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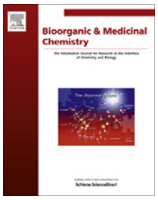

Synthesis and biological evaluation of novel anticancer bivalent colchicine–tubulizine hybrids

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ABSTRACT

A series of novel antimitotic hybrids were synthesized in good yields by linking of azide-containing colchicine congeners with acetylene-substituted tubulizine-type derivatives using copper-mediated 1,3-dipolar cycloaddition. Obtained compounds exhibit good cytotoxicity against HBL100 epithelial cell lines (IC_{50} = 0.599–2.93 μM). Several newly synthesized compounds are the substoichiometric inhibitors of microtubule assembly (R = 0.41–0.78). The results highlight the importance of the length of spacer linking the tubulin binding ligands in heterodimeric molecules.

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1. Introduction

Microtubules, formed by tubulin molecules, are essential components of the cytoskeleton in eukaryotic cells and are involved in many important cellular processes including mitosis. As components of the mitotic spindle, microtubules have emerged as a strategic target in anticancer therapy.[1] Depending on the molecular structure of tubulin poison (Fig. 1) it is able to act with different binding sites on tubulin: with the vinca, taxane, colchicine and tubulizine domains.[2] Despite existence of the broad range of antimitotic agents, only few examples reached so far clinical and commercial success.[2] The failure of the plurality of these molecules could be attributed to poor therapeutic indexes—the balance between efficiency and toxicity, perhaps related to pharmacokinetics, to the solubility problems, low affinity of therapeutic agents to tubulin molecules in vivo trials and other unrecognized factors.[1a] One way to improve some of these pharmacological parameters is to construct therapeutic molecules in accordance with the concept of multivalency.[3]

This theory is based on the observation that in nature various biological interactions are mediated multivalently, so that individual ligands bind multiple receptors simultaneously to produce a greater biological effect.[4] This can be exemplified by the attachment of viruses and bacteria to cellular surfaces, by binding of a cell to another cell, or by interactions of antibodies with pathogens.[5] Several natural compounds possessing multiple binding sites are represented by the homo- or heterodimers of bis-indole alkaloid vincristine,[6] bis-steroidal pyrazine cephalostatine,[7] dimeric sesquiterpene absinthine[8] or bifunctional duocarmycin derivatives.[9] In fact, polyvalent interactions are normally much stronger than the corresponding monovalent contacts. They can provide the basis for the drug-receptor interactions, which are fundamentally different from those available in monovalent systems.[3]

The idea of combining different monovalent agents into bivalent hybrids was used for the construction of several types of novel antimitotic agents like taxoid-colchicine[4c] (thiocolchicine[10]) or vinca alkaloids-taxoid[11] hybrids and podophyllotoxin-thiocolchicine[12] dynamic combinatorial library. Some of obtained dimeric agents, however, were shown to be even less active in comparison with their monomeric precursors. This can be explained by the use of rather rigid and short linkers between the individual ligands, making simultaneous binding of both ligands to tubulin impossible.

We report herein the synthesis of new antimitotic bivalent ligands, connected by flexible spacers, prepared by linking of

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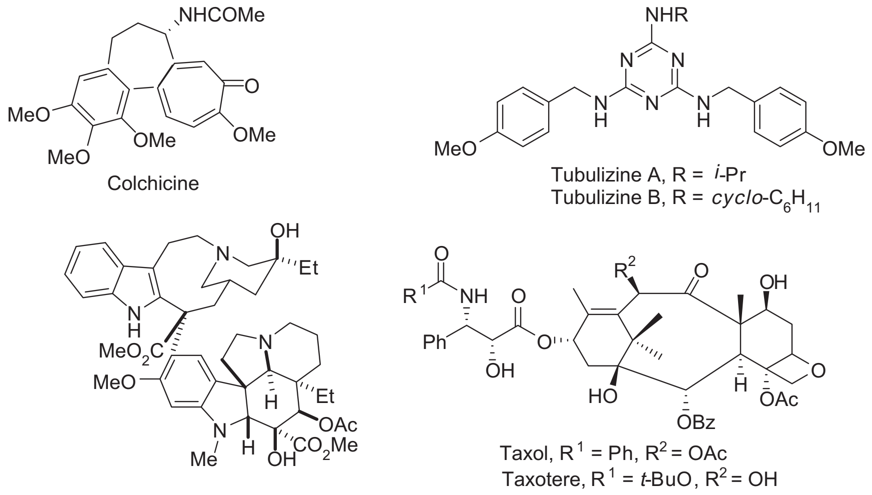
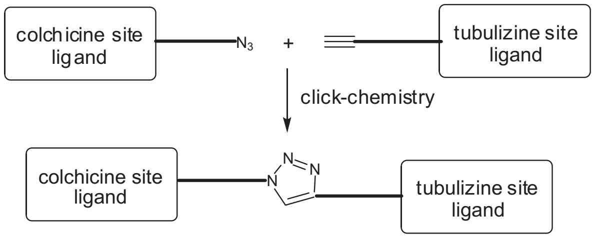


Figure 1. Structure of some compounds with high affinity for different tubulin binding sites.



Scheme 1. Synthesis of colchicine–tubulizine hybrids.

azide-containing deacetylcolchicine and acetylene-substituted tubulizine entities using copper-mediated[13] 1,3-dipolar cycloaddition (Scheme 1). The colchicine binding site includes 239Cysß of ß-tubulin,[1b,14] while tubulizine interacts with 12Cysß of the same protein molecule.[2c] 12Cysß is known to be a part of the exchangeable GTP-binding site.[2c] Techniques for measuring fluorescence resonance energy transfer (FRET) has been used to determine the distance between colchicine and GTP-binding sites.[15] However, no FRET was observed between ligands bound to the colchicine and GTP sites, indicating that these binding sites are at least 40 Å apart.[15b] The exact distance between colchicine and GTP-binding sites is unknown due to long range and allosteric effect.[15a] Tubulizines' binding to tubulin induces conformational changes of protein.[2c] Therefore, we decided to synthesize several colchicine–tubulizine hybrids with various length of the linker, in order to find the optimal distance between the affine units and to realize their simultaneous binding to colchicine and tubulizine domains of the tubulin molecule.

Recently, it has been shown that the functionalization of the colchicine core in the position 7[16] as well as the tubulizine molecule in the NH-alkyl fragment[17] can be done without a significant loss of antitumor activity. Therefore, a range of acylated deacetylcolchicines **4a–c** (Scheme 2) and tubulizines **9a–e** (Scheme 3) were proposed as building blocks for the heterodimers construction.

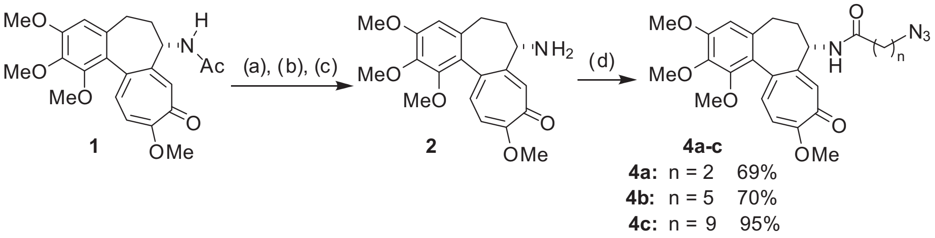
2. Results and discussion

2.1. Synthesis of colchicine–tubulyzine heterodimers

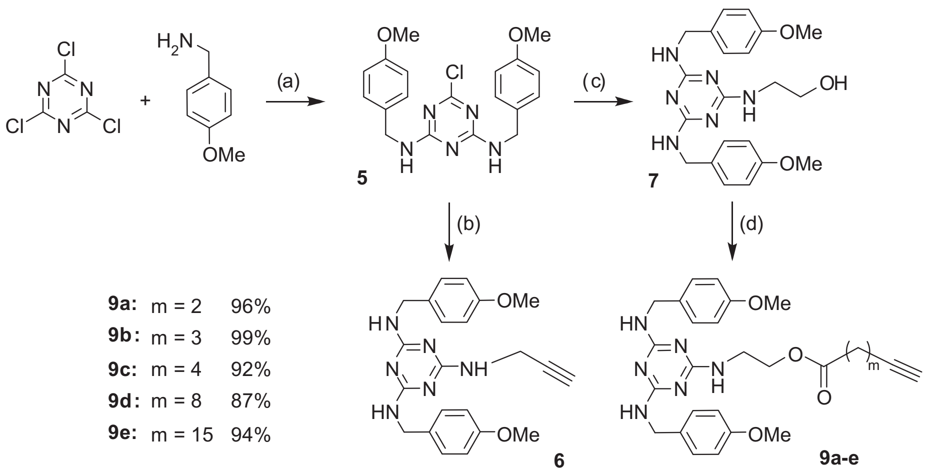
The colchicine site ligands **4a–c**, bearing linkers of various lengths, were synthesized from commercially available colchicine **1** in several steps (Scheme 2). The one-pot deacetylation of colchicine[18] afforded deacetylcolchicine **2** in 94% overall yield. It was subjected to the Steglich amidation[18a] with a row of ω-azidocarboxylic acids $N_3(CH_2)_nCO_2H$ (**3a–c**, n = 2, 5, 9), prepared from the corresponding ω-bromocarboxylic acids).[19] Related ω-azidoamides **4a–c** were obtained in good to high yields (69–95%).

Alkyne-substituted tubulizine analogs **6** and **9a–f** were prepared from commercially available 2,4,6-trichloro-1,3,5-triazine (cyanuric chloride, Scheme 3). Its reaction with *p*-methoxybenzylamine in aqueous acetone led to 2-chloro-4,6-di-(*p*-methoxybenzylamino)-1,3,5-triazine **5** in 92% yield.[2c] Its treatment with propargylamine in dioxane at 100 °C gave alkynyl analog of tubulizine A (**6**) in a good yield. Triazine **5** reacted with 2-aminoethanol to give hydroxyl-substituted tubulizine analog **7** in 71% yield. Esterification of **7** with alkynoic acids (**8a–e**, m = 2, 3, 4, 8, 15) afforded acetylenic esters **9a–e** in excellent yields (Scheme 3).

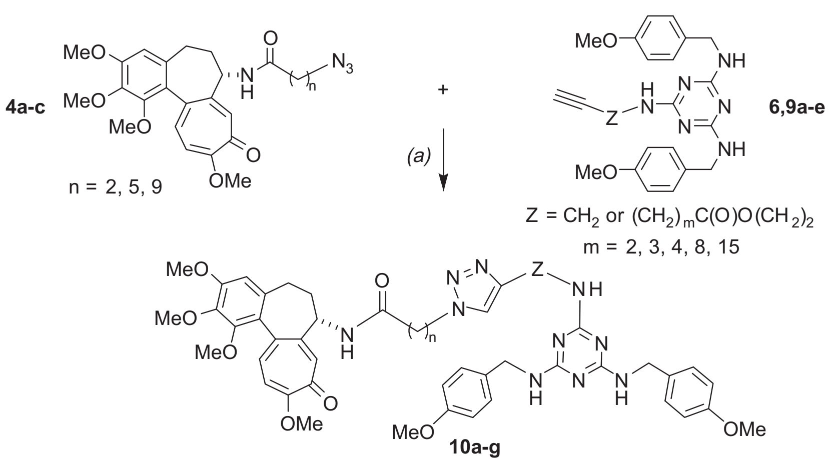
The tubuline ligands **4a–c**, **6** and **9a–e**, bearing correspondingly terminal azide- and alkyne-fragments, were linked using 'click' [3+2] cycloaddition reaction,[13] applying copper sulfate/magnesium ascorbate catalytic system in THF-water solution (Scheme 4). This methodology allows to synthesize exclusively 1,4-regioisomeric triazoles **10a–g** in 47–82% yields (Table 1).



Scheme 2. Synthesis of colchicine derivatives containing an azide group. Reagents and conditions: (a) $(Boc)_2O$, DMAP, Et_3N, CH_3CN, 100 °C, 4 h; (b) MeONa, MeOH, rt, 1.5 h; (c) TFA, CH_2Cl_2, rt, 2 h, 94% (yield of 3 steps); (d) $N_3(CH_2)_nCOOH$ (**3a–c**, *n* = 2, 5, 9), EDC, DMAP, CH_2Cl_2, 0 °C—1 h, rt—4 h, 69–95%.



Scheme 3. Synthesis of tubulizine derivatives containing a terminal alkyne group. Reagents and conditions: (a) acetone, H_2O, 0 °C—20 min, rt—20 h, 92%; (b) $NH_2CH_2C\equiv CH$, dioxane, 100 °C, 60 h, 61%; (c) $NH_2(CH_2)_2OH$, dioxane, 100 °C, 60 h, 71%; (d) $HC\equiv C(CH_2)_mCOOH$ (**8a–e**, *m* = 2, 3, 4, 8, 15), DCC, DMAP, THF, rt, 24 h, 87–99%.



Scheme 4. Synthesis of triazole-joined colchicine–tubulizine hybrids using click chemistry. Reagents and conditions: $CuSO_4{\cdot}5H_2O$, 0.1 equiv, MgAsc, 0.2 equiv, THF–H_2O (1:1), rt, 24 h, 57–82%.

Table 1
The yields of colchicines-tubulizine hybrids

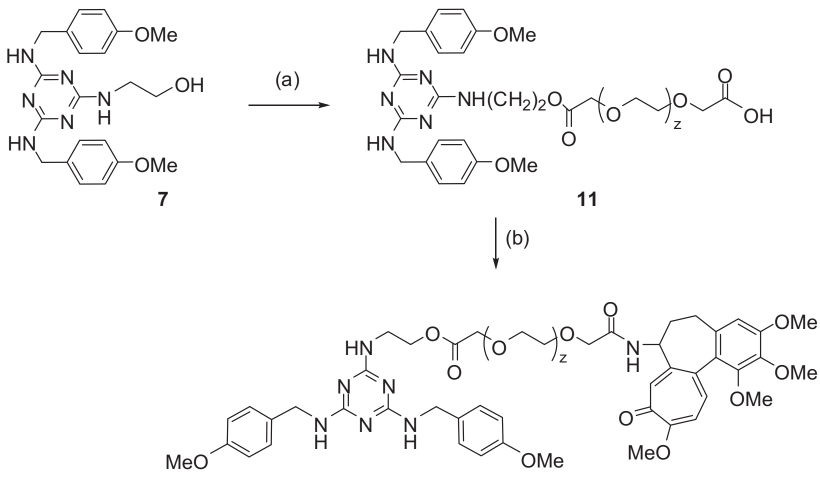
Compound	*n*	*m*	*l*[a]	Yields (%)
10a	9	–	16	47
10b	2	2	14	57
10c	2	4	16	82
10d	5	3	18	73
10e	2	8	20	63
10f	9	3	22	69
10g	5	15	30	64

[a] Linker length (*l*) was calculated as the number of atoms between two NH-groups inclusively.

Additionally to **10a–g** triazole-linked compounds, polyethyleneglycol-bridged colchicine–tubulizine heterodimer **10h** with the longest spacer, containing 24 carbon and 11 oxygen atoms, was prepared using two-step synthetic procedure (Scheme 5). At the first stage, tubulizine analog 7 was subjected to the Steglich esterification with poly(ethylene glycol)-600 bis-dicarboxylic acid, affording intermediate **11** in 55% yield. The subsequent reaction of **11** with deacetylcolchicine **2** led to PEG-bridged colchicine–tubulizine dimer **10h** in 37% overall yield (Scheme 5).

2.2. Evaluation of in vitro biological activity

In vitro cytotoxicity of the synthesized colchicine–tubulizine hybrids **10a–h** as well as the modified tubuline ligands **2**, and **7** was investigated toward HBL100 human mammary cell line. A tetrazolium-based assay was used for determination of the drug concentration required to inhibit cell growth by 50% after the incubation in the culture medium for 72 h. The obtained values are summarized in Table 2. Furthermore, to confirm that the potential activity of these compounds are due to interaction with tubulin binding site, studies on their effect on microtubules assembly were performed. As an example, Figure 2 shows the effects of **10e** on the 4′,6-diamidino-2-phenylindole (DAPI) fluorimetric time course of in vitro microtubule assembly from pure tubulin. A clear inhibition was observed, and the rate of assembly as well as the final amount of microtubules was lower in the presence of **10e** than in the control experiment. The inset of Figure 2 show that the extent of inhibition increased monotonically with the mole ratio of the total ligand to total tubulin in the solution (*R*). The activity of microtubule assembly inhibition for hybrids **10b–h** decreases along with the linker length. Very importantly, that hybrids with *l = 14–20* are the substoichiometric inhibitors of microtubule assembly (*R* = 0.41–0.71, Table 2) with the same order of activity as diacetylcolchicine



10h z = 10

Scheme 5. Synthesis of PEG-joined colchicine–tubulizine hybrid. Reagents and conditions: (a) PEG-diacid, DCC, DMAP, THF, rt, 24 h, 55%; (b) deacetylcolchicine, EDC, DMAP, CH_2Cl_2, 0 °C—1 h, rt—4 h, 68%.

Table 2
Cytotoxicity and inhibitory effects on microtubule assembly of colchicine–tubulizine hybrids

Compound	l	IC_{50} (μM)	R^a	Log$D$
2	–	0.045 ± 0.002	0.17	–
7	–	3.14 ± 0.24	0.34	–
10a	16	0.699 ± 0.021	0.99	8.465
10b	14	2.59 ± 0.03	0.48	4.815
10c	16	1.62 ± 0.09	0.41	5.591
10d	18	1.86 ± 0.07	0.48	6.367
10e	20	0.687 ± 0.013	0.71	7.612
10f	22	1.01 ± 0.06	3.40	8.738
10g	30	0.599 ± 0.06	2.75	9.814
10h	40	2.93 ± 0.01	3.44	2.568

[a] R—half inhibitory molar ratio (ligand/tubulin) of microtubule formation in vitro.

2 (R = 0.17, Table 2). The decrease of tubulin inhibition by heterodimers with l = 22–40 can be argued by increase of the steric encumbrance adversely influencing the effective binding to the catalytic sites of protein in the case of **10f–h** agents. On the contrary, the cytotoxic activity of the synthesized compounds depends on hydrophobicity (Log$D$) of heterodimers. The cell growth inhibiting activity increases with the rise of hydrophobicity of hybrids what can be explained by augment of membranotropicity for these compounds. While heterodimers **10a–h** manifested relatively high cytotoxic activity (IC_{50} =*0.599–2.93* μM), all of them appeared to be less active than deacetylcolchicine **2**, but more active compare to tubulizine **7** (Table 2). Such decrease of the activity in comparison with the parent compound can be attributed to such factors as cell availability and fast biological transformations of the newly prepared compounds. Compound **10e** with l = 20, contain, probably, the optimal spacer between the tubulin-binding units, as it manifest the best combination of antiproliferative and tubulin binding activity (IC_{50} = *0.687 ± 0.013* μM, R = 0.71, Table 2). Hybrid **10a** without ester group between triazole fragment and tubulizine core showed relatively good cytotoxic and tubulin binding activity (IC_{50} = 0.699 ± 0.021 μM, R = 0.99), like **10e** compound containing such ester linkage. The lowest cytotoxicity and inhibition of microtubule formation were found for the long chain PEG-bridged colchicine–tubulizine dimer **10h** which characterized with low hydrophobicity (IC_{50} = *2.93 ± 0.01* μM, R = *3.44,* Table 2).

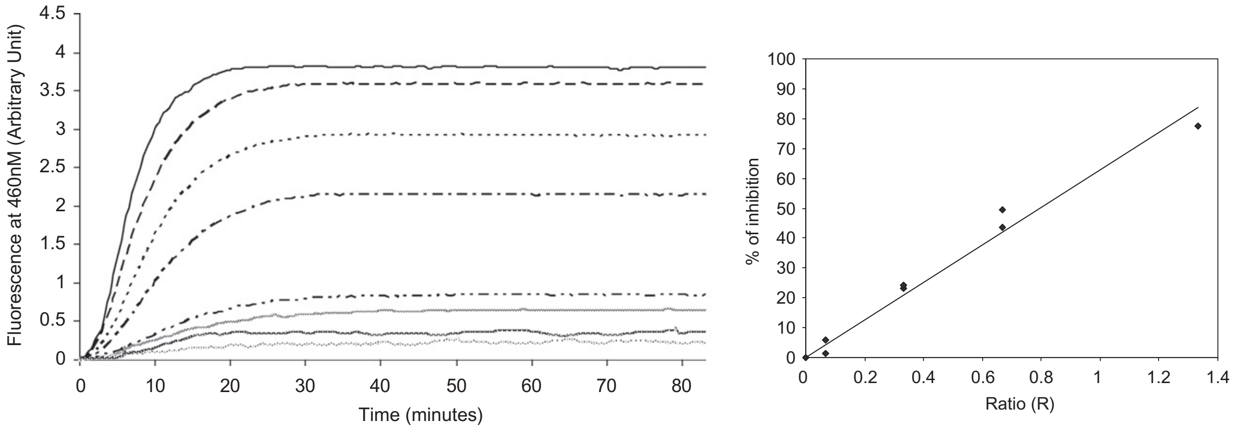


Figure 2. Effect of **10e** on the 4′,6-diamidino-2-phenylindole (DAPI) fluorimetric time course of in vitro microtubule assembly (excitation at 355 nm). The reaction was started by warming the solution to 37 °C. Panel shows tubulin polymerization at 15 μM alone (line 1) and with 1, 5, 10, 20, 30 μM of **10e** (line 2–6). Colchicine derivative 2 (line 7) and tubulizine derivative 7 (line 8) were used as positive controls at 5 and 10 μM, respectively. Inset shows the percentage of fluorescence inhibition as a function of the molar ratio of ligand to tubulin in the solution (R).

3. Conclusions

Several new hybrid tubulin-interacting agents were synthesized by linking the modified small tubuline ligands, using modular approach and the 'click' chemistry. Exclusively 1,4-triazole regioisomers of colchicine–tubulizine hybrids **10a–g** were obtained in 47–82% yields. The cytotoxic activity of the synthesized heterodimers with several different linker lengths was investigated toward HBL100 human mammary cell line. All of the tested heterodimers presented substantial cytotoxic activity (IC_{50} = *0.599–2.93* μM), all of them appeared to be less active than deacetylcolchicine **2**, but more active compare to tubulizine **7**. Analysis of the structure–activity relationship for synthesized hybrids showed that their activity of microtubule formation inhibition decreases along with the linker length and cytotoxic activity depends on hydrophobicity of the molecule. Several newly synthesized compounds **10b–e** are the substoichiometric inhibitors of microtubule assembly (R = 0.41–0.71). The highest activity among the heterodimers was achieved for ligand **10e** (IC_{50} = *0.687 ± 0.013* μM, R = 0.71). The proposed modular synthesis and simple 'click' chemistry methodology makes it easy to expand the bivalent hybrid library with a variety of new members.

4. Experimental section

4.1. Chemistry

4.1.1. General information

^{1}H and ^{13}C NMR spectra were recorded on 300 and 600 MHz spectrometers Varian NMR-System INOVA 300, INOVA 600. Chemical shifts are given as ppm relative to the residual solvent peak (chloroform-d1: 7.26/77.0 ppm). Column chromatography purification was performed on Merck silica gel 60 (0.040–0.063 mm, 230–400 mesh ASTM). Melting points are uncorrected and were measured on a Büchi B.540 apparatus. Mass spectra were recorded on a Finnigan MAT 90 and Finnigan MAT 95Q instrument. Commercial reagents were used without prior purification.

4.1.2. Synthesis of *N*-deacetylcolchicine (2)

Di(*tert*-butyl) pyrocarbonate (6 g, 28 mmol) was added portionwise to a mixture of colchicine **1** (2.4 g, 6 mmol), Et_3N (1.7 mL, 12 mmol), and DMAP (0.73 g, 6 mmol) in CH_3CN (50 mL), and the mixture was stirred for 4 h at 100 °C. The reaction was quenched by the addition of 150 mL of CH_2Cl_2 and was washed with 3 × 100 mL of saturated aqueous citric acid. The combined aqueous layers were back extracted with CH_2Cl_2, and the organic layers combined. The organic layer was washed with saturated brine and then concentrated to a brown solid. 0.65 ml of 2 M solution of NaOMe in MeOH was added to a solution of *N*-boccolchicine in 3 ml of MeOH. The reaction mixture was stirr0d for 1.5 h at room temperature. It was then neutralized by the addition of NH_4Cl and and extracted with CH_2Cl_2. The organic layer was washed with saturated brine and then concentrated to a brown solid. The residue was purified by flash chromatography on silica gel (AcOEt/acetone, 4:1) to give of *N*-[(*tert*-butoxy)carbonyl]deacetylcolchicine (2.67 g, 5.8 mmol) as a pale yellow solid. A solution of *N*-[(*tert*-Butoxy)carbonyl]deacetylcolchicine (2.67 g, 5.8 mmol) in CH_2Cl_2 (50 mL) containing TFA (5 mL) was stirred for 3 h at room temperature. Toluene was then added, the mixture was concentrated in vacuo, and the residue was purified by flash chromatography on silica gel (CH_2Cl_2/EtOH, 9:1) to give deacetylcolchicine **2** (2.03 g, 5.7 mmol, 95%) as a yellow solid, mp 172.4–178.8 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.15 (br s, 2H), 7.69 (s, 1H), 7.37 (d, J = 11.3 Hz, 1H), 6.95 (d, J = 11.3 Hz, 1H), 6.54 (s, 1H), 4.05 (s, 1H), 3.98–3.78 (m, 9H), 3.55 (s, 3H), 2.69–2.47 (m, 2H), 2.46–2.11 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 179.44, 163.93, 154.28, 151.00, 147.29, 141.71, 137.17, 137.07, 133.78, 131.65, 124.37, 114.48, 107.81, 61.28, 61.15, 56.78, 56.15, 53.93, 35.78, 29.50. MS (ESI), m/z (%): 356 (13) $[M-H]^+$, 249 (95). HRMS (ESI), m/z: Calcd for $C_{20}H_{24}NO_5$ 358.1654. Found 358.1649 $[M+H]^+$.

4.1.3. General procedure for the preparation of colchicine deriv atives (4a–c)

A solution of 3-azidopropionic acid (173 mg, 1.5 mmol) in CH_2Cl_2 (2 ml) was added to **2** (268 mg, 0.75 mmol), EDC·HCl (288 mg, 1.5 mmol) and DMAP (46 mg, 0.375 mmol) at 0 °C under nitrogen atmosphere. The obtained solution was stirred for 1 h at 0 °C, then for 4 h at room temperature. The solvent was removed and the residue was purified by flash chromatography on silica gel (EtOAc/acetone, 2:1, then EtOAc/acetone/EtOH, 2:1:1) to give *N*-(3-azidopropionyl)deacetylcolchicine **4a** (163 mg, 0.359 mmol, 48%) as a yellow solid, mp 150.0–152.1 °C.

^{1}H NMR (599 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.66 (s, 1H), 7.39 (d, J = 10.8 Hz, 1H), 6.93 (d, J = 10.8 Hz, 1H), 6.52 (s, 1H), 4.74–4.64 (m, 1H), 3.99 (s, 3H), 3.92 (s, 3H), 3.88 (s, 3H), 3.64 (s, 3H), 3.57–3.42 (m, 2H), 2.61–2.43 (m, 3H), 2.39–2.20 (m, 2H), 1.92–1.82 (m, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 179.68, 169.93, 163.79, 153.77, 153.30, 151.20, 141.70, 137.78, 135.96, 134.39, 131.04, 125.41, 113.70, 107.52, 61.68, 61.46, 56.66, 56.20, 52.42, 47.24, 36.72, 35.06, 29.97. MS (ESI), m/z (%): 453 (16) $[M-H]^+$, 249 (14). HRMS (ESI), m/z: Calcd for $C_{23}H_{25}N_4O_6$ 453.1774. Found 453.1760 $[M-H]^+$.

4.1.4. *N*-(6-Azidocaproyl)deacetylcolchicine (4b)

Yellow solid (70%), mp 85.5–89.5 °C, ^{1}H NMR (599 MHz, $CDCl_3$) δ 7.52 (s, 1H), 7.38 (s, 1H), 7.32 (d, J = 10.8 Hz, 1H), 6.85 (d, J = 10.8 Hz, 1H), 6.50 (s, 1H), 4.73–4.54 (m, 1H), 3.98 (s, 3H), 3.91 (s, 3H), 3.87 (s, 3H), 3.63 (s, 3H), 3.36–3.10 (m, 2H), 2.63–2.08 (m, 5H), 1.95–1.24 (m, 7H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 179.56, 172.59, 164.11, 153.62, 152.25, 151.30, 141.76, 136.83, 135.58, 134.31, 130.68, 125.68, 112.88, 107.45, 61.71, 61.49, 56.51, 56.21, 52.36, 51.30, 36.89, 35.95, 30.03, 28.64, 26.47, 24.96. MS (ESI), m/z (%): 495 (51) $[M-H]^+$, 340 (6), 156 (17).HRMS (ESI), m/z: Calcd for $C_{26}H_{33}N_4O_6$ 497.2400. Found 497.2392 $[M+H]^+$.

4.1.5. *N*-(10-Azidodecanoyl)deacetylcolchicine (4c)

Yellow oil (95%), ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.68 (s, 1H), 7.40 (d, J = 10.8 Hz, 1H), 7.18 (br s, 1H), 6.92 (d, J = 10.8 Hz, 1H), 6.53 (s, 1H), 4.75–4.60 (m, 1H), 4.01 (s, 3H), 3.93 (s, 3H), 3.89 (s, 3H), 3.64 (s, 3H), 3.29–3.13 (m, 2H), 2.61–2.15 (m, 5H), 2.03–1.83 (m, 1H), 1.68–1.45 (m, 4H), 1.44–1.14 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.18, 173.09, 164.11, 153.78, 153.25, 151.29, 141.80, 137.68, 136.31, 134.42, 130.72, 125.58, 113.68, 107.56, 61.77, 61.49, 56.62, 56.24, 52.37, 51.57, 37.09, 36.35, 30.08, 29.38, 29.33, 29.31, 29.16, 28.91, 26.76, 25.49. MS (ESI), m/z (%): 553 (100) $[M+H]^+$, 523 (4). HRMS (ESI), m/z: Calcd for $C_{30}H_{41}N_4O_6$ 553.3026. Found 553.3023 $[M+H]^+$.

4.1.6. General procedure for the preparation of (6,7)

A suspension of cyanuric chloride (17.8 g, 97 mmol) in acetone (100 mL) was cooled on ice and treated dropwise with 4-methoxybenzylamine (25 mL, 193 mmol). After the mixture was stirred at that temperature for 20 min, 200 mL of a 1 N sodium hydroxide aqueous solution was added dropwise at rt, followed by stirring for 20 h at room temperature. The precipitate was collected by filtration and washed with EtOH to obtain 2-chloro- 4,6-di (4-methoxybenzylamino)-1,3,5-triazine **5** (34 g, 89 mmol, 92%) as a white solid. To a suspension of **5** (0.75 g, 2 mmol) and monopropargyliamine (0.35 ml, 5 mmol) in dioxane (5 mL) was added DIPEA (1 ml, 3 mmol). The mixture was heated for 60 h at 100 °C and the solvent was removed on vacue. The compound was isolated by flash chromatography on silica gel (EtOAc/EtOH 10:1) to give 2,4-di(4′-methoxybenzylamino)-6-(propargylamino)-1,3,5-triazine **6** (0.49 g, 61%) as a yellow oil. ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.20 (d, J = 8.5 Hz, 4H), 6.82 (d, J = 8.5 Hz, 4H), 5.68 (br s, 3H), 4.47 (s, 4H), 4.15 (br s, 2H), 3.77 (s, 6H), 2.16 (s, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 165.51, 164.47, 158.93, 131.23, 129.00, 114.03, 71.14, 67.20, 55.38, 44.22, 30.47. MS (EI), *m*/*z* (%): 404 (100) [M]$^+$, 403 (43), 283 (24), 136 (20), 121 (62). HRMS, *m*/*z*: Calcd for $C_{22}H_{24}N_6O_2$ 404.1961. Found 404.1966 [M]$^+$.

4.1.7. 2-(2′-Hydroxyethylamino)-4,6-di(4′-methoxybenzylamino)-1,3,5-triazine (7)

White solid (71%), mp 106.4–108.0 °C, ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.17 (d, J = 7.9 Hz, 4H), 6.80 (d, J = 7.9 Hz, 4H), 6.52 (br s, 1H), 5.98 (br s, 1H), 4.95 (br s, 2H), 4.44 (s, 4H), 3.76 (s, 6H), 3.69 (s, 2H), 3.46 (br s, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 164.44, 163.82, 158.85, 130.71, 128.84, 113.94, 62.87, 55.26, 44.11, 43.75. MS (EI), *m*/*z* (%): 410 (90) [M]$^+$, 380 (12), 289 (19), 245 (14), 136 (40), 121 (100). HRMS, *m*/*z*: Calcd for $C_{21}H_{26}N_6O_3$ 410.2066. Found 410.2056 [M]$^+$.

4.1.8. General procedure for the preparation of tubulyzine derivatives (9a–e)

A solution of **7** (1 g, 2.4 mmol), 4-pentinoic acid (0.27 g, 2.6 mmol), DCC (0.7 g, 3.1 mmol) and DMAP (0.37 g, 3 mmol) in THF (4 ml) was stirred for 24 h at room temperature under nitrogen atmosphere. The solvent was removed and the residue was purified by flash chromatography on silica gel (EtOAc/pentane, 1:1, then EtOAc) to give 2,4-di(4′-methoxybenzylamino)-6-[2′-(4″-pentynoyloxy)ethylamino]-1,3,5-triazine **9a** (1.12 g, 2.3 mmol, 96%) as a white oil. ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.19 (d, J = 8.5 Hz, 4H), 6.82 (d, J = 8.5 Hz, 4H), 5.88–5.20 (m, 3H), 4.47 (s, 4H), 4.19 (t, J = 5.1 Hz, 2H), 3.78 (s, 6H), 3.61 (br s, 2H), 2.64–2.35 (m, 4H), 1.99 (t, J = 2.5 Hz, 1H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 171.77, 165.37, 165.18, 158.93, 131.25, 128.91, 114.04, 82.60, 69.34, 63.83, 55.38, 44.23, 39.69, 33.34, 14.47. MS (ESI), *m*/*z* (%): 489 (100) [M−H]$^+$, 391 (16), 269 (53), 223 (29). HRMS (ESI), *m*/*z*: Calcd for $C_{26}H_{31}N_6O_4$ 491. 2407. Found 491.2399 [M+H]$^+$.

4.1.9. 2-[2′-(5″-Hexynoyloxy)ethylamino]-4,6-di(4′-methoxybenzylamino)-1,3,5-triazine (9b)

Colorless oil (99%), ^{1}H NMR (300 MHz, CDCl$_3$ 6.82 (d, J = 8.4 Hz, 4H), 5.97–5.16 (m, 3H), 4.47 (s, 4H), 4.17 (t, J = 5.2 Hz, 2H), 3.78 (s, 6H), 3.60 (br s, 2H), 2.44 (t, J = 7.3 Hz, 2H), 2.24 (td, J = 6.9, 2.6 Hz, 2H), 1.97 (t, J = 2.6 Hz, 1H), 1.91–1.74 (k, J = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.10, 165.31, 164.36, 158.90, 131.16, 128.93, 114.05, 83.34, 69.37, 63.46, 55.38, 44.22, 39.79, 32.84, 23.61, 17.97. MS (ESI), *m*/*z* (%): 503 (100) [M−H]$^+$, 450 (30), 391 (14). HRMS (ESI), *m*/*z*: Calcd for $C_{27}H_{33}N_6O_4$ 505.2563. Found 505.2555 [M+H]$^+$.

4.1.10. 2-[2′-(6″-Heptynoyloxy)ethylamino]-4,6-di(4′-methoxybenzylamino)-1,3,5-triazine (9c)

Colorless oil (92%), ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.19 (d, J = 8.3 Hz, 4H), 6.82 (d, J = 8.3 Hz, 4H), 5.80–5.26 (m, 3H), 4.47 (s, 4H), 4.16 (t, J = 5.2 Hz, 2H), 3.77 (s, 6H), 3.60 (br s, 2H), 2.31 (t, J = 7.3 Hz, 2H), 2.19 (td, J = 7.0, 2.6 Hz, 2H), 1.95 (t, J = 2.6 Hz, 1H), 1.82–1.63 (m, 2H), 1.62–1.45 (m, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.39, 165.63, 164.71, 158.93, 131.23, 128.92, 114.03, 84.05, 68.80, 63.40, 55.37, 44.22, 39.79, 33.68, 27.91, 24.00, 18.22. MS (ESI), *m*/*z* (%): 517 (100) [M−H]$^+$, 391 (14). HRMS (ESI), *m*/*z*: Calcd for $C_{28}H_{35}N_6O_4$ 519.2720. Found 519.2716 [M+H]$^+$.

4.1.11. 2,4-Di(4′-methoxybenzylamino)-6-[2′-(10″-undecynoyloxy)ethylamino]-1,3,5-triazine (9d)

Colorless oil (87%), ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.20 (d, J = 8.3 Hz, 4H), 6.83 (d, J = 8.3 Hz, 4H), 6.13–5.52 (m, 3H), 4.49 (s, 4H), 4.17 (t, J = 5.2 Hz, 2H), 3.78 (s, 6H), 3.62 (br s, 2H), 2.30 (t, J = 7.6 Hz, 2H), 2.17 (td, J = 7.0, 2.6 Hz, 2H), 1.93 (t, J = 2.6 Hz, 1H), 1.73–1.13 (m, 12H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.86, 159.07, 130.73, 129.02, 114.12, 84.85, 68.25, 63.10, 55.41, 44.33, 39.90, 34.26, 29.22, 29.19, 29.01, 28.78, 28.55, 24.96, 18.50, 1.14. MS (ESI), *m*/*z* (%): 573 (100) [M−H]$^+$, 285 (16), 283 (21), 269 (25). HRMS (ESI), *m*/*z*: Calcd for $C_{32}H_{43}N_6O_4$ 575.3346. Found 575.3342 [M+H]$^+$.

4.1.12. 2,4-Di(4′-methoxybenzylamino)-6-[2′-(17″-octadecynoyloxy)ethylamino]-1,3,5-triazine (9e)

Yellow solid (94%), mp 63.5–64.3 °C, ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.20 (d, J = 8.5 Hz, 4H), 6.82 (d, J = 8.5 Hz, 4H), 5.70–5.45 (m, 3H), 4.47 (s, 4H), 4.16 (t, J = 5.2 Hz, 2H), 3.78 (s, 6H), 3.60 (br s, 3H), 2.28 (t, J = 7.6 Hz, 2H), 2.17 (td, J = 7.0, 2.6 Hz, 2H), 1.93 (t, J = 2.6 Hz, 1H), 1.70–0.70 (m, 26H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 173.89, 165.64, 164.02, 158.97, 131.11, 128.95, 114.05, 84.93, 68.16, 63.24, 55.37, 44.28, 39.86, 34.29, 29.77, 29.76, 29.72, 29.62, 29.58, 29.47, 29.40, 29.28, 29.23, 28.88, 28.62, 25.00, 18.51, 14.23. MS (ESI), *m*/*z* (%): 671 (63) [M−H]$^+$, 390 (100). HRMS (ESI), *m*/*z*: Calcd for $C_{39}H_{57}N_6O_4$ 673.4441. Found 673.4439 [M+H]$^+$.

4.1.13. General procedure for the preparation of triazole-joined hybrids (10a–g)

Compound **4a** (55.2 mg, 0.1 mmol) and **6** (44.5 mg, 0.11 mmol) were dissolved in THF mixture (1 mL). 0.04 M aqueous solution of magnesium ascorbate (0.5 ml, 0.02 mmol) was added to a virgiously stirred mixture, followed by addition of 0.02 M aqueous solution of CuSO$_4$ (0.5 ml, 0.01 mmol). The resulting mixture was stirred for 24 at room temperature. After distillation of the solvent, the corresponding product was isolated by flash chromatography on silica gel (CH$_2$Cl$_2$/EtOH, 10:1) to give **10a** (45 mg, 0.047 mmol, 47%), as a yellow solid, mp 128.0–130.8 °C. ^{1}H NMR (599 MHz, CDCl$_3$) δ 7.48 (s, 1H), 7.29 (d, J = 10.7 Hz, 1H), 7.25–6.98 (m, 5H), 6.92–6.70 (m, 5H), 6.51 (s, 1H), 5.89 (br s, 3H), 4.73–4.34 (m, 7H), 4.31–4.02 (m, 3H), 3.96 (s, 3H), 3.93 (s, 3H), 3.88 (s, 3H), 3.75 (s, 6H), 3.64 (s, 3H), 2.49 (dd, J = 13.6, 6.5 Hz, 1H), 2.37 (td, J = 13.2, 6.8 Hz, 1H), 2.31–2.17 (m, 3H), 1.90–1.67 (m, 3H), 1.61–1.50 (m, 2H), 1.36–1.15 (m, 9H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 179.55, 172.93, 165.34, 164.74, 164.10, 158.77, 158.00, 153.56, 151.85, 151.33, 141.76, 136.62, 135.29, 134.33, 131.42, 130.88, 128.86, 128.47, 125.79, 122.16, 113.95, 112.55, 107.45, 77.37, 77.16, 76.95, 61.72, 61.50, 56.44, 56.22, 55.36, 52.23, 51.59, 50.35, 44.09, 43.97, 36.90, 36.28, 30.21, 30.05, 29.24, 29.08, 28.84, 26.37, 25.40. MS (ESI), *m*/*z* (%): 955 (31) [M−H]$^+$, 786 (40), 616 (28). HRMS (ESI), *m*/*z*: Calcd for $C_{52}H_{65}N_{10}O_8$ 957.4987. Found 957.4983 [M+H]$^+$.

4.1.14. 4-{3′-[3″,5″-Di(4‴-methoxybenzylamino)-2″,4″,6″-triazinylaminoethyloxy]-3′-oxopropyl}-1-{3′-[(1″,2″,3″,10″-tetramethoxy-9″-oxo-5″,6″,7″,9″-tetrahydrobenzo[*a*]heptalen-7″-yl)amino]-3′-oxopropyl}-1,2,3,-triazole (10b)

Yellow solid (57%), mp 138.8–140.6 °C, ^{1}H NMR (599 MHz, CDCl$_3$) δ 8.01 (br s, 1H), 7.41 (br s, 2H), 7.28 (d, J = 10.7 Hz, 1H), 7.17 (br s, 4H), 6.88–6.70 (m, 5H), 6.52 (s, 1H), 5.94–5.09 (m, 3H), 4.64–4.34 (m, 7H), 4.15 (br s, 2H), 3.94 (s, 3H), 3.93 (s, 3H), 3.88 (s, 3H), 3.76 (s, 6H), 3.69–3.47 (m, 5H), 2.99 (br s, 2H), 2.88 (br s, 2H), 2.65 (br s, 2H), 2.53–2.43 (m, 1H), 2.41–2.30 (m, 1H), 2.21–2.09 (m, 1H), 1.95–1.79 (m, 1H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 179.42, 172.77, 169.31, 164.20, 164.00, 158.85, 158.80, 157.65, 153.59, 151.22, 141.67, 138.57, 136.57, 135.34, 134.38, 131.34, 131.11, 128.97, 125.71, 122.49, 113.98, 112.57, 107.48, 63.66, 61.56, 61.49, 56.41, 56.21, 55.38, 52.53, 46.18, 44.22, 39.76, 36.55, 36.38, 33.83, 30.01, 21.14. MS (ESI), *m*/*z* (%): 943 (42) [M−H]$^+$, 604 (11), 532 (9). HRMS (ESI), *m*/*z*: Calcd for $C_{49}H_{57}N_{10}O_{10}$ 945.4259. Found 945.4257 [M+H]$^+$.

4.1.15. 4-{5′-[3″,5″-Di(4‴-methoxybenzylamino)-2″,4″,6″-triazinylaminoethyloxy]-5′-oxopentyl}-1-{3′-[(1″,2″,3″,10″-tetramethoxy-9″-oxo-5″,6″,7″,9″-tetrahydrobenzo[*a*]heptalen-7″-yl)amino]-3′-oxopropyl}-1,2,3,-triazole (10c)

Yellow solid (82%), mp 143.6–147.8 °C, ^{1}H NMR (599 MHz, CDCl$_3$) δ 7.86 (br s, 1H), 7.41 (s, 1H), 7.31 (s, 1H), 7.28 (d, *J* = 10.7 Hz, 1H), 7.17 (s, 4H), 6.91–6.67 (m, 5H), 6.52 (s, 1H), 5.43 (br s, 3H), 4.64–4.31 (m, 7H), 4.15 (s, 2H), 3.94 (s, 3H), 3.93 (s, 3H), 3.89 (s, 3H), 3.76 (s, 6H), 3.64 (s, 3H), 3.65–3.46 (m, 2H), 2.98–2.79 (m, 2H), 2.67 (s, 2H), 2.53–2.44 (m, 1H), 2.42–2.23 (m, 3H), 2.23–2.10 (m, 1H), 1.95–1.81 (m, 1H), 1.62 (s, 4H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 179.40, 173.57, 169.29, 165.57, 164.08, 158.83, 153.58, 151.37, 151.23, 147.78, 141.67, 138.53, 136.51, 135.34, 134.37, 131.42, 131.10, 128.96, 125.72, 122.07, 113.96, 112.53, 107.46, 63.45, 61.55, 61.49, 56.41, 56.20, 55.38, 52.52, 46.05, 44.20, 39.87, 36.59, 36.20, 33.95, 29.99, 28.80, 25.26, 24.42. MS (ESI), *m*/*z* (%): 971 (37) [M−H]$^+$, 669 (5), 560 (12). HRMS (ESI), *m*/*z*: Calcd for C$_{51}$H$_{61}$N$_{10}$O$_{10}$ 973.4572. Found 973.4565 [M+H]$^+$.

4.1.16. 4-{4′-[3″,5″-Di(4‴-methoxybenzylamino)-2″,4″,6″-triazinylaminoethyloxy]-4′-oxobutyl}-1-{6′-[(1″,2″,3″,10″-tetramethoxy-9″-oxo-5″,6″,7″,9″-tetrahydrobenzo[*a*]heptalen-7″-yl)amino]-6’-oxohexyl}-1,2,3,-triazole (10d)

Yellow solid (73%), mp 102.8–105.7 °C, ^{1}H NMR (599 MHz, CDCl$_3$) δ 7.61–7.35 (m, 3H), 7.29 (d, *J* = 10.7 Hz, 1H), 7.18 (s, 4H), 6.89–6.70 (m, 5H), 6.52 (s, 1H), 5.42 (br s, 3H), 4.65–4.56 (m, 1H), 4.56–4.38 (m, 4H), 4.28 (s, 2H), 4.16 (s, 2H), 3.96 (s, 3H), 3.93 (s, 3H), 3.89 (s, 3H), 3.76 (s, 6H), 3.64 (s, 3H), 3.63–3.48 (m, 2H), 2.75 (s, 2H), 2.54–2.45 (m, 1H), 2.44–2.27 (m, 3H), 2.26–2.12 (m, 3H), 1.99 (s, 2H), 1.93–1.74 (m, 3H), 1.69–1.57 (m, 1H), 1.57–1.45 (m, 1H), 1.33–1.17 (m, 2H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 179.46, 173.50, 172.44, 164.13, 158.87, 153.59, 151.61, 151.33, 146.76, 141.78, 138.62, 138.48, 136.56, 135.32, 134.30, 131.42, 131.01, 128.98, 125.79, 114.05, 113.99, 112.52, 107.47, 63.49, 61.70, 61.51, 56.45, 56.23, 55.40, 52.41, 50.18, 44.24, 39.80, 39.62, 36.79, 35.95, 33.52, 30.03, 29.82, 26.11, 24.90, 24.63. MS (ESI), *m*/*z* (%): 999 (15) [M−H]$^+$, 774 (10), 696 (23), 661 (29), 660 (100). HRMS (ESI), *m*/*z*: Calcd for C$_{53}$H$_{65}$N$_{10}$O$_{10}$ 1001.4885. Found 1001.4877 [M+H]$^+$.

4.1.17. 4-{9′-[3″,5″-Di(4‴-methoxybenzylamino)-2″,4″,6″-triazinylaminoethyloxy]-9′-oxononyl}-1-{3′-[(1″,2″,3″,10″-tetramethoxy-9″-oxo-5″,6″,7″,9″-tetrahydrobenzo[*a*]heptalen-7″-yl)amino]-3′-oxopropyl}-1,2,3,-triazole (10e)

Yellow solid (63%), mp 115.0–117.7 °C, ^{1}H NMR (599 MHz, CDCl$_3$) δ 7.78 (br s, 1H), 7.43 (s, 1H), 7.31 (s, 1H), 7.28 (d, *J* = 10.7 Hz, 1H), 7.19 (s, 4H), 6.88–6.71 (m, 5H), 6.52 (s, 1H), 5.33 (br s, 3H), 4.68–4.35 (m, 7H), 4.16 (s, 2H), 3.95 (s, 3H), 3.93 (s, 3H), 3.89 (s, 3H), 3.76 (s, 6H), 3.65 (s, 3H), 3.63–3.51 (m, 2H), 3.01–2.90 (m, 1H), 2.90–2.80 (m, 1H), 2.64 (s, 2H), 2.49 (dd, *J* = 13.3, 6.5 Hz, 1H), 2.38 (td, *J* = 13.3, 6.5 Hz, 1H), 2.31–2.22 (m, 2H), 2.23–2.13 (m, 1H), 1.94–1.81 (m, 1H), 1.64–1.51 (m, 4H), 1.37–1.16 (m, 8H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 179.44, 173.92, 169.27, 164.25, 158.86, 153.59, 151.33, 151.25, 141.69, 138.58, 136.49, 135.35, 134.35, 131.47, 131.36, 131.19, 128.96, 125.74, 121.80, 113.98, 112.53, 110.13, 107.46, 63.37, 61.55, 61.49, 56.42, 56.21, 55.38, 52.49, 46.07, 44.24, 39.85, 36.67, 36.18, 34.31, 32.04, 30.01, 29.81, 29.42, 29.18, 29.12, 25.64, 24.96, 22.81. MS (ESI), *m*/*z* (%): 1027 (38) [M−H]$^+$, 761 (4). HRMS (ESI), *m*/*z*: Calcd for C$_{55}$H$_{69}$N$_{10}$O$_{10}$ 1029. 5198. Found 1029.5198 [M+H]$^+$.

4.1.18. 4-{4′-[3″,5″-Di(4‴-methoxybenzylamino)-2″,4″,6″-triazinylaminoethyloxy]-4′-oxobutyl}-1-{10′-[(1″,2″,3″,10″-tetramethoxy-9″-oxo-5″,6″,7″,9″-tetrahydrobenzo[*a*]heptalen-7″-yl)amino]-10′-oxodecanyl}-1,2,3,-triazole (10f)

Yellow solid (69%), mp 110.2–112.3 °C, ^{1}H NMR (599 MHz, CDCl$_3$) δ 7.50 (s, 1H), 7.29 (d, *J* = 10.6 Hz, 1H), 7.21 (br s, 5H), 6.82 (s, 5H), 6.52 (s, 1H), 5.49 (br s, 3H), 4.68–4.61 (m, 1H), 4.50 (s, 4H), 4.27 (s, 2H), 4.18 (s, 2H), 3.95 (s, 3H), 3.93 (s, 3H), 3.89 (s, 3H), 3.77 (s, 6H), 3.71–3.52 (m, 5H), 2.75 (s, 2H), 2.56–2.45 (m, 1H), 2.43–2.31 (m, 3H), 2.31–2.12 (m, 3H), 2.09–1.93 (m, 2H), 1.93–1.73 (m, 3H), 1.63–1.47 (m, 2H), 1.41–1.01 (m, 10H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 179.53, 173.36, 172.84, 164.08, 159.00, 153.56, 151.67, 151.34, 141.77, 138.75, 138.56, 136.62, 135.19, 134.35, 131.06, 130.79, 129.05, 128.74, 125.82, 114.06, 112.50, 112.44, 107.48, 63.19, 61.71, 61.51, 56.43, 56.23, 55.40, 52.14, 50.38, 44.31, 44.08, 39.92, 37.04, 36.42, 33.46, 30.23, 30.08, 29.22, 29.06, 28.82, 26.39, 25.44, 24.95, 24.66. MS (ESI), *m*/*z* (%): 1055 (21) [M−H]$^+$, 717 (32), 716 (100), 668 (12). HRMS (ESI), *m*/*z*: Calcd for C$_{57}$H$_{73}$N$_{10}$O$_{10}$ 1057.5511. Found 1057.5511 [M+H]$^+$.

4.1.19. 4-{16′-[3″,5″-Di(4‴-methoxybenzylamino)-2″,4″,6″-triazinylaminoethyloxy]-16′-oxohexadecanyl}-1-{6′-[(1″,2″,3″,10″-tetramethoxy-9″-oxo-5″,6″,7″,9″-tetrahydrobenzo[*a*]heptalen-7″-yl)amino]-6′-oxohexyl}-1,2,3,-triazole (10g)

Yellow solid (64%), mp 88.0–91.7 °C, ^{1}H NMR (599 MHz, CDCl$_3$) δ 7.42 (s, 1H), 7.34 (s, 1H), 7.30 (d, *J* = 10.7 Hz, 1H), 7.19 (s, 4H), 7.01 (br s, 1H), 6.92–6.69 (m, 5H), 6.52 (s, 1H), 5.46 (br s, 3H), 4.67–4.56 (m, 1H), 4.48 (s, 4H), 4.34–4.21 (m, 2H), 4.15 (s, 2H), 3.97 (s, 3H), 3.93 (s, 3H), 3.89 (s, 3H), 3.77 (s, 6H), 3.70–3.46 (m, 5H), 2.73–2.62 (m, 2H), 2.51 (dd, *J* = 13.5, 6.3 Hz, 1H), 2.39 (td, *J* = 13.2, 6.8 Hz, 1H), 2.34–2.13 (m, 5H), 1.94–1.76 (m, 4H), 1.72–1.48 (m, 6H), 1.43–1.06 (m, 23H). ^{13}C NMR (151 MHz, CDCl$_3$) δ 179.47, 173.93, 172.38, 164.13, 158.87, 153.59, 151.61, 151.33, 148.39, 141.78, 136.54, 135.36, 134.26, 131.52, 131.38, 130.88, 128.93, 125.76, 121.02, 120.85, 113.99, 112.52, 107.45, 63.37, 61.68, 61.50, 56.46, 56.22, 55.38, 52.39, 51.67, 51.47, 50.05, 44.25, 39.83, 39.74, 38.97, 36.85, 35.85, 34.31, 30.12, 30.02, 29.81, 29.77, 29.75, 29.71, 29.69, 29.64, 29.57, 29.52, 29.45, 29.38, 29.27, 26.17, 25.85, 25.01, 24.89. MS (ESI), *m*/*z* (%): 1167 (23) [M−H]$^+$, 943 (7), 829 (51). HRMS (ESI), *m*/*z*: Calcd for C$_{65}$H$_{89}$N$_{10}$O$_{10}$ 1169.6763. Found 1169.6753 [M+H]$^+$.

4.1.20. Synthesis of poly(ethylene glycol) 2-[3′,5′-di(4″-methoxybenzylamino)-2′,4′,6′-triazinylaminoethyloxy]-2-oxoethyl 2-[(1′,2′,3′,10′-tetramethoxy-9′-oxo-5′,6′,7′,9′-tetrahydrobenzo[*a*]heptalen-7′-yl)amino]-2-oxoethyl ether (10h)

A solution of **7** (0.409 g, 1 mmol), poly(ethylene glycol) bis(carboxymethyl) ether (average $M_n = 600$) (1.2 g, 2 mmol), DCC (0.206 g, 1 mmol) and DMAP (0.061 g, 0.5 mmol) in THF (5 ml) was stirred for 3 h at room temperature under nitrogen atmosphere. The solvent was evaporated, followed by addition of CH$_2$Cl$_2$. The precipitate was removed and the solution was washed with water, dried over Na$_2$SO$_4$ and concentrated to colorless oil. It was purified by flash chromatography on silica gel (CH$_2$Cl$_2$/MeOH, 1:1) to give **11** (0.544 g, 0.55 mmol, 55%) as a colorless oil. ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.20 (d, *J* = 8.2 Hz, 4H), 6.81 (d, *J* = 8.2 Hz, 4H), 4.55–4.38 (m, 4H), 4.23 (t, *J* = 5.4 Hz, 2H), 4.13 (br s, 2H), 3.90 (br s, 2H), 3.77 (s, 6H), 3.73–3.49 (m, 42H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 175.39, 175.20, 170.60, 170.56, 158.88, 131.39, 128.95, 113.99, 71.02–68.65, 63.87, 55.38, 44.18, 39.63. MS (ESI), *m*/*z* (%): 965 (98) [M−H]$^+$, 921 (89), 877 (70), 833 (46), 789 (26), 735 (18), 691 (11). HRMS (ESI), *m*/*z*: Calcd for C$_{45}$H$_{71}$N$_6$O$_{17}$ 967.4876. Found 967.4876 [M+H]$^+$. A solution of **11** (238 mg, 0.24 mmol) in CH$_2$Cl$_2$ (2 ml) was added to **2** (86 mg, 0.24 mmol), EDC·HCl (50 mg, 0.26 mmol) and DMAP (15 mg, 0.12 mmol) at 0 °C under nitrogen atmosphere. The obtained solution was stirred for 1 h at 0 °C, then for 48 h at room temperature. The solvent was removed and the residue was purified by flash chromatography on silica gel (CH$_2$Cl$_2$/EtOH, 4:1) to give **10h** (213 mg, 0.16 mmol, 68%) as a yellow solid, mp 58.5–60.8 °C. ^{1}H NMR (300 MHz, CDCl$_3$) δ 7.98 (br s, 1H), 7.48 (s, 1H), 7.39–7.13 (m, 6H), 6.96–6.70 (m, 5H), 6.55 (s, 1H), 4.76–4.59 (m, 2H), 4.62–4.37 (m, 4H), 4.37–3.29 (m, 63H), 2.55 (dd, J = 10.4, 6.2 Hz, 1H), 2.51–2.33 (m, 1H), 2.33–2.07 (m, 2H), 2.11–1.85 (m, 2H). ^{13}C NMR (75 MHz, CDCl$_3$) δ 179.56, 159.10, 153.82, 153.65, 151.26, 141.73, 136.12, 134.46, 134.44, 132.99, 129.11, 114.12, 114.12, 70.15–68.11, 63.43, 61.54, 61.48, 56.25, 56.25, 55.41, 44.37, 36.58, 29.85. MS (ESI), m/z (%): 1307 (40) [M+H]$^+$, 1263 (33), 1175 (18), 1131 (9), 775 (29), 731 (56), 709 (99), 687 (98), 643 (67), 621 (39) 610 (32). HRMS (ESI), m/z: Calcd for C$_{65}$H$_{92}$N$_7$O$_{21}$1306.6346. Found 1306.6353 [M+H]$^+$.

4.2. Biology

4.2.1. Cell cultures and survival assay

Human epithelial mammary HBL100 cells were grown in DMEM (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin (Gibco) and maintained in a humidified incubator at 37 °C with 5% CO$_2$. Compounds were dissolved in DMSO at a concentration of 10 mM, and diluted in culture medium before use. For experiments, cells were seeded in 96-well plates for 24 h before treatment with compounds during 72 h. Growth inhibition of cell line was estimated by using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay, and absorbance was measured at 550 nm with a Dynatech MR 7-000 plate reader. At least three independent experiments were performed, and the IC$_{50}$ values (i.e., concentration half-inhibiting cell proliferation) were graphically determined.

4.2.2. Purification of lamb brain tubulin

Tubulin was extracted from lamb brain by ammonium sulfate fractionation and ion-exchange chromatography. The protein was stored in liquid nitrogen and prepared as described.[20] Protein concentrations were determined spectrophotometrically with a Perkin-Elmer spectrophotometer Lambda 800 at 275 nm with an extinction coefficient of 1.07 L g^{-1} cm^{-1} in neutral aqueous buffer or 1.09 L g^{-1} cm^{-1} in 6 M guanidine hydrochloride.

4.2.3. Microtubules assembly monitored by fluorescence

Microtubules assembly was performed on a Fluoroscan Ascent FL spectrofluorometer (labsystems) using a 96-well plate. The excitation wavelength was set at 355 nm and the emission wavelength was set at 460 nm. Experiments were carried out at 37 °C and performed with 7.5 μM Dapi, 15 μM tubulin in 20 mM sodium phosphate buffer, 1 mM EGTA, 10 mM MgCl$_2$ and 3.4 M glycerol, pH 6.5. Under these conditions, the Dapi fluorescence enhancement is directly proportional to the concentration of polymerised tubulin and was monitored as a function of time.[21] DMSO concentration was maintained below 1% in all samples and controls. Experiments were done in triplicate.

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