C*1″). IR γ_{max} (neat): 2942, 2871, 1453, 1367, 1244, 1162, 1025, 978, 881. MS (ESI): 671.3 (100, $[M + HOCOCMe_3]^+$), 571.5 (11, $[M + H]^+$). Analysis for $C_{35}H_{58}N_2O_4$ (570.859): C, 73.64; H, 10.24; N, 4.91; found: C, 69.88; H, 9.31; N, 2.49.

*4.1.9. Preparation of κN,N′-(3-O-acetylbetulinic (1,3-diamino 2-propyl)ester) dichloro platinum(II) [**5(PtCl$_2$)**]*

Dichlorobis(dimethyl sulfoxide)platinum(II) (200 mg, 0.47 mmol) was suspended in CH_3OH (10 mL), and a solution of **5** (300 mg, 0.53 mmol) in CH_3OH (2 mL) was added dropwise. The mixture was stirred for 2 h, the forming precipitate was filtered off and the solution was concentrated under reduced pressure onto 2 mL. The solution was stored in the fridge for 40 h, then filtered again, and the solution was concentrated onto 0.5 mL under reduced pressure. The forming precipitate was filtered off, washed with water, and dried to give a yellow powder (142 mg, 36% yield).

^{1}H NMR (400 MHz, $CDCl_3$): 0.81 (3H, s, *H*-25), 0.83 (3H, s, *H*-24), 0.84 (3H, s, *H*-26), 0.92 (3H, s, *H*-27), 0.96 (3H, s, *H*-23), 1.68 (3H, s, *H*-30), 1.96 (2H, m, *H*-12), 2.03 (3H, s, −OAc), 2.17 (1H, m, *H*-2), 2.26 (1H, m, *H*-13), 2.99 (1H, dt, *H*-19, $^3J^d_{H-H}$ = 4.6 Hz, $^3J^t_{H-H}$ = 10.7 Hz), 3.53–3.10 (5H, m, *H*-1′ + *H*-2′ + *H*-3′), 4.46 (1H, dd, *H*-3, $^3J_{H-H}$ = 10.3 Hz, 6.0 Hz), 4.46 and 4.60 (1H each, s, *H*-29). ^{13}C NMR (100 MHz, $CDCl_3$): 14.6 (*C*27), 16.0 (*C*25), 16.1 (*C*26), 16.4 (*C*24), 18.1 (*C*6), 19.3 (*C*30), 20,8 (*C*11), 21.3 (*C*2″), 23.7 (C2), 25.4 (*C*12), 27.9 (*C*23), 29.7 (*C*21), 30.6 (*C*15), 32.2 (*C*16), 34.2 (*C*4), 37.0 (*C*7), 37.1 (*C*10), 37.8 (*C*1), 38.4 (*C*22), 38.4 (*C*13), 40.7 (*C*8), 42.4 (*C*14), 44.8 (*C*1′ + *C*3′), 46.9 (*C*19), 49.3 (*C*18), 50.4 (*C*9), 55.4 (*C*5), 56.4 (*C*17), 72.8 (*C*2′), 81.0 (*C*3), 109.7 (*C*29), 150.4 (*C*20), 171.1 (*C*1″), 182.2 (*C*28). IR γ_{max} (neat): 2943, 2871, 1718, 1642, 1451, 1368, 1245, 1150, 1130, 1022, 978, 882, 752, 732, 438.

*4.1.10. Preparation of chloro κN,N′-(1,3-diamino 2-propanol) κS-dimethyl sulfoxide platinum(II) chloride [**6(PtClS)**]*

Dichlorobis(dimethyl sulfoxide)platinum(II) (100 mg, 0.24 mmol) was suspended in CH_3OH (10 mL) and a solution of 1,3-diamino-2-propanol (22 mg, 0.24 mmol) in CH_3OH (2 mL) was added dropwise. The mixture was stirred for 3 h, then concentrated onto 2 mL under reduced pressure and stored in the fridge for 40 h. The forming precipitate was filtered off and the solution was allowed to dry under air. The forming crystals were washed carefully with CH_3OH and dried to yield colorless, hygroscopic crystals (63 mg, 61 % yield).

^{1}H NMR (400 MHz, CD_3OD): 2.66 (6H, s, dmso), 3.30 (1H, m, HOC*H*), 3.45 and 3.48 (2H each, m, $-CH_2-$). ^{13}C NMR (100 MHz, CD_3OD): 40.7 ($-CH_2-$), 44.2 (DMSO), 66.3 (HOCH). ^{195}Pt NMR (86 MHz, CD_3OD): −3283. IR γ_{max} (neat): 3142, 3071, 1315, 1239, 1085, 1026, 853, 559, 438. MS (ESI): 399.1 (60, $[M - Cl]^+$). Analysis for $C_5H_{16}ClN_2O_2PtS$ (366.738): C, 13.83; H, 3.71; N, 6,45; S, 7.38; found: C, 14.38; H, 3.98; N, 6.16; S, 9.06.

*4.1.11. Preparation of dichloro κN,N′-(1,3-diamino-2-propanol) platinum(II) [**6(PtCl$_2$)**]*

Compound **6(PtClS)** (200 mg, 0.46 mmol) was dissolved in water (5 mL) and LiCl (98 mg, 2.31 mmol) was added. The solution was stirred for 2 h at 80 °C, then concentrated and dried under reduced pressure. The remaining solid was suspended in water (1 mL) and stored in the fridge for 20 h. The forming crystals were filtered off, washed with water and ethanol, and dried to yield a pale yellow powder (43 mg, 26% yield).

^{1}H NMR (400 MHz, DMSO-d_6): 2.58–2.76 (4H, m, $-CH_2-$), 3.49 (1H, m, HOC*H*). ^{13}C NMR (100 MHz, DMSO-d_6): 47.5 ($-CH_2-$), 65.4 (HOCH).

4.2. Biological studies

4.2.1. Cell lines and culture conditions

The cell lines 518A2 (melanoma), A2780 (ovarian), A549 (lung), MCF-7 (breast) and 8505C (anaplastic thyroid tumor) were included in this study. Cultures were maintained as monolayer in RPMI 1640 (PAA Laboratories, Pasching, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Sigma–Aldrich Chemie GmbH, Germany) and penicillin/streptomycin (PAA Laboratories, Pasching, Austria) at 37 °C in a humidified atmosphere of 5% CO_2.

4.2.2. In vitro antitumoral studies

The cytotoxicity of all compounds was evaluated by sulforhodamine-B (SRB) (Sigma–Aldrich) microculture colorimetric assay. Since the investigated compounds were insoluble in water, they were initially dissolved in DMSO or DMF and further diluted with culture medium for analysis. The final concentration of DMSO or DMF never exceeded 0.5%, which was found to be non-toxic to the cells. 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 experiment. After 24 h, the cells were treated with serial dilutions of the compounds (0–100 μM) for 96 h. The percentages of surviving cells relative to untreated controls were determined 96 h after the beginning of drug exposure. After 96 h treatment, the supernatant medium from the 96-well plates was discarded and the cells were fixed with 10% TCA. For a thorough fixation plates were allowed to stand at 4 °C for at least 2 h. After fixation the cells were washed in a plate washer (Tecan Austria GmbH, Austria). The washing step was done five times with water using alternate dispensing and aspiration procedures. The plates were then dyed with 100 μL of 0.4% SRB for about 45 min. After dying the plates were washed with 1% acetic acid to remove the dye and allowed to air-dry overnight. Hundred microliters of 10 mM Tris base solution was added to each well and the absorbance was measured at 570 nm using a plate reader (TECAN Infinite F200 PRO, Tecan GmbH, Austria).

Acknowledgements

This work was supported by “Gruenderwerkstatt-Biowissenschaften” and Univations from the University Halle.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.01.031.

References

[1] L. Bai, F. Meng, L. Zhao, M. Zhao, The anti-inflammatory activity of pentacyclic triterpenes in vitro, Appl. Mech. Mater. 138–139 (Pt. 2) (2012) 1174–1178.

[2] I.D. Bori, H.Y. Hung, K. Qian, C.H. Chen, S.L. Morris-Natschke, K.H. Lee, Anti-AIDS agents 88. Anti-HIV conjugates of betulin and betulinic acid with AZT prepared via clickchemistry, Tetrahedron Lett. 53 (15) (2012) 1987–1989.

[3] W.F. de Moraes, P.M. Galdino, M. Nascimento, F.A. Vanderlinde, T.F. Bara Maria, E.A. Costa, J.R. de Paula, Triterpenes involved in the anti-inflammatory effect of ethanolic extract of Pterodon emarginatus Vogel stem bark, J. Nat. Med. 66 (1) (2011).

[4] P. Gonzalez, I. Mader, A. Tchoghandjian, S. Enzenmueller, S. Cristofanon, F. Basit, K.M. Debatin, S. Fulda, Impairment of lysosomal integrity by B10, a glycosylated derivative of betulinic acid, leads to lysosomal cell death and converts autophagy into a detrimental process, Cell Death Differ. 19 (8) (2012) 1337–1346.

[5] R. Graziose, P. Rojas-Silva, T. Rathinasabapathy, C. Dekock, M.H. Grace, A. Poulev, M. Ann Lila, P. Smith, I. Raskin, Antiparasitic compounds from *Cornus florida* L. with activities against *Plasmodium falciparum* and *Leishmania tarentolae*, J. Ethnopharmacol. 142 (2) (2012) 456–461.

[6] M. Bache, S. Bernhardt, A. Hein, S. Passin, M. Zschornak, R. Paschke, D. Vordermark, Betulinic acid derivatives for in vitro glioblastoma therapy, Exp. Strahlenther. Klin. Strahlenbiol. 22 (2013) 43–47.

[7] H. Kommera, G.N. Kaluderovic, S. Dittrich, J. Kalbitz, B. Draeger, T. Mueller, R. Paschke, Carbamate derivatives of betulinic acid and betulin with selective cytotoxic activity, Bioorg. Med. Chem. Lett. 20 (11) (2010) 3409–3412.

[8] H. Kommera, G.N. Kaluderovic, M. Bette, J. Kalbitz, P. Fuchs, S. Fulda, W. Mier, R. Paschke, In vitro anticancer studies of Î± and Î²−glucopyranose betulin anomers, Chem. Biol. Interact 185 (2) (2010) 128–136.

[9] H. Kommera, G.N. Kaluderovic, J. Kalbitz, R. Paschke, Lupane triterpenoids-betulin and betulinic acid derivatives induce apoptosis in tumor cells, Invest. New Drugs 29 (2) (2011) 266–272.

[10] M. Willmann, V. Wacheck, J. Buckley, K. Nagy, J. Thalhammer, R. Paschke, T. Triche, B. Jansen, E. Selzer, Characterization of NVX-207, a novel betulinic acid-derived anti-cancer compound, Eur. J. Clin. Invest. 39 (5) (2009) 384–394.

[11] G.N. Kaluderovic, A. Dietrich, H. Kommera, J. Kuntsche, K. Maeder, T. Mueller, R. Paschke, Liposomes as vehicles for water insoluble platinum-based potential drug: 2-(4-(tetrahydro-2H-pyran-2-yloxy)-undecyl)-propane-1,3-diamminedichloroplatinum(II), Eur. J. Med. Chem. 54 (2012) 567–572.

[12] R. Paschke, A. Dietrich, T. Mueller, B. Kalinowski, G.N. Kaluderovic, Novel platinum compound that overcomes cisplatin resistance and induces apoptosis, Phys. Chem. (2010) 529–531. Proceedings of the Tenth International Conference on Fundamental and Applied Aspects of Physical Chemistry, 2010.

[13] R. Paschke, W. Voigt, T. Mueller, J. Kalbitz, K. Maeder, A. Dietrich, Platinum (II) compounds from 2-substituted propane-1,3-diamines for use as antitumor chemotherapeutic drugs, Martin-Luther-Universitaet Halle-Wittenberg, Germany, 2005, p. 9, 2005-102005047308[102005047308]. DE. 30-9-2005.

[14] Wilfried Roth, Apoptoseresistenz in malignen Tumoren. Neue Apoptose-basierte Therapieansätze, Pathologe 30 (2010) 113–116.

[15] I. Ben Sahra, K. Laurent, S. Giuliano, F. Larbret, G. Ponzio, P. Gounon, Y. Le Marchand-Brustel, S. Giorgetti-Peraldi, M. Cormont, C. Bertolotto, M. Deckert, P. Auberger, J.F. Tanti, F. Bost, Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells, Cancer Res. 70 (6) (2010) 2465–2475.

[16] G.q. Chen, Z.w. Yao, W.p. Zheng, L. Chen, H. Duan, Y. Shen, Combined anti-tumor effect of ursolic acid and 5-fluorouracil on human esophageal carcinoma cell Eca-109 in vitro, Chin. J. Cancer Res. 22 (1) (2010) 62–67.

[17] W. Guo-Bao, C. Xiao-Qin, G. Qi-Rong, L. Jie, L. Gui-Nan, L. Yue, Arsenic Trioxide overcomes cell adhesion-mediated drug resistance through down-regulating the expression of beta(1)-integrin in K562 chronic myelogenous leukemia cell line, Leuk. Lymphoma 51 (6) (2010) 1090–1097.

[18] J. Puente, A. Manzano, M. Martin, S. Lopez-Tarruella, E. az-Rubio, Breast cancer: complete response with the combination of sunitinib and trastuzumab in a patient with grade III ductal carcinoma, Anti-Cancer Drugs 21 (Suppl. 1) (2010) S19–S22.

[19] R.A. Smith, H. Yuan, R. Weissleder, L.C. Cantley, L. Josephson, A Wortmannin-Cetuximab as a double drug, Bioconjugate Chem. 20 (11) (2009) 2185–2189.

[20] T. Gianferrara, A. Bergamo, I. Bratsos, B. Milani, C. Spagnul, G. Sava, E. Alessio, Ruthenium-porphyrin conjugates with cytotoxic and phototoxic antitumor activity, J. Med. Chem. 53 (12) (2010) 4678–4690.

[21] P. Lan, J. Wang, D.M. Zhang, C. Shu, H.H. Cao, P.H. Sun, X.M. Wu, W.C. Ye, W.M. Chen, Synthesis and antiproliferative evaluation of 23-hydroxybetulinic acid derivatives, Eur. J. Med. Chem. 46 (6) (2011) 2490–2502.

[22] R. Paschke, J. Kalbitz, C. Paetz, Novel spacer linked bile acid-cisplatin compounds as a model for specific drug delivery, synthesis and characterization, Inorg. Chim. Acta 304 (2) (2000) 241–249.

[23] R.J. Holmes, M.J. McKeage, V. Murray, W.A. Denny, W.D. McFadyen, *cis*-Dichloroplatinum(II) complexes tethered to 9-aminoacridine-4-carboxamides: synthesis and action in resistant cell lines in vitro, J. Inorg. Biochem 85 (2-3) (2001) 209–217.

[24] W. Wong. An optimized relaxivity and specificity hepatobiliary MRI contrast agent. The University of Hong Kong, Peop. Rep. China. 2006-CN3127 [2007056961], 61 pp. WO. 21-11-2006.

[25] J.H. Price, A.N. Williamson, R.F. Schramm, B.B. Wayland, Palladium(II) and platinum(II) alkyl sulfoxide complexes. Examples of sulfur-bonded, mixed sulfur- and oxygen-bonded, and totally oxygen-bonded complexes, Inorg. Chem. 11 (6) (1972) 1280–1284.

[26] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, J. Natl. Cancer Inst. 82 (13) (1990) 1107–1112.