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ARTICLE

Spermine amides of selected triterpenoid acids: Dynamic supramolecular systems formation influences cytotoxicity of the drugs

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Cancer has been a global disease of great importance, and a need for novel cytotoxic drugs has still been eminent. A series of spermine amides of several selected triterpene acids (betulonic, heterobetulonic, oleanolic, ursolic and platanic acid) has been synthesized to search for new cytotoxic and antimicrobial agents. The compounds have also been subjected to the investigation of their physico-chemical characteristics (ability to self-assemble), and to an *in silico* comparative calculation of their physico-chemical and ADME parameters. In the *in vitro* screening tests with several target compounds (**8a–8c** and **11c**), their cytotoxicity changed with the prolonged time, which effect appeared to be a result of a formation of dynamic supramolecular networks. This phenomenon is important in investigation of the effect of self-assembly on biological activity. The most important compounds in this series were spermine derivatives of heterobetulonic acid (**3b**) and ursolic acid (**8b**), showing cytotoxicity < 5 μM and < 10 μM , respectively, on all tested cancer cell lines. Comparable cytotoxicity was also displayed by **13b**, formerly a model compound prepared for testing of the synthetic procedures, the 1,2-diaminoethane derivative. The target compounds **3b** and **8b** displayed antimicrobial activity on *Staphylococcus aureus*, *Streptococcus mutans* and *Listeria monocytogenes* at a concentration 6.25 μM . Supramolecular characteristics of several compounds were documented by the TEM and SEM micrographs showing fibrous, partially helical, networks, and UV measurements showing changes in the intensity of UV signals, also indicating a formation of supramolecular systems.

Introduction

Phytochemicals in their variety represent a rich and important source of natural products capable of displaying their potential in the area of cancer chemotherapy.¹ Having dealt with a phytochemical research, we have already synthesized and investigated a series of spermine amides of betulonic acid,

searching for their pharmacological activity and supramolecular characteristics.^{2,3} The compounds showed their cytotoxicity to several cancer cell lines,² displayed antimicrobial activity,² as well as their ability to self-assemble in aqueous media and in organic solvents, showing generally fibrous supramolecular networks documented by AFM, SEM and TEM images, and supported by the DOSY NMR and UV-VIS measurements.³ Triterpenoid acids are mostly natural products available by extraction from different plant sources. Some of them are rarely present in natural sources, e.g., betulonic acid (**1a**) and heterobetulonic acid (**1b**) that are semisynthetic compounds. Betulonic acid (**1a**) is accessible by oxidation of betulin with the Jones reagent.⁴ Heterobetulonic acid (**1b**) can be prepared from heterobetulinic acid (first isolated from *Calyptanthus pallens*) or from betulin.⁴ Oleanolic acid (**4a**) was found in the leaves of *Olea europaea* (olive tree),⁵ and ursolic acid (**4b**) can be found in medicinal plants like *Rosmarinus officinalis* (rosemary) or *Glechoma hederaceae* (tunhoof).⁶ Platanic acid (**4c**) was found in *Syzgium claviflorum* (trumpet satinash),⁴ in bark of *Platanus hybrida* (London plane),⁷ in *Leptospermum scoparium* (New Zealand teatree),⁸ Brazilian plant *Eugenia moraviana*⁹ or *Melaleuca leucadendron* (weeping paperbark).¹⁰

Due to their structures, these plant products are almost insoluble in water. Regardless of this finding, they display different types of pharmacological effects because nature itself is able to form their conjugates, mostly with oligosaccharides, to increase bioavailability of final conjugates.⁴ Moreover,

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Electronic Supplementary Information (ESI) available: Analytical data and NMR spectra of the prepared compounds, and the UV spectra demonstrating self-assembly of the studied compounds are presented therein. See DOI: 10.1039/x0xx00000x

supramolecular characteristics of triterpenoid acids have potentially enhancing effect on mediating leukemia cell death.¹¹ Spermine belongs among polyamines present in all living cells.¹² Polyamines bring a positive charge at physiological pH value, due to which characteristics they are able to bind to the negatively charged DNA macromolecules. Generally, polyamines are engaged in any interaction of ionic character in living systems. Polyamines also neutralize negative charge of phosphates. During angiogenesis, the tumour cells produce growth factors of new vessels providing the tumour with nutrients. Polyamine conjugates are able to inhibit that vessel formation, and to cause tumour death.^{13,14}

Based on the results we obtained with spermine amides of betulinic acid, the most recent investigation in our team has been focused on spermine amides of several other selected triterpenoid acids to investigate similarity and/or difference. Besides oleanolic, ursolic and platanic acid (i.e., C(3)-hydroxy-triterpenoid acids), betulonic and heterobetulonic acid (i.e., C(3)-oxo-triterpenoid acids) were also included into this investigation. Its objectives were specified in five partial targets: (a) A preparation of a series of amides with the selected triterpenoid acids; (b) investigation of their cytotoxicity and antimicrobial activity; (c) investigation of their ability to form supramolecular networks; (d) investigation of the possible differences in supramolecular characteristics of amides derived from the C(3)-hydroxy- and C(3)-oxo-triterpenoid acids; and, (e) investigation and evaluation of the relationship between biological activity and self-assembly of the target compounds in screening assays against cancer cell cultures *in vitro*.

Experimental

General

The ¹H NMR and the ¹³C NMR spectra were recorded on a Bruker AVANCE 600 MHz spectrometer at 600.13 MHz and 150.90 MHz in CDCl₃ or CD₃OD, using tetramethylsilane (δ = 0.0) as internal reference. ¹H 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 ¹H and ¹³C signals 2D NMR ¹H,¹³C gHSQC and gHMBC spectra were measured using standard parameters sets and pulse programs delivered by producer of the spectrometer. 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 or a negative ESI mode. A 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.). Rapid analysis was made on TLC silica gel plates (Merck 60F₂₅₄) 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 in combination with a mobile phase formed by chloroform/methanol (100/0 to 95/5) mixtures. Triterpenoid

acids were purchased from Dr. Jan Šarek – Betulinines (www.betulinines.com), all other chemicals and solvents from regular commercial sources in analytical grade, and the solvents were purified by general methods before use. All analytical data of the prepared compounds are presented in the Supplementary material.

Synthetic procedures

Synthesis of 2a and 2b

Oxalyl chloride (1.54 mmol) was added to a solution of **1a** or **1b** (0.22 mmol) in DCM (CH₂Cl₂; 2 mL), and the mixture was stirred for 3 h at r.t. under argon. The solvent was evaporated to dryness to get crude acyl chloride, which was then dissolved in DCM (2 mL), and EDIPA (ethyl diisopropyl amine; 0.57 mmol) and *N*¹,*N*⁴,*N*⁹-tri-Boc-spermine (0.24 mmol) were added. The mixture was stirred at r.t. for 15 h. The solvent was evaporated, and the residue was purified by column chromatography, yielding the products **2a** or **2b** in 90-97 % yield.

Synthesis of 3a and 3b

A solution of HCl (g) in ethyl acetate (1 M; 3 mL) was added to a solution of **2a** or **2b** (0.067 mmol) in 1,4-dioxane, and the mixture was shaken at r.t. for 8 h. The solvents were evaporated and the residue was purified by column chromatography, yielding the products **3a** or **3b** (isolated as hydrochlorides) in 90-97 % yield.

Synthesis of 5a–5c

EDIPA (0.4 mL), DMAP (0.014 g; 0.114 mmol) and acetic anhydride (0.13 g; 1.268 mmol) were added to a solution of **4a**, **4b** or **4c** (0.2 g; 0.44 mmol) in THF (10 mL). The reaction mixture was heated in a water bath at 80 °C for 4 h. Then the mixture was cooled to r.t., distilled water (10 mL) was added, and stirring continued for an additional 1 h. Extraction was made by chloroform (5 × 10 mL), the extract was dried over sodium sulphate. After removing the inorganics by filtration, the solvent was evaporated and the residue was purified by column chromatography. The products **5a**, **5b** or **5c** were obtained in 88-93 % yields.

Synthesis of 6a–6c

Oxalyl chloride (1.101 mL; 2.02 mmol) was added to a solution of **5a**, **5b** or **5c** (0.157 g; 0.315 mmol) in DCM (6 mL), and the mixture was stirred at r.t. under argon atmosphere for 3 h. The solvent was evaporated to dryness to get crude acyl chloride, which was then dissolved in DCM (6 mL), and EDIPA (0.143 mL; 0.819 mmol) and *N*¹,*N*⁴,*N*⁹-tri-Boc-spermine (0.174 g; 0.347 mmol) were added. The mixture was stirred at r.t. under argon atmosphere for 15 h. The solvent was evaporated, and the residue was purified by column chromatography, yielding the products **6a**, **6b** or **6c** in 90-97 % yields.

Synthesis of 7a–7c

LiOH·H₂O (0.056 g; 1.344 mmol) was added to a solution of **6a**, **6b** or **6c** (0.261 g; 0.266 mmol) in methanol (26 mL), and the mixture was stirred at 80 °C under argon atmosphere for 24 h.

Scheme 1 Synthetic procedure. Reagents used: i: (a) oxalyl chloride, DCM; (b) N^1, N^4, N^9 -tri-Boc-spermine, EDIPA, DCM; ii: HCl (g) in ethyl acetate; iii: Ac_2O , EDIPA, DMAP, THF; iv: $\text{LiOH} \cdot \text{H}_2\text{O}$, MeOH; v: succinic anhydride, DMAP, pyridine; vi: N^1, N^4, N^9 -tri-Boc-spermine, T3P, pyridine.

Then the mixture was filtered, the inorganic salts were washed with chloroform, and the solvents were finally evaporated. The residue was purified by column chromatography yielding the products **7a**, **7b** or **7c** in 80-90 % yields.

Synthesis of **8a–8c**

A solution of HCl (g) in ethyl acetate (1 M; 3 mL) was added to a solution of **7a**, **7b** or **7c** (0.085 g; 0.09 mmol) in 1,4-dioxane, and the mixture was shaken at r.t. for 17 h. Then the reaction mixture was filtered, and the solid phase was washed with ethyl acetate and ether. The solid phase collected on the sintered glass was dissolved in methanol, and then the solvent was evaporated. The residue was purified by column chromatography, yielding the products **8a**, **8b** or **8c** (isolated as hydrochlorides) in 90-97 % yields.

Synthesis of **9a–9c**

Succinic anhydride (0.28 g; 2.8 mmol) and DMAP (0.032 g; 0.26 mmol) were added to a solution of **4a**, **4b** or **4c** (0.2 g; 0.44 mmol) in dry pyridine (2 mL). The reaction mixture was stirred at r.t. in an inert atmosphere for 7 days. It was poured onto ice, washed with chloroform, and the pH value was adjusted to 7.0 using diluted hydrochloric acid. The organic layer was extracted with chloroform (5 × 30 mL), and dried over sodium sulphate. Then, the solvent was evaporated and the residue was purified by column chromatography, yielding the products **9a**, **9b** or **9c** in 89-92 % yields.

Synthesis of **10a–10c**

N^1, N^4, N^9 -tri-Boc-Spermine (0.197 g; 0.392 mmol) and T3P (1-propanephosphonic anhydride; 1.05 mL; 3.354 mmol) were added to a solution of **9a**, **9b** or **9c** (0.196 g; 0.352 mmol) in dry pyridine (4 mL), and the solution was stirred at r.t. under argon atmosphere for 20 h. Then, saturated solution of NaHCO_3 (4 mL) was added, and the mixture was stirred for an additional 1 h. Extraction was made by chloroform (5 × 10 mL), the extract was dried over sodium sulphate, and then the solvent was evaporated. The crude residues were purified by column chromatography, yielding the products **10a**, **10b** or **10c** in 70-80 % yields.

Synthesis of **11a–11c**

A solution of HCl (g) in ethyl acetate (1 M; 6.9 mL) was added to a solution of **10a**, **10b** or **10c** (0.239 g; 0.23 mmol) in 1,4-dioxane, and the mixture was shaken at r.t. for 53 h. The reaction mixture was filtered, and the solid phase was washed with ether on the sintered glass. Then the solid phase was dissolved in methanol, the solvents were evaporated, and the residue was purified by column chromatography, yielding the products **11a**, **11b** or **11c** (isolated as hydrochlorides) in 90-97 % yields.

Synthesis of **13b**

(a) Using the synthetic procedure described for the synthesis of **2a** and **2b**, and using 1-Boc-1,2-diaminoethane, **12b** was prepared in a 91 % yield.

Scheme 2 Synthetic procedure for **13b**. Reagents used: i: (a) oxalyl chloride, DCM; (b) 1-Boc-diaminoethane, EDIPA, DCM; ii: HCl (g) in ethyl acetate.

Fig. 1 TEM (top) and SEM (bottom) micrographs: Compound **8a** formed low-contrast agglomerates from methanol in TEM (top left) and fibrous networks in 1-butanol in SEM (bottom left), while compound **8b** formed partly helical structures from methanol in TEM (top right) and in 1-butanol in SEM (bottom right).

(b) Using the synthetic procedure described for the synthesis of **3a** and **3b**, **13b** (isolated as the hydrochloride) was prepared in an 80 % yield.

Analytical and biological procedures

Transmission electron microscopy (TEM)

The TEM micrographs were taken on a Tecnai G2 Spirit Twin 120 kV (FEI, Czech Republic). The studied compounds (2 μ L droplets; methanol solution, concentration 5 mg·mL⁻¹), were dropped onto a copper TEM grid (300 mesh), which was coated with thin, electron-transparent carbon film. After 2 min the rest of the solution was removed by touching the bottom of the grid with filtering paper. Then the sample was left to dry completely at the ambient temperature. The transmission electron micrographs were obtained using bright field imaging at electron accelerating voltage of 120 kV (Fig. 1).

Scanning electron microscopy (SEM)

Gel structures were imaged with a Zeiss Sigma VP scanning electron microscope (acceleration voltage of 1.5 keV) to investigate surface morphology of formed gels (Fig. 1). Prior to

the imaging Pd/Pt coating was sputtered onto sample under vacuum conditions with LEICA EM ACE600 (35 mA, 4 nm thickness). Gel samples (1.0 %; 10 μ L) prepared in selected solvents were put onto the carbon tape placed on an aluminum stub and left to dry at room temperature.

UV spectrometry

UV spectra were measured on a Specord 210 spectrometer (Jena Analytical, Germany) in the wavelength range of 200–400 nm. First, stock solutions of the studied compounds were prepared at a concentration of 1 mg·mL⁻¹, in water. A series of water/acetonitrile mixtures were then prepared starting from water/acetonitrile ratio 0/100 up to 100/0 in 10 % steps. The stock solutions of the studied compounds (0.15 mL) were added separately to each vial containing water/acetonitrile mixtures (3 mL), and the UV spectra recorded in the wavelength range of 200–400 nm. Spectra were recorded every 24 h for 8 days. The resulting sets of spectra of were worked-up and the changes are shown in the Supplementary Material (Fig. S1).

Cytotoxicity tests

In the cytotoxicity screening tests, cancer cell lines of T-lymphoblastic leukemia (CEM), breast carcinoma (MCF7), cervical carcinoma (HeLa), malignant melanoma (G-361) were used, all of which were obtained from the European Collection of Authenticated Cell Cultures (ECACC, London, UK). Human foreskin fibroblasts (BJ) were purchased 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 % foetal bovine serum, 2mM L-glutamine, and 1 % penicillin-streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5 % CO₂ in a humid environment. Cells were sub-cultured twice or three times a week using the standard trypsinization procedure. Description of the experimental procedure used in cytotoxicity screening tests was already published.² IC₅₀ values of cytotoxicity obtained with the target compounds **3a**, **3b**, **8a–8c**, **11a–11c** and **13b** are shown in Table 1.

Antimicrobial tests

Standard reference bacterial strains, *Staphylococcus aureus* CCM 3953, *Enterococcus faecalis* CCM 4224, *Pseudomonas aeruginosa* CCM 3955 and *Escherichia coli* CCM 3954 from the Czech Collection of Microorganisms (CCM), Faculty of Science, Masaryk University Brno, were used for testing of antimicrobial activity. Bacterial suspensions were prepared as follows. Bacterial strains were inoculated on blood agar (Trios, Czech Republic), and incubated for 24 h at 37 °C. Three well isolated colonies were dissolved in 2 mL of Mueller Hinton broth (Himedia) and incubated for 2 h at 37 °C. Antimicrobial efficacy was tested by standard dilution micro-method and minimal inhibition concentrations (MIC) were determined. Compounds were diluted with Mueller Hinton broth (Himedia), and tested in microtiter plates. Mueller Hinton broth was used as a cultivation medium. Plates were inoculated into a standard

Table 1. Cytotoxicity (IC₅₀ [μM]) of **3a**, **3b**, **8a–8c**, **11a–11c** and **13b** in four cancer cell lines and normal human fibroblasts after 72 h ^a

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Compound	MW ^b	Cytotoxicity (IC ₅₀ [μM])				
		CEM	MCF7	HeLa	G-361	BJ
3a	639.03	31.5 ± 9.8 <i>24.6/42.6/27.6</i>	33.4 ± 6.6 <i>40.7/27.8/31.8</i>	34.3 ± 0.0 <i>34.3/34.3</i>	43.2 ± 4.2 <i>45.6/45.7/38.4</i>	12.0 ± 1.7 <i>10.8/13.2</i>
3b	639.03	2.8 ± 0.6	3.3 ± 0.1	4.8 ± 1.9	3.4 ± 1.0	3.7 ± 1.1
8a	641.04	25.1 ± 8.2 <i>18.8/31.8/32.6/17.2</i>	11.2 ± 1.3 <i>12.1/10.2</i>	27.0 ± 0.2 <i>26.8/27.1</i>	27.5 ± 0.1 <i>27.6/27.4</i>	12.1 ± 1.3 <i>11.2/13.0</i>
8b	641.04	7.5 ± 1.6	6.3 ± 0.1	7.9 ± 1.1	8.1 ± 1.3	6.4 ± 0.5
8c	643.00	33.3 ± 4.0 <i>36.1/30.5</i>	45.9 ± 1.1 <i>45.1/46.6</i>	31.4 ± 0.7 <i>31.9/30.9</i>	44.7 ± 1.0 <i>45.4/44.0</i>	43.3 ± 4.3 <i>40.2/46.3</i>
11a	741.10	29.9 ± 1.9 <i>31.2/28.5</i>	46.1 ± 0.7 <i>46.6/45.6</i>	34.0 ± 3.3 <i>36.3/31.7</i>	33.2 ± 1.4 <i>32.2/34.2</i>	18.5 ± 1.3 <i>17.6/19.4</i>
11b	741.10	45.1 ± 4.5 <i>48.3/41.9</i>	>50 <i>>50/>50</i>	>50 <i>>50/>50</i>	20.8 ± 3.0 <i>37.2/22.9/18.7</i>	45.2 ± 4.5 <i>42.0/48.3</i>
11c	743.07	28.0 ± 4.7 <i>31.3/24.6</i>	>50 <i>46.6/>50</i>	>50 <i>>50/>50</i>	>50 <i>>50/48.7</i>	46.0 ± 0.8 <i>46.6/45.4</i>
13b	496.78	8.8 ± 1.2	9.0 ± 0.1	8.2 ± 1.1	2.0 ± 0.2	12.9 ± 0.0

^a Numbers in italics represent the values of cytotoxicity (IC₅₀ [μM]) calculated for each replicate of the biological experiment. Only several tested compounds displayed this kind of behaviour (cf. the main text); ^b Molecular mass calculated without hydrochlorides.

quantity of microbe (106 CFU·mL⁻¹). The final concentration of the tested compounds ranged from 0 to 50 μg·mL⁻¹. MIC/MBC values were determined after 24 h of incubation at 37 °C.

Results and Discussion

Synthesis

The synthetic protocol used for the synthesis of the target compounds was identical for the compounds **3a**, **3b** and **8a–8c** (Scheme 1). Small modification was applied for **11a–11c** (Scheme 1) due to the incorporation of the spacer – succinic acid unit. The procedure had already been used earlier in our research, and it has been used here with several improvements incorporated on the basis of our experience.² Thus, **1a** or **1b** were converted into their acyl chlorides using oxalyl chloride in DCM, and the crude acyl chlorides were allowed to react with *N*¹,*N*⁴,*N*⁹-tri-Boc-spermine to get **2a** or **2b**. The protecting Boc-groups were removed by HCl (g) in ethyl acetate, yielding the target compounds **3a** or **3b**. Using **4a–4c** as starting triterpenes, their C(3)-OH group had to be protected by forming the acetates **5a–5c** in the first step. Then the procedure described for preparation of **2a** and **2b** was applied to get **6a–6c**, followed by a removal of the protecting acetate group by a methanolysis using lithium hydroxide to get **7a–7c**, from which the protecting Boc-groups were removed as above to get the target **8a–8c**. Using **4a–4c** to prepare the target **11a–11c**, no protection of C(17)-COOH is necessary due its low reactivity, caused by its hindered location between the rings D and E. Then, the C(3)-OH can be directly esterified with succinic anhydride in pyridine in the presence of DMAP to prepare **9a–9c**. To get **10a–10c**, T3P as condensing agent was applied to enable amide bond formation between **9a–9c** and *N*¹,*N*⁴,*N*⁹-tri-Boc-spermine. The target **11a–11c** were prepared using the identical procedure described above for preparation of **3a** or **3b**. The procedure

described for the synthesis of **2a** and **2b** was originally elaborated to get **12b** as a model compound to set the synthetic procedure, using 1-Boc-1,2-diaminoethane for formation of the amide bond. Removal of the Boc-protecting group of **12b** by HCl (g) in ethyl acetate afforded **13b** (Scheme 2). Even if the compounds **12b** and **13b** were prepared for setting the details in the synthetic procedure, later on, **13b** was also subjected to the cytotoxicity screening tests, which resulted in surprisingly good cytotoxicity found.

Pharmacological activity, supramolecular self-assembly and their relationship

All target compounds showed cytotoxicity *in vitro*, and the values were high for **3b**, **8b** and **13b**. However, the cytotoxicity of all target compounds towards the cancer cell lines CEM, MCF7, HeLa and G-361, and towards the reference human fibroblasts (BJ) was comparable, which makes all those compounds less valuable than expected (Table 1). In contrary, the results obtained with cytotoxicity of **13b**, synthesized as the model compound in optimizing the reaction conditions, were the most promising, because **13b** showed high cytotoxicity (IC₅₀ = 2.0 μM) towards G-361 cancer cell line (therapeutic index, TI = 6). Comparable cytotoxicity values in G-361, found with **13b**, were observed only with **3b** and **8b**, however, those compounds showed TI = 1 (Table 1).

Comparing the current results of cytotoxicity of the target compounds (Table 1) with those published earlier with the corresponding amides of betulinic acid,² compounds **3b**, **8b** and **11b** showed comparable spectrum of cytotoxicity with the corresponding amides of betulinic acid. The disadvantage of **3b** and **8b** consists in their high cytotoxicity in normal human fibroblasts, comparable with their activity in the target cancer cell lines, which was also found for the amides of betulinic acid earlier.² The cytotoxicity of all other target compounds was surprisingly low. Certain selectivity was found with **11b** (for G-

Table 2. Physico-chemical and ADME parameters of the compounds **3a**, **3b**, **8a–8c**, **11a–11c** and **13b** calculated *in silico* using the ACD/iLabs software.¹⁹ DOI: 10.1039/C9TB01957J

Compd. or recom. range	MW ^a	Physico-chemical and ADME parameters ^b									
		log <i>P</i>	log <i>D</i> (pH 7.4)	log <i>S</i> (pH 7.4)	bioav. [%]	log <i>PS</i> [*] <i>f</i> _{u, brain}	log <i>PS</i>	log <i>PB</i>	log <i>BB</i>	PPB [%]	H _{acc} / H _{don} / n.m.b.
3a	639.01	+5.74	+0.42	−0.31	30–70	−4.8	−2.7	+0.70	+0.64	96.52	6/5/14
3b	639.01	+7.15	+1.83	−0.37	30–70	−5.8	−3.6	+0.68	+0.68	97.00	6/5/13
8a	641.03	+7.34	+2.02	−0.26	30–70	−6.2	−4.0	+0.64	+0.68	97.26	6/6/13
8b	641.03	+7.20	+1.88	−0.48	30–70	−6.1	−3.8	+0.65	+0.68	97.18	6/6/13
8c	643.00	+4.64	−0.67	+0.19	30–70	−4.6	−2.7	+0.55	+0.59	95.46	7/6/14
11a	741.10	+7.36	+3.35	−4.92	< 30	−6.5	−4.3	+0.42	+0.50	98.36	9/6/18
11b	741.10	+7.23	+3.22	−5.02	< 30	−6.4	−4.2	+0.45	+0.50	98.22	9/6/18
11c	743.07	+4.58	+0.57	−3.55	< 30	−4.9	−3.0	+0.49	+0.47	95.85	10/6/19
13b	496.78	+6.94	+5.06	−4.44	30–70	−5.0	−2.8	+0.43	+0.50	98.32	4/3/3
recom. range	180/500– 550	−0.4/+5.6	–	−6.5/+0.5	–	–	–	−1.5/+1.5	−3.0/+1.2	–	10/5/–

^a Molecular mass calculated without hydrochlorides; ^b 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; limits given in literature^{20,21} for several physico-chemical parameters: MW max. 500 (ref.^{20,21}); log *P* max. 5.0 (ref.²⁰); max. 5.6 (ref.²¹); n.m.b. max. 10 / max. 5 / – (ref.²⁰).

361) and **11c** (for CEM; Table 1). Comparing **3a** and **3b**, big difference in their cytotoxicity was observed, **3b** was more cytotoxic than **3a**. Comparing **3a** and **3b** versus **8a–8c**, i.e. the target compounds bearing amide bond at the C(28)-carboxyl group, certain analogy in cytotoxicity can be seen according to the structural analogy of these compounds. Thus, cytotoxicity values of **3a** and **8c** are similar, as well as those of **3b** and **8b**. Cytotoxicity values of **8a** seem to be between those of **8b** and **8c**. Comparing **8a–8c** versus **11a–11c**, **8a–8c** were found to show higher cytotoxicity values, however, **11a–11c** showed certain selectivity, even if with lower cytotoxicity (see above and in Table 1).

During the cytotoxicity data measurement and evaluation, irregularity in cytotoxicity of **3a**, **8a** (most remarkable) and **11b** was observed (Table 1). The stock solution of each tested compound was prepared at the very beginning of the experiments. These solutions were stored in a refrigerator before being used in the second, the third, and, occasionally, in the fourth biological replicate. Each experiment took 5 days, and on the day 8, each following biological replicate started using the stock solution of the tested compound kept in refrigerator up to that moment. The values of cytotoxicity of **3a** on CEM and MCF7, **8a** on CEM and **11b** on G-361 showed varying values, which can be explained by a formation of dynamic supramolecular structures. Comparing **3a** and **3b** versus **8a–8c** again from this point of view, no influence of supramolecular structures formation was observed with **3b** and **8b**. In contrary, **3a**, **8a** and **8c** showed important irregularity in their cytotoxicity values. Compounds **11a–11c** showed certain selectivity in displaying cytotoxicity values irregularity as well.

The formation, existence, and dynamic change from one supramolecular form to another one indicates the advantages and disadvantages of different supramolecular structures formed in those solutions and/or after application to the cancer cell cultures. The supramolecular systems differ in their

bioavailability, which results in apparently illogical system of cytotoxicity values. However, experiments with supramolecular drug release forms have already been known.^{11,15–17} Higher efficacy of self-assembled betulinic acid in comparison with non-assembled one had already been described.¹¹ Herewith the results found with the dynamic supramolecular systems formed by the drugs themselves in convenient media and conditions are presented and discussed above.

Antimicrobial activity at lower concentration than 10 μM was found only for **3a** and **8a**. Their MIC/MBC values were 6.25 μM for *Staphylococcus aureus*, *Streptococcus mutans* and *Listeria monocytogenes*, identically for both compounds (**3a** and **8a**) and for all three microorganisms. No influence of self-assembly was observed during the antimicrobial activity studies.

Self-assembly of the target compounds was studied by the UV measurements and by recording TEM micrographs. When self-assembly was studied by UV measurements, hyperchromic shifts were observed in certain time intervals. This finding indicates a formation of supramolecular networks as a result of H-bond formation on the basis of amine groups (hydrogen atoms) and carboxyl groups (carbonyl oxygen)¹⁸ (Fig. S1). To prove a formation of supramolecular network in a visible form, TEM and SEM measurements were made, and the TEM and SEM micrographs were recorded. We have not observed a formation of supramolecular networks by the spermine amides (**3a** and **3b**) derived from the 3-oxoacids (**1a** and **1b**) under the tested conditions. However, based on the cytotoxicity data (cf. Table 1) it seems **3a** forms complex systems, changing dynamically in time. Taking cytotoxicity data for **3a** on CEM and MCF7, the changing values of cytotoxicity obtained in different time since the preparation of the solution for application to the cancer cells (time 0 h), show big differences (cf. numbers in italics in Table 1). The supramolecular networks were identified with polyamine derivatives of 3-hydroxyacids. The compound **8a** seemed to form loose agglomerates or loose network

structures with low contrast in TEM (from methanol), but it formed clear fibrous networks in SEM when dissolved in 1-butanol before taking micrographs (Fig. 1). The compound **8b** formed partly helical structures and fibrous networks visible both in TEM and SEM micrographs (Fig. 1). Comparing again the cytotoxicity data (Table 1), differences in the biological activity can be recognized for CEM (**8a**, **8c**, **11b** and **11c**) MCF7 (**8a**), HeLa (**11a**) and G-361 (**8b** and **11b**) cancer cell lines. CEM cell lines seem to be the most sensitive cell line to the formed supramolecular networks of the target compounds. Generally, the cytotoxicity values given in Table 1 should be understood as average values calculated based on the values changing according to the time of application since the time of preparation of the solutions for application to the cancer cells. Therefore, the real cytotoxicity of the target compounds can be higher than the average value, if applied in the right time.

In silico calculated physico-chemical and ADME parameters

The experimental data determined for the target compounds **3a**, **3b**, **8a–8c**, **11a–11c** and **13b** were compared with the *in silico* calculated physico-chemical and ADME parameters, using ACD/iLabs software and databases.¹⁹ The calculated physico-chemical parameters were further compared with the Lipinski rule of five²⁰ and with the Ghose rule.²¹ 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. We described those rules and their importance in designing pharmacologically prospective structures recently.²² The target compounds **3a**, **3b**, **8a–8c**, **11a–11c** and **13b** do not correspond to the whole range given for any of the Lipinski²⁰ and Ghose²¹ rules, except of several parameters (Table 2). Despite those facts, all target compounds display cytotoxicity, and especially **3b**, **8b** and **13b** display relatively high cytotoxicity (cf. Table 1).

The most important molecular descriptors are the partition coefficient ($\log P$) and the distribution coefficient ($\log D$).²³ 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 dependence on the pH of the matrix. Hydrophobic drugs are then preferentially distributed to hydrophobic compartments (e.g., lipid bilayer of cells), while hydrophilic drugs are preferentially distributed to hydrophilic compartments (e.g., blood serum). The distribution coefficient is a pH dependent value, and, therefore, the value at pH = 7.4 (the physiological pH value of blood serum) is of particular importance (Table 2). Thus, $\log P$ expresses a ratio of concentrations of non-ionized compound between two phases, non-polar (octanol) and polar (water), while $\log D$ expresses the ratio of the sum of the concentrations of all forms of the compound (ionized and non-ionized) in each of the two phases. In pharmacology, $\log P$ and $\log D$ indicate how easily the drug can reach its intended target in the body, how strong its effect will be once it reaches its target and how long it will remain in

the body in an active form. The $\log P$ values calculated for **3b**, **8b** and **13b** show that their $\log P$ values do not appear within the interval given by the Lipinski²⁰ and Ghose²¹ rules (Table 2), and, theoretically, should display no or low cytotoxicity. Standard range for $\log P$ is $-0.4/+5.6$.²⁴ However, another supportive parameter is the predicted aqueous solubility, $\log S$, in which S (in mol·dm⁻³) is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid, and it is a pH dependent parameter. Standard range for $\log S$ at pH 7.4 is $-6.5/+0.5$.²⁴ All three highly cytotoxic compounds **3b**, **8b** and **13b** show the $\log S$ values within this range (Table 2).

The blood brain barrier (BBB) and plasma protein binding are two of the important factors, affecting distribution of the compound in the human body. Several parameters assist in evaluation of each potential drug for its BBB transport.²⁵ The rate of brain penetration, $\log PS$, is a logarithm of the permeability-surface area coefficient that measures the ability of a drug to cross the BBB and 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). Most of the current drugs show the $\log PB$ value up to +2 (active on CNS) or down to -2 (inactive on CNS).²⁴ The $\log PB$ values calculated for not only **3b**, **8b** and **13b** but for all target compounds of this series indicate that the values calculated for these compounds are within the given range, however the compounds will be inactive on CNS (Table 2). However, the complex of the BBB parameters is completed by the brain/plasma equilibration rate, expressed as $\log PS^*f_{u,brain}$ that is a mathematical modelling 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 $-3.0/+1.2$.^{24,25} The $\log BB$ values calculated for all target compounds appear in the required range of this parameter. Neither of the target compounds seem to be suitable for treating neurodegenerative diseases. A supportive parameter, plasma protein binding (PPB) gives calculated quantity of a drug bound to a protein. The compounds **11c**, **8c** and **3a** show real protein binding activity. Another important ADME parameter is bioavailability. Among the prepared compounds, the calculated bioavailability appears in the range 30–70 % for all compounds except for **11a–11c**. The experimental data for all the studied compounds are in correspondence with this calculation.

Conclusions

Prepared cytotoxic amide derivatives of selected triterpenoid acids were subjected to a detailed investigation. Their antimicrobial activity and cytotoxicity were tested and studied *in vitro*. Compounds **3b** and **8b** displayed antimicrobial activity

on *Staphylococcus aureus*, *Streptococcus mutans* and *Listeria monocytogenes* at a concentration of 6.25 μM . The same compounds showed high cytotoxicity towards all tested cancer cell lines, but they showed high activity in the reference human fibroblasts as well. Compound **13b** showed the highest cytotoxicity from the studied compounds in the human malignant melanoma cell line G-361 (TI = 6). The experimentally observed cytotoxicity data were supported by the *in silico* calculated physico-chemical and ADME parameters, which appeared for the compounds **3b**, **8b** and **13b** in the recommended ranges for those parameters in the majority of studied parameters. According to the calculated data, compounds **11c**, **8c** and **3a** show real protein binding activity. Moreover, due to the ability to self-assemble, several compounds of this series displayed differences in cytotoxicity values, if the stored solutions of the tested compounds were applied to the cancer cell lines in different time since its preparation. Supramolecular systems formed are not static but dynamic systems with properties changed in time. Their bioavailability values are different, and, therefore their tests resulted in different values of cytotoxicity in different biological replicates of the experiments. Therefore, irregularity in cytotoxicity values of **3a**, **8a** and **11b** was observed, caused by their dynamic ability to self-assemble into the aggregates, the bioavailability of which changed based on the supramolecular structure formed. The most remarkable changes were observed in tests of **8a** and **3a** in CEM, and **11b** in G-361. Phenomenon similar to this one had already been observed, and it should be investigated in more details in a future. Self-assembly of several compounds of this series measured under different conditions that are conditions for cytotoxicity screening tests proved a formation of supramolecular networks. Compound **8a** formed low-contrast agglomerates or networks, while **8b** formed partly helical structures according to the results of the TEM imaging.

Conflicts of interest

There are no conflicts to declare.

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Graphical Abstract

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Cytotoxicity data: **8a**: IC_{50} [μ M]: 18.8 / 31.8 / 32.6 / 17.2 (25.1 ± 8.2), dynamic self-assembly, low-contrast networks (TEM), cytotoxicity values varied according to the supramolecular system formed;
8b: IC_{50} [μ M]: 7.5 ± 1.6 , dynamic self-assembly not detected, partly helical structures (TEM).