Accepted Manuscript

Picolyl amides of betulinic acid as antitumor agents causing tumor cell apoptosis

Uladzimir Bildziukevich, Lucie Rárová, David Šaman, Zdeněk Wimmer

PII: S0223-5234(17)31126-1

DOI: 10.1016/j.ejmech.2017.12.096

Reference: EJMECH 10074

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 1 November 2017

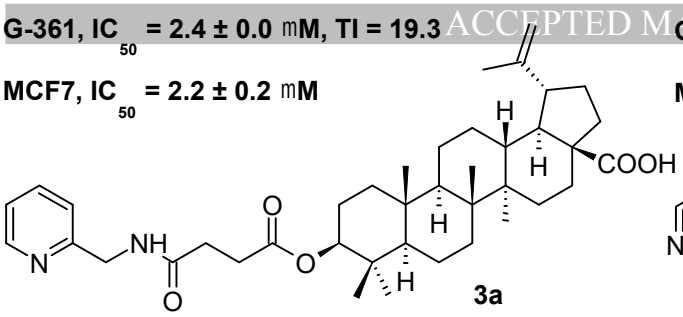
Revised Date: 13 December 2017

Accepted Date: 30 December 2017

Please cite this article as: U. Bildziukevich, L. Rárová, D. Šaman, Zdeně. Wimmer, Picolyl amides of betulinic acid as antitumor agents causing tumor cell apoptosis, *European Journal of Medicinal Chemistry* (2018), doi: 10.1016/j.ejmech.2017.12.096.

G-361, IC_{50} = 2.4 ± 0.0 μM, TI = 19.3

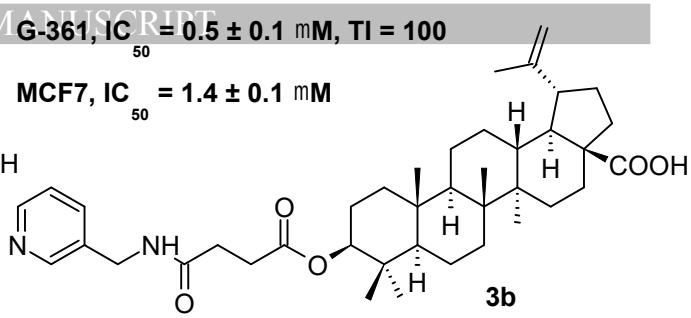
MCF7, IC_{50} = 2.2 ± 0.2 μM



3a

G-361, IC_{50} = 0.5 ± 0.1 μM, TI = 100

MCF7, IC_{50} = 1.4 ± 0.1 μM



3b

Picolyl amides of betulinic acid as antitumor agents causing tumor cell apoptosis

Uladzimir Bildziukevich,[a,b] Lucie Rárová,[c] David Šaman,[d] and Zdeněk Wimmer [a,b,*]

[a] University of Chemistry and Technology, Department of Chemistry of Natural Compounds, Technická 5, 166 28 Prague 6, Czech Republic;

[b] Institute of Experimental Botany AS CR, v.v.i., Isotope Laboratory, Vídeňská 1083, 142 20 Prague 4, Czech Republic;

[c] Palacký University, Centre of the Region Haná for Biotechnological and Agricultural Research, Department of Chemical Biology and Genetics, Šlechtitelů 27, 783 71 Olomouc, Czech Republic;

[d] Institute of Organic Chemistry and Biochemistry AS CR, v.v.i., Flemingovo náměstí 2, 166 10 Prague 6, Czech Republic

Corresponding author at: University of Chemistry and Technology, Department of Chemistry of Natural Compounds, Technická 5, 166 28 Prague 6 & Institute of Experimental Botany AS CR, v.v.i., Isotope Laboratory, Vídeňská 1083, 14220 Prague 4, Czech Republic.

Tel.: +420 220 444 398, +420 241 062 457; fax: +420 220 444 260.

E-mail addresses: Zdenek.Wimmer@vscht.cz, wimmer@biomed.cas.cz (Z. Wimmer).

Abstract

A series of picolyl amides of betulinic acid (**3a**–**3c** and **6a**–**6c**) was prepared and subjected to the cytotoxicity screening tests. Structure-activity relationships studies resulted in finding differences in biological activity in dependence on *o*-, *m*- and *p*-substitution of the pyridine ring in the target amides, when cytotoxicity data of **3a**–**3c** and **6a**–**6c** were obtained and compared. The amides **3b** and **3a** displayed cytotoxicity (given in the IC_{50} values) in G-361 (0.5 ± 0.1 µM and 2.4 ± 0.0 µM, respectively), MCF7 (1.4 ± 0.1 µM and 2.2 ± 0.2 µM, respectively), HeLa (2.4 ± 0.4 µM and 2.3 ± 0.5 µM, respectively) and CEM (6.5 ± 1.5 µM and 6.9 ± 0.4 µM, respectively) tumor cell lines, and showed weak effect in the normal human fibroblasts (BJ). Selectivity against all tested cancer cells was determined and compared to normal cells with therapeutic index (TI) between 7 and 100 for compounds **3a** and **3b.** The therapeutic index (TI = 100) was calculated for human malignant melanoma cell line (G-361) *versus* normal human fibroblasts (BJ). The cytotoxicity of other target amides (**3c** and **6a**–**6c**) revealed lower effects than **3a** and **3b** in the tested cancer cell lines.

Key words: betulinic acid; picolyl amine; amide; cytotoxicity; therapeutic index

1. Introduction

In the past years, we have published a paper dealing with the investigation of heteroaromatic amides of selected steryl hemiesters [1]. Several of those compounds were based on *o*-, *m*- and *p*-picolyl amines, displaying only moderate cytotoxicity. Those compounds suffered from low polarity of the steryl skeleton. Nevertheless, even in that range of biological activity, difference in cytotoxicity values could be seen, based on *o*-, *m*- or *p*-substitution in picolyl amides. More recently, our attention has been focused at the investigation of triterpenoid acid and their derivatives, which mostly showed considerable cytotoxicity and high and sometimes

selective antimicrobial activity. The compounds completed a majority of ADME parameters required for prediction of potential drugs, and – in several cases – displayed important supramolecular characteristics, among which formation of supramolecular hydrogels was one of the most important features [2,3].

Betulinic acid, (3β)-3-hydroxylup-20(29)-en-28-oic acid, is practically insoluble in water, mainly due to the presence of large lipophilic backbone and inadequate number of hydrophilic groups [4]. Even with this feature, which means, in fact, that this natural product does not correspond to the Lipinski [5] and Ghose [6] rules describing potentially biologically active compounds, it displays anti-HIV activity [7], antitumor [8,9] and antidiabetic activity [10,11]. Nature itself possesses natural mechanisms for increasing bioavailability of natural products by forming their water-soluble conjugates (e.g. glycosides) [12]. To improve its pharmacological characteristics in forms of the novel compounds has always been a challenge for researchers, to find its new derivatives and related compounds [12]. Betulinic acid is a plant product, to be found in genus *Betulla*, *Diospiros*, *Paeonia*, *Syzigium* or *Ziziphus* [13]. Concerning the antitumor effect, derivatives of betulinic acid have been used against a variety of tumor cell lines: malignant brain tumor, primitive neuroectodermal tumor [14], human chronic myelogenous leukemia, and against most of prevalent human cancer types, such as cervical, prostate, breast, lung or colorectal cancer [12].

Many bioactive secondary metabolites of plants, including betulinic acid, induce apoptosis pathway in cancer cells to exert their selective cytotoxic effects [15]. The cysteine-aspartic acid protease (caspase) family proteins play a central role in the execution phase of apoptosis, and the mechanism of initiation of apoptosis is mediated by the caspase cascade activation [16]. Caspase-3 and caspase-7 are downstream factors activated by caspase-8 and caspase-9, which – in turn – are activated predominantly by the extrinsic (death receptor) and intrinsic (mitochondrial) pathways, respectively [17]. Caspase-3 is an executioner protease that results

in cleavage of poly-(ADP-ribose) polymerase (PARP), subsequent DNA degradation and apoptotic death [18]. Previous studies showed that betulinic acid also displays anti-inflammatory, anti-HIV, anti-helminthic, anti-nociceptive activities [19]. Several studies were done to explain the molecular mechanisms of betulinic acid mediated antitumor activity. The process seems to be largely dependent on the ability of betulinic acid to trigger the mitochondrial pathway of apoptosis in cancer cells. Betulinic acid shows direct effect on mitochondria. Successive treatment of betulinic acid on cancer cells disrupts the mitochondrial transmembrane potential, which facilitates the cells to undergo apoptosis. Involvement of excessive amount of reactive oxygen species was found to be the most important factor for loss of mitochondrial membrane integrity of melanoma cells treated with betulinic acid. It directly targets to mitochondria, which – in turn – regulates the downstream caspase activation and side by side overcomes resistance property. Thus, betulinic acid has not developed resistance in cancer cells, and, thereby, it became permissible agents for future cancer therapy [15].

Aminomethylpyridines (picolyl amines), and related heteroaromatic amines, including numbers of their derivatives, and also the derived *N*-oxides, had already been investigated for their pharmacological activity [1,20-22], as markers of solid tumors [23] or agents in supramolecular self-assembly [24]. Lipophilic derivation of picolyl amines have resulted in obtaining conjugates, bearing ester and amide bond in each molecule; that type of study was made in our previous work [1]. The importance of such compounds consists in enabling transportation of potentially biologically active compounds through biomembrane and they often form cationic immune-stimulating complexes [25,26]. The derivatives of picolyl amines have also been found to be involved in activation of the caspase cascade and promote cell death [27].

The objectives of the current research consist in (a) a synthesis of a series of picolyl amides of betulinic acid both, at C(17)-COOH and at C(3)-OH (in the latter subseries using succinic acid as a junction unit), and (b) investigation of cytotoxicity of the target compounds. A comparison of the current series of compounds with those published earlier [1] will also be discussed.

2. Experimental part

2.1. General

The ^{1}H NMR and the ^{13}C NMR spectra were recorded on a Bruker AVANCE 600 MHz spectrometer at 600.13 MHz and 150.90 MHz in deuterochloroform, using tetramethylsilane ($\delta = 0.0$) as internal reference. ^{1}H NMR data are presented in the following order: chemical shift (δ) expressed in ppm, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants in Hertz, number of protons. For unambiguous assignment of both ^{1}H and ^{13}C signals 2D NMR ^{1}H,^{13}C gHSQC and gHMBC spectra were measured using standard parameters sets and pulse programs delivered by producer of the spectrometer. Copies of the NMR spectra are presented in the Supplementary material. Infrared spectra (IR) were measured with a Nicolet 205 FT-IR spectrometer. Mass spectra (MS) were measured with a Waters ZMD mass spectrometer in a positive ESI mode. Optical rotation was measured on an Autopol IV instrument (Rudolph Research Analytical, USA) at 589 nm wavelength, and the value was corrected to 20°C. The PE 2400 Series II CHNS/O Analyzer (Perkin Elmer, USA) was used for simultaneous determination of C, H, and N (accuracy of CHN determination better than 0.30 % abs.). TLC was carried out on silica gel plates (Merck $60F_{254}$) and the visualization was performed by the UV detection and by spraying with the methanolic solution of phosphomolybdic acid (5%) followed by heating. For column chromatography, silica gel 60 (0.063-0.200 mm) from Merck was used. All chemicals and

solvents were purchased from regular commercial sources in analytical grade and the solvents were purified by general methods before use.

2.2. (3β)-3-[(3-Carboxypropanoyl)oxy]lup-20(29)-en-28-oic acid (**2**)

Succinic anhydride (1.62 g; 16.2 mmol; 3.5 eq) and DMAP (170 mg; 1.39 mmol, 0.30 eq) were added to a solution of betulinic acid (**1**; 2.1 g; 4.6 mmol) in dry pyridine (20 mL). The reaction mixture was stirred at r.t. for 5 days. After stopping the reaction, the resulting mixture was poured onto ice, hydrochloric acid was added to adjust pH = 7, the organic layer was extracted with chloroform, and the extract was dried over sodium sulfate. Evaporation of the solvent afforded a solid that was purified by column chromatography, yielding 1.93 g (75%) of **2**.

^{1}H NMR: δ 0.79 (3H, s, H23), 0.79 (3H, s, H24), 0.80 (3H, s, H25), 0.87 (3H, s, H26), 0.94 (3H, s, H27), 1.64 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.4 Hz, H29), 2.09-2.13 (2H, m, H16), 2.22 (1H, ddd, J_1 = 3.6 Hz, J_2 = 11.6 Hz, J_3 = 12.9 Hz, H13), 2.44-2.52 (4H, m, H2'-H3'), 2.95 (1H, dt, J_1 = 4.8 Hz, J_2 = 11.0 Hz, J_3 = 11.0 Hz, H19), 4.38 (1H, dd, J_1 = 4.7 Hz, J_2 = 11.6 Hz, H3), 4.56 (1H, dq, J_1 = 1.4 Hz, J_2 = 1.4 Hz, J_3 = 1.4 Hz, J_4 = 2.4 Hz, H30), 4.69 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.4 Hz, H30). ^{13}C NMR: δ 14.31 (q, C27), 15.65 (q, C24), 15.81 (q, C25), 16.35 (q, C26), 17.67 (t, C6), 18.90 (q, C29), 20.42 (t, C11), 23.27 (t, C2), 25.00 (t, C12), 27.56 (q, C23), 28.77 (t, C2'), 29.15 (t, C21), 29.15 (t, C3'), 30.06 (t, C15), 31.64 (t, C16), 33.68 (t, C22), 36.28 (t, C1), 36.60 (s, C10), 37.38 (s, C4), 37.54 (d, C13), 37.67 (t, C7), 40.21 (s, C8), 41.99 (s, C14), 46.57 (d, C19), 48.50 (d, C18), 49.61 (d, C9), 54.62 (d, C5), 55.36 (s, C17), 79.98 (d, C3), 109.58 (t, C30), 150.26 (s, C20), 171.56 (s, C1'), 173.32 (s, C4'), 177.17 (s, C28). MS: *m/z* 555.2 $[M-H]^-$. IR (KBr; cm^{-1}): 2946 (C–H val.), 2872 (–CH_3 val.), 1734 (–C=O val.). For $C_{34}H_{52}O_6$ (556.77) calculated C (73.34), H (9.41), found C (73.37), H (9.40). M.p. 244-246 ^{o}C.

2.3. (3β)-3-({4-Oxo-4-[(pyridin-n-ylmethyl)amino]butanoyl}oxy)lup-20(29)-en-28-oic acid [**3a** (n=2), **3b** (n=3) and **3c** (n=4)]

o-, *m*- or *p*-Picolyl amine (20 µl; 0.198 mmol; 1.1 eq) and T3P (0.32 ml; 0.54 mmol; 3 eq) were added to a solution of **2** (100 mg; 0.18 mmol) in dry pyridine (2 mL), and the reaction mixture was stirred at r.t. for 1 day. After stopping the reaction, saturated solution of sodium bicarbonate (4 mL) was added to reaction mixture, and stirring continued for several more hours. The resulting mixture was extracted with chloroform, and dried over sodium sulfate. Evaporation of the solvent afforded a solid, which was purified by column chromatography. Yields: **3a** (85 %), **3b** (91 %), **3c** (89 %).

3a: ^{1}H NMR: δ 0.77 (3H, s, H23), 0.78 (3H, s, H24), 0.80 (3H, s, H25), 0.87 (3H, s, H26), 0.95 (3H, s, H27), 1.65 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.3 Hz, H29), 2.09-2.14 (2H, m, H16), 2.23 (1H, ddd, J_1 = 4.7 Hz, J_2 = 11.7 Hz, J_3 = 12.9 Hz, H13), 2.44-2.55 (4H, m, H2'-H3'), 2.95 (1H, dt, J_1 = 5.3 Hz, J_2 = 11.0 Hz, J_3 = 11.0 Hz, H19), 4.31 (1H, dd, J_1 = 6.0 Hz, J_2 = 15.9 Hz, H5'), 4.35 (1H, dd, J_1 = 6.0 Hz, J_2 = 15.9 Hz, H5'), 4.38 (1H, dd, J_1 = 4.8 Hz, J_2 = 11.6 Hz, H3), 4.56 (1H, dq, J_1 = 1.3 Hz, J_2 = 1.3 Hz, J_3 = 1.3 Hz, J_4 = 2.4 Hz, H30), 4.69 (1H, bd, J = 2.4 Hz, H30), 7.24 (1H, dddt, J_1 = 0.6 Hz, J_2 = 0.6 Hz, J_3 = 1.2 Hz, J_4 = 4.8 Hz, J_5 = 7.5 Hz, H8'), 7.26 (1H, dtt, J_1 = 0.6 Hz, J_2 = 0.6 Hz, J_3 = 1.1 Hz, J_4 = 1.1 Hz, J_5 = 7.8 Hz, H10'), 7.72 (1H, dt, J_1 = 1.8 Hz, J_2 = 7.7 Hz, J_3 = 7.7 Hz, H9'), 8.45 (1H, t, J = 6.0 Hz, NH), 8.48 (1H, ddd, J_1 = 0.9 Hz, J_2 = 1.8 Hz, J_3 = 4.8 Hz, H7'). ^{13}C NMR: δ 14.32 (q, C27), 15.66 (q, C24), 15.82 (q, C25), 16.36 (q, C26), 17.67 (t, C6), 18.90 (q, C29), 20.43 (t, C11), 23.29 (t, C2), 25.01 (t, C12), 27.58 (q, C23), 29.16 (t, C21), 29.35 (t, C2'), 29.87 (t, C3'), 30.06 (t, C15), 31.66 (t, C16), 33.70 (t, C22), 36.29 (t, C1), 36.59 (s, C10), 37.38 (s, C4), 37.54 (d, C13), 37.69 (t, C7), 40.22 (s, C8), 41.99 (s, C14), 44.17 (t, C5'), 46.58 (d, C19), 48.50 (d, C18), 49.62 (d, C9), 54.63 (d, C5), 55.37 (s, C17), 79.87 (d, C3), 109.58 (t, C30), 120.88 (d, C10'), 121.97 (d, C8'),

136.56 (d, C9'), 148.71 (d, C7'), 150.28 (s, C20), 158.64 (s, C6'), 170.88 (s, C1'), 171.88 (s, C4'), 177.18 (s, C28). MS: *m/z* 647.3 $[M+H]^+$, 645.0 $[M-H]^-$. IR (KBr; cm^{-1}): 3377 (N–H val. amide), 2945 (C–H val.), 2871 (–CH_3 val.), 1728 (–C=O val. ester), 1659 (–C=O val. amide). For $C_{40}H_{58}N_2O_5$ (646.90) calculated C (74.27), H (9.04), N (4.33), found C (74.29), H (9.03), N (4.35). M.p. 121-124 ^oC. $[\alpha]_D^{20}$ = +15.5 (*c* 0.323).

3b: ^1H NMR: δ 0.70-0.73 (1H, m, H5), 0.76 (3H, s, H24), 0.76 (3H, s, H23), 0.78 (3H, d, *J* = 0.6 Hz, H25), 0.88 (3H, s, H26), 0.92 (3H, d, *J* = 0.7 Hz, H27), 1.63 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.3 Hz, H29), 2.17 (1H, ddd, J_1 = 3.7 Hz, J_2 = 11.6 Hz, J_3 = 12.9 Hz, H13), 2.22 (2H, ddd, J_1 = 2.9 Hz, J_2 = 4.0 Hz, J_3 = 13.1 Hz, H16), 2.46 (2H, t, *J* = 6.6 Hz, H3'), 2.58-2.66 (2H, m, H2'), 2.94 (1H, dt, J_1 = 5.0 Hz, J_2 = 11.0 Hz, J_3 = 11.0 Hz, H19), 4.39 (1H, dd, J_1 = 5.9 Hz, J_2 = 14.9 Hz, H5'), 4.41 (1H, dd, J_1 = 5.7 Hz, J_2 = 14.9 Hz, H5'), 4.42 (1H, dd, J_1 = 6.2 Hz, J_2 = 10.1 Hz, H3), 4.55 (1H, dq, J_1 = 1.3 Hz, J_2 = 1.3 Hz, J_3 = 1.3 Hz, J_4 = 2.4 Hz, H30), 4.68 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.3 Hz, H30), 6.20 (1H, bt, *J* = 5.8 Hz, NH), 7.23 (1H, ddd, J_1 = 0.8 Hz, J_2 = 4.8 Hz, J_3 = 7.8 Hz, H10'), 7.63 (1H, ddt, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 1.8 Hz, J_4 = 2.1 Hz, J_5 = 7.8 Hz, H11'), 8.48 (1H, bdd, J_1 = 1.8 Hz, J_2 = 4.8 Hz, H9'), 8.50 (1H, bs, H7'). ^{13}C NMR: δ 14.71 (q, C27), 16.13 (q, C24), 16.17 (q, C25), 16.51 (q, C26), 18.24 (t, C6), 19.41 (q, C29), 20.99 (t, C11), 23.77 (t, C2), 25.60 (t, C12), 28.02 (q, C23), 29.78 (t, C21), 29.78 (t, C2'), 30.72 (t, C3'), 31.25 (t, C15), 32.29 (t, C16), 34.38 (t, C22), 37.09 (t, C1), 37.23 (s, C10), 37.95 (s, C4), 38.49 (d, C13), 38.49 (t, C7), 40.85 (t, C5'), 41.09 (s, C8), 42.56 (s, C14), 47.01 (d, C19), 49.46 (d, C18), 50.57 (d, C9), 55.56 (d, C5), 56.38 (s, C17), 81.68 (d, C3), 109.64 (t, C30), 123.82 (d, C10'), 134.65 (s, C6'), 136.46 (d, C11'), 147.92 (d, C9'), 148.35 (d, C7'), 150.45 (s, C20), 171.74 (s, C1'), 172.73 (s, C4'), 179.99 (s, C28). MS: *m/z* 647.3 $[M+H]^+$, 645.3 $[M-H]^-$. IR (KBr; cm^{-1}): 3350 (N–H val. amide), 2946 (C–H val.), 2871 (–CH_3 val.), 1729 (–C=O val. ester), 1657 (–C=O val. amide). For $C_{40}H_{58}N_2O_5$ (646.90)

calculated C (74.27), H (9.04), N (4.33), found C (74.30), H (9.02), N (4.35). M.p. 131-134 ^{o}C. $[\alpha]_D^{20}$ = +12.8 (*c* 0.383).

3c: ^{1}H NMR: δ 0.70-0.74 (1H, m, H5), 0.76 (3H, s, H23), 0.76 (3H, s, H24), 0.78 (3H, s, H25), 0.87 (3H, s, H26), 0.91 (3H, bd, H27), 0.98 (2H, dq, J_1 = 4.0 Hz, J_2 = 13.0 Hz, J_3 = 13.0 Hz, J_4 = 13.0 Hz, H12), 1.62 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.3 Hz, H29), 2.16 (1H, ddd, J_1 = 3.6 Hz, J_2 = 11.5 Hz, J_3 = 12.8 Hz, H13), 2.21 (2H, ddd, J_1 = 3.2 Hz, J_2 = 3.8 Hz, J_3 = 13.0 Hz, H16), 2.48 (1H, dt, J_1 = 6.5 Hz, J_2 = 6.5 Hz, J_3 = 15.0 Hz, H3'), 2.50 (1H, dt, J_1 = 5.7 Hz, J_2 = 5.7 Hz, J_3 = 15.0 Hz, H3'), 2.62 (1H, dt, J_1 = 5.7 Hz, J_2 = 5.7 Hz, J_3 = 17.3 Hz, H2'), 2.66 (1H, dt, J_1 = 6.6 Hz, J_2 = 6.6 Hz, J_3 = 17.3 Hz, H2'), 2.95 (1H, dt, J_1 = 5.0 Hz, J_2 = 10.9 Hz, J_3 = 10.9 Hz, H19), 4.38 (1H, bdd, J_1 = 6.1 Hz, J_2 = 16.0 Hz, H5'), 4.42 (1H, bdd, J_1 = 6.1 Hz, J_2 = 16.0 Hz, H5'), 4.43 (1H, dd, J_1 = 5.5 Hz, J_2 = 11.0 Hz, H3), 4.54 (1H, dq, J_1 = 1.3 Hz, J_2 = 1.3 Hz, J_3 = 1.3 Hz, J_4 = 2.3 Hz, H30), 4.67 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.3 Hz, H30), 6.30 (1H, t, *J* = 6.1 Hz, NH), 7.16-7.18 (2H, m, H7'), 8.47-8.49 (2H, m, H8'). ^{13}C NMR: δ 14.68 (q, C27), 16.06 (q, C24), 16.15 (q, C25), 16.49 (q, C26), 18.18 (t, C6), 19.37 (q, C29), 20.92 (t, C11), 23.75 (t, C2), 25.50 (t, C12), 28.01 (q, C23), 29.73 (t, C21), 29.94 (t, C2'), 30.64 (t, C3'), 31.16 (t, C15), 32.26 (t, C16), 34.28 (t, C22), 37.08 (t, C1), 37.16 (s, C10), 37.90 (s, C4), 38.38 (t, C7), 38.40 (d, C13), 40.75 (s, C8), 42.42 (s, C14), 42.48 (t, C5'), 46.97 (d, C19), 49.33 (d, C18), 50.47 (d, C9), 55.48 (d, C5), 56.34 (s, C17), 81.73 (d, C3), 109.63 (t, C30), 122.47 (d, C7'), 148.49 (s, C6'), 149.08 (d, C8'), 150.49 (s, C20), 171.98 (s, C1'), 172.91 (s, C4'), 180.46 (s, C28). MS: *m/z* 647.2 $[M+H]^+$, 645.3 $[M-H]^-$. IR (KBr; cm^{-1}): 3358 (N–H val. amide), 2946 (C–H val.), 2870 (–CH_3 val.), 1729 (–C=O val. ester), 1663 (–C=O val. amide). For $C_{40}H_{58}N_2O_5$ (646.90) calculated C (74.27), H (9.04), N (4.33), found C (74.26), H (9.05), N (4.31). M.p. 145-147 ^{o}C. $[\alpha]_D^{20}$ = +12.8 (*c* 0.188).

2.4. (3β)-3-(Acetyloxy)lup-20(29)-en-28-oic acid (**4**)

Acetic anhydride (0.45 mL; 4.7 mmol; 1.44 eq), DMAP (52 mg; 0.43 mmol, 0.13 eq) and EDIPA (1 mL) were added to a solution of betulinic acid (**1**; 1.5 g; 3.3 mmol) in dry THF (10 mL). The reaction mixture was refluxed for 3 h. After stopping the reaction, water (10 mL) was added and the resulting mixture was stirred for 1 hour. The resulting mixture was extracted with chloroform, and the extract was dried over sodium sulfate. Evaporation of the solvent afforded a solid, which was purified by column chromatography, resulting finally in 1.55 g (95 %) of **4**.

^{1}H NMR: δ 0.83 (3H, s, H23), 0.84 (3H, s, H25), 0.85 (3H, bs, H24), 0.93 (3H, s, H26), 0.97 (3H, bs, H27), 1.05 (2H, dq, J_1 = 4.9 Hz, J_2 = 13.0 Hz, J_3 = 13.0 Hz, J_4 = 13.0 Hz, H12), 1.19 (2H, dt, J_1 = 3.2 Hz, J_2 = 3.2 Hz, J_3 = 13.6 Hz, H21), 1.70 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.4 Hz, H29), 2.04 (3H, s, OAc), 2.13 (2H, ddd, J_1 = 3.7 Hz, J_2 = 11.6 Hz, J_3 = 12.8 Hz, H1), 2.27 (2H, dt, J_1 = 3.8 Hz, J_2 = 3.8 Hz, J_3 = 13.1 Hz, H16), 3.01 (1H, dt, J_1 = 5.0 Hz, J_2 = 10.9 Hz, J_3 = 10.9 Hz, H19), 4.47 (1H, dd, J_1 = 5.5 Hz, J_2 = 10.9 Hz, H3), 4.61 (1H, dq, J_1 = 1.4 Hz, J_2 = 1.4 Hz, J_3 = 1.4 Hz, J_4 = 2.3 Hz, H30), 4.74 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.3 Hz, H30). ^{13}C NMR: δ 14.64 (q, C27), 16.02 (q, C24), 16.17 (q, C25), 16.46 (q, C26), 18.15 (t, C6), 19.32 (q, OAc), 19.33 (q, C29), 20.84 (t, C11), 23.68 (t, C2), 25.43 (t, C12), 27.94 (q, C23), 29.68 (t, C21), 30.54 (t, C15), 32.13 (t, C16), 34.22 (t, C22), 37.03 (d, C13), 37.11 (s, C10), 37.79 (t, C7), 38.37 (s, C4), 38.39 (t, C1), 40.68 (s, C8), 42.41 (s, C14), 46.92 (d, C19), 49.25 (d, C18), 50.38 (d, C9), 55.40 (d, C5), 56.35 (s, C17), 80.94 (d, C3), 109.74 (t, C30), 150.36 (s, C20), 171.06 (s, OAc), 181.47 (s, C28). MS: *m/z* 497.1 $[M-H]^-$. IR (KBr; cm^{-1}): 2945 (C–H val.), 2871 (–CH_3 val.), 1735 (–C=O val. ester), 1694 (–C=O val. conjugated acid). For $C_{32}H_{50}O_4$ (498.74) calculated C (77.06), H (10.10), found C (77.09), H (10.08). M.p. 263-266 ^{o}C.

2.5. (3β)-28-Oxo-28-[(pyridin-n-ylmethyl)amino]lup-20(29)-en-3-yl acetate [**5a** (n=2), **5b** (n=3) and **5c** (n=4)]

A solution of oxalyl chloride in dry dichloromethane (2 M; 1.4 mL; 2.8 mmol; 7 eq) was added to a solution of **4** (200 mg; 0.4 mmol) in dry dichloromethane (4 mL), the reaction mixture was stirred for 3 h, then evaporated and dissolved again in dry dichloromethane (4 mL). EDIPA (0.182 mL; 1.04 mmol; 2.6 eq) and *o*-, *m*- or *p*-picolyl amine (0.049 mL; 0.48 mmol; 1.2 eq) were added, and the reaction mixture was stirred at r.t. through night (for 12 h). After stopping the reaction, evaporation of the solvent afforded a solid, which was purified by column chromatography. Yields: **5a** (86 %), **5b** (99 %), **5c** (87 %).

5a: ^{1}H NMR: δ 0.72 (1H, dd, J_1 = 2.1 Hz, J_2 = 11.7 Hz, H5), 0.75 (3H, s, H24), 0.77 (3H, s, H25), 0.77 (3H, s, H23), 0.78 (3H, s, H26), 0.90 (3H, d, J = 0.6 Hz, H27), 1.08 (2H, dt, J_1 = 3.2 Hz, J_2 = 3.2 Hz, J_3 = 13.4 Hz, H21), 1.64 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.4 Hz, H29), 1.99 (3H, s, H2'), 2.04 (2H, dt, J_1 = 3.3 Hz, J_2 = 3.3 Hz, J_3 = 13.6 Hz, H16), 2.38 (1H, ddd, J_1 = 3.7 Hz, J_2 = 11.5 Hz, J_3 = 12.9 Hz, H13), 3.10 (1H, dt, J_1 = 4.6 Hz, J_2 = 11.1 Hz, J_3 = 11.1 Hz, H19), 4.41 (1H, dd, J_1 = 5.5 Hz, J_2 = 10.0 Hz, H3), 4.46 (1H, dd, J_1 = 5.2 Hz, J_2 = 15.9 Hz, H3'), 4.52 (1H, dd, J_1 = 5.2 Hz, J_2 = 15.9 Hz, H3'), 4.54 (1H, dq, J_1 = 1.4 Hz, J_2 = 1.4 Hz, J_3 = 1.4 Hz, J_4 = 2.4 Hz, H30), 4.69 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.4 Hz, H30), 6.85 (1H, t, J = 5.2 Hz, NH), 7.17 (1H, m, J_1 = 0.6 Hz, J_2 = 0.6 Hz, J_3 = 1.2 Hz, J_4 = 4.9 Hz, J_5 = 7.5 Hz, H7'), 7.29 (1H, m, J_1 = 0.6 Hz, J_2 = 0.6 Hz, J_3 = 1.2 Hz, J_4 = 7.8 Hz, H9'), 7.64 (1H, dt, J_1 = 1.8 Hz, J_2 = 7.6 Hz, J_3 = 7.6 Hz, H8'), 8.49 (1H, ddd, J_1 = 0.8 Hz, J_2 = 1.8 Hz, J_3 = 4.9 Hz, H6'). ^{13}C NMR: δ 14.58 (q, C27), 15.98 (q, C24), 16.17 (q, C25), 16.45 (q, C26), 18.15 (t, C6), 19.42 (q, C29), 20.91 (t, C11), 21.31 (q, C2'), 23.67 (t, C2), 25.56 (t, C12), 27.91 (q, C23), 29.41 (t, C21), 30.86 (t, C15), 33.65 (t, C16), 34.26 (t, C22), 37.08 (s, C10), 37.71 (d, C13), 37.76 (s, C4), 38.33 (t, C1), 38.35 (t, C7), 40.70 (s, C8), 42.45 (s, C14), 44.17 (t, C3'), 46.78 (d, C19), 50.06 (d, C18), 50.49 (d, C9), 55.40 (d, C5), 55.76 (s, C17),

80.93 (d, C3), 109.35 (t, C30), 122.34 (d, C7'), 122.64 (d, C9'), 137.12 (d, C8'), 148.50 (d, C6'), 150.98 (s, C20), 157.08 (s, C4'), 171.01 (s, C1'), 176.39 (s, C28). MS: *m/z* 589.2 $[M+H]^+$, 587.2 $[M-H]^-$. IR (KBr; cm^{-1}): 2946 (C–H val.), 2870 ($–CH_3$ val.), 1734 (–C=O val. ester), 1651 (–C=O val. amide). For $C_{38}H_{56}N_2O_3$ (588.86) calculated C (77.51), H (9.59), N (4.76), found C (77.53), H (9.58), N (4.75). M.p. 110-115 ^oC.

5b: ^1H NMR: δ 0.72 (1H, dd, J_1 = 2.1 Hz, J_2 = 11.0 Hz, H5), 0.77 (3H, s, H24), 0.78 (3H, d, J = 0.7 Hz, H25), 0.78 (3H, s, H23), 0.79 (3H, s, H26), 0.90 (3H, s, H27), 1.07 (2H, dt, J_1 = 3.3 Hz, J_2 = 3.3 Hz, J_3 = 13.5 Hz, H21), 1.64 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.4 Hz, H29), 1.89 (2H, dt, J_1 = 3.4 Hz, J_2 = 3.4 Hz, J_3 = 13.7 Hz, H16), 1.99 (3H, s, H2'), 2.39 (1H, ddd, J_1 = 3.7 Hz, J_2 = 11.5 Hz, J_3 = 12.9 Hz, H13), 3.09 (1H, dt, J_1 = 4.5 Hz, J_2 = 11.2 Hz, J_3 = 11.2 Hz, H19), 4.31 (1H, dd, J_1 = 5.9 Hz, J_2 = 14.9 Hz, H3'), 4.41 (1H, dd, J_1 = 5.3 Hz, J_2 = 9.6 Hz, H3), 4.48 (1H, dd, J_1 = 6.1 Hz, J_2 = 14.9 Hz, H3'), 4.55 (1H, dq, J_1 = 1.4 Hz, J_2 = 1.4 Hz, J_3 = 1.4 Hz, J_4 = 2.4 Hz, H30), 4.69 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.4 Hz, H30), 6.08 (1H, t, J = 6.0 Hz, NH), 7.24 (1H, ddd, J_1 = 0.9 Hz, J_2 = 4.9 Hz, J_3 = 7.8 Hz, H8'), 7.66 (1H, bddd, J_1 = 1.8 Hz, J_2 = 2.2 Hz, J_3 = 7.8 Hz, H9'), 8.47 (1H, dd, J_1 = 1.8 Hz, J_2 = 4.9 Hz, H7'), 8.52 (1H, bd, J = 2.2 Hz, H5'). ^{13}C NMR: δ 14.57 (q, C27), 16.01 (q, C24), 16.21 (q, C25), 16.45 (q, C26), 18.14 (t, C6), 19.45 (q, C29), 20.91 (t, C11), 21.31 (q, C2'), 23.67 (t, C2), 25.56 (t, C12), 27.91 (q, C23), 29.39 (t, C21), 30.81 (t, C15), 33.68 (t, C16), 34.29 (t, C22), 37.09 (s, C10), 37.68 (d, C13), 37.77 (s, C4), 38.32 (t, C1), 38.37 (t, C7), 40.71 (t, C3'), 40.73 (s, C8), 42.44 (s, C14), 46.65 (d, C19), 50.10 (d, C18), 50.51 (d, C9), 55.43 (d, C5), 55.66 (s, C17), 80.91 (d, C3), 109.48 (t, C30), 123.66 (d, C8'), 135.15 (s, C4'), 136.22 (d, C9'), 148.24 (d, C7'), 148.73 (d, C5'), 150.74 (s, C20), 171.03 (s, C1'), 176.26 (s, C28). MS: *m/z* 589.2 $[M+H]^+$, 587.2 $[M-H]^-$. IR (KBr; cm^{-1}): 3270 (N–H val. amide), 2941 (C–H val.), 2871 ($–CH_3$ val.), 1725 (–C=O val. ester), 1659 (–C=O val. amide). For $C_{38}H_{56}N_2O_3$ (588.86) calculated C (77.51), H (9.59), N (4.76), found C (77.50), H (9.60), N (4.78). M.p. 241-244 ^oC.

5c: ^{1}H NMR: δ 0.72 (1H, dd, J_1 = 2.1 Hz, J_2 = 11.1 Hz, H5), 0.78 (3H, s, H24), 0.79 (3H, s, H25), 0.79 (3H, s, H23), 0.82 (3H, s, H26), 0.91 (3H, s, H27), 1.11 (2H, dt, J_1 = 3.4 Hz, J_2 = 3.4 Hz, J_3 = 13.5 Hz, H21), 1.64 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.4 Hz, H29), 1.91 (2H, dt, J_1 = 3.3 Hz, J_2 = 3.3 Hz, J_3 = 13.9 Hz, H16), 1.99 (3H, s, H2'), 2.39 (1H, ddd, J_1 = 3.7 Hz, J_2 = 11.6 Hz, J_3 = 13.0 Hz, H13), 3.08 (1H, dt, J_1 = 4.5 Hz, J_2 = 11.1 Hz, J_3 = 11.1 Hz, H19), 4.31 (1H, dd, J_1 = 6.0 Hz, J_2 = 15.6 Hz, H3'), 4.42 (1H, dd, J_1 = 5.9 Hz, J_2 = 10.7 Hz, H3), 4.45 (1H, dd, J_1 = 6.1 Hz, J_2 = 15.6 Hz, H3'), 4.55 (1H, dq, J_1 = 1.4 Hz, J_2 = 1.4 Hz, J_3 = 1.4 Hz, J_4 = 2.4 Hz, H30), 4.69 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.4 Hz, H30), 6.10 (1H, t, J = 6.1 Hz, NH), 7.18-7.21 (2H, m, H5'), 8.48-8.51 (2H, m, H6'). ^{13}C NMR: δ 14.58 (q, C27), 16.09 (q, C24), 16.22 (q, C25), 16.45 (q, C26), 18.15 (t, C6), 19.45 (q, C29), 20.91 (t, C11), 21.31 (q, C2'), 23.66 (t, C2), 25.55 (t, C12), 27.91 (q, C23), 29.46 (t, C21), 30.80 (t, C15), 33.72 (t, C16), 34.34 (t, C22), 37.09 (s, C10), 37.68 (d, C13), 37.77 (s, C4), 38.38 (t, C7), 38.38 (t, C1), 40.77 (s, C8), 42.24 (s, C14), 42.46 (t, C3'), 46.60 (d, C19), 50.10 (d, C18), 50.52 (d, C9), 55.44 (d, C5), 55.72 (s, C17), 80.91 (d, C3), 109.55 (t, C30), 122.57 (d, C5'), 148.98 (s, C4'), 149.41 (d, C6'), 150.65 (s, C20), 171.04 (s, C1'), 176.38 (s, C28). MS: *m/z* 589.2 $[M+H]^+$, 587.2 $[M-H]^-$. IR (KBr; cm^{-1}): 3378 (N–H val. amide), 2946 (C–H val.), 2869 (–CH_3 val.), 1734 (–C=O val. ester), 1642 (–C=O val. amide). For $C_{38}H_{56}N_2O_3$ (588.86) calculated C (77.51), H (9.59), N (4.76), found C (77.49), H (9.57), N (4.78). M.p. 105-108 ^{o}C.

2.6. (3β)-3-Hydroxy-*N*-(pyridin-n-ylmethyl)lup-20(29)-en-28-amide [**6a** (n=2), **6b** (n=3) and **6c** (n=4)]

LiOH·H_2O (54 mg; 1.3 mmol; 5 eq) was added to a solution of **5a**, **5b** or **5c** (151 mg; 0.26 mmol) in methanol (15 mL), and the reaction mixture was refluxed for 5 h. After stopping the reaction, the solvent was evaporated, and the residue was dissolved in chloroform and filtered.

Evaporation of the solvent afforded a solid, which was purified by column chromatography. Yields: **6a** (82 %), **6b** (84 %), **6c** (83 %).

6a: ^{1}H NMR: δ 0.62 (1H, dd, J_1 = 2.3 Hz, J_2 = 11.5 Hz, H5), 0.70 (3H, s, H24), 0.74 (3H, d, J = 0.8 Hz, H25), 0.75 (3H, s, H23), 0.90 (3H, s, H26), 0.91 (3H, d, J = 0.6 Hz, H27), 1.08 (2H, dt, J_1 = 3.1 Hz, J_2 = 3.1 Hz, J_3 = 13.5 Hz, H21), 1.64 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.4 Hz, H29), 2.04 (2H, dt, J_1 = 3.3 Hz, J_2 = 3.3 Hz, J_3 = 13.5 Hz, H16), 2.38 (1H, ddd, J_1 = 3.6 Hz, J_2 = 11.6 Hz, J_3 = 13.1 Hz, H13), 3.10 (1H, dt, J_1 = 4.6 Hz, J_2 = 11.1 Hz, J_3 = 11.1 Hz, H19), 3.13 (1H, dd, J_1 = 4.9 Hz, J_2 = 11.5 Hz, H3), 4.46 (1H, dd, J_1 = 5.1 Hz, J_2 = 12.0 Hz, H1'), 4.53 (1H, dd, J_1 = 5.1 Hz, J_2 = 12.0 Hz, H1'), 4.54 (1H, dq, J_1 = 1.4 Hz, J_2 = 1.4 Hz, J_3 = 1.4 Hz, J_4 = 2.4 Hz, H30), 4.69 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.4 Hz, H30), 6.85 (1H, bt, J = 5.1 Hz, NH), 7.17 (1H, ddd, J_1 = 1.3 Hz, J_2 = 5.0 Hz, J_3 = 7.6 Hz, H5'), 7.29 (1H, bdt, J_1 = 1.0 Hz, J_2 = 1.0 Hz, J_3 = 7.7 Hz, H7'), 7.64 (1H, dt, J_1 = 1.8 Hz, J_2 = 7.6 Hz, J_3 = 7.6 Hz, H6'), 8.49 (1H, ddd, J_1 = 1.0 Hz, J_2 = 1.8 Hz, J_3 = 5.0 Hz, H4'). ^{13}C NMR: δ 14.63 (q, C27), 15.34 (q, C24), 15.98 (q, C25), 16.12 (q, C26), 18.28 (t, C6), 19.46 (q, C29), 20.90 (t, C11), 25.61 (t, C12), 27.40 (t, C2), 27.99 (q, C23), 29.43 (t, C15), 33.67 (t, C16), 33.88 (t, C21), 34.34 (t, C22), 37.17 (s, C10), 37.74 (d, C13), 38.34 (t, C7), 38.69 (t, C1), 38.83 (s, C4), 40.69 (s, C8), 42.46 (s, C14), 44.21 (t, C1'), 46.77 (d, C19), 50.07 (d, C18), 50.59 (d, C9), 55.34 (d, C5), 55.77 (s, C17), 78.95 (d, C3), 109.30 (t, C30), 122.33 (d, C4'), 122.61 (d, C7'), 137.05 (d, C6'), 148.58 (d, C3'), 151.02 (s, C20), 157.12 (s, C2'), 176.36 (s, C28). MS: *m/z* 547.3$[M+H]^+$, 545.3 $[M-H]^-$. IR (KBr; cm^{-1}): 3368 (N–H val. amide), 2942 (C–H val.), 2868 (–CH_3 val.), 1649 (–C=O val. amide). For $C_{36}H_{54}N_2O_2$ (546.83) calculated C (79.07), H (9.95), N (5.12), found C (79.09), H (9.93), N (5.14). M.p. 210-211 ^{o}C. $[\alpha]_D^{20}$ = +10.3 (*c* 0.350).

6b: ^{1}H NMR: δ 0.61 (1H, dd, J_1 = 2.3 Hz, J_2 = 11.0 Hz, H5), 0.70 (3H, s, H24), 0.76 (3H, d, J = 0.7 Hz, H25), 0.79 (3H, s, H23), 0.91 (3H, s, H26), 0.91 (3H, d, J = 0.8 Hz, H27), 1.07 (2H,

dt, J_1 = 3.2 Hz, J_2 = 3.2 Hz, J_3 = 13.4 Hz, H21), 1.63 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.4 Hz, H29), 1.88 (2H, dt, J_1 = 3.3 Hz, J_2 = 3.3 Hz, J_3 = 13.5 Hz, H16), 2.39 (1H, ddd, J_1 = 3.7 Hz, J_2 = 11.5 Hz, J_3 = 13.0 Hz, H13), 3.09 (1H, dt, J_1 = 4.4 Hz, J_2 = 11.0 Hz, J_3 = 11.0 Hz, H19), 3.12 (1H, dd, J_1 = 4.9 Hz, J_2 = 11.4 Hz, H3), 4.31 (1H, dd, J_1 = 5.8 Hz, J_2 = 15.0 Hz, H1'), 4.47 (1H, dd, J_1 = 6.1 Hz, J_2 = 15.0 Hz, H1'), 4.55 (1H, dq, J_1 = 1.4 Hz, J_2 = 1.4 Hz, J_3 = 1.4 Hz, J_4 = 2.4 Hz, H30), 4.69 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.4 Hz, H30), 6.06 (1H, bt, J = 6.0 Hz, NH), 7.22 (1H, ddd, J_1 = 0.9 Hz, J_2 = 4.9 Hz, J_3 = 7.9 Hz, H6'), 7.64 (1H, bddd, J_1 = 1.7 Hz, J_2 = 2.3 Hz, J_3 = 7.6 Hz, H7'), 8.49 (1H, bdd, J_1 = 1.7 Hz, J_2 = 4.9 Hz, H5'), 8.50 (1H, bdq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.3 Hz, H3'). ^{13}C NMR: δ 14.62 (q, C27), 15.34 (q, C24), 16.02 (q, C25), 16.15 (q, C26), 18.26 (t, C6), 19.49 (q, C29), 20.90 (t, C11), 25.60 (t, C12), 27.39 (t, C2), 27.96 (q, C23), 29.41 (t, C15), 30.83 (t, C21), 33.70 (t, C16), 34.37 (t, C22), 37.17 (s, C10), 37.71 (d, C13), 38.33 (t, C7), 38.70 (t, C1), 38.83 (s, C4), 40.42 (s, C8), 40.72 (t, C1'), 42.45 (s, C14), 46.63 (d, C19), 50.12 (d, C18), 50.61 (d, C9), 55.35 (d, C5), 55.67 (s, C17), 78.94 (d, C3), 109.42 (t, C30), 123.62 (d, C6'), 135.04 (s, C2'), 136.06 (d, C7'), 148.41 (d, C5'), 148.83 (d, C3'), 150.77 (s, C20), 176.23 (s, C28). MS: *m/z* 547.3$[M+H]^+$, 545.3 $[M-H]^-$. IR (KBr; cm^{-1}): 3284 (N–H val. amide), 2933 (C–H val.), 2867 (–CH_3 val.), 1640 (–C=O val. amide). For $C_{36}H_{54}N_2O_2$ (546.83) calculated C (79.07), H (9.95), N (5.12), found C (79.05), H (9.96), N (5.11). M.p. 254-256 ^oC. $[\alpha]_D^{20}$ = +11.1 (*c* 0.307).

6c: ^{1}H NMR: δ 0.62 (1H, dd, J_1 = 2.2 Hz, J_2 = 11.1 Hz, H5), 0.71 (3H, s, H24), 0.77 (3H, bs, H25), 0.82 (3H, s, H23), 0.92 (3H, bs, H27), 0.92 (3H, s, H26), 1.12 (2H, dt, J_1 = 3.5 Hz, J_2 = 3.5 Hz, J_3 = 13.5 Hz, H21), 1.64 (3H, dd, J_1 = 0.7 Hz, J_2 = 1.4 Hz, H29), 1.74 (2H, ddd, J_1 = 0.9 Hz, J_2 = 8.1 Hz, J_3 = 12.3 Hz, H7), 2.39 (1H, ddd, J_1 = 3.6 Hz, J_2 = 11.6 Hz, J_3 = 12.8 Hz, H13), 3.09 (1H, dt, J_1 = 4.6 Hz, J_2 = 11.1 Hz, J_3 = 11.1 Hz, H19), 3.13 (1H, dd, J_1 = 4.9 Hz, J_2 = 11.4 Hz, H3), 4.32 (1H, dd, J_1 = 6.0 Hz, J_2 = 15.6 Hz, H1'), 4.45 (1H, dd, J_1 = 6.1 Hz, J_2 =

15.6 Hz, H1'), 4.55 (1H, dq, J_1 = 1.4 Hz, J_2 = 1.4 Hz, J_3 = 1.4 Hz, J_4 = 2.4 Hz, H30), 4.69 (1H, dq, J_1 = 0.7 Hz, J_2 = 0.7 Hz, J_3 = 0.7 Hz, J_4 = 2.4 Hz, H30), 6.05 (1H, bt, J = 6.0 Hz, NH), 7.16-7.18 (2H, m, H4'), 8.49-8.51 (2H, m, H3'). ^{13}C NMR: δ 14.63 (q, C27), 15.33 (q, C24), 16.10 (q, C25), 16.16 (q, C26), 18.27 (t, C6), 19.49 (q, C29), 20.90 (t, C11), 25.60 (t, C12), 27.39 (t, C2), 27.96 (q, C23), 29.47 (t, C15), 30.82 (t, C21), 33.75 (t, C16), 34.42 (t, C22), 37.18 (s, C10), 37.71 (d, C13), 38.39 (t, C7), 38.71 (t, C1), 38.84 (s, C4), 40.76 (s, C8), 42.21 (t, C1'), 42.48 (s, C14), 46.59 (d, C19), 50.12 (d, C18), 50.61 (d, C9), 55.37 (d, C5), 55.73 (s, C17), 78.96 (d, C3), 109.49 (t, C30), 122.47 (d, C3'), 148.49 (s, C2'), 149.78 (d, C4'), 150.70 (s, C20), 176.23 (s, C28). MS: *m/z* 547.3$[M+H]^+$, 545.3 $[M-H]^-$. IR (KBr; cm^{-1}): 3352 (N–H val. amide), 2942 (C–H val.), 2871 (–CH_3 val.), 1670 (–C=O val. amide). For $C_{36}H_{54}N_2O_2$ (546.83) calculated C (79.07), H (9.95), N (5.12), found C (79.06), H (9.94), N (5.15). M.p. 246-248 ^{o}C. $[\alpha]_D^{20}$ = +3.7 (*c* 0.454).

2.7. Cell Cultures

The screening cell lines: T-lymphoblastic leukemia CEM; breast carcinoma MCF7; cervical carcinoma HeLa; malignant melanoma G-361; and human foreskin fibroblasts BJ were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Sigma, MO, USA). Media used were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin-streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in humid environment. Cells were subcultured twice or three times a week using the standard trypsinization procedure.

2.8. Cytotoxicity screening tests

Description of the experimental procedure used in cytotoxicity test was already published

[1,2]. IC_{50} values obtained with the compounds **3a**–**3c** and **6a**–**6c** are shown in Table 1.

2.9. Flow cytometry analysis

G-361 cancer cells were trypsinized, seeded in 100 mm culture dishes, and after 24 h of stabilization incubated with the tested compounds. After additional 24 h, the cells were again detached with trypsin, washed, fixed and stained in 0.1% [v/v] Triton X-100, 0.2 $mg.mL^{-1}$ RNase A and 10 $\mu g.mL^{-1}$ propidium iodide in PBS. Their DNA contents were then assessed by a flow cytometer (FACS Verse™, Becton Dickinson, NJ, USA), and the distribution of cells in subG_1 ("apoptotic cells"), G_0/G_1, S and G_2/M peaks were quantified by histogram analysis using BD FACSuite software (Becton Dickinson, NJ, USA). The experiments were repeated three times. The differences between control and treated cells were analyzed by one-way analysis of variance ANOVA using Microsoft Excel 2010. Signification was marked with asterisk ($p < 0.05$).

2.10. SDS–polyacrylamide gel electrophoresis and immunoblotting

Cells (G-361) were seeded into culture medium in 100-mm culture dishes at a density of 1.5×10^6 $cells.mL^{-1}$ and after 24 h of stabilization treated with tested compounds. DMSO was used as a vehicle for controls. After another 24 h of treatment, the cells were washed three times with cold PBS (10 mM, pH 7.4) and lysed in ice-cold RIPA protein extraction buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 mM PMSF, 1 mM DTT, 10 $mg.mL^{-1}$ of aprotinin and leupeptin). The lysate was collected into a microfuge tube and incubated on ice for 1 h. It was then cleared by centrifugation at 18,000 × g for 30 min at 4 ºC and the supernatant was collected. Proteins in lysates were quantified by the Bradford method and diluted with Laemmli electrophoresis buffer. The proteins were then separated on 10% or 12% SDS-polyacrylamide gels,

transferred to nitrocellulose membranes (Bio-Rad Laboratories, CA, USA) and stained with Ponceau S (Sigma Aldrich, St. Louis, MO, USA) to check equal protein loading. The membranes were blocked with 5% (w/v) non-fat dry milk and 0.1% Tween-20 in PBS for 2 h and probed with specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA; Santa Cruz Biotechnology, CA, USA) overnight. Anti-caspase-3 and anti-caspase-7 recognized zymogen and also its fragment. After washing in PBS and PBS with 0.1% Tween-20, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies and visualized with West Pico Supersignal chemiluminescent detection reagent (Thermo Fisher Scientific, Rockford, USA). Signal was detected by a CCD camera (Fujifilm, Tokio, Japan). To confirm equal protein loading, immunodetection was performed with anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, CA, USA). The experiments were repeated three times. The protein expression in treated cells was compared to untreated controls.

2.11. Activities of caspase 3/7

Treated G-361 cells were harvested by centrifugation and homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, plus protease inhibitors: aprotinin, leupeptin, PMSF; pH 7.4) on ice for 20 min. The resulting homogenates were clarified by centrifugation at 18,000 × g for 20 min at 4°C. The protein contents of the samples were quantified by the Bradford method and they were diluted to equivalent protein concentrations. Lysates were then incubated for 1 h with 100 mM Ac-DEVD-AMC as a substrate (Sigma, MO, USA) in an assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 mM Ac-DEVD-CHO (Sigma, MO, USA) as a caspase-3/7 inhibitor. The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems, Vantaa, Finland) at 346/442 nm (ex/em). Experiments were repeated three times in triplicates. The differences

between control and treated cells were analyzed by one-way analysis of variance ANOVA using Microsoft Excel 2010. Signification was marked with asterisk ($p < 0.05$).

3. Results and Discussion

3.1. Synthetic protocol

The synthesis of both series of the target picolyl amides (**3a**–**3c** and **6a**–**6c**) is efficient and easy. To get **3a**–**3c** (Scheme 1), betulinic acid (**1**) was first esterified by succinic anhydride [2,26] to prepare its hemiester **2**, which was then subjected to a formation of an amide bond with *o*-, *m*- and *p*-picolyl amines, using T3P as condensation agent [2,27]. To get **6a**–**6c** (Scheme 2), protecting of the C(3)-OH group as acetate ester was required, and achieved by reacting betulinic acid (**1**) with acetic anhydride in dry THF, using EDIPA as a base and DMAP as reaction promotor [2]. Free carboxylic group of **1** was then converted to betulinic acyl chloride by means of oxalyl chloride in dichloromethane [2,28], and used subsequently without isolation in a formation of amide with *o*-, *m*- and *p*-picolyl amines, using again EDIPA as a base [27], affording **5a**–**5c**. Protecting acetate group was removed by $LiOH.H_2O$ in methanol [2], yielding the required amides **6a**–**6c**.

Cytotoxicity: The experimental data obtained (Table 1) revealed high importance of **3b** and **3a**, while **3c** and **6a**–**6c** displayed only moderate cytotoxicity. The cytotoxicity data of **3a**–**3c** clearly demonstrate the importance of substitution of pyridine ring in structure-activity relationship studies: while *o*- and *m*-substitution of the pyridine ring resulted in highly cytotoxic compounds **3a** and **3b**, *p*-substitution revealed its disadvantage in **3c**. Both cytotoxic compounds, **3a** and **3b**, showed very low cytotoxicity in normal fibroblasts, and became potential candidates for cytotoxic agents against G-361 (2.4 ± 0.0 µM and 0.5 ± 0.1 µM, respectively), MCF7 (2.2 ± 0.2 µM and 1.4 ± 0.1 µM, respectively), HeLa (2.3 ± 0.5 µM and 2.4 ± 0.4 µM, respectively) and CEM (6.9 ± 0.4 µM and 6.5 ± 1.5 µM, respectively) cancer

cell lines. Moreover, **3b** was the most active in melanoma cancer cell line G-361 (IC_{50} 0.5 ± 0.1 μM) compared to other tested cancer cell lines with weak activity in normal human cells (BJ) that is important for wide therapeutic window. We determined selectivity against all tested cancer cells compared to normal cells with therapeutic index between 7 and 100 for compounds **3a** and **3b.** The highest therapeutic index (TI = 100) was calculated for human malignant melanoma cell line (G-361) *versus* normal human fibroblasts (BJ) with **3b**. Difluoroderivatives of dihydrobetulinic acid showed selectivity with therapeutic index between 2 and 8 against cancer lines [29]. In another study, therapeutic index of 9.1 was measured for conjugate of (3β)-3-*O*-acetylbetulinic acid with a triazole ring further substituted by benzaldehyde [30]. Based on the literature search, no derivative of betulinic acid was as highly active in melanoma cancer cell line G-361 as **3b**. In a future, we would like to design other compounds with higher selectivity, mainly based on the structure of **3b**. Together with obtained results, these derivatives could be promising for *in vivo* experiments for development new anticancer drugs.

Concerning **6a**–**6c**, their lower cytotoxicity has been expected due to the substitution of carboxyl group in these derivatives of betulinic acid. However, the great disadvantage of **6a**–**6c** is their cytotoxicity in normal fibroblasts, which is sometimes even higher than that in the cancer cell lines. The cytotoxicity of **6a**–**6c** in CEM, MCF7 and HeLa cancer cell lines is comparable with the cytotoxicity of the compounds investigated earlier [1]. The equal moderate cytotoxicity with no selectivity as for **6a**–**6c** was also shown for saponins of betulinic acid in different cancer cell lines [33].

3.2. Picolyl amides of betulinic acid caused apoptosis

We have observed whether compounds **3a**–**3c** influence the cell cycle and cause apoptosis in human malignant melanoma cells G-361. Cells were treated with 0.5, 2.5, 12.5 or 25 μM of

three substances (**3a**–**3c**) for 24 h. Then the cells were harvested, stained and analyzed by flow cytometry, western blotting or caspase activation assay. Flow cytometric analysis showed an increase in the subG$_1$ phase of the cell cycle (apoptotic cells) in G-361 cell line after treatment with **3a** and **3b**. In turn, **3c** did not enhance the number of subG$_1$ cells and caused no changes in the cell cycle distribution. In these cells, treatment with **3a** and **3b** increased the proportion of cells in the G$_0$/G$_1$ phase and decreased their proportion in the concomitant phases, mainly G$_2$/M (Figure 1). Apoptotic markers were detected by western blotting to examine the antiproliferative activities of tested compounds **3a**–**3c**. The initiation of apoptosis is mediated by caspase cascade activation [34]. Caspase-3 and caspase-7 are executioner proteases that result in cleavage of poly-(ADP-ribose) polymerase (PARP) and subsequent DNA degradation and apoptotic death [35]. The western blots showed degradation of caspase-7 into cleaved fragments (a part of the apoptotic cascade) after treatments with **3a** and **3b** in G-361 cells (Figure 2). PARP cleavage, a final step of caspase activation, was observed in human malignant melanoma cells treated by **3a** and **3b** (Figure 2). pRb was downregulated by 12.5 µM and 25 µM of **3a** and **3b**, but total Rb also decreased in those treatments. It is probably due to reaching the cytotoxicity towards human melanoma cells. No changes in the expressions of anti-apoptotic proteins Bcl-2 and Mcl-1 and also p53 were observed. Downregulation of phospho-p44/42 MAPK (Erk 1/2) (Thr202/Tyr204) was detected after the treatment with 12.5 and 25 µM of **3a** and **3b** for 24 h. Total level of p44/42 was not changed. This kinase is responsible for proliferation, differentiation, motility, and death of cells. The compounds **3a** and **3b** inhibited those activities mediated by Erk 1/2. These data were supplemented by estimation of caspase-3/7 activity in G-361 treated cells using the fluorogenic substrate Ac-DEVD-AMC and/or caspase-3/7 inhibitor Ac-DEVD-CHO. Compound **3a** increased activity of caspase-3/7 after the treatment with 25 µM up to eighteen fold. Compound **3b** induced the activity of caspase-3/7; after 24 h treatment with

concentration 25 μM, a twelve fold increase in the effector caspases was observed compared to the untreated control (Figure 3). Unlike **3a** and **3b**, compound **3c** did not affect the activity of caspases-3/7 after 24 h. These results show that **3a** and **3b** can induce apoptosis by caspase-3 activation, and target proliferation and differentiation in G-361 cells.

3.3. Physico-chemical parameters

To support rational design of the target compounds investigated in this work, their physico-chemical parameters have been calculated using ACD/iLabs software and databases [36]. The parameters calculated for **3a**–**3c** and **6a**–**6c** were compared with the Lipinski [5] rule of five and with the Ghose [6] rules. The rules describe molecular properties important for a small molecule drug pharmacokinetics in the human body, including their absorption, distribution, metabolism and excretion (known as ADME parameters). However, the rules do not predict displaying of the pharmacological activity. Lipinski [5] rule of five considers partition coefficient (log *P*, range -0.4 to +5.6), molar refractivity (range 40 to 130), molecular weight (range 180 to 500), number of atoms in the molecule (20 to 70) and polar surface area (up to 14 nm). The data for comparison are presented in Table 2. However, the target compounds **3a**–**3c** and **6a**–**6c** do not correspond to the range given for any of the above Lipinski and Ghose rules [5,6], except of the parameter giving the number of atoms in the molecule. Despite those facts, **3a**–**3c** and **6a**–**6c** display cytotoxicity. Exceptions are already known, where pharmacologically active compounds do not correspond to all rules, we have observed such result as well [1,2].

The most important molecular descriptors are the partition coefficient (log *P*) and the distribution coefficient (log *D*) [37]. In chemical and pharmaceutical sciences, both, log *P* and log *D* are measures of hydrophilicity or hydrophobicity of the studied compound, and are useful for estimating distribution of a drug within the body, where log *D* shows the

548 dependence on the pH of the matrix. Hydrophobic drugs are then preferentially distributed to
549 hydrophobic compartments (e.g., lipid bilayer of cells), while hydrophilic drugs are
550 preferentially distributed to hydrophilic compartments (e.g., blood serum). The distribution
551 coefficient is a pH dependent value, and, therefore, the value at pH = 7.4 (the physiological
552 pH value of blood serum) is of particular importance (see also Table 2). Thus, log *P* expresses
553 a ratio of concentrations of non-ionized compound between two phases, non-polar (octanol)
554 and polar (water), while log *D* expresses the ratio of the sum of the concentrations of all forms
555 of the compound (ionized and non-ionized) in each of the two phases. In pharmacology, log *P*
556 and log *D* indicate how easily the drug can reach its intended target in the body, how strong
557 its effect will be once it reaches its target and how long it will remain in the body in an active
558 form. The log *P* values calculated for either **3a**–**3c** or **6a**–**6c** are identical, and they exceed the
559 values given by the Lipinski and Ghose rules [5,6] (Table 2). Another supportive parameter is
560 the predicted aqueous solubility, log *S*. *S* (in mol dm^{-3}) is the concentration of the solute in a
561 saturated solution that is in equilibrium with the crystalline solid, and it is a pH dependent
562 parameter. Standard range for log *S* at pH 7.4 is –6.5/+0.5. Among the prepared compounds,
563 only **3a**–**3c** show values for log *S* in this range (Table 2).
564
565 3.4. ADME parameters:
566 The importance of some of the ADME parameters for evaluation of pharmacokinetic
567 properties of the prepared compounds is summarized in Table 2. The blood brain barrier
568 (BBB) and plasma protein binding are two of the important factors affecting distribution of
569 the compound in the human body. Several parameters assist in evaluation of each potential
570 drug for its BBB transport [38] The rate of brain penetration, log *PS*, is a logarithm of the
571 permeability-surface area coefficient that measures the ability of a drug to cross the BBB and
572 to move into brain tissue over time. It is one of the relevant parameters for evaluation of the

rate of BBB penetration. The extent of brain penetration parameter, log *PB*, indicates if the drug might be active or inactive on the central nervous system (CNS). The typical values for log *PB* are –1.5/+1.5. Most of the current drugs show the log PB value up to +2 (active on CNS) or down to –2 (inactive on CNS). The log *PB* values calculated for **3a**–**3c** and **6a**–**6c** indicate that these compounds will be inactive on CNS (Table 2). However, the complex of the BBB parameters is completed by the brain/plasma equilibration rate, the parameter expressed as log $PS * f_{u,\,brain}$ that is a mathematical modeling parameter based on time required for reaching brain equilibrium. It is dependent on the brain unbound fraction ($f_{u,\,brain}$). This value indicates if the drug may potentially be active on CNS together with the log *BB* parameter, the predicted brain/blood partition coefficient (cf. Supplementary material). The parameter log *BB* is a hybrid parameter determined by permeability, plasma and brain tissue binding, and active transport mechanism, standard range –3.0/+1.2. The log BB values calculated for **3a**–**3c** and **6a**–**6c** appear in the required range of this parameter. Nevertheless, there are numerous exceptions already known. A supportive parameter, plasma protein binding (PPB) gives calculated quantity of a drug bound to a protein.

Another important ADME parameter is bioavailability. Among the prepared compounds, the calculated bioavailability for **3a**–**3c** is lower than that for **6a**–**6c**. However, the experimental data show that **3a**–**3c** are generally more cytotoxic to the tested cancer cell lines than **6a**–**6c**. The rules [5,6] describe molecular properties important for a small molecule drug pharmacokinetics in the human body, including their absorption, distribution, metabolism and excretion (ADME parameters). However, the rules do not predict whether a compound will display pharmacological activity. Nevertheless, the importance of several ADME parameters, namely blood-brain barrier and plasma protein binding, reflects the distribution of the compound in the human body [39]. A comparison of the calculated physico-chemical properties with the measured cytotoxicity shows that every time a new class of compound is

598 being investigated, no available experimental screening may be intentionally omitted (Tables
599 1 and 2).
600
601 3.5. A comparison of **3a**–**3c** and **6a**–**6c** with the compounds prepared earlier [1, 2]
602 Earlier prepared compound, bearing picolyl amine moiety in the molecule, derived either
603 from lanosterol, cholesterol or sitosterol, displayed cytotoxicity in the range of IC_{50} = 25–45
604 μM, or were inactive (IC_{50} > 50 μM). The earlier published data also show that the physico-
605 chemical and ADME parameters calculated for those compounds, corresponded to the
606 Lipinski and Ghose rules [5,6] partially only. The current compounds **3a**–**3c** and **6a**–**6c** show
607 generally higher cytotoxicity than the earlier series of compounds. Only **3a** and **3b** display
608 considerable cytotoxicity, and **3b** show the IC_{50} value on G361 in the range IC_{50} = 0.5 ± 0.1
609 μM, with the therapeutic index TI = 100. This finding augments the importance of this
610 particular compound **3b**. In turn, even **3a** and **3b** corresponded to a limited number of
611 calculated physico-chemical and ADME parameters. Any calculation of those parameters is
612 useful and often desired in the drug design. However, the conclusions of the calculations
613 should always be supported by the experimental data for confirmation or correction.
614
615 **4. Conclusion**
616 Amides **3b** and **3a** represent the most important members of this series of compounds because
617 of their high cytotoxicity against human melanoma cell line G-361 (Table 1). The former of
618 this couple of amides (**3b**) is active in concentration 0.5 ± 0.1 μM, and, subsequently it does
619 not damage healthy cells. It also displays high cytotoxicity in other tested cell lines.
620 Investigating structure-activity relationship in dependence on substitution of pyridine ring, the
621 conclusions are different for each subseries. In the former subseries (**3a**–**3c**), the cytotoxicity
622 of amides bearing *m*- and *o*-substituted pyridine ring (**3b** and **3a**) showed high cytotoxicity in

all tested cell lines, while *p*-substitution of the pyridine ring (**3c**) resulted in weak cytotoxicity (Table 1). Our hypothesis is that this result might be connected with different mechanism of action of **3b** and **3a** in comparison with **3c** due to the modifications in their structures. This hypothesis will be focused in a future investigation, because its confirmation may be important for better understanding of structure-activity relationship. However, in the latter subseries (**6a**–**6c**), amides bearing *p*- and *o*-substituted pyridine ring showed either comparable cytotoxicity, or *p*-substituted amide was more active, and the *m*-substituted derivative displayed the lowest cytotoxicity (Table 1). The most important finding with **6a**–**6c** was their high activity in the normal human cells. Due to that finding, **6a**–**6c** are much less suitable anti-cancer agents than **3a**–**3c** that are, in turn, potential anti-cancer candidate agents.

Supplementary material is available on the web page of the journal.

Acknowledgment: The authors are grateful to the grants FV10599 (MPO; UB; ZW) and 14-27669P (GACR; LR) for financial support. Financial support from the specific university (UCT) research (MSMT No. 20-SVV/2017) is also acknowledged (UB; ZW).

References

[1] U. Bildziukevich, L. Rárová, D. Šaman, L. Havlíček, P. Drašar, Z. Wimmer, Amides derived from heteroaromatic amines and selected steryl hemiesters, Steroids 78 (2013) 1347–1352.

[2] U. Bildziukevich, N. Vida, L. Rárová, M. Kolář, D. Šaman, L. Havlíček, P. Drašar, Z. Wimmer, Polyamine derivatives of betulinic acid and β-sitosterol: a comparative investigation, Steroids 100 (2015) 27–35.

[3] U. Bildziukevich, E. Kaletová, D. Šaman, E. Sievänen, E.T. Kolehmainen, M. Šlouf, Z. Wimmer, Spectral and microscopic study of self-assembly of novel cationic spermine amides of betulinic acid, Steroids 117 (2017) 90–96.

[4] B.G. Bag, C. Garai, R. Majumdar, M. Laguerre, Natural triterpenoids as renewable nanos, Struct. Chem. 23 (2012) 393–398.

[5] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and developmental settings. Adv. Drug Deliv. Rev. 46 (2001) 3–26.

[6] A.K. Ghose, V.N. Viswanadhan, J.J. Wendoloski, A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery. 1. A qualitative and quantitative characterization of known drug databases. J. Combin. Chem. 1 (1999) 55–68.

[7] Y. Kashiwada, J. Chiyo, Y. Ikeshiro, T. Nagao, H. Okabe, L.M. Cosentino, K. Fowke, K.H. Lee, 3,28-Di-*O*-(dimethylsuccinyl)-betulin isomers as anti-HIV agents, Bioorg. Med. Chem. Lett. 11 (2001) 183–185.

[8] V. Zuco, R. Supino, S.C. Righetti, L. Cleris, E. Marchesi, C. Gambacorti-Passerini, F. Formelli, Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells, Cancer Lett. 175 (2002) 17–25.

[9] Y.J. You, Y. Kim, N.H. Nam, B.Z. Ahn, Synthesis and cytotoxic activity of A-ring modified betulinic acid derivatives, Bioorg. Med. Chem. Lett. 13 (2003) 3137–3140.

[10] S. Fulda, Betulinic acid for cancer treatment and prevention, Int. J. Mol. Sci. 9 (2008) 1096–1107.

[11] C. Genet, A. Strehle, C. Schmidt, G. Boudjelal, A. Lobstein, K. Schoonjans, M. Souchet, J. Auwerx, R. Saladin, A. Wagner, Structure-activity relationship study of betulinic acid, a novel and selective TGR5 agonist, and its synthetic derivatives: potential impact in diabetes, J. Med. Chem. 53 (2010) 178–190.

672 [12] J. Šarek, M. Kvasnica, M. Vlk, M. Urban, P. Džubák, M. Hajdúch, The potential of
673 triterpenoids in the treatment of melanoma. In: Research on melanoma – a glimpse into
674 current directions and future trends. (M. Murph, Ed.). Chapter 7, pp. 125–158. InTech, Rijeka,
675 Croatia, 2011.
676 [13] R.H. Cichewicz, S.A. Kouzi, Chemistry, biological activity, and chemotherapeutic
677 potential of betulinic acid for the prevention and treatment of cancer and HIV infection, Med.
678 Res. Rev. 24 (2004) 90–114.
679 [14] Y. Iwamoto, Diagnosis and treatment of Ewing's sarcoma, Jpn. J. Clin. Oncol. 37 (2007)
680 79–89.
681 [15] S.K. Dash, S.S. Dash, S. Chattopadhyay, T. Ghosh, S. Tripathy, S.K. Mahapatra, B.G.
682 Bag, D. Das, S. Roy, Folate decorated delivery of self-assembled betulinic acid nano fibers: a
683 biocompatible antileukemic therapy, RSC Adv. 5 (2015) 24144–24157.
684 [16] I. Budihardjo, H. Oliver, M. Lutter, X. Luo, X. Wang, Biochemical pathways of caspase
685 activation during apoptosis, Annu. Rev. Cell Dev. Biol. 15 (1999) 269–290.
686 [17] M. Nihal, C.T. Roelke, G.S. Wood, Anti-melanoma effects of vorinostat in combination
687 with polyphenolic antioxidant (–)-epigallocatechin-3-gallate (EGCG), Pharm. Res. 27 (2010)
688 1103–1114.
689 [18] R.T. Allen, M.W. Cluck, D.K. Agrawal, Mechanisms controlling cellular suicide: role of
690 Bcl-2 and caspases, Cell Mol. Life Sci. 54 (1998) 427–445.
691 [19] P. Džubák, M. Hajdúch, D. Vydra, A. Hustová, M. Kvasnica, D. Biedermann, L.
692 Marková, M. Urban, J. Šarek, Pharmacological activities of natural triterpenoids and their
693 therapeutic implications, Nat. Prod. Rep. 23 (2006) 394–411.
694 [20] K. Kaczanowska, K.-H. Wiesmüller, A.-P. Schaffner, Design, synthesis, and in vitro
695 evaluation of novel aminomethyl-pyridines as DPP-4 inhibitors, ACS Med. Chem. Lett. 1
696 (2010) 530–535.

[21] B. Baptiste, C. Douat-Casassus, K. Laxmi-Reddy, F. Godde, I. Huc, Solid phase synthesis of aromatic oligoamides: application to helical water-soluble foldamers, J. Org. Chem. 75 (2010) 7175–7185.

[22] E. Rajanarendar, S. Raju, M.N. Reddy, S.R. Krishna, L.H. Kiran, A.R.N. Reddy, Y.N. Reddy, Multi-component synthesis and in vitro and in vivo anticancer activity of novel arylmethylene bis-isoxazolo[4,5-b]pyridine-*N*-oxides, Eur. J. Med. Chem. 50 (2012) 274–279.

[23] W. Zhu, M. Dai, Y. Xu, X. Qian, Novel nitroheterocyclic hypoxic markers for solid tumor: synthesis and biological evaluation, Bioorg. Med. Chem. 16 (2008) 3255–3260.

[24] K.V. Ahonen, M.K. Lahtinen, M.S. Löfman, A.M. Liesilä, A.M. Valkonen, E.I. Sievänen, Nonappa, E.T. Kolehmainen, Structural studies of five novel bile acid-4-aminopyridine conjugates, Steroids 77 (2012) 1141–1151.

[25] H.L. Pham, B.P. Ross, R.P. McGeary, P.N. Shaw, N.M. Davies, Synthesis of cationic derivatives of Quil A and the preparation of cationic immune-stimulating complexes (ISCOMs), Int. J. Pharm. 376 (2009) 123–133.

[26] H. Svobodová, Nonappa, M. Lahtinen, Z. Wimmer, E. Kolehmainen, A steroid-based gelator of $A(LS)_2$ type: tuning gel properties by metal coordination, Soft Matter 8 (2012) 7840–7847.

[27] E. Lee, R. Enomoto, K. Takemura, Y. Tsuda, Y. Okada, A selective plasmin inhibitor, *trans*-aminomethylcyclohexanecarbonyl-L-(*O*-picolyl)tyrosine-octylamide (YO-2), induces thymocyte apoptosis, Biochem. Pharmacol. 63 (2002) 1315–1323.

[28] S.N. Khan, H.-S. Kim, Synthesis and biological activities of 3-polyamino-5β-cholane-7α,24-diols, J. Ind. Eng. Chem. 15 (2009) 561–565.

[29] J.R. Dunetz, Y. Xiang, A. Baldwin, J. Ringling, General and scalable amide bond formation with epimerization-prone substrates using T3P and pyridine, Org. Lett. 13 (2011) 5048–5051.

722 [30] K. Qian, D. Yu, C.-H. Chen, L. Huang, S.L. Morris-Natschke, T.J. Nitz, K. Salzwedel,
723 M. Reddick, G.P. Allaway, K.-H. Lee, Anti-AIDS agents. 78. Design, synthesis, metabolic
724 stability assessment, and antiviral evaluation of novel betulinic acid derivatives as potent anti-
725 human immunodeficiency virus (HIV) agents, J. Med. Chem. 52 (2009) 3248–3258.
726 [31] L. Borková, L. Jasíková, J. Řehulka, K. Frisonsová, M. Urban, I. Frydrych, I. Popa, M.
727 Hajdúch, N. J. Dickinson, M. Vlk, P. Džubák, J. Šarek, Synthesis of cytotoxic 2,2-
728 difluoroderivatives of dihydrobetulinic acid and allobetulin and study of their impact on
729 cancer cells, Eur. J. Med. Chem. 96 (2015) 482–490.
730 [32] V. Sidová, P. Zoufalý, J. Pokorný, P. Džubák, M. Hajdúch, I. Popa, M. Urban, Cytotoxic
731 conjugates of betulinic acid and substituted triazoles prepared by Huisgen cycloaddition from
732 30-azidoderivatives, PLoS One 12 (2017) e0171621.
733 [33] P. Cmoch, Z. Pakulski, J. Swaczynová, M. Strnad, Synthesis of lupane-type saponins
734 bearing mannosyl and 3,6-branched trimannosyl residues and their evaluation as anticancer
735 agents, Carbohydr. Res. 343 (2008) 995–1003.
736 [34] I. Budihardjo, H. Oliver, M. Lutter, X. Luo, X. Wang, Biochemical pathways of caspase
737 activation during apoptosis, Annu. Rev. Cell Dev. Biol. 15 (1999) 269-290.
738 [35] R. T. Allen, M. W. Cluck, D. K. Agrawal, Mechanisms controlling cellular suicide: role
739 of Bcl-2 and caspases, Cell Mol. Life Sci. 54 (1998) 427-445.
740 [36] Advanced Chemistry Development, software ACD/iLabs, version 12.02. (2011).
741 [37] J. Kujawski, H. Popielarska, A. Myka, B. Drabinska, M.K. Bernard, The log P parameter
742 as a molecular descriptor in the computer-aided drug design – an overview. Comp. Methods
743 Sci. Technol. 18 (2012) 81–88.
744 [38] R. Zhao, J.C. Kalvass, G.M. Pollack, Assessment of blood–brain barrier permeability
745 using the in situ mouse brain perfusion technique. Pharm. Res. 26 (2009) 1657–1664.

[39] K. Kalani, D.K. Yadav, F. Khan, S.K. Srivastava, N. Suri, Pharmacophore, QSAR, and ADME based semisynthesis and in vitro evaluation of ursolic acid analogs for anticancer activity. J. Mol. Model. 18 (2012) 3389–3413.

Abbreviations: CEM, cells of human T-lymphoblastic leukemia; MCF7, cells of human breast adenocarcinoma; HeLa, cells of human cervical cancer; G-361, cells of human malignant melanoma; BJ, normal human fibroblasts; T3P, propylphosphonic anhydride; EDIPA, ethyl diisopropyl amine.

[Scheme and Figure Captions]

Scheme 1

i: succinic anhydride, DMAP, dry pyridine; ii: 2-, 3- or 4-aminomethylpyridine, T3P, dry pyridine

Scheme 2

i: acetanhydride, EDIPA, DMAP, THF; ii: oxalyl chloride, dichloromethane; iii: 2-, 3- or 4-aminomethylpyridine, EDIPA, dichloromethane; iv: $LiOH.H_2O$, methanol

Figure 1

Histograms with the distributions of G-361 cells in the G_0/G_1, S, and G_2/M cell cycle phases (**A**), and the $subG_1$ fraction of cells (**B**) measured by flow cytometric analysis, after 24 h treatment with **3a, 3b** and **3c** relative to untreated controls. Data indicate the percentages (%) of the number of cells in respective phases. Experiments were repeated three times in triplicates. Error bars are omitted for clarity. (**A**) Analysis of variance (one-way ANOVA) between control and treated cells failed due to high biological variability in replicates. (**B**) Differences between control and treated cells among $subG_1$ phase were significant ($p < 0.05$) *.

Figure 2

Western blot analysis of apoptotic proteins (PARP, pRb, Rb, zymogen and fragment of caspase-7, pAKT, AKT, pp44/42 MAPK (Erk 1/2) (Thr202/Tyr204), p44/42 MAPK (Erk 1/2) in human malignant melanoma cells G-361 treated with amides. The expression of proteins in cells treated with 0.5, 2.5, 12.5 and 25 μM of compounds **3a, 3b** and **3c** for 24 h were

compared with the protein expression of untreated control cells (0+ means control with DMSO). The expression of β-actin was used as a protein loading marker.

Figure 3

Activity of caspase-3/7 in malignant melanoma cells G-361 treated with 0.5, 2.5, 12.5 and 25 μM amides **3a, 3b** and **3c** compared with untreated control cells for 24 h. The data indicate the relative increase in caspase-3/7 activity. Experiments were repeated three times in triplicates. * Differences between control and treated cells were significant ($p < 0.05$).

Table 1. IC_{50} [μM] values in four cancer cell lines and normal human fibroblasts for **3a**–**3c** and **6a**–**6c** after 72 h

Compound	Cytotoxicity (IC_{50} [μM])					TI [a]
	CEM [b]	MCF7 [c]	HeLa [d]	G-361 [e]	BJ [f]	
3a	6.9 ± 0.4	2.2 ± 0.2	2.3 ± 0.5	2.4 ± 0.0	46.2 ± 2.8	19.3
3b	6.5 ± 1.5	1.4 ± 0.1	2.4 ± 0.4	0.5 ± 0.1	50.0 ± 0.0	100.0
3c	22.6 ± 5.9	>50	40.9 ± 4.7	32.1 ± 2.1	>50	ND [g]
6a	18.6 ± 1.0	27.0 ± 5.5	14.7 ± 0.2	16.4 ± 1.6	15.8 ± 1.4	1.0
6b	25.3 ± 6.9	38.7 ± 1.0	23.6 ± 2.3	18.1 ± 0.1	17.5 ± 2.7	1.0
6c	18.6 ± 3.7	21.1 ± 4.3	14.8 ± 0.2	11.6 ± 1.6	11.2 ± 1.4	1.0

[a] Therapeutic index (TI) calculated for G-361 line *versus* fibroblasts BJ; [b] CEM, cells of human T-lymphoblastic leukemia; [c] MCF7, cells of human breast adenocarcinoma; [d] HeLa, cells of human cervical cancer; [e] G-361, human malignant melanoma cell line [f] BJ, normal human fibroblasts; [g] ND = not determined.

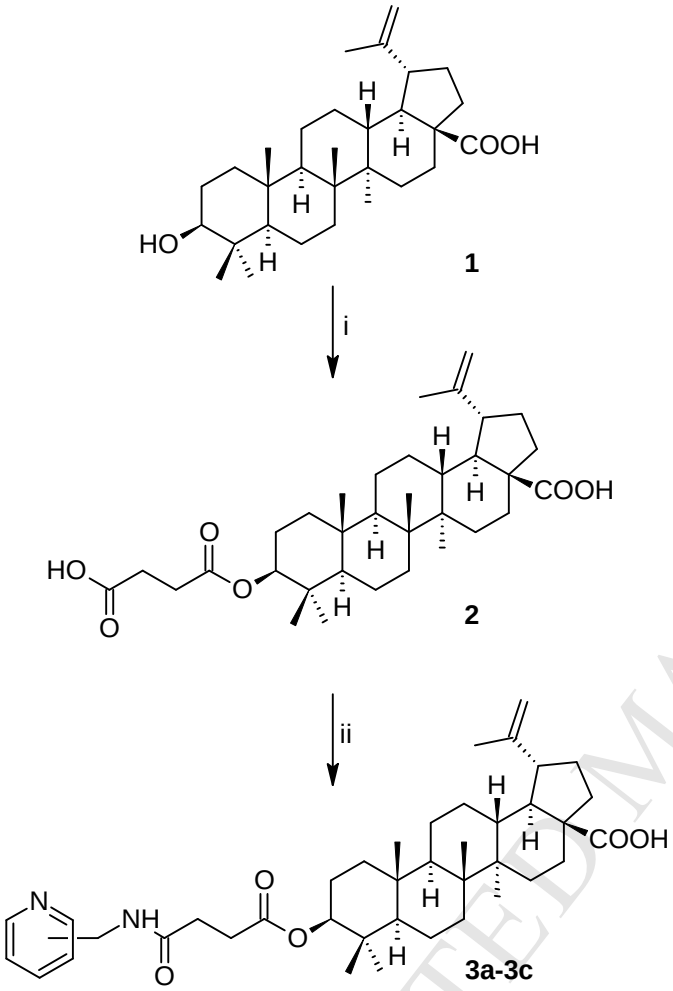
863 Table 2. Physico-chemical and ADME parameters of the target compounds **3a**–**3c** and **6a**–**6c** calculated using the ACD/iLabs software [36]

Compd. or ref. No.	MW	Physico-chemical and ADME parameters [a]									
		log *P*	log *D* (pH 7.4)	log *S* (pH 7.4)	bioav. [%]	log *PS* * $f_{u,\,brain}$	log *PS*	log *PB*	log *BB*	PPB [%]	H_{acc} / H_{don} / n.m.b.
3a	646.90	7.56	4.78	-5.01	< 30	-5.8	-4.0	-0.89	-0.0	99.80	7/2/9
3b	646.90	7.56	5.26	-5.04	< 30	-5.8	-3.9	-0.89	-0.0	99.80	7/2/9
3c	646.90	7.56	5.07	-5.48	< 30	-5.8	-3.9	-0.88	-0.0	99.80	7/2/9
6a	546.83	7.00	7.00	-6.76	30-70	-4.1	-1.8	-0.30	-0.0	99.75	4/2/4
6b	546.83	7.00	7.00	-6.61	30-70	-4.1	-1.8	-0.30	-0.0	99.75	4/2/4
6c	546.83	7.00	6.99	-6.58	30-70	-4.1	-1.8	-0.30	-0.0	99.75	4/2/4
ref. [32]	max. 500	max. 5.0									max. 10 / max. 5 / -
ref. [33]	Max. 500	Max. 5.6									

864

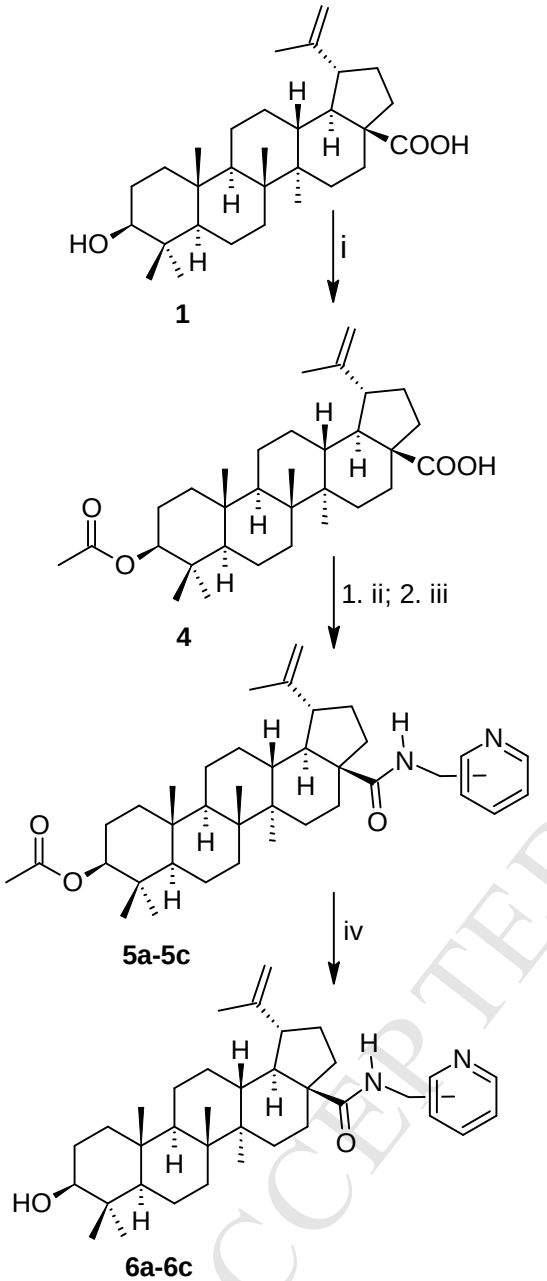
[a] log *P* – partition coefficient; log *D* – distribution coefficient; log *S* – predicted aqueous solubility; bioav. = bioavailability – the degree of availability of a chemical by the target tissue; log *PS* * $f_{u,\,brain}$ – the brain/plasma equilibration rate, the parameter that is a mathematical modeling parameter based on time required for reaching brain equilibrium; log *PS* – logarithm of the permeability-surface area coefficient; log *PB* – the extent of brain penetration parameter; log *BB* – a hybrid parameter determined by permeability, plasma and brain tissue binding, and active transport mechanism; PPB – plasma protein binding; H_{acc} / H_{don} / n.m.b. = number of H-bond acceptors / number of H-bond donors / number of movable bonds.

Scheme 1



893 Scheme 2

894



895

896

897

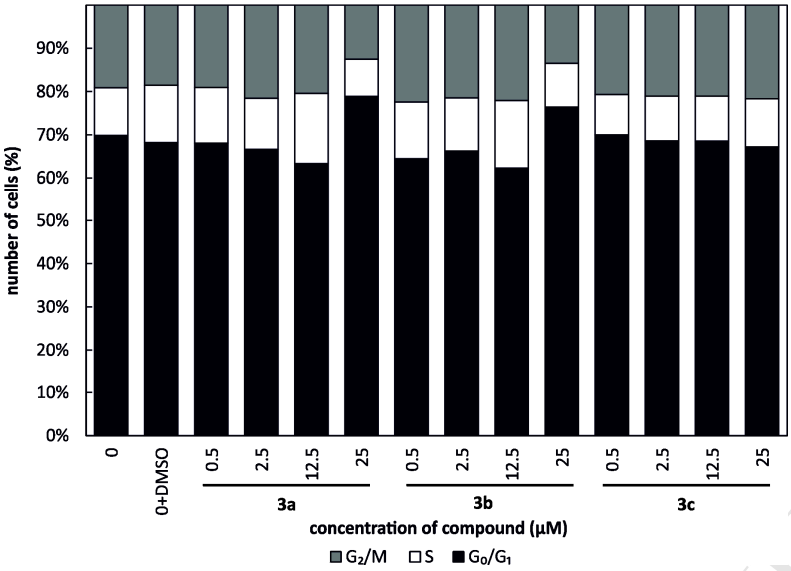
898

899

900

Figure 1

A


90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
number of cells (%)
0
0+DMSO
0.5
2.5
12.5
25
3a
3b
3c
concentration of compound (μM)
G2/M
S
G0/G1

B

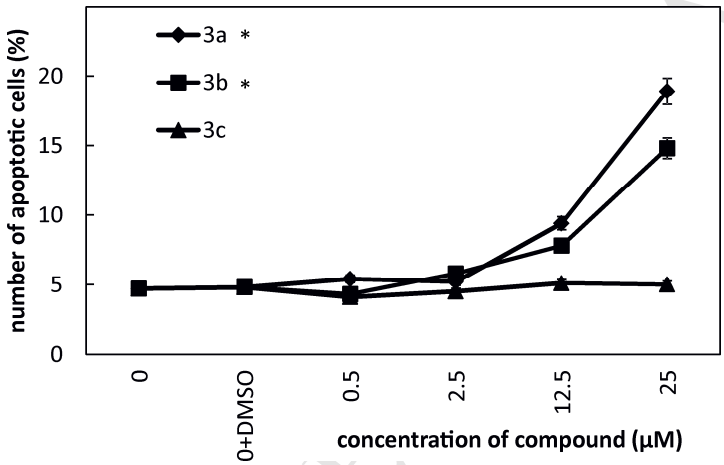

3a *
3b *
3c
number of apoptotic cells (%)
20
15
10
5
0
0
0+DMSO
0.5
2.5
12.5
25
concentration of compound (μM)

Figure 2

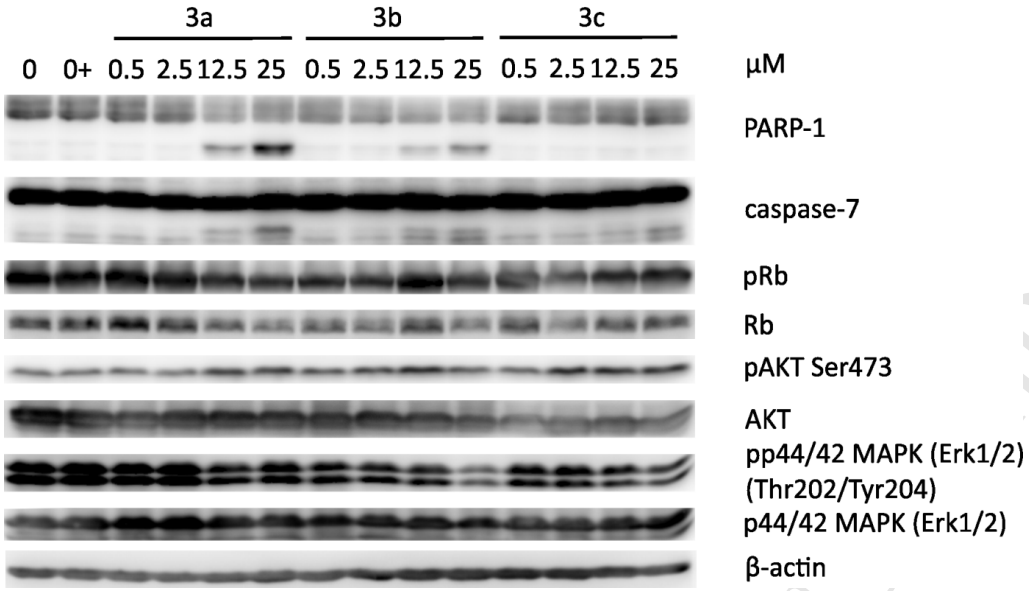
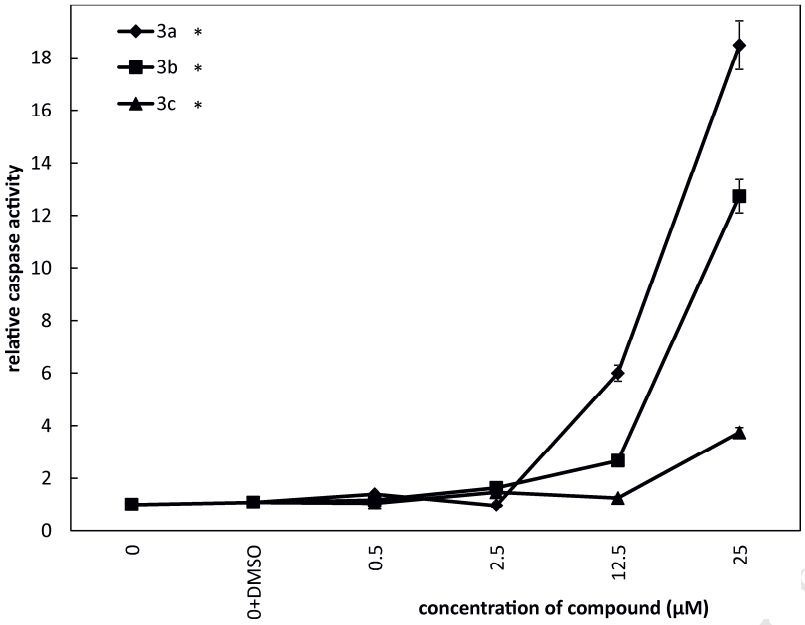


Figure 3

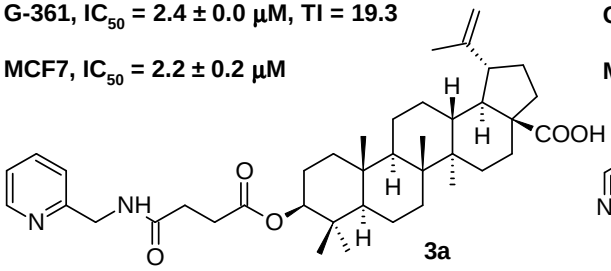


950 Graphical Abstract

951

G-361, IC_{50} = 2.4 ± 0.0 µM, TI = 19.3

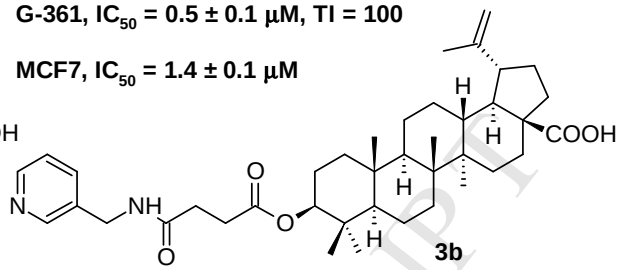
MCF7, IC_{50} = 2.2 ± 0.2 µM



3a

G-361, IC_{50} = 0.5 ± 0.1 µM, TI = 100

MCF7, IC_{50} = 1.4 ± 0.1 µM



3b

952
953

954

955

Table 1. IC_{50} [µM] values in four cancer cell lines and normal human fibroblasts for **3a**–**3c** and **6a**–**6c** after 72 h

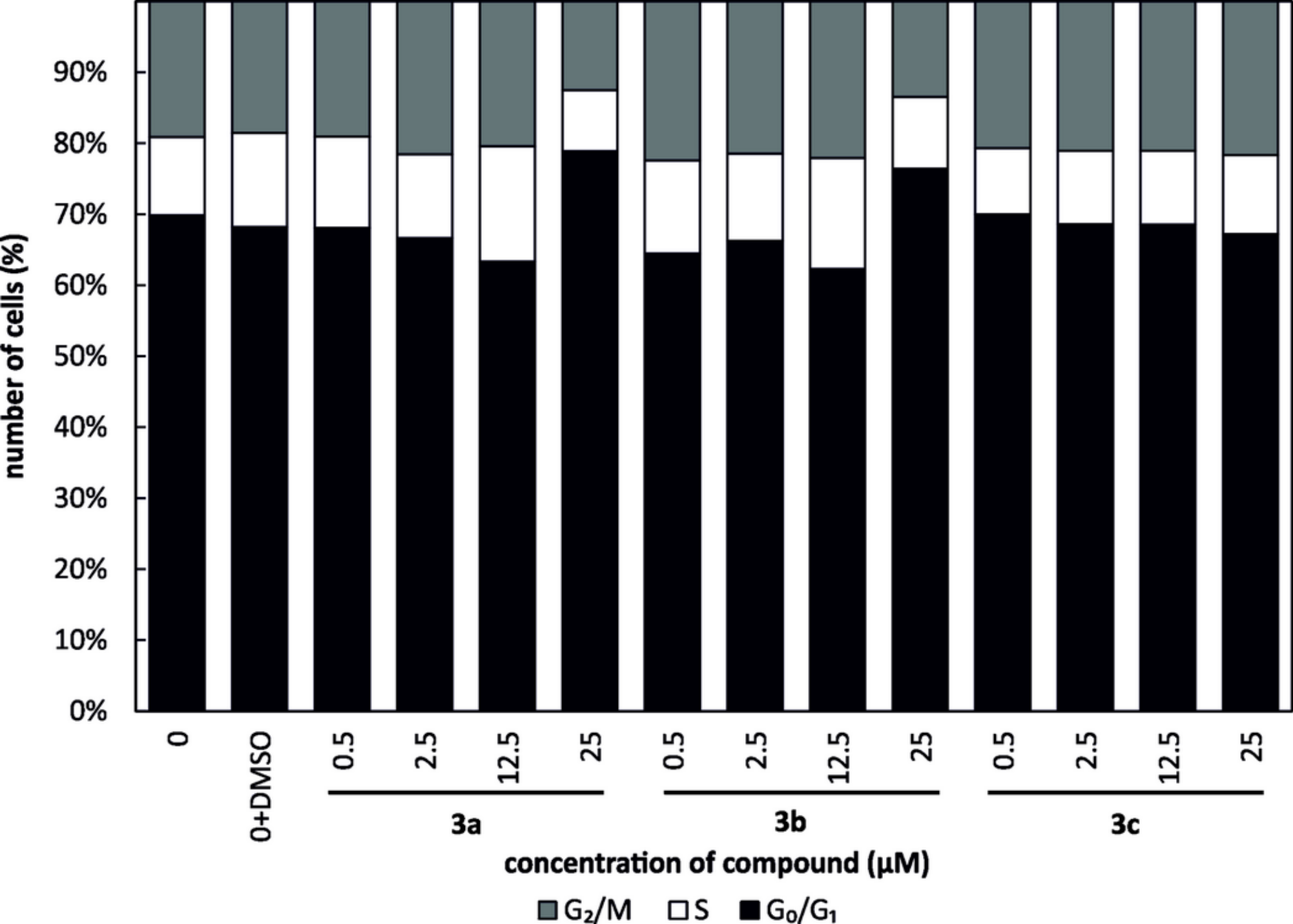
Compound	Cytotoxicity (IC_{50} [µM])					TI [a]
	CEM [b]	MCF7 [c]	HeLa [d]	G-361 [e]	BJ [f]	
3a	6.9 ± 0.4	2.2 ± 0.2	2.3 ± 0.5	2.4 ± 0.0	46.2 ± 2.8	19.3
3b	6.5 ± 1.5	1.4 ± 0.1	2.4 ± 0.4	0.5 ± 0.1	50.0 ± 0.0	100.0
3c	22.6 ± 5.9	>50	40.9 ± 4.7	32.1 ± 2.1	>50	ND [g]
6a	18.6 ± 1.0	27.0 ± 5.5	14.7 ± 0.2	16.4 ± 1.6	15.8 ± 1.4	1.0
6b	25.3 ± 6.9	38.7 ± 1.0	23.6 ± 2.3	18.1 ± 0.1	17.5 ± 2.7	1.0
6c	18.6 ± 3.7	21.1 ± 4.3	14.8 ± 0.2	11.6 ± 1.6	11.2 ± 1.4	1.0

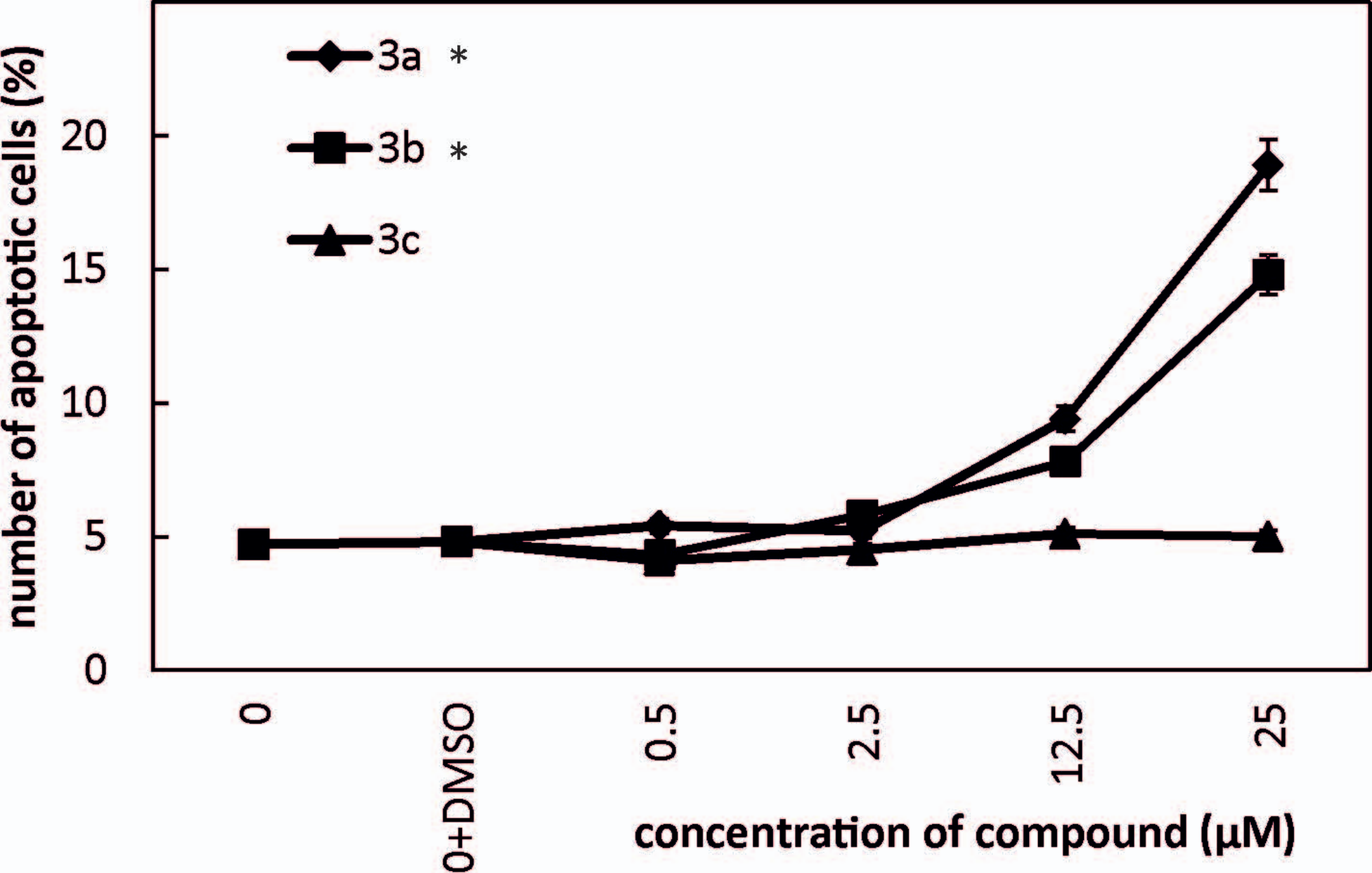
[a] Therapeutic index (TI) calculated for G-361 line *versus* fibroblasts BJ; [b] CEM, cells of human T-lymphoblastic leukemia; [c] MCF7, cells of human breast adenocarcinoma; [d] HeLa, cells of human cervical cancer; [e] G-361, human malignant melanoma cell line [f] BJ, normal human fibroblasts; [g] ND = not determined

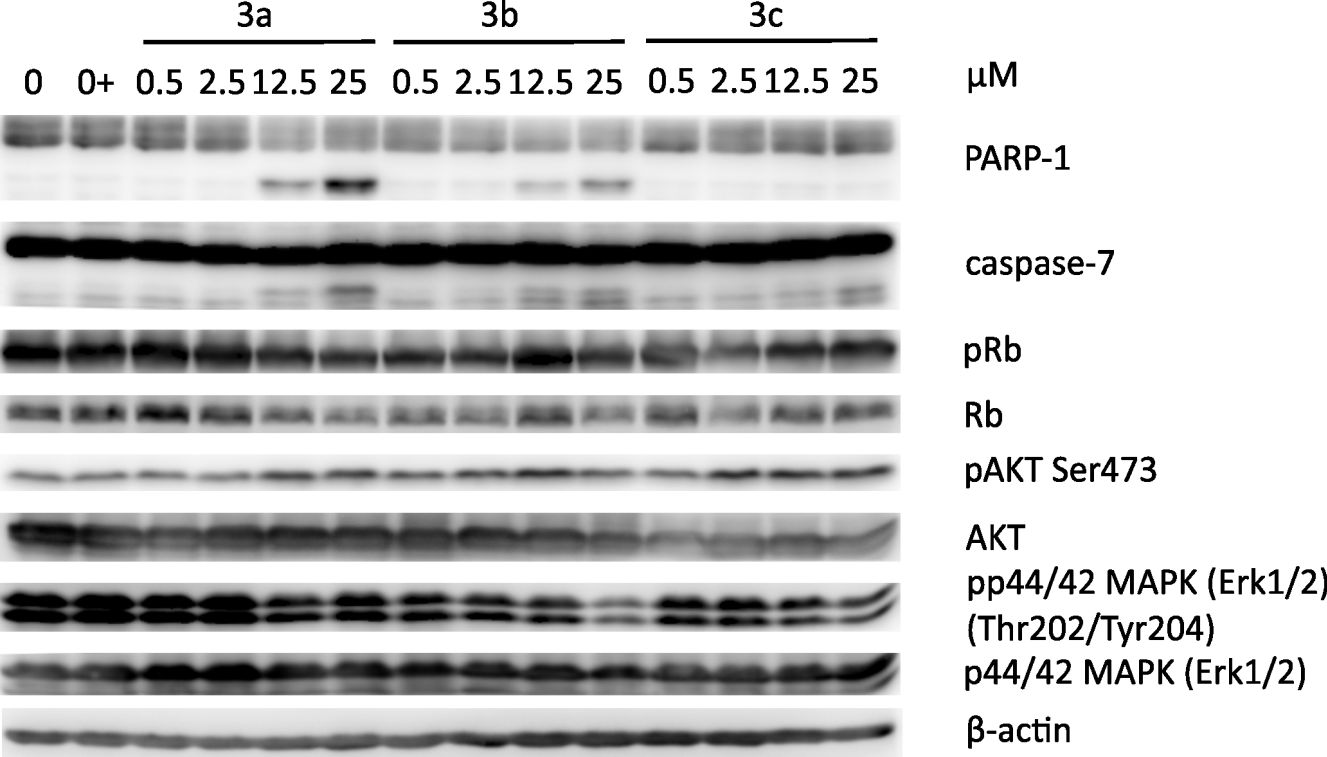
Table 2. Physico-chemical and ADME parameters of the target compounds **3a**–**3c** and **6a**–**6c** calculated using the ACD/iLabs software [36]

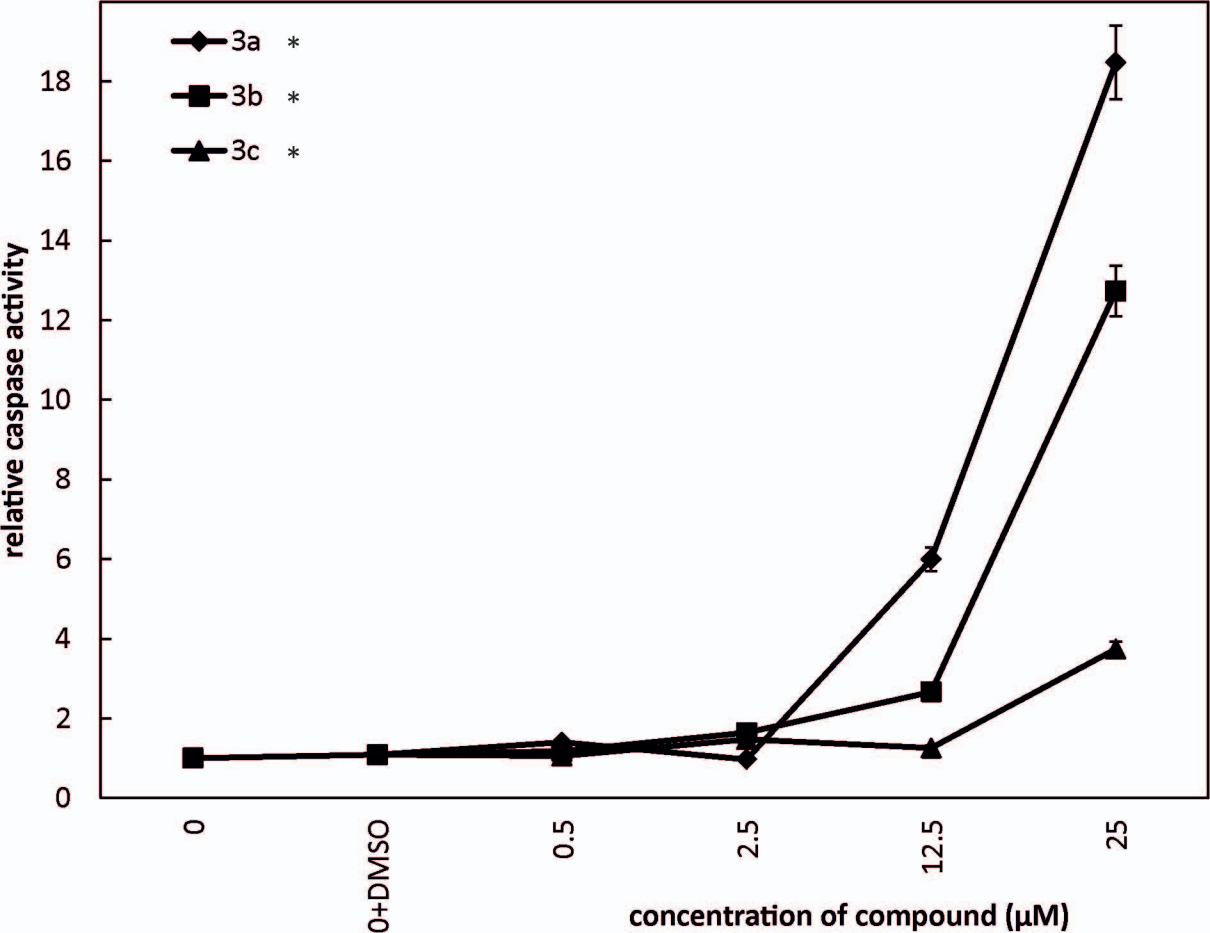
Compd. or ref. No.	MW	Physico-chemical and ADME parameters [a]									
		log *P*	log *D* (pH 7.4)	log *S* (pH 7.4)	bioav. [%]	log *PS* * $f_{u,\,brain}$	log *PS*	log *PB*	log *BB*	PPB [%]	H_{acc} / H_{don} / n.m.b.
3a	646.90	7.56	4.78	-5.01	< 30	-5.8	-4.0	-0.89	-0.0	99.80	7/2/9
3b	646.90	7.56	5.26	-5.04	< 30	-5.8	-3.9	-0.89	-0.0	99.80	7/2/9
3c	646.90	7.56	5.07	-5.48	< 30	-5.8	-3.9	-0.88	-0.0	99.80	7/2/9
6a	546.83	7.00	7.00	-6.76	30-70	-4.1	-1.8	-0.30	-0.0	99.75	4/2/4
6b	546.83	7.00	7.00	-6.61	30-70	-4.1	-1.8	-0.30	-0.0	99.75	4/2/4
6c	546.83	7.00	6.99	-6.58	30-70	-4.1	-1.8	-0.30	-0.0	99.75	4/2/4
ref. [32]	max. 500	max. 5.0									max. 10 / max. 5 / -
ref. [33]	Max. 500	Max. 5.6									

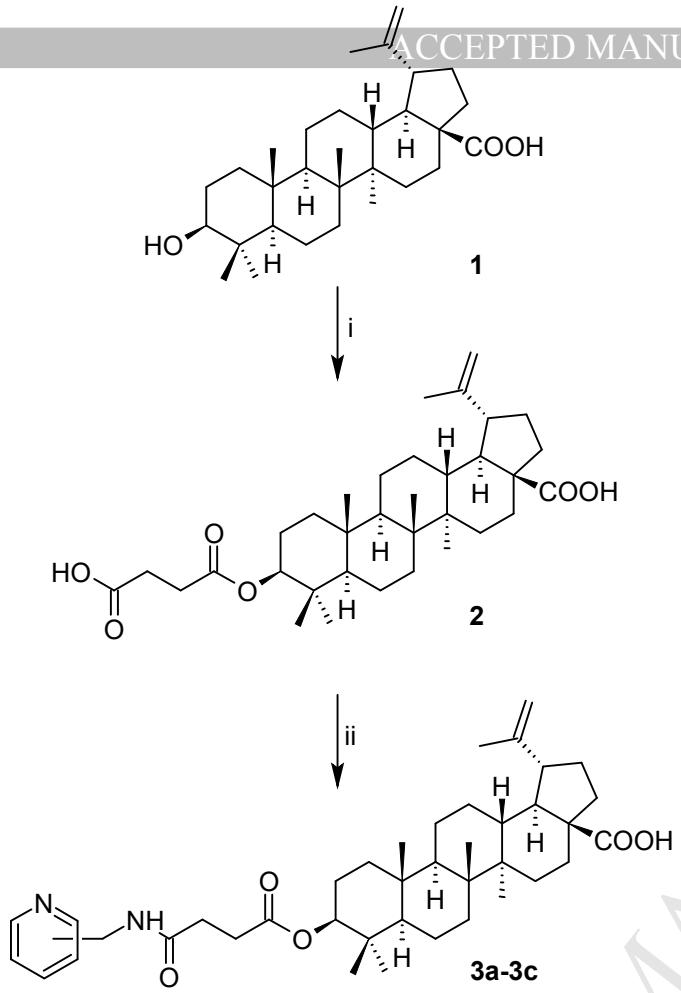
[a] log *P* – partition coefficient; log *D* – distribution coefficient; log *S* – predicted aqueous solubility; bioav. = bioavailability – the degree of availability of a chemical by the target tissue; log *PS* * $f_{u,\,brain}$ – the brain/plasma equilibration rate, the parameter that is a mathematical modeling parameter based on time required for reaching brain equilibrium; log *PS* – logarithm of the permeability-surface area coefficient; log *PB* – the extent of brain penetration parameter; log *BB* – a hybrid parameter determined by permeability, plasma and brain tissue binding, and active transport mechanism; PPB – plasma protein binding; H_{acc} / H_{don} / n.m.b. = number of H-bond acceptors / number of H-bond donors / number of movable bonds.

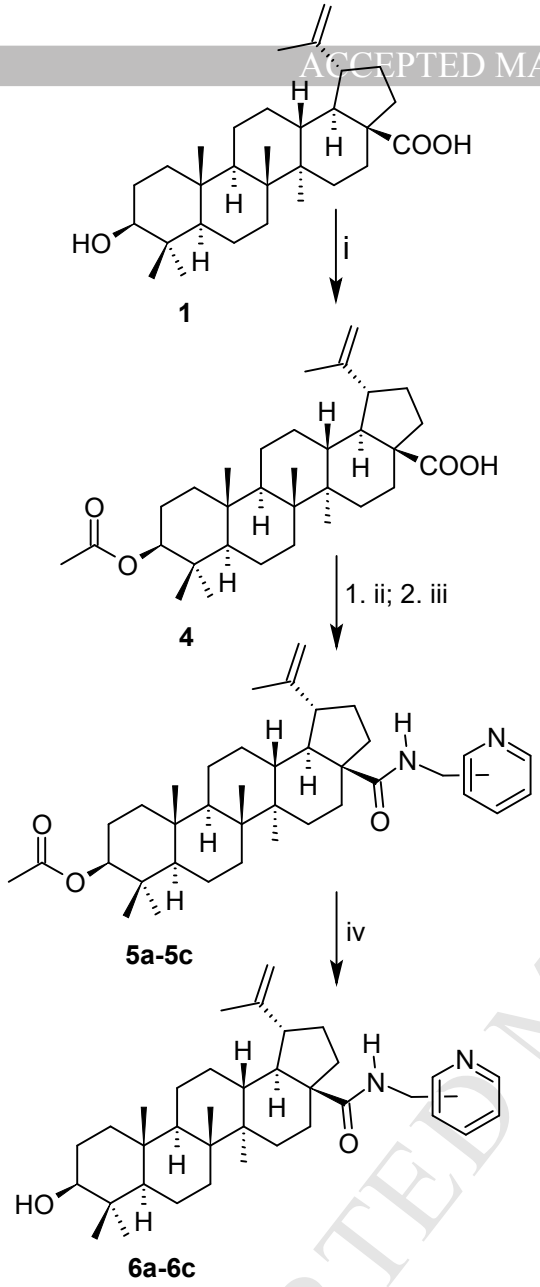

number of cells (%)
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
0+DMSO
0.5
2.5
12.5
25
3a
0.5
2.5
12.5
25
3b
0.5
2.5
12.5
25
3c
concentration of compound (μM)
G2/M
S
G0/G1


3a *
3b *
3c
number of apoptotic cells (%)
0
5
10
15
20
0
0+DMSO
0.5
2.5
12.5
25
concentration of compound (μM)


3a
3b
3c
0
0+
0.5
2.5
12.5
25
0.5
2.5
12.5
25
0.5
2.5
12.5
25
µM
PARP-1
caspase-7
pRb
Rb
pAKT Ser473
AKT
pp44/42 MAPK (Erk1/2)
(Thr202/Tyr204)
p44/42 MAPK (Erk1/2)
β-actin


3a *
3b *
3c *
relative caspase activity
0
2
4
6
8
10
12
14
16
18
0
0+DMSO
0.5
2.5
12.5
25
concentration of compound (μM)





Highlights

Picolyl amides of betulinic acid as antitumor agents causing tumor cell apoptosis

Uladzimir Bildziukevich, Lucie Rárová, David Šaman, and Zdeněk Wimmer

- Picolyl amides of betulinic acid cause human malignant melanoma cell apoptosis;
- Comparison of substitution at the C(3)-OH and C(17)-COOH on cytotoxicity studied;
- Facilitating cellular uptake and enhancing antitumor properties;
- Cytotoxicity of one of the target compounds on G-361 found with TI = 100.