European Journal of Medicinal Chemistry 145 (2018) 41–50

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Research paper

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

Uladzimir Bildziukevich [a, b], Lucie Rárová [c], David Šaman [d], 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, 16610 Prague 6, Czech Republic

ARTICLE INFO

Article history:
Received 1 November 2017
Received in revised form
13 December 2017
Accepted 30 December 2017
Available online 4 January 2018

Keywords:
Betulinic acid
Picolyl amine
Amide
Cytotoxicity
Therapeutic index

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.

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].

* Corresponding author. Institute of Experimental Botany AS CR, v.v.i., Isotope Laboratory, Vídeňská 1083, 14220 Prague 4, Czech Republic.
E-mail addresses: Zdenek.Wimmer@vscht.cz, wimmer@biomed.cas.cz (Z. Wimmer).

https://doi.org/10.1016/j.ejmech.2017.12.096
0223-5234/

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 °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,