



Adamantyl-substituted ligands of colchicine binding site in tubulin: different effects on microtubule network in cancer cells

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Abstract

Four novel conjugates of adamantane connected at a bridgehead position via four-bond linker to structurally different ligands interacting with colchicine binding site of tubulin were synthesized. All compounds were found to inhibit cancer cell proliferation and, notably, to promote the formation of different atypical tubulin assemblies — clusters, curly, or “entangled” microtubules. The distinction in the observed effects for conceptually equivalent structural templates can be partially explained on the basis of computer molecular modeling.

Keywords Tubulin polymorphism · “Entangled” microtubules · Adamantane · Colchicine binding site · Human lung carcinoma cells A549

Introduction

Cellular dimeric protein α, β -tubulin plays a crucial role in the formation of cytoskeleton and the process of cell division [1, 2]. The protein is known for its ability to constitute assemblies of different type and shape. Besides typical structures, i.e., microtubules (MT) formed by tubulin polymerization in cells, tubulin makes atypical assemblies — rings, tubulin sheets, paracrystals, and others [3]. The latter are composed only under special media conditions (pH, different cations, temperature, etc.) in vitro or in the presence of some anticancer agents [3–6]. For example, interaction of taxol with its binding site in β -subunit of tubulin stabilizes preexisted MT and promotes the protein assembly into stable MT bundles [4]. Another group of tubulin ligands binds with vinca-domain at the border of two α, β -dimers. This binding causes MT

destabilization and a formation of either tubulin paracrystals (e.g., vinblastine) or other tubulin aggregates (cryptophycins, phomopsin etc.) [5]. There exists also a big group of compounds, which interact with binding site of colchicine (Fig. 1) and normally promote MT depolymerization only [6]. However, these compounds can cause the formation of tubulin assemblies under special conditions (e.g., [7]). Last decade several new derivatives of ligands of colchicine binding site displaying unusual effect on MT network were described (see [8] and ref. herein and [9–13] for recent examples).

Most of these new compounds represent colchicine derivatives with lipophilic (alicyclic or aromatic) substituents attached via linker chain to C⁷ atom of the parent molecule and are able to promote MT disassembly with further tubulin assembly into clusters [8–12]. The arising of this ability is attributed to additional interactions of lipophilic group with α -tubulin subunit. For colchicine—adamantane conjugates like tubuloelustin (Fig. 1(1b)) —the morphology of unusual tubulin assemblies was studied and direct correlation of the clusterization strength caused by compounds and their mitostatic activity in cancer cells was demonstrated (see [8] and ref. herein). Tubulin assemblies induced by derivatives of other than colchicine ligands of colchicine binding site are much less studied. Recently, we demonstrated that podophyllotoxin and 2-methoxyestradiol analogues of tubuloelustin cause the formation of weak clusters or slightly curved MT correspondingly [12, 13]. This observation made

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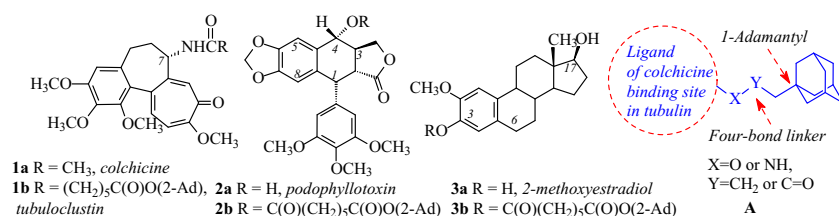


Fig. 1 Examples of the ligands of colchicine binding site (**1a**, **2a**, **3a**) and their derivatives with ability to promote unusual effect on MT network (**1b**, **2b**, **3b**, 2-Ad = 2-adamantyl); (A) represents a general structural type of conjugates synthesized and tested for MT-effect in the present work

interesting additional investigations in this field. In the present work, we synthesized novel conjugates of colchicine, podophyllotoxin, and 2-methoxyestradiol with adamantane connected at a bridged position via rather short “four-bond” linker of approximately equal length. The linker was attached to the main molecule so that the substituent should be pointed at the direction of α -tubulin subunit of tubulin dimer (according to pharmacophore model of colchicine domain inhibitors [14]). The effect of the compounds on the MT net of cancer cells was studied and molecular modeling was carried out to explain the results of biotests.

Experimental part

Chemistry

All reaction temperatures correspond to internal temperatures unless otherwise noted. The high-grade commercial starting materials were used without further purification, the solvents were technical grade and were purified by standard procedures prior to use. Liquid column chromatography was performed on silica gel Acros (40–60 μ m). 1D and 2D (gHSQC, gHMBC) NMR spectra were recorded on spectrometer Agilent 400-MR (400.0 MHz for ¹H; 100.6 MHz for ¹³C) at room temperature; chemical shifts were measured with reference to the solvent (CDCl₃, $\delta_{\text{H}} = 7.24$ ppm, $\delta_{\text{C}} = 77.0$ ppm). Chemical shifts are given in ppm (δ); multiplicities are indicated by s (singlet), d (doublet), t (triplet), and m (multiplet) and spin-spin coupling constants (J) are reported in Hz. Assignment of NMR signals of colchicine fragment in the target conjugates was made using the atomic numbering of colchicine, podophyllotoxin, and 2-methoxyestradiol given in Fig. 1. Electron impact mass spectra were obtained with typical voltage of 70 eV. Elemental analysis was performed on CNH analyzer Carlo-Erba ER-20.

N-[2-(1-adamantyl)acetyl]-N-deacetylcolchicine (4) To a solution of N-deacetylcolchicine (50 mg, 0.140 mmol) in CH₂Cl₂, 2-(1-adamantyl)acetic acid (33 mg, 0.169 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (50 mg, 0.202 mmol) were added. The mixture was stirred for 48 h. The filtrate was evaporated and chromatographed

with a gradient (ethyl acetate/petroleum ether (40–70 °C) 1:2, then CH₂Cl₂/methanol in a gradient ratio from 99:1 to 98:2). Yield 30% (0.026 g), yellowish oily liquid. **NMR** ¹H (CDCl₃, δ , ppm, J /Hz): 1.54–1.65 (12H, m, Ad), 1.76–1.84 (1H, m), 1.95 (5H, m, Ad+CH₂), 2.21–2.29 (1H, m), 2.40–2.45 (1H, m), 2.50–2.55 (1H, m), 3.65 (3H, s, OCH₃), 3.91 (3H, s, OCH₃), 3.94 (3H, s, OCH₃), 3.99 (3H, s, OCH₃), 4.65–4.71 (1H, m, H⁷), 6.20 (1H, m, NH), 6.54 (1H, s, Ar), 6.82 (1H, d, $J = 10.8$ Hz, Ar), 7.30 (1H, d, $J = 10.8$ Hz, Ar), 7.50 (1H, d, $J = 19.5$ Hz, Ar). **NMR** ¹³C (CDCl₃, δ , ppm): 28.52; 29.94; 32.78; 36.61; 37.35; 42.51; 51.44; 51.77; 56.01; 56.27; 61.34; 61.60; 107.22; 112.27; 125.60; 131.13; 134.13; 135.06; 136.33; 141.53; 151.19; 151.31; 153.34; 163.91; 170.28 (CONH); 179.24 (C=O). **MS (EI)**, m/z : 533 (M⁺), 556 (M⁺ + Na). Anal. calcd. for C₃₂H₃₉NO₆, %: C, 72.02; H, 7.37. Found: C, 72.08; H, 7.40.

4-O-(1-adamantylacetyl)-L-podophyllotoxin (5) To the solution of 0.046 g (0.239 mmol) 2-(1-adamantyl)acetic acid in CH₂Cl₂ (10 ml), 0.080 g (0.193 mmol) of podophyllotoxin was added at room temperature. To the resulting mixture, 0.068 g (0.330 mmol) of DCC and a catalytic amount of DMAP (0.01 g) were added. After stirring at room temperature for 12 h, 5–10 μ l of acetic acid was added and then, after 15 min, the solvent was removed in vacuo. The residue was dissolved in ethyl acetate (10–20 ml) and kept at 0–4 °C for 1 h. The N, N'-dicyclohexylurea precipitate was filtered and washed with cold ethyl acetate (2 \times 10 ml), and the filtrate was washed by saturated NaCl solution (10 ml), water (10 ml), then dried with Na₂SO₄ and evaporated. The residue was chromatographed [eluent: ethyl acetate—petroleum ether (40–70 °C) 1:8–1:5]. Obtained 0.065 g of **5** (yield 57%), waxy solid, m.p. 123 °C. **NMR** ¹H (CDCl₃, δ , ppm, J /Hz): 1.62–1.68 (9H, m, Ad), 1.72–1.75 (3H, m, Ad), 2.00 (3H, m, Ad), 2.18 (1H, d, $J = 13.0$, AdCH₂), 2.21 (1H, d, $J = 13.0$, AdCH₂), 2.77–2.87 (1H, m, H³), 2.94 (1H, dd, $J = 14.5, 4.3$, H²), 3.76 (6H, s, OCH₃), 3.82 (3H, c, OCH₃), 4.24 (1H, dd, $J = 10.3, 9.4$, H^{3a}), 4.33 (1H, dd, $J = 9.4, 6.9$, H^{3a}), 4.61 (1H, d, $J = 4.3, H^j$), 5.87 (1H, d, $J = 9.1, H^j$), 5.98 (1H, d, $J = 1.0, OCH_2O$), 6.00 (1H, d, $J = 1.0, OCH_2O$), 6.40 (2H, s, H^{2',6'}), 6.54 (1H, s, H⁸), 6.50 (1H, s, H⁵). **NMR** ¹³C (CDCl₃, δ , ppm): 28.45, 33.00, 36.56, 38.87, 42.53, 43.69, 45.55, 48.88 (AdCH₂CO₂), 56.04 (2OCH₃), 60.71 (OCH₃), 71.58, 73.25

(C^4), 101.53 (OCH₂O), 107.17, 108.00, 109.62, 128.40, 132.28, 134.80, 137.06, 147.51, 148.03, 152.60, 172.18, 173.64 (AdCH₂CO₂). Mass (MALDI-TOF), m/z : 590 [M]⁺, 613 [M+Na]⁺, 629 [M+K]⁺. Found, %: C 69.10; H 6.52. Calculated, %: C₃₄H₃₈O₉, %: C 69.14; H 6.48.

4-O-[2-(adamant-1-yl)ethyl]-L-podophyllotoxin (6 α) and 4-O-[2-(adamant-1-yl)ethyl]-L-epipodophyllotoxin (6 β) Twenty-nine microliters of boron trifluoride etherate was added to the solution of 0.070 g (0.169 mmol) of podophyllotoxin in 4 ml of dry CH₂Cl₂ at 0 °C. After 5 min, 0.046 g (0.256 mmol) of 2-adamant-1-yl ethanol was added. The reaction mixture was stirred for 6 h at room temperature, then 20 ml of water was added and extracted with methylene chloride (3 × 20 ml). The organic layers were combined and dried over Na₂SO₄, and the solvent was evaporated in vacuo. The residue was chromatographed (eluent ethyl acetate: petroleum ether 40–70 °C gradient 1:6–1:4). Obtained 0.062 g of compound 46 (64% yield) in a 3:2 ratio (according to ¹H NMR spectroscopy), a colorless oily liquid. **6 α , β** : NMR ¹H (CDCl₃, δ , J/Hz, ppm); chemical shifts for **6 β** are in square brackets): 1.40 (1H, m, AdCH₂), 1.52 (7H, m, Ad), 1.60–1.63 (3H, m, Ad), 1.69–1.72 (4H, m, Ad), 1.94 (3H, m, Ad), 2.79–2.95 (2H, m, AdCH₂CH₂O+H³), 3.37–3.42 (0.40H, m, H²), 3.47–3.57 (1.60H, m, AdCH₂CH₂O+H²), 3.74 (3.6H, s, OCH₃) [3.73 (2.4H, s, OCH₃)], 3.81 (1.8H, s, OCH₃) [3.79 (1.2H, s, OCH₃)], 4.32–4.35 (0.6H, m H^{3a}) [4.39 (0.4H, m, J=3.2 Hz, H^{3a})], 4.56–4.59 (1.2H, m, H^{1+3a}) [4.60–4.63 (0.80H, m, H^{1+3a})], 5.97 (1H, d, J=1.3, OCH₂O) [5.96 (1H, d, J=1.3, OCH₂O)], 6.38 (1.2H, s, H^{2',6'}) [6.25 (0.80H, s, H^{2',6'})], 6.49 (0.60H, s, H⁸) [6.54 (0.40H, s, H⁸)], 7.06 (0.60H, s, H⁵) [6.82 (0.40H, s, H⁵)]. NMR ¹³C (CDCl₃, δ , ppm); chemical shifts for **6 β** are in square brackets): 28.50 [28.53], 31.64 [31.60], 36.95 [36.96], 37.89 [38.30], 42.73 [42.71], 43.91 [43.88], 44.09 [43.82] (AdCH₂), 45.47 [41.09], 56.04 [56.16] (2OCH₃), 60.68 (OCH₃), 64.05 [66.26] (AdCH₂CH₂O), 71.57 [67.61], 79.82 [74.46] (C^4), 101.29 [101.37] (OCH₂O), 107.02 [110.70], 107.99 [108.15], 109.47 [109.45], 131.13 [129.59], 131.48 [132.14], 135.41 [135.49], 136.90 [137.02], 147.48 [146.59], 147.58 [148.17], 152.49 [152.42], 174.11 [175.08] (C=O). (MALDI-TOF), m/z : 576 (M⁺), 599 (M⁺ + Na), 615 (M⁺ + K). Found, %: C 70.78; H 7.04. Calculated, %: C₃₄H₄₀O₈, %: C 70.81; H 6.99.

2-(Adamant-1-yl)- (17 β)-17-hydroxy-2-methoxyestra-1,3,5(10)-triene-3-yl acetate (7) A solution of 2-(1-adamantyl)acetic acid (32.2 mg, 0.166 mmol) and DCC (34.15 mg, 0.166 mmol) in CH₂Cl₂ was added dropwise to a solution of 2-methoxyestradiol (50 mg, 0.166 mmol) and DMAP (0.02 mg) in CH₂Cl₂ for 15 min. After addition, the mixture was stirred for 30 min and then the solvent was evaporated, and ethyl acetate was added and cooled to 4 °C. The

precipitated N,N'-dicyclohexylurea was filtered off, and the filtrate was evaporated and chromatographed first using column chromatography on silica gel (petroleum ether (40–70 °C): ethyl acetate 1:15–1:10) and then on TLS glass plates pre-coated with silica gel with fluorescent indicator (Macherey–Nagel, Germany). Yield 15% (0.012 g), colorless oily liquid. NMR ¹H (CDCl₃, δ , ppm, J/Hz): 0.79 (3H, s), 0.84–0.89 (1H, m), 1.16–1.24 (1H, m), 1.31–1.57 (9H, m), 1.67–1.73 (6H, m), 1.76–1.77 (7H, m), 1.85–1.89 (1H, m), 1.95–2.00 (1H, m), 2.02 (3H, m), 2.11–2.15 (1H, m), 2.16–2.29 (1H, m), 2.32 (2H, s), 2.77–2.81 (2H, m), 3.74 (1H, m), 3.80 (1H, c), 6.72 (1H, s), 6.90 (1H, s). NMR ¹³C (CDCl₃, δ , ppm): 11.86, 23.58, 26.32, 27.31, 28.54, 30.14, 30.45, 33.32, 36.56, 37.92, 38.75, 41.82, 42.24, 43.46, 44.58, 49.78, 55.68, 85.70, 110.24, 121.68, 129.28, 136.84, 138.96, 148.15, 168.55 (AdCH₂CO₂). (MALDI-TOF), m/z : 478 (M⁺), 501 (M⁺ + Na), 517 (M⁺ + K).

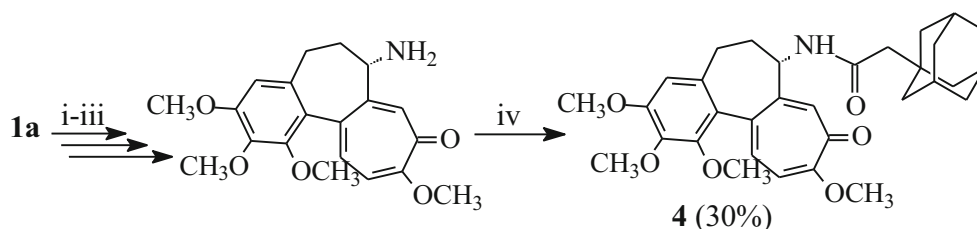
Biology

Cytotoxicity was measured using quantitative colorimetric MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-2H-tetrazoliumbromid, Roth GmbH, Karlsruhe, Germany) assay [15]. A549 human lung epithelial carcinoma cells (CCL-185™) were cultured with Dulbecco's modified Eagle's 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 nM–100 μ M (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, USA). For each compound, experiments were repeated three times and EC₅₀ values were determined by sigmoid curve fitting using Excel-based software.

Cell growth inhibition assay A549 cells were incubated with 1 μ M or 10 μ M of each tested (or control) compound during 24 and 48 h (0.5% DMSO was used as a negative control). After culturing, the cells were resuspended in PBS and counted directly by phase-contrast microscopy using hemocytometer.

Immunofluorescence staining of cellular MT A549 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 10 and 100 μ M for 24 and 48 h (0.5% DMSO served as a negative control). The cells were fixed and stained as described in [16]. Fixed cells were labeled for tubulin with mouse monoclonal antibody against α -tubulin at a dilution of 1:300 (Sigma, St.

Scheme 1 Reagents and conditions: (i) Boc_2O , NEt_3 , 4-DMAP, CH_3CN , 82°C , 12 h; (ii) 1. CH_3ONa , MeOH , rt, 1 h. 2. H_2O ; (iii) 1. CF_3COOH , CH_2Cl_2 , rt, 1 h; 2. NaOH , H_2O ; and (iv) 2-(1-adamantyl)acetic acid, EEDQ, CH_2Cl_2 , rt, 12 h



Louis, USA), followed by incubation of Alexa Fluor488 labeled goat anti-mouse IgG at a dilution of 1:300 (Invitrogen, Germany). 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).

Molecular modeling

Computer molecular modeling of ligand–tubulin interactions was performed with AutoDock 4.2 software [17], using a model of the colchicine binding site in tubulin (PDB ID: 1SA1). The structures of the compounds were previously submitted to a conformational MMFF force field optimization and then automated molecular docking was carried out. The best model was selected based on the scoring functions calculated by Autodock 4.2.

Results and discussion

The synthesis of colchicine derivative **4** was carried out by EEDQ-assisted amidation reaction of 2-(1-adamantyl)acetic acid with N-deacetylcolchicine, obtained from initial molecule in three steps using slightly modified procedure described in [18] and depicted on Scheme 1. The target conjugate **4** was obtained in a 15% yield, the formation of amide bond having been proved by the downfield shift of C^7 -proton resonance in its ^1H NMR spectra (4.65–4.71 ppm) in comparison with the corresponding peak of N-deacetylcolchicine (~3.71 ppm).

Podophyllotoxin ester **5** was synthesized by Steglich esterification of initial lignan **2a** with 2-(1-adamantyl)acetic acid in

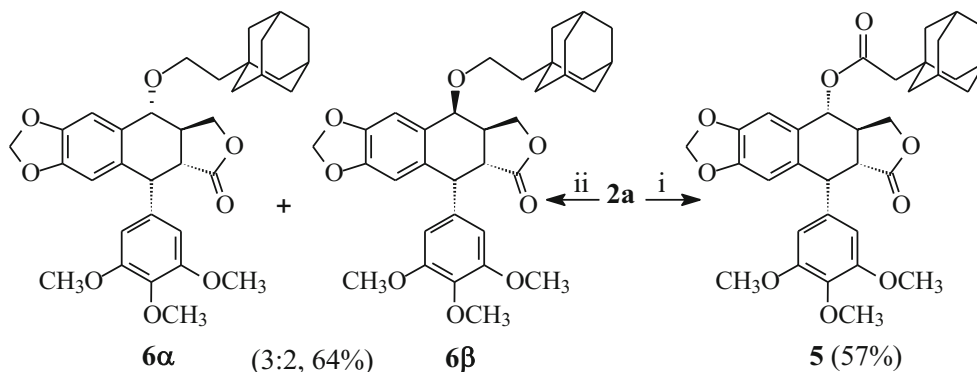
the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (4-DMAP) (Scheme 2). In ^1H NMR spectra of compound **5**, the resonance of C^4 -proton in podophyllotoxin fragment is observed at 5.87 ppm; in ^{13}C NMR spectra, atom C^4 is displayed at 73.25 ppm. The corresponding to ester **5** ether derivative of podophyllotoxin was obtained as diastereomeric mixture **6 α , β** by reaction of **2a** with (adamantan-1-yl)ethanol in the presence of boron trifluoride diethyl etherate (Scheme 2).

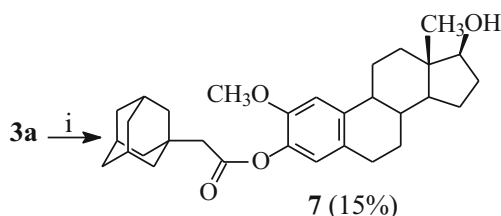
Though subjected to column chromatography on silica gel, the diastereomeric mixture was not resolved to individual isomers due to almost identical values of retardation factor of individual α and β isomers of **6**; the isomeric ratio was 3:2. In ^1H NMR spectra of compound **6**, the resonances of protons $-\text{OCH}_2\text{CH}_2\text{Ad}$ are observed at 3.47–3.57 ppm for α -isomer and at 3.37–3.42 ppm for β -isomer correspondingly. In ^{13}C NMR spectra of ether **6**, the resonance of atom C^4 is shifted upfield with respect to the corresponding peaks of C^4 in podophyllotoxin and revealed at 79.82 ppm for **6 α** and 74.46 ppm for **6 β** .

Steglich esterification of 2-methoxyestradiol **3a** with 2-(1-adamantyl)acetic acid in equivalent amounts led to a target compound **7** (Scheme 3) in a low yield 15% due to the double purification procedure. As though ester **7** partially decomposed during column chromatography, it was additionally purified using thin layer chromatography on glass plates.

The ability of synthesized conjugates to promote a formation of atypical tubulin assemblies in human epithelial lung carcinoma cell line A549 was investigated using immunofluorescence microscopy. The compounds were also evaluated for determination of their cytotoxicity and ability to inhibit the

Scheme 2 Reagents and conditions: (i) 2-(1-adamantyl)acetic acid, DCC, 4-DMAP, CH_2Cl_2 , rt, 12 h and (ii) (adamantan-1-yl)ethanol, $\text{BF}_3 \cdot \text{Et}_2\text{O}$, CH_2Cl_2 , 0°C , then rt, 6 h, column chromatography





Scheme 3 Reagents and conditions: (i) 2-(1-adamantyl)acetic acid, DCC, 4-DMAP, CH_2Cl_2 , rt, 12 h; (added dropwise to 2-methoxyestradiol)

cell growth correspondingly in a standard calorimetric MTT assay and using microscopy for direct cell counting over 24 h and 48 h of culturing. The results are presented in Table 1 and on Fig. 2.

The results of biotests indicated that the behavior of all compounds in the cells of carcinoma A549 was different. At 10 μM , colchicine derivative **4** caused complete depolymerization of microtubules and also stimulated the formation of small clusters typical for some less active tubuloclustin analogues [12] (Fig. 2c). This result was interesting, because the tubulin-clustering ability was mostly associated with the colchicine binding site derivatives with long linker chain (see, e.g., [9–11]). Present data reveal that this effect can be manifested by compound with rather short linker. However, since the clusterization was observed at high concentrations, this linker length seems to be minimal for the effect observation. Conjugate **4** was more cytotoxic than parent molecule (see Table 1) in accordance with the previous observation that the ability to cause tubulin clustering correlates with cytotoxicity level [12].

Interesting result was observed for podophyllotoxin ester **5**: at 10 μM , it stimulated the formation of tubulin associates curled and involuted so intensively that they

reminded a “tangle of microtubules” (Fig. 2f). The type of tubulin assemblies was totally different from the tubulin clusters induced by compound **4** or podophyllotoxin analogue of tubuloclustin (**2a**) [12]. The shape of the observed associates did not resemble either regular vinblastine-induced paracrystals or taxol-induced MT bundles. Structurally similar to ester **5**, podophyllotoxin ethers **6 α,β** at concentration 10 μM were found to cause MT depolymerization and the formation of some shortened and slightly curled microtubules (Fig. 2d). However, at high concentrations (100 μM), ethers **6 α,β** promoted the same effect as ester **5** (Fig. 2e). Thus, instead of either clustering typical for the action of tubuloclustin analogues or diffused tubulin pattern (see Fig. 2b) specific for the action of all classical colchicine binding site ligands in cells, e.g., podophyllotoxin (**2a**), 2-methoxyestradiol (**3a**), nocodazole, or combretastatin A-4, we observed the network of unusual MT after cell treatment with compounds **5** and **6** (Fig. 2e, f).

Computer molecular docking of compounds **5** and **6 α** into the model of the colchicine binding site and intradimer region of α/β -tubulin (PDB ID: 1SA1) indicates that carbonyl oxygen of the ester bond in **5** does not form any hydrogen bonds with protein (Fig. 3a), and adamantane moieties of both compounds are located in the very close areas at the interface of α and β -subunits. So, the differences in concentrations necessary for the formation of “entangled assemblies” seem to relate to various conformational flexibility of linker chains of **5** and **6 α** .

According to the computer molecular modeling data the position of adamantane core in podophyllotoxin, ester **5** does not match the one in colchicine derivative **4** (Fig. 3), which might explain the differences in the structural type of the tubulin associates induced by the compounds.

Table 1 Effect of the compounds 4–7 on A549 cells

Compound	Cytotoxicity, EC_{50} (nM), ^a cell growth inhibition, %	Effect on microtubules (MT) (10 μM , 48 h if not specially indicated)
4	20 \pm 4 1 μM (48 h): 100%	MT depolymerized, very few tiny tubulin clusters, at 100 μM (24 h): spot-like tubulin clusters
5	6200 \pm 600 10 μM (48 h): 100%	Strongly “entangled” MT
6α,β (3:2)	8000 \pm 2000 10 μM (48 h): 55%	MT depolymerized, some shortened and slightly curled MT, at 100 μM (24 h): “entangled” MT
7	n.d. 10 μM (48 h): 100%	No effect on MT, at 100 μM (24 h): slightly curled MT
1a	30 \pm 3	MT depolymerized
Colchicine	1 μM (48 h): 100%	
2a	10 \pm 3	MT depolymerized
Podophyllotoxin	1 μM (48 h): 100%	
3a	100 \pm 3	MT depolymerized
2-Methoxyestradiol	1 μM (48 h): 100%	

^a The average of three to six experiments

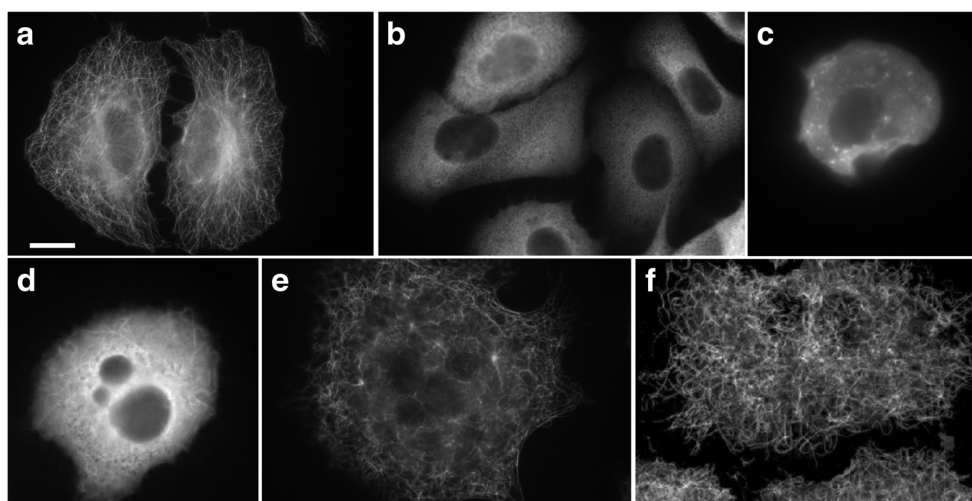


Fig. 2 Immunofluorescence microscopy images of the MT network in human lung carcinoma cells A549 treated with tested conjugates or DMSO or control compounds. **a** Intact MT (0.5% DMSO, negative control). **b** MT depolymerized (**2a**, positive control; the same for **1a** and **3a**); **c** Spot-like tubulin clusters (**4**, 100 μM). **d** MT depolymerized,

some shortened and slightly curled MT (**6 α,β** , 10 μM). **e** “Entangled” microtubules (**6 α,β** , 100 μM), the rests of MT around centrosomes are observed as star-like structures. **f** Strongly “entangled” MT (**5**, 10 μM). Bar 20 μm

2-Methoxyestradiol conjugate **7** did not cause any effect on MT network at concentration 10 μM ; however, at 100 μM , slight MT “curling” was observed in A549 cells and this action reminds the effect of 2-methoxyestradiol analogue of tubuloclastin (**3a**) on microtubule cytoskeleton [13].

In general, the studied structural modification of the ligands of colchicine binding site enables their ability to induce the formation of the assemblies under the conditions when no aggregates are formed by parent molecules. The precise mechanism of the conformational changes in tubulin leading to the curling effect is worth additional study and this work is now in progress.

Conclusion

Four conjugates of adamantane connected at a bridge-head position to structurally different ligands, interacting with colchicine binding site in tubulin via short linker of equal length, were synthesized. Immunofluorescence microscopy study revealed the ability of compounds to promote the formation of different atypical tubulin assemblies—clusters, curly, or “entangled” microtubules in cancer cells A549. The distinction in the observed effects for conceptually equivalent structural templates was partially explained by computer molecular modeling.

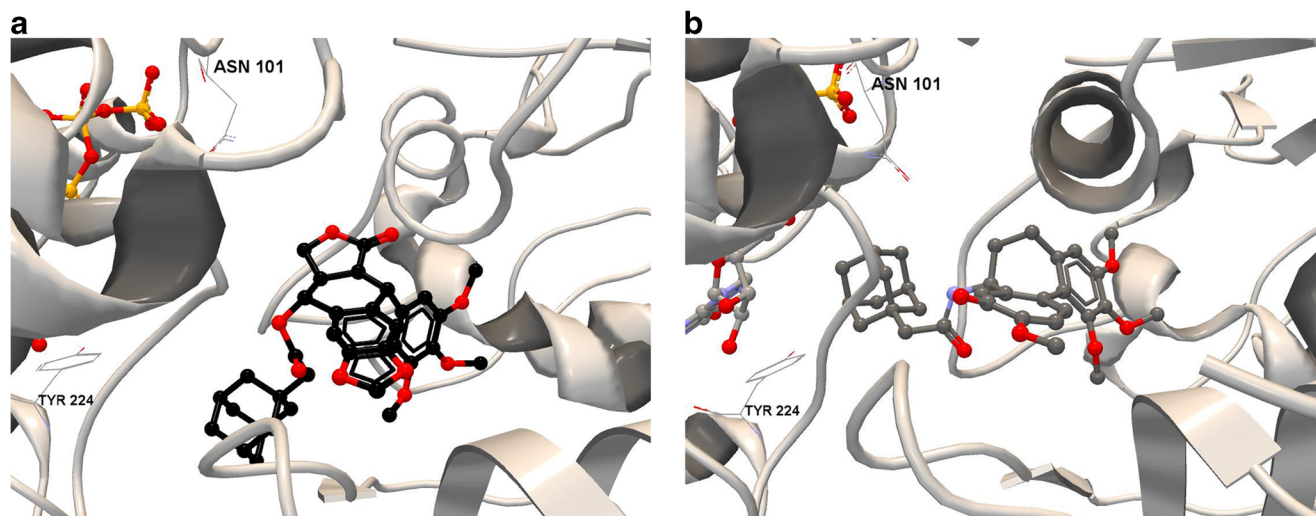


Fig. 3 Binding modes of conjugates **5** (**a**) and **4** (**b**) in tubulin dimer as predicted by automated docking (AutoDock 4.2; visualized using CLC Drug Discovery Workbench). α -Subunit is presented at the left and β -subunit is at the right (hydrogen atoms are omitted for clarity)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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