

Conference paper

Olga N. Zefirova*, Evgenia V. Nurieva, Birgit Wobith, Svetlana Schulz,
Nikolay A. Zefirov and Sergei A. Kuznetsov

Novel colchicine conjugate with unusual effect on the microtubules of cancer cells

<https://doi.org/10.1515/pac-2019-1215>

Abstract: Colchicine derivative bearing substituted bispidine moiety, namely N-{7-(3,7-Di-(tert-butoxycarbonyl)-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-yl)-oxy-7-oxoheptanoyl}-N-deacetylcolchicine, was synthesized and tested for its effect on the net of microtubules (MT) in lung cancer cells A549. The compound induced not only MT depolymerization but stimulated the formation of small tubulin aggregates and long tubulin fibrils localized mainly around nuclei. The assemblies were morphologically different from tubulin clusters induced by structurally related anticancer agent tubuloclustin. The biotests data demonstrate that the depolymerization takes place for both pure tubulin and tubulin in cellulo, while fibrils are formed only in the cells. The research data of structure–activity relationship for several similar colchicine derivatives synthesized in the work give evidence for the proposition that the initial conjugate may interact not only with tubulin and MT in the cells, but also with MT-associated proteins, involved in the process of tubulin polymerization. The ability to affect simultaneously MAP – tubulin interactions opens attractive prospects in the design of novel anticancer agents.

Keywords: 3,7-diazabicyclo[3.3.1]nonane; carcinoma A549; colchicine; Mendeleev-21; tubulin; tubuloclustin.

Introduction

Cell dimeric protein α,β -tubulin is a validated molecular target for many anticancer agents [1]. It plays an important role in the formation of cytoskeleton and in the process of cell division, when tubulin polymerizes to microtubules (MTs) [2]. Different types of atypical tubulin assemblies compose under special conditions or in the presence of some metal ions or small molecules [3]. The type of particular assembly induced by tubulin-targeted anticancer drug depends upon its binding site at the protein. Thus, binding of taxol at β -subunit leads to the formation of MT bundles [4]. Ligands of vinca domain at the interface of two neighbor dimers may cause both depolymerization of MTs with the following formation of tubulin paracrystals [5] and assembly to rings sheets etc. (e.g. phalloidin A, dolastatin 10) [6].

Article note: A collection of invited papers based on presentations at 21st Mendeleev Congress on General and Applied Chemistry (Mendeleev-21), held in Saint Petersburg, Russian Federation, 9–13 September 2019.

***Corresponding author: Olga N. Zefirova**, Department of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russian Federation; and Institute of Physiologically active compounds, Russian Academy of Sciences, 142432 Chernogolovka, Noginsk Area, Moscow Region, Russian Federation, Tel.: +74959391243, E-mail: olgaz@med.chem.msu.ru

Evgenia V. Nurieva: Department of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russian Federation
Birgit Wobith, Svetlana Schulz and Sergei A. Kuznetsov: Institute of Biological Sciences, University of Rostock, D-18059 Rostock, Germany

Nikolay A. Zefirov: Department of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russian Federation; and Institute of Physiologically active compounds, Russian Academy of Sciences, 142432 Chernogolovka, Noginsk Area, Moscow Region, Russian Federation

The majority of anticancer agents interacting with colchicine binding site (located in β -tubulin at the intradimeric interface of the heterodimer) under normal conditions are causing depolymerization of MTs only [7]. However, some ligands of colchicine domain can in addition stimulate the formation of atypical tubulin assemblies [8–16]. Many of these ligands represent colchicine (**1a**, Fig. 1) derivatives, able to provide hydrophobic contacts with α -tubulin subunit [10–14]. Tubuloclustin (**1b**) is a typical example of such compound: it initiates MT depolymerization with the following formation of tubulin clusters [10, 14]. Importantly, tubuloclustin is much more cytotoxic to cancer cells than colchicine and has better toxicological profile *in vivo* [10, 11].

Structure – activity relationship in a series of tubuloclustin analogs indicates that the volume of terminal substituent plays a crucial role in clustering [9, 10, 16]. Thus, isopropyl derivative **1c** (Fig. 1) stimulates “point-like” clusterization, while conjugates with voluminous and branched moieties, like adamantane and its derivatives stimulate the formation of long “wavy” tubulin clusters [9, 16]. The strength of clustering effect of the compounds correlates with their cytotoxicity to cancer cells and this fact makes interesting additional studies in the field. In the present work we investigated whether an essential enhancement of the bulkiness and branching of the terminal substituent in tubuloclustin would lead to increased clusterization and cytotoxicity. We synthesized and tested novel tubuloclustin analogue – compound **2a** (Fig. 2). Unexpectedly, its action on tubulin was qualitatively different: in addition to small tubulin clusters the compound induced the formation of long straight filamentous tubulin structures (Fig. 3, *vide infra*). This observation stimulated synthesis of compounds **2b–d** to determine the structural requirements necessary for manifestation of the unusual effect.

Target compound **2a** was synthesized as depicted on Scheme 1. Initial ketone **3a** was obtained using a ring opening reaction in 5,7-dimethyl-1,3-diazaadamantane-6-one by action of Boc_2O in the presence of HSO_3NH_2 and then reduced to the corresponding alcohol **4a** [18]. The alcohol **4a** was subjected to esterification with polyanhydride of pimelic acid [19] to give ester **5a**, and reaction of the latter with N-deacetylcolchicine [20] in the presence of EEDQ led to target conjugate **2a**.

The action of compound **2a** on the network of MTs in human lung carcinoma A549 cells was studied by immunofluorescence microscopy and gave as it was mentioned above the unexpected results (see Table 1 and Fig. 3). The morphological effect caused by **2a** on cellular tubulin was different from that of both tubuloclustin and colchicine (Fig. 3b,c). At 10 μM of **2a** the pattern observed represented small aggregates and tubulin filaments localized mainly around nuclei in about 50 % of the cells (Fig. 3g). At 100 μM of **2a** these

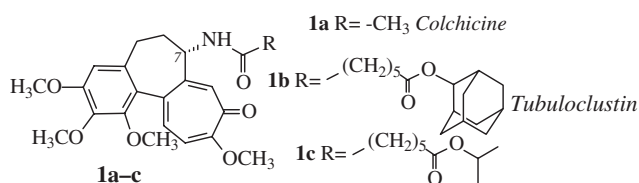


Fig. 1: Structures of colchicine (**1a**) and its derivatives **1b** and **1c**. Compound **1b** (tubuloclustin) stimulates the formation of long tubulin clusters in cancer cells and is more cytotoxic than the parent molecule **1a**. Compound **1c** with less voluminous than adamantane isopropyl moiety stimulates the formation of “point-like” tubulin clusters and is less cytotoxic than **1b**.

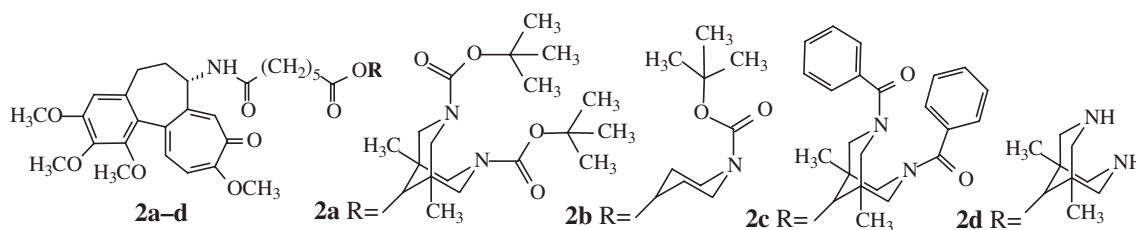


Fig. 2: Structures of tubuloclustin analogues synthesized in the present work.

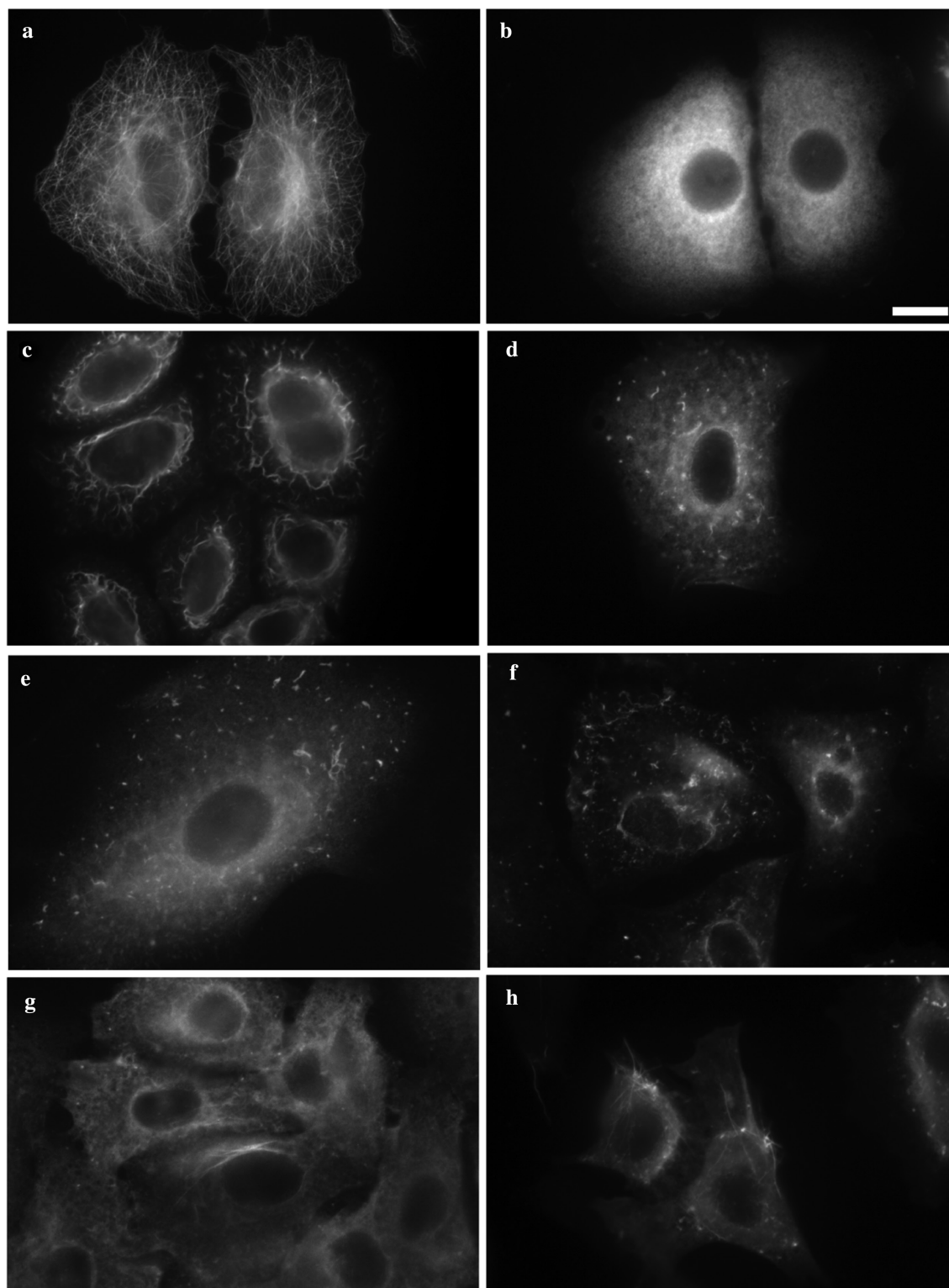
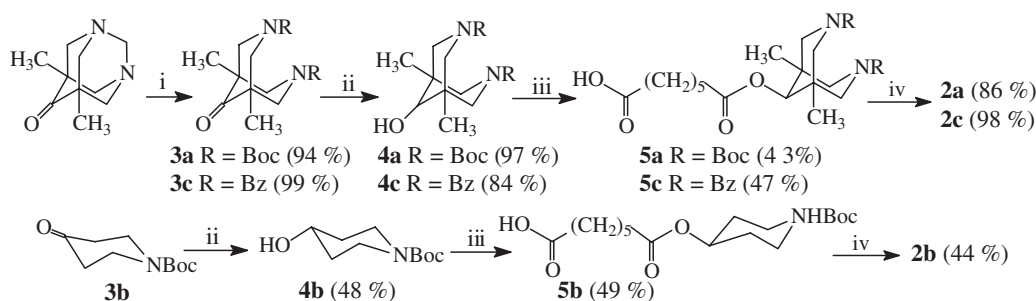


Fig. 3: Fluorescence microscopy of MTs and tubulin structures in human lung carcinoma cells A549 treated with: (a) 0.5 % DMSO (negative control) – intact microtubules; (b) 1 μ M of colchicine (positive control) – depolymerization of MTs, no clustering; (c) 1 μ M of tubuloclastin (**1**) – long wavy clusters; (d) 10 μ M **2b** – point-like and short wavy clusters; (e) 10 μ M **2c** – point-like and short wavy clusters; (f) 100 μ M **2d** – point-like and short wavy clusters; (g) 10 μ M **2a** – tiny aggregates and long tubulin filaments; (h) 100 μ M **2a** – small clusters and many long tubulin fibrils. Bar 10 μ m.



Scheme 1: Reagents and conditions: (i) for **3a**: Boc_2O , HSO_3NH_2 ; for **3c**: PhCOCl , NaHCO_3 , $\text{H}_2\text{O}-\text{CHCl}_3$; (ii) NaBH_4 , CH_3OH , rt, 12 h; (iii) polyanhydride of pimelic acid, 4-DMAP, CH_2Cl_2 , rt; (iv) N-deacetylcolchicine, EEDQ, CH_2Cl_2 , rt.

Table 1: Cytotoxicity and effect on microtubule network of subject compounds.

Compound	A549 EC_{50} (nM) ^a	Effect on A549 cells MT net (10 μM , 24 h)
2a	430 ± 90	MT depolymerization Small aggregates and tubulin filaments
2b	54 ± 5	MT depolymerization Point-like and short wavy clusters
2c	710 ± 90	MT depolymerization Point-like clusters and short wavy clusters
1	6	MT depolymerization Long wavy clusters
Colchicine	30	MT depolymerization

^aThe average of three to six experiments.

long tubulin fibrils were detected in almost 100 % of observed cells (Fig. 3h). Cytotoxicity of compound **2a** (measured in MTT test [20]) was two orders of magnitude lower than that of tubuloclustin (**1**), but still in nanomolar range (Table 1).

Literature search for the recent publications concerning mitostatic compounds able to first depolymerize MT and to stimulate the following formation of assemblies from tubulin dimer revealed the data about so called Janus compounds, which bind to colchicine site and in addition to MTs depolymerization stimulate the formation of long filamentous structures of tubulin in cells, sometimes very similar to tubulin fibrils promoted by **2a** [21]. However, in contrast to Janus compounds, **2a** cannot promote the MT formation from pure tubulin in vitro (data not shown). Moreover, Janus compounds belong to a pyrimido[4,5-*b*]indole structural type, totally different from that of **2a** [21]. Therefore, we studied the structural requirements necessary for the manifestation of the “fibrillation” effect by conjugate **2a**.

To check the role of the bridged bicyclic moiety in **2a** we synthesized its monocyclic analogue **2b**. The synthesis was fulfilled using standard procedure: after reduction of ketone **3b** to alcohol **4b** [22] the latter was subjected to reaction with polyanhydride of pimelic acid to give monoester **5b** (Scheme 1). Its following amidation with N-deacetylcolchicine led to the target conjugate **2b**. Compound **2b** was one order of magnitude more cytotoxic, than **2a** and was as cytotoxic as colchicine (Table 1). Immunofluorescence microscopy study demonstrated that at 10 μM compound **2b** caused MT depolymerization in cancer cells and a formation of point-like and short wavy clusters (Fig. 3d) typical to that of less active tubuloclustin analogues [9]. No tubulin filaments were observed for compound **2b**, giving evidence that bicyclic moiety in the conjugate **2a** was important for unusual action of the latter on MT.

To check whether the filaments could be observed in the presence of analogues of **2a** with other substituents in bispidine core, we synthesized N,N-dibenzoyl-conjugate **2c** as depicted on Scheme 1. A ring opening

reaction in 5,7-dimethyl-1,3-diazaadamantane-6-one was carried out by action of chloroanhydride of benzoic acid in the presence of aqueous solution of NaHCO_3 [23]. Obtained ketone **3c** was reduced to the corresponding alcohol **4c** as described in [17] with slight modifications. The esterification of alcohol **4c** by polyanhydride of pimelic acid gave monoester **5c**, and amidation of the latter with N-deacetylcolchicine gave target conjugate **2c**. Cytotoxicity of compound **2c** was close to that of **2a** (see Table 1), however it did not cause the formation of tubulin fibrils, but stimulated MT depolymerization and formation of point-like and short wavy tubulin clusters analogously to that of “monocyclic” conjugate **2b** and other less active tubuloclastin analogues (Table 1 and Fig. 3e).

The biotesting data gave a reason for the proposition, that unusual action of compound **2a** on MT of cancer cells was a result of a cleavage of biodegradable carbamate moiety in the cells. This cleavage should lead to a formation of a conjugate with unsubstituted bispidine (3,7-diazabicyclo[3.3.1]nonane) moiety, able to form complexes with ions of metals [24] and influence tubulin polymerization (sensitive to the presence of divalent cations [25]). However, hydrolysis of compound **2a** with trifluoroacetic acid and the following treatment of the A549 cells with the corresponding base **2d** indicated that tubulin fibrils still were not formed in these cells. Instead the point-like and short wavy clusters, very similar to that formed in the presence of **2b** or **2c** were observed (Fig. 3f). This fact indicates that the base **2d** penetrates into the cell, but acts differently from the parent molecule **2a**.

As though the *tert*-butoxycarbonyl moiety of **2a** seems not to cleave in the cells, the differences in effect might be explained by different binding type of conjugates **2a** and **2c** with tubulin or by possibility of compound **2a** to interact with another biotarget besides tubulin. To check the latter hypothesis, we carried out a study of pure tubulin polymerization in the presence of conjugate **2a** under the conditions of MT forming (in the presence of 10 % DMSO). The obtained microscopy picture (Fig. 4) showed that **2a** prevented MT formation in vitro (only small tubulin aggregates were observed).

The data demonstrate that compound **2a** stimulates depolymerization of both pure tubulin and tubulin in cellulo, while fibrils are formed (most probably, from depolymerized tubulin) only in the cells. This fact gives evidence for the proposition that conjugate **2a** may interact not only with tubulin and MT in the cells, but also with MT-associated proteins, involved in the process of tubulin polymerization. This makes the conjugate under discussion interesting for the additional studies of MAPs binding and its role in altering MT dynamics. The ability of MT-targeting agent to affect simultaneously MAP – tubulin interactions will open attractive prospects in the design of novel agents in anti-cancer therapy.

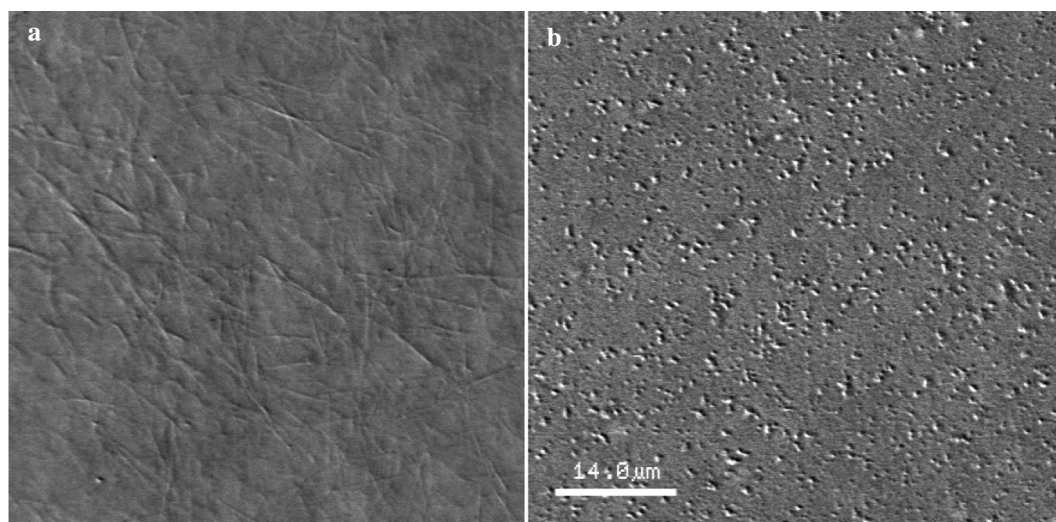
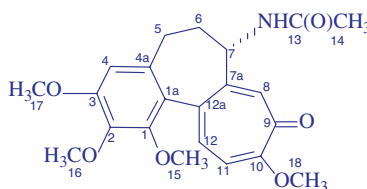


Fig. 4: AVEC-DIC microscopy of purified tubulin after induction its polymerization by 10 % DMSO: (a) without addition of **2a** – MTs and (b) in the presence of **2a** at concentration 50 μM – small aggregates.

Experimental part

Chemistry

Liquid column chromatography was performed using silica gel Acros (40–60 μm). Thin-layer chromatography (TLC) was performed on Silufol-UV254 silica gel sheets, and spots were visualized with UV light ($\lambda = 254 \text{ nm}$) or stained with iodine vapor or aqueous potassium permanganate solution. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer at 28°C in CDCl_3 at 400 and 100 MHz correspondingly. Chemical shifts (δ) are reported in parts per million (ppm) referenced to residual protonated solvent signal (CDCl_3 , $\delta = 7.26$ for ^1H NMR) or to carbon signals in the NMR solvent (CDCl_3 , $\delta = 77.0$ for ^{13}C NMR). The following abbreviations were used to designate multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, dd = double-doublet, ddd = double-double-doublet. Electron impact mass spectra (EI-MS) were recorded on a Finnigan MAT INCOSOO spectrometer (electron impact, 70 eV). Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass-spectra were taken on a Bruker Autoflex II mass spectrometer with 2,5-dihydroxybenzoic acid (DHB). CHN elemental analysis was performed using a Carlo-Erba ER-20 analyser. Infrared spectra (IR) were registered on a Thermo Nicolet IR200 apparatus in the range $900\text{--}3500 \text{ cm}^{-1}$ using KBr disks. Melting points were determined using a capillary melting point apparatus and were uncorrected.



Di-tert-butyl 9-oxo-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonane-3,7-dicarboxylate (3a). 5,7-dimethyl-1,3-diazaadamantane-6-one (1.0 g, 5.56 mmol) was mixed with Boc_2O (1.42 g, 6.51 mmol) and catalytic amount of sulfamic acid (0.015 ml, 0.33 mmol). After overnight stirring at room temperature ethyl acetate (40 ml) was added and the organic layer was washed with washed with water ($3 \times 20 \text{ ml}$) and brine ($2 \times 20 \text{ ml}$) and dried over anhydrous Na_2SO_4 . The solvent was distilled off under vacuum, the residue was purified by column chromatography: 10 % methanol in CH_2Cl_2 . Yield 1.92 g (94 %), white solid, m.p. $200\text{--}202^\circ\text{C}$. ^1H NMR (CDCl_3 , δ , ppm, J/Hz): 0.99 (6H, s, 1,5- CH_3), 1.47 (18H, s, *t*Bu), 2.96 (2H, d, $J = 13.5$), 3.00 (2H, d, $J = 13.5$), 4.28 (2H, d, $J = 12.6$), 4.43 (2H, d, $J = 12.6$). ^{13}C NMR (CDCl_3 , δ , ppm): 16.89 (1,5- CH_3), 28.36 ($\text{C}(\text{CH}_3)_3$), 45.96 (C-1,5), 55.97 (CH_2), 56.76 (CH_2), 80.38 ($\text{C}(\text{CH}_3)_3$), 154.36 (C=O), 213.65 ($\text{C}^9=\text{O}$). Anal. calcd. for, %: $\text{C}_{19}\text{H}_{32}\text{N}_2\text{O}_5$, %: C 61.93, H 8.75, N 7.60. Found, %: C 61.90, H 8.78, N 7.53.

Di-tert-butyl 9-hydroxy-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonane-3,7-dicarboxylate (4a). To the solution of ketone **3a** (1.0 g, 2.72 mmol) in methanol (10 ml) NaBH_4 (0.150 g, 3.97 mmol) was added portionwise and the reaction mixture was stirred 12 h at room temperature. The solvent was evaporated at reduced pressure and the residue was purified by column chromatography: 5 % methanol in CH_2Cl_2 . Yield 0.980 g (97 %), colorless oily liquid. ^1H NMR (CDCl_3 , δ , ppm, J/Hz): 0.90 (6H, s, 1,5- CH_3), 1.41 (18H, s, *t*Bu), 2.64 (1H, d, $J = 13.2$), 2.70 (1H, d, $J = 12.7$), 2.94 (1H, d, $J = 13.0$), 3.02 (1H, d, $J = 12.7$), 3.21 (1H, s, H-9), 3.61 (1H, d, $J = 12.7$), 3.73 (1H, d, $J = 12.7$), 3.92 (1H, d, $J = 13.0$), 4.05 (1H, d, $J = 13.2$). ^{13}C NMR (CDCl_3 , δ , ppm): 20.79 (1,5- CH_3), 28.33 ($\text{C}(\text{CH}_3)_3$), 34.88 (C-1,5), 46.75 (CH_2), 47.55 (CH_2), 53.78 (CH_2), 54.80 (CH_2), 79.23 ($\text{C}(\text{CH}_3)_3$), 79.56 (C-9), 154.45 (C=O), 154.92 (C=O). Anal. calcd. for, %: $\text{C}_{19}\text{H}_{34}\text{N}_2\text{O}_5$, %: C 61.60, H 9.25, N 7.56. Found, %: C 61.58, H 9.21, N 7.50.

Tert-butyl 4-hydroxypiperidine-1-carboxylate (4b) was obtained by literature procedure [22].

Polyanhydride of pimelic acid (PPA) was obtained by literature procedure [18].

3,7-Dibenzoyl-1,5-dimethyl-9-oxo-3,7-diazabicyclo[3.3.1]nonane (3c) was synthesized from 5,7-dimethyl-1,3-diazaadamantane-6-one and benzoyl chloride by literature procedure [23].

3,7-Dibenzoyl-9-hydroxy-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonane (4c). To the solution of ketone **3c** (0.4 g, 1.06 mmol) in methanol (20 ml) NaBH₄ (0.133 g, 3.59 mmol) was added portionwise and the reaction mixture was stirred 12 h at room temperature. The solvent was evaporated at reduced pressure and the residue was dissolved in CH₂Cl₂ (30 ml), washed by water (3 × 10 ml), dried over Na₂SO₄, filtered and evaporated. Yield 0.336 g (84 %), white solid. M.p. 238 – 240 °C. ¹H NMR (CDCl₃, δ, ppm, J/Hz): 0.83 (3H, s, CH₃), 0.84 (3H, s, CH₃), 2.69 (1H, d, J = 13.8, CH₂), 2.91 (1H, br s, OH), 2.95 (1H, d, J = 13.2, CH₂), 3.05 (1H, d, J = 13.8, CH₂), 3.21 (1H, s, C^H), 3.35 (1H, d, J = 13.2, CH₂), 3.39 (1H, d, J = 13.0, CH₂), 3.72 (1H, d, J = 13.0, CH₂), 4.30 (1H, d, J = 13.6, CH₂), 4.55 (1H, d, J = 13.6, CH₂), 7.40–7.50 (10H, m, Ph). ¹³C NMR (CDCl₃, δ, ppm): 20.34 (Me), 20.53 (Me), 35.46 (C-Me), 35.40 (C-Me), 45.09 (CH₂), 50.89 (CH₂), 51.82 (CH₂), 57.81 (CH₂), 77.92 (C-9), 127.08 (CH^{Ph}), 127.18 (CH^{Ph}), 128.57 (CH^{Ph}), 129.34 (CH^{Ph}), 129.52 (CH^{Ph}), 135.66 (C^{Ph}), 136.09 (C^{Ph}), 171.08 (C=O), 170.89 (C=O). IR (cm⁻¹): 3321 br s (O–H), 3058–2844 (C–H), 1635 (C=O), 1600 (C=O), 1571 (C–H aryl), 1442, 1429, 1317, 1082, 1020, 916.

General procedure A for the preparation of monoesters of dicarboxylic acids. To a solution of an alcohol in CH₂Cl₂ (3–5 ml) was added polyanhydride of pimelic acid and catalytic amount (0.01 g) of 4-dimethylaminopyridine (DMAP). The mixture was stirred at room temperature for 48 h and then the solvent was evaporated under reduced pressure. The residue was purified by column chromatography.

7-((3,7-Bis(*tert*-butoxycarbonyl)-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-yl)oxy)-7-oxo-heptanoic acid (5a) synthesized according to general procedure A from **4a** (0.573 g, 1.547 mmol) and PPA (0.506 g, 3.563 mmol). Column chromatography: ethyl acetate/petroleum ether (40–70 °C) in a gradient mixture 1:6–1:4. Yield 0.340 g (43 %), colorless oily liquid. ¹H NMR (CDCl₃, δ, ppm, J/Hz): 0.74 (6H, s, 1,5-CH₃), 1.37–1.45 (2H, m, γ-CH₂), 1.38 (9H, s), 1.39 (9H, s, *t*Bu), 1.60–1.69 (4H, m, β-CH₂), 2.32 (2H, t, J = 7.4, CH₂CO₂H), 2.37 (2H, t, J = 7.4, CH₂CO₂H), 2.70 (1H, d, J = 13.3, CH₂ bicycl.), 2.78 (1H, d, J = 13.0, CH₂ bicycl.), 2.83 (1H, d, J = 13.3, CH₂ bicycl.), 2.89 (1H, d, J = 13.0, CH₂ bicycl.), 3.67 (1H, d, J = 12.7, CH₂ bicycl.), 3.79 (1H, d, J = 12.7, CH₂ bicycl.), 3.94 (1H, d, J = 13.2, CH₂ bicycl.), 4.09 (1H, d, J = 13.2, CH₂ bicycl.), 4.74 (1H, s, H-9 bicycl.), 8.75–9.08 (1H, br s., CO₂H). ¹³C NMR (CDCl₃, δ, ppm): 20.32 (1,5-CH₃), 24.18 (β-CH₂), 24.64 (β-CH₂), 28.21 (CCH₃), 28.28 (CCH₃), 28.47 (γ-CH₂), 33.61 (α-CH₂), 33.94 (α-CH₂), 34.24 (C-1,5 bicycl.), 47.53 (CH₂ bicycl.), 48.42 (CH₂ bicycl.), 53.41 (CH₂ bicycl.), 54.50 (CH₂ bicycl.), 77.50 (C-9 bicycl.), 79.66 (CCH₃), 79.86 (CCH₃), 154.22 (*t*BuOC=O), 154.86 (*t*BuOC=O), 172.92 (C=O), 178.86 (CO₂H). MS (MALDI-TOF), *m/z*: 512 [M]⁺, 535 [M + Na]⁺, 551 [M + K]⁺. Anal. calcd. for, %: C₂₆H₄₄N₂O₈, %: C 60.92, H 8.65, N 5.46. Found, %: C 60.96, H 8.59, N 5.42.

7-((1-(*Tert*-butoxycarbonyl)piperidin-4-yl)oxy)-7-oxoheptanoic acid (5b) synthesized according to general procedure A from **4b** (0.500 g, 2.49 mmol) and PPA (0.700 g, 4.929 mmol). Column chromatography: ethyl acetate/petroleum ether (40–70 °C) – 1:8. Yield 0.420 g (49 %), pale yellow oily liquid. ¹H NMR (CDCl₃, δ, ppm, J/Hz): 1.35–1.42 (2H, m, γ-CH₂), 1.46 (9H, s, *t*Bu), 1.55–1.70 (6H, m, β-CH₂ + H-3,5 piper.), 1.80–1.87 (2H, m, H-3,5 piper.), 2.32 (2H, t, J = 7.4, CH₂CO₂H), 2.36 (2H, t, J = 7.0, CH₂CO₂H), 3.23 (2H, ddd, J = 13.6, 8.6, 3.9, H-2,6 piper.), 3.67–3.72 (2H, m, H-2,6 piper.), 4.92 (1H, tt, J = 8.2, 3.9, H-4 piper.). ¹³C NMR (CDCl₃, δ, ppm): 24.24 (β-CH₂), 24.55 (β-CH₂), 28.36 (CCH₃), 28.41 (γ-CH₂), 30.52 (CH₂CH₂N), 33.70 (α-CH₂), 34.26 (α-CH₂), 40.95 (br s., CH₂N), 69.55 (C-4), 79.75 (CCH₃), 154.76 (*t*BuOC=O), 172.87 (C=O), 179.05 (CO₂H). MS (MALDI-TOF), *m/z*: 343 [M]⁺, 366 [M + Na]⁺, 382 [M + K]⁺. Anal. calcd. for, %: C₁₇H₂₉NO₆, %: C 59.46; H 8.51. Found, %: C 59.53; H 8.57.

7-((N,N'-dibenzoyl-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-yl)oxy)-7-oxoheptanoic acid (5c) synthesized according to general procedure A from **4c** (0.201 g, 0.53 mmol) and PPA (0.230 g, 1.62 mmol). Column chromatography: ethyl acetate/petroleum ether (40–70 °C) in a gradient mixture 1:5 – 1:1. Yield 0.129 g (47 %), white solid, m.p. 93–95 °C. ¹H NMR (CDCl₃, δ, ppm, J/Hz): 0.77 s (3H, CH₃), 0.78 s (3H, CH₃), 1.40 m (2H, γ-CH₂), 1.64–1.73 m (4H, β-CH₂), 2.33 t (2H, J = 7.3, CH₂CO₂H), 2.43 t (2H, J = 7.6, CH₂CO₂H), 2 bicycl.), 3.27 d (1H, J = 12.9, CH₂ bicycl.), 3.56 d (1H, J = 12.9, CH₂ bicycl.), 3.81 d (1H, J = 13.4, CH₂ bicycl.), 4.41 d (1H, J = 13.9, CH bicycl.), 4.67 d (1H, J = 13.9, CH₂ bicycl.), 4.91 s (1H, C^H–O), 7.38–7.54 m (10H, Ph). ¹³C NMR (CDCl₃, δ, ppm): 20.13 (CH₃), 20.23 (CH₃), 24.20 (β-C), 24.68 (β-C), 28.50 (γ-C), 33.59 (CH₂CO₂H), 33.94 (CH₂–CO₂R), 34.82 (CCH₃), 34.89 (CCH₃), 46.0 (CH₂ bicycl.), 51.66 (CH₂ bicycl.), 51.82 (CH₂ bicycl.), 57.67 (CH₂ bicycl.), 77.52 (C^H–O), 127.16 (CH *m*-Ph), 128.56 (CH *o*-Ph), 129.51 (CH *p*-Ph), 129.64 (CH *p*-Ph), 135.33 (C *ipso*-Ph), 135.78 (C *ipso*-Ph), 170.88 (PhC=O), 171.31

(PhC=O), 172.80 (C₂R), 178.16 (CO₂H). IR (cm⁻¹): 3199 br (O–H), 3059–2856 (C–H), 1732(C=O), 1641–1595 (C–H aryl), 1454, 1323, 1192, 1024, 937. MS (MALDI-TOF), *m/z*: 543 [M + Na]⁺, 559 [M + K]⁺.

General procedure B for the preparation of conjugates 2a–c. To a solution of compounds **5a–c** in CH₂Cl₂ (5 ml) was added *N*-deacetylcolchicine and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). After stirring for 24 h at room temperature, the solvent was removed under reduced pressure, and the residue was purified by column chromatography (ethyl acetate/petroleum ether (40–70 °C) 1:1, then 1 % methanol in CH₂Cl₂).

N-{7-(3,7-Di-(*tert*-butoxycarbonyl)-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-yl)-oxy-7-oxoheptanoyl}-N-deacetylcolchicine (2a**)** synthesized according to general procedure B from **5a** (0.240 g, 0.469 mmol), *N*-deacetylcolchicine (0.155 g, 0.434 mmol) and EEDQ (0.116 g, 0.470 mmol). Yield 0.317 g (86 %), pale yellow oily liquid. ¹H NMR (CDCl₃, δ, ppm, *J*/Hz): 0.75 (6H, s, 1,5-CH₃ bicycl.), 1.29–1.37 (2H, m, γ-CH₂), 1.41 (9H, s, *t*Bu), 1.42 (9H, s, *t*Bu), 1.56–1.66 (4H, m, β-CH₂), 1.85 (1H, td, *J*=12.1, 6.6, H-6 colch.), 2.23 (2H, t, *J*=7.4, CH₂CONH), 2.27–2.31 (1H, m, *J*=12.1, 6.5, H-6 colch.), 2.35 (2H, t, *J*=7.3, CH₂CO₂), 2.42 (1H, m, H-5 colch.), 2.52 (1H, dd, *J*=13.1, 6.1, H-5 colch.), 2.72 (1H, d, *J*=13.3, CH₂ bicycl.), 2.79 (1H, d, *J*=12.6, CH₂ bicycl.), 2.83 (1H, d, *J*=12.6, CH₂ bicycl.), 2.90 (1H, d, *J*=13.3, CH₂ bicycl.), 3.65 (3H, s, OCH₃), 3.68 (1H, s, *J*=13.2, CH₂ bicycl.), 3.80 (1H, d, *J*=12.6, CH₂ bicycl.), 3.90 (3H, c, OCH₃), 3.94 (3H, s, OCH₃), 3.99 (1H, d, *J*=12.6, CH₂ bicycl.), 4.00 (3H, s, OCH₃), 4.09 (1H, d, *J*=13.2, CH₂ bicycl.), 4.64 (1H, ddd, *J*=11.1, 7.1, 5.6, H-7 colch.), 4.73 (1H, s, H-9 bicycl.), 6.53 (1H, s, H-4 colch.), 6.86 (1H, d, *J*=10.6, H-11colch), 7.07 (1H, br.s, NH), 7.34 (1H, d, *J*=10.6, H-12 colch.), 7.51 (1H, s, H-8 colch.). ¹³C NMR (CDCl₃, δ, ppm): 20.44 (1,5-CH₃ bicycl.), 24.79 (β-CH₂), 24.87 (β-CH₂), 28.30 (CCH₃), 28.37 (CCH₃), 28.74 (γ-CH₂), 29.85 (C-5 colch.), 33.97 (α-CH₂), 34.30 (C-1,5 bicycl.), 35.79 (α-CH₂), 36.79 (C-6 colch.), 47.61 (CH₂ bicycl.), 48.53 (CH₂ bicycl.), 52.15 (C-7 colch.), 53.55 (CH₂ bicycl.), 54.63 (CH₂ bicycl.), 56.05 (OCH₃), 56.33 (OCH₃), 61.30 (OCH₃), 61.51 (OCH₃), 77.52 (C-9 bicycl.), 79.47 (CCH₃), 79.71 (CCH₃), 107.30 (C-4 colch.), 112.66 (C-11 colch.), 125.54, 130.54 (C-8 colch.), 134.12, 135.45, 136.65, 141.63, 151.16, 151.89, 153.47, 154.21 (*t*BuOC=O), 154.78 (*t*BuOC=O), 163.95 (C-10 colch.), 172.33 (C=O), 173.02 (C=O), 179.15 (C-9 colch.). MS (MALDI-TOF), *m/z*: 852 [M]⁺, 875 [M + Na]⁺, 891 [M + K]⁺. Anal. calcd. for, %: C₄₆H₆₅N₃O₁₂, %: C 64.84, H 7.69, N 4.93. Found, %: C 64.79, H 7.60, N 4.92. *N,N'*-di-Boc-derivative **2a** (10 mg, 0.011 mmol) dissolved in CH₂Cl₂ (2 ml) was treated with trifluoroacetic acid (10 μL, 0.130 mmol) and stirred at room temperature for 1 day. The reaction mass was concentrated under reduced pressure at 40 °C, diluted with CH₂Cl₂ (10 ml), washed with saturated solution of NaHCO₃ (5 ml), dried over anhydrous Na₂SO₄. The solvent was distilled off under vacuum to afford compound **6** mg of **N-{7-(1,5-Dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-yl)-oxy-7-oxoheptanoyl}-N-deacetylcolchicine (**2d**)**.

N-{7-[1-(*tert*-butoxycarbonyl)piperidin-4-yloxy]-7-oxoheptanoyl}-N-deacetylcolchicine (2b**)** synthesized according to general procedure B from **5b** (0.055 g, 0.160 mmol), *N*-deacetylcolchicine (0.050 g, 0.140 mmol) and EEDQ (0.050 g, 0.202 mmol). Yield 0.042 g (44 %), pale yellow oily liquid. ¹H NMR (CDCl₃, δ, ppm, *J*/Hz): 1.30–1.37 (2H, m, γ-CH₂), 1.46 (9H, C, *t*Bu), 1.58–1.65 (6H, m, β-CH₂ + H-3,5 piper.), 1.78–1.86 (3H, m, H-3,5 piper. + H-6 colch.), 2.23 (2H, t, *J*=7.5, CH₂CONH), 2.24–2.28 (1H, m, H-6 colch.), 2.30 (2H, t, *J*=7.3, CH₂CO₂), 2.42 (1H, m, td, *J*=13.1, 7.0, H-5 colch.), 2.54 (1H, dd, *J*=13.1, 6.1, H-5 colch.), 3.22 (2H ddd, *J*=13.8, 8.5, 3.2, H-2,6 piper.), 3.67 (3H, s, OCH₃), 3.68–3.74 (2H, m, H-2,6 piper.), 3.92 (3H, s, OCH₃), 3.96 (3H, s, OCH₃), 4.01 (3H, s, OCH₃), 4.66 (1H, ddd, *J*=11.7, 7.0, 5.6, H-7 colch.), 4.91 (1H, tt, *J*=8.1, 4.1, H-4 piper.), 6.35 (1H, br. s, NH), 6.55 (1H, s, H-4 colch.), 6.84 (1H, d, *J*=10.9, H-11 colch.), 7.32 (1H, d, *J*=10.9, H-12 colch.), 7.41 (1H, s, H-8 colch.). ¹³C NMR (CDCl₃, δ, ppm): 24.58 (β-CH₂), 24.95 (β-CH₂), 28.36 (γ-CH₂), 28.39 (CCH₃), 29.66 (C-3 piper.), 29.87 (C-5 colch.), 30.56 (C-5 piper.), 34.24 (α-CH₂), 35.04 (α-CH₂), 36.97 (C-6 colch.), 40.24 (br., C-2,6 piper.) 52.04 (C-7 colch.), 56.08 (OCH₃), 56.33 (OCH₃), 61.37 (OCH₃), 61.56 (OCH₃), 69.51 (C-4 piper.), 79.62 (CCH₃), 107.31 (C-4 colch.), 112.40 (C-11 colch.), 125.60, 130.65 (C-8 colch.), 134.08, 135.24, 136.34, 141.66, 151.22, 151.28, 153.44, 154.71 (*t*BuOC=O), 163.97 (C-10 colch.), 172.28 (C=O), 172.94 (C=O), 179.34 (C-9 colch.). MS (MALDI-TOF), *m/z*: 682 [M]⁺, 705 [M + Na]⁺, 721 [M + K]⁺. Anal. calcd. for, %: C₃₇H₅₀N₂O₁₀, %: C 65.08, H 7.38, N 4.10. Found, %: C 65.05, H 7.35, N 4.07.

N-[7-(3,7-dibenzoyl-1,5-dimethyl-3,7-diazabicyclo[3.3.1]nonan-9-yl)-oxy-7-oxoheptanoyl]-N-deacetylcolchicine (2c) synthesized according to general procedure A from **5c** (0.043 g, 0.08 mmol), EEDQ (0.031 g, 0.12 mmol) and 0.038 g N-deacetylcolchicine (0.11 mmol). Yield 0.069 g **4** (98 %), pale orange solid, m.p. 174–176 °C. ¹H NMR (CDCl₃, δ, ppm, J/Hz): 0.76 (6H, s, CH₃ bicycl.), 1.35 (2H, m, γ-CH₂), 1.63 (4H, m, β-CH₂), 1.84 (1H, m, H-6 colch.), 2.24 (2H, t, J = 7.1, CH₂CO₂H), 2.24–2.27 (1H, m, H-6 colch.), 2.36–2.42 (1H, m, H-5 colch.), 2.41 (2H, t, J = 7.5, CH₂CO₂R), 2.53 (1H, dd, J = 13.1, 5.6, H-5 colch.), 2.84 (1H, d, J = 13.9, CH₂ bicycl.), 2.95 (1H, d, J = 13.9, CH₂ bicycl.), 3.13 (1H, d, J = 13.1, CH₂ bicycl.), 3.26 (1H, d, J = 13.1, CH₂ bicycl.), 3.53 (1H, d, J = 12.6, CH₂ bicycl.), 3.66 (3H, s, OCH₃), 3.80 (1H, d, J = 13.1, CH₂ bicycl.), 3.91 (3H, s, OCH₃), 3.95 (3H, s, OCH₃), 4.00 (3H, s, OCH₃), 4.41 (1H, d, J = 13.1, CH₂ bicycl.), 4.64 – 4.67 (2H, d + m, J = 13.1, CH₂ bicycl. + H-7 colch.), 4.89 (1H, s, HOC-9 bicycl.), 6.54 (1H, s, H-4 colch.), 6.69 (1H, br. s, NH), 6.85 (1H, d, J = 11.2, H-11 colch.), 7.32 (1H, d, J = 11.2, H-12 colch.), 7.40–7.51 (11H, m, Ph + H-8 colch.). ¹³C NMR (CDCl₃, δ, ppm): 20.12 (CH₃ bicycl.), 20.22 (CH₃ bicycl.), 24.81 (β-CH₂), 28.69 (β-CH₂), 29.82 (γ-CH₂), 33.91, 34.79, 34.83, 35.65, 36.63, 45.87 (CH₂ bicycl.), 51.81 (CH₂ bicycl.), 51.82 (CH₂ bicycl.), 52.15 (C-7 colch.), 56.03 (OCH₃), 56.32 (OCH₃), 57.66 (CH₂ bicycl.), 61.31 (OCH₃), 61.49 (OCH₃), 78.45 (C-9 bicycl.), 107.21 (C-4 colch.), 112.51 (C-11 colch.), 125.50, 127.10, 128.52, 129.38, 129.54, 130.52, 134.09, 135.30, 135.94, 136.49, 141.54, 151.10, 151.78, 153.40, 163.88, 170.74 (PhCONH), 170.98 (PhCONH), 172.31 (C=O), 172.84 (C=O), 179.33 (C-9 colch.). IR (cm⁻¹, KBr): 3300 br (N–H), 3059–2858 (C–H), 1738 (C=O), 1701 (C=O), 1671 (C=O amide), 1637, 1624 (C–H aryl), 1448, 1250, 1140, 1022. MS (MALDI-TOF), m/z: 883 [M + Na]⁺, 899 [M + K]⁺.

Biology

MTT cytotoxicity assay

The MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-2H-tetrazolium bromide, Roth GmbH, Karlsruhe, Germany) quantitative colorimetric assay was used to measure the cytotoxicity, viability and metabolic activity [20]. A549 human lung epithelial carcinoma cells (CCL-185™) were cultured with Dulbecco's Modified Eagle medium (DMEM) containing 10 % fetal bovine serum and 1 % antibiotic penicillin/streptomycin at 37 °C under a 5 % CO₂ humidified atmosphere. The cells were seeded in 96-well plates at a density of 3000 cells per well. Stock solutions of test compounds were prepared in DMSO at concentration 20 mM. Cells were treated for 24 h with selected compounds at 1–2400 nM (8 wells for each concentration). DMSO (0.5 %) served as a negative control. Optical density was measured at 550 nm with 690 nm reference filter using EL808 Ultra Microplate Reader (BioTek Instruments, Winooski, VT, USA). Experiments for all compounds were repeated at least 3 times and EC₅₀ values were determined by sigmoid curve fitting using Excel-based software.

Immunofluorescence staining of cellular microtubules and nuclei. For this staining A549 human lung epithelial carcinoma cells were cultured in 12-well plates on small glass coverslips (11 mm diameter) at a density of 20 000 cells per coverslip. Cells were incubated with tested compounds at concentrations of 100–1200 nM for 24 and 48 h. 0.5 % DMSO served as a negative control. The cells were fixed and stained as described in [26]. Fixed cells were labelled for tubulin with mouse monoclonal antibody against β-tubulin at a dilution of 1:300 (Sigma, St. Louis, MO, USA), followed by incubation of Alexa Fluor488 labelled goat anti-mouse IgG at a dilution of 1:300 (Invitrogen, Germany). In order to analyze the compound effect on the apoptosis induction effect, the cell nuclei were stained with the Hoechst Nr. 33258 (Sigma, St. Louis, MO, USA) at concentration 5 µg/ml. Images of all samples were acquired with a Nikon Diaphot 300 inverted microscope (Nikon GmbH, Düsseldorf, Germany) equipped with a cooled charge-couple device camera system (SenSys; Photometrics, Munich, Germany).

Acknowledgements: This work was supported by Russian Foundation for Basic Research (project no. 18-03-00524). Initial reagent 5,7-dimethyl-1,3-diazaadamantane-6-one was synthesized within the State assignment AAAA-A16-116032250004-2. Authors acknowledge the German organization DAAD (German Academic Exchange Service).

References

- [1] G. L. Regina, A. Coluccia, V. Naccarato, R. Silvestri. *Eur. J. Pharm. Sci.* **131**, 58 (2019).
- [2] M. O. Steinmetz, A. E. Prota. *Trends Cel. Biol.* **28**, 776 (2018).
- [3] E. Unger, K. J. Bohm, W. Vater. *Electron Microsc. Rev.* **3**, 355 (1990).
- [4] D. F. Albertini; J. I. Clark. *Cell Biol. Int. Rep.* **5**, 387 (1981).
- [5] E. Vassal, C. Barette, X. Fonrose, R. Dupont, E. Sans-Soleilhac, L. Lafanechère. *J. Biomol. Screen.* **11**, 377 (2006).
- [6] J. A. Hadfield, S. Ducki, N. Hirst, A. T. McGown. *Prog. Cell Cycle Res.* **5**, 309 (2003).
- [7] B. Bhattacharyya, D. Panda, S. Gupta, M. Banerjee. *Med. Res. Rev.* **28**, 155 (2008).
- [8] N. A. Zefirov, A. Kruth, B. Wobith, E. V. Nurieva, S. Riyaz, Ch. V. R. Reddy, S. A. Kuznetsov, O. N. Zefirova. *Mendeleev Commun.* **28**, 475 (2018).
- [9] N. A. Zefirov, Y. A. Evteeva, B. Wobith, S. A. Kuznetsov, O. N. Zefirova. *Struct. Chem.* **30**, 465 (2019).
- [10] O. N. Zefirova, H. Lemcke, M. Lantow, E. V. Nurieva, B. Wobith, G. E. Onishchenko, A. Hoenen, G. Griffiths, N. S. Zefirov, S. A. Kuznetsov. *ChemBioChem.* **14**, 1444 (2013).
- [11] O. N. Zefirova, E. V. Nurieva, Y. S. Glazkova, N. A. Zefirov, A. V. Mamaeva, B. Wobith, V. I. Romanenko, N. A. Lesnaia, H. M. Treshalina, S. A. Kuznetsov. *Pharm. Chem. J.* **48**, 373 (2014).
- [12] P. Thomopoulou, J. Sachs, N. Teusch, A. Mariappan, J. Gopalakrishnan, H. G. Schmalz. *ACS Med. Chem. Lett.* **7**, 188 (2015).
- [13] C. Vilanova, S. Díaz-Oltra, J. Murga, E. Falomir, M. Carda, J. M. Redondo-Horcajo, J. F. Díaz, I. Barasoain, A. Marco. *J. Med. Chem.* **57**, 10391 (2014).
- [14] S. K. Kim, S. M. Cho, H. Kim, H. Seok, S. O. Kim, T. K. Kwon, J. S. Chang. *Exp. Mol. Med.* **45**, E19 (2013).
- [15] O. N. Zefirova, E. V. Nurieva, D. V. Shishov, I. I. Baskin, F. Fuchs, H. Lemcke, F. Schröder, D. G. Weiss, N. S. Zefirov, S. A. Kuznetsov. *Bioorg. Med. Chem.* **19**, 5529 (2011).
- [16] O. N. Zefirova, E. V. Nurieva, B. Wobith, V. V. Gogol, N. A. Zefirov, A. V. Ogonkov, D. V. Shishov, N. S. Zefirov, S. A. Kuznetsov. *Mol. Divers.* **21**, 547 (2017).
- [17] Ts. Ye. Aghajanian, G. L. Arutyunian, R. K. Shahkhatuni. *Chem. Heterocycl. Com.* **39**, 767 (2003).
- [18] J. W. Hill, W. H. Carothers. *J. Am. Chem. Soc.* **55**, 5023 (1933).
- [19] J. D. Bagnato, A. L. Eilers, R. A. Horton, C. B. Grissom. *J. Org. Chem.* **69**, 8987 (2004).
- [20] D. Gerlier, N. Thomasset. *J. Immunol. Methods*, **94**, 57 (1986).
- [21] C. C. Rohena, A. L. Risinger, R. K. V. Devambatla, N. F. Dybdal-Hargreaves, R. Kaul, S. Choudhary, A. Gangjee, S. L. Mooberr. *Molecules*, **21**, 1661 (2016).
- [22] P. J. Rayner, P. O'Brien, R. A. J. Horan. *J. Am. Chem. Soc.* **135**, 8071 (2013).
- [23] Ts. E. Agadzhanian, A. D. Arutyunyan, G. L. Arutyunyan. *Chem. Heterocycl. Com.* **28**, 772 (1992).
- [24] P. Comba, M. Kerscher, W. Schiek. *Prog. Inorg. Chem.* **55**, 613 (2007).
- [25] M. Schliwa, *J. Cell Biol.* **70**, 527 (1976).
- [26] A. Al-Haddad, M. A. Shonn, B. Redlich, A. Blocker, J. K. Burkhardt, H. Yu, J. A. Hammer, D. G. Weiss, W. Steffen, G. Griffiths, S. A. Kuznetsov. *Mol. Biol. Cell.* **12**, 2742, (2001).

Graphical abstract

Olga N. Zefirova, Evgenia V. Nurieva,
Birgit Wobith, Svetlana Schulz, Nikolay A.
Zefirov and Sergei A. Kuznetsov

Novel colchicine conjugate with unusual effect on the microtubules of cancer cells

<https://doi.org/10.1515/pac-2019-1215>
Pure Appl. Chem. 2020; x(x): xxx–xxx

Conference paper:
Colchicine derivative bearing substituted bispidine moiety induces not only depolymerization of microtubules of cancer cells, but stimulates the formation of small tubulin aggregates and long tubulin fibrils localized mainly around nuclei. The assemblies are morphologically different from tubulin clusters induced by structurally related anticancer agent tubuloclustin.

Keywords:
3,7-diazabicyclo[3.3.1]
nonane; carcinoma
A549; colchicine;
Mendelev-21; tubulin;
tubuloclustin.

