European Journal of Medicinal Chemistry 45 (2010) 219–226

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

Synthesis and biological evaluation of novel thiocolchicine–podophyllotoxin conjugates

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ARTICLE INFO

Article history:
Received 26 June 2009
Received in revised form
23 September 2009
Accepted 28 September 2009
Available online 6 October 2009

Keywords:
Thiocolchicine
Podophyllotoxin
Tubulin polymerization
Microtubule network
Anticancer drugs

ABSTRACT

The synthesis and biological evaluation of 9 dimeric compounds obtained by condensation of thiocolchicine and/or podophyllotoxin with 6 different dicarboxylic acids is described. In particular, tubulin assembly assay and immunofluorescence analysis results are reported. The biological data highlighted three compounds as being more active than the others, having a marked ability to inhibit the polymerization of tubulin *in vitro* and causing significant disruption to the microtubule network *in vivo*. The spacer unit was found to have a significant effect on biological activity, reinforcing the importance of the design of conjugate compounds to create new biologically active molecules in which the spacer could be useful to improve the solubility and to modulate the efficacy of well known anticancer drugs.

1. Introduction

Microtubules are the key components of the mitotic spindle of eukaryotic cells and they play an important role in cell division. The discovery of natural and synthetic substances capable of interfering with the polymerization or depolymerization of microtubules has gained much attention because microtubules are an attractive pharmacological target for the design of anticancer drugs. Indeed, a large number of tubulin binding agents are in clinical development, and the recent finding that some tubulin binding agents target the vascular system of tumors (antiangiogenic activity) has extended their use still further. [1–4] There is conclusive evidence that microtubule disruption causes cell cycle arrest mainly at the G2/M phase, and subsequently programmed cell death. In this regard, thiocolchicine **1** [5] and podophyllotoxin **2** [6] behave in the same way as colchicine **3** [7] (Fig. 1): their binding to tubulin results in partial unfolding of the secondary structure of β-tubulin at the carboxy terminus and prevents polymerization. [8]

The importance of tubulin in cell replication, the promising application of antiangiogenic therapies and the recent discovery that antiangiogenic agents can normalize abnormal tumor vasculature, leading to more efficient penetration of chemotherapeutic drugs [9], justify the need for new tubulin-targeted compounds [10]. In particular, there is a need for compounds with increased selectivity to improve the antitumor activity and to reduce toxicity, which is often severe [11].

Various strategies are being developed to increase the effects of different antitubulin drugs. [12] We have elected to exploit bifunctional small molecules [13], an idea based on the concept of multivalency [14], the dominant interaction in biological systems. Multivalency is now accepted as an effective strategy for designing ligands, inhibitors and drugs, and it has proved useful in enhancing the activity and selectivity of some monomeric leads by forming divalent homo- and heterodimers. [15] As part of our research on the synthesis of new potential antitumor compounds [16], we reported previously the synthesis of taxoid–thiocolchicine hybrids

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doi:10.1016/j.ejmech.2009.09.047

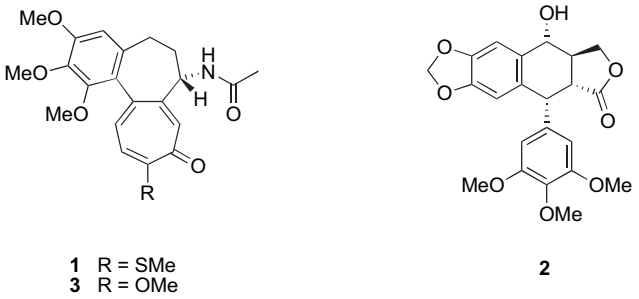


Fig. 1. Cochicine, thiocolchicine and podophyllotoxin.

[17], and more recently we have prepared binary adducts of Vinca alkaloids with podophyllotoxin, thiocolchicine and baccatin III [18]. We have also approached the problem by a target-assisted dynamic combinatorial library of thiocolchicine–podophyllotoxin derivatives based on the disulfide bond exchange reaction [19]. Biological evaluations revealed that some of the compounds that we prepared behave as multifunctional tubulin inhibitors and could be interesting probes to investigate not only tubulin biology but also the complex, and so far poorly understood, relationship between tubulin and cytotoxicity. In addition, quite surprising biological properties were displayed, probably due to the interaction with targets other than tubulin. These results encouraged us to continue our program directed towards the synthesis of hybrids of naturally occurring antimitotic products.

Here we report the synthesis of six new conjugates of thiocolchicine and podophyllotoxin, and their detailed biological evaluation along with three other conjugates that we have previously prepared [19].

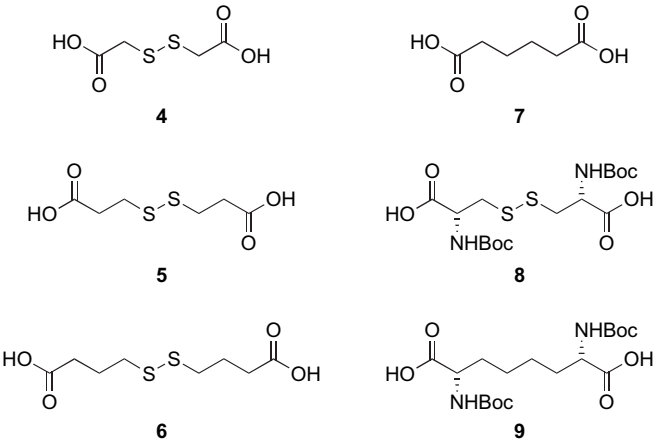
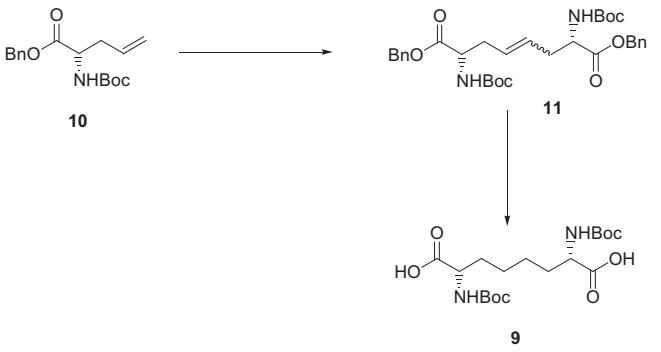


Fig. 2. The dicarboxylic acids used as linkers.



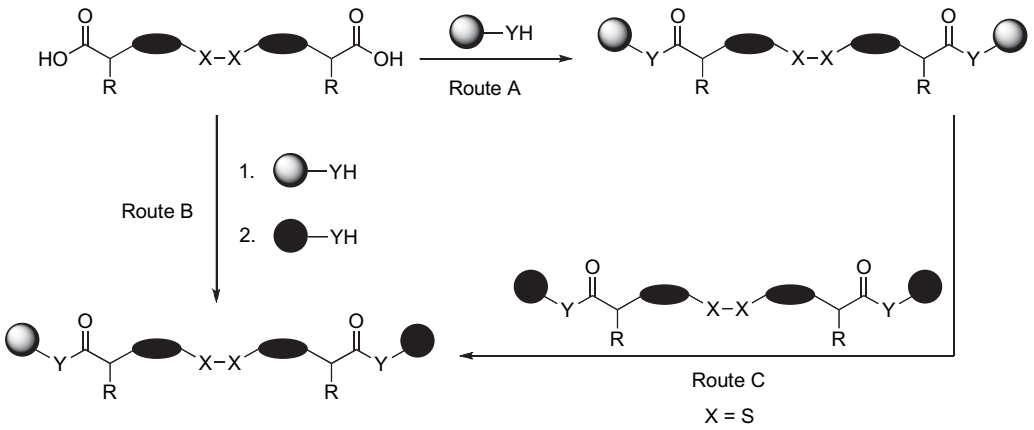
Scheme 1. Preparation of the linker **9**.

2. Results and discussion

2.1. Chemistry

Six different dicarboxylic acids, four of which contain a disulfide bond, (Fig. 2) were used as the linker molecules to form the dimers. The dicarboxylic acids were: dithiodiglycolic acid **4**; dithiodipropionic acid **5**; dithiodibutyric acid **6**; adipic acid **7**; *N*-Boc-cystine **8**; and 2,7-bis(*tert*-butoxycarbonylamino)octanedioic acid **9**. It was hoped that the amino groups in the latter two dicarboxylic acids would enhance the water solubility of the target dimers.

Dicarboxylic acid **9** was synthesised from L-*N*-Boc-allylglycine. Protection of the carboxylic acid as a benzyl ester (Scheme 1) provided compound **10** that was submitted to a cross-metathesis reaction using Grubbs' 2nd generation catalyst, giving a mixture of *E* and *Z* isomers of **11** in 64% yield. Similar yields were obtained when microwaves were used in place of conventional heating. Hydrogenolysis of the benzyl esters and reduction of the double bond was accomplished by catalytic hydrogenation (Pd/C) using ethyl acetate as solvent. There was no evidence in the NMR spectra of epimerization during this synthetic sequence.



Scheme 2. The three routes used for homo- and heterodimer synthesis.

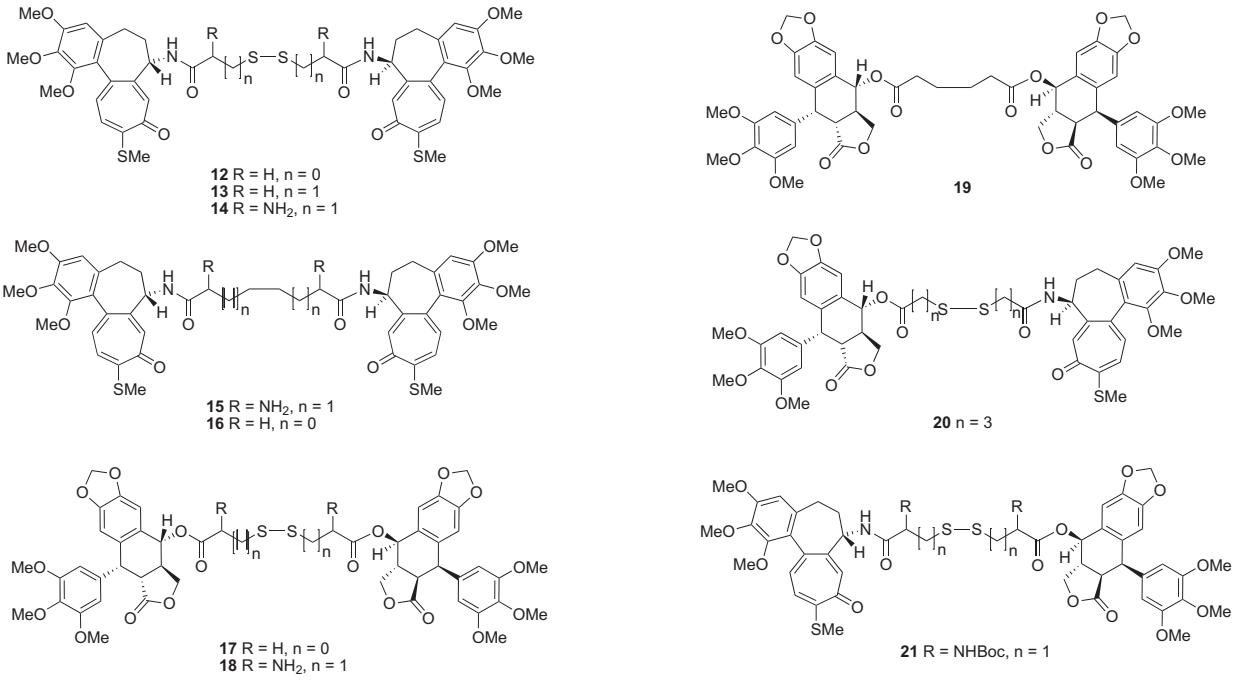


Fig. 3. The homo- and heterodimers.

The synthesis of the homo- and heterodimers was accomplished according to Scheme 2. Route A shows the preparation of a homodimer by a simple coupling reaction; route B illustrates the preparation of a heterodimer via sequential coupling reactions; route C, applicable when the linker contains a disulfide bond, shows the preparation of a heterodimer by disufide exchange between two homodimers.

The formation of the homodimers **12**–**19** (Fig. 3) was straightforwardly achieved by condensation of the dicarboxylic acids with podophyllotoxin or deacetylthiocolchicine, mediated by DCC and DMAP. The removal of the *tert*-butoxycarbonyl group from the compounds obtained by condensation with *N*-Boc-cystine **8** and 2,7-bis(*tert*-butoxycarbonylamino)octanedioic **9** acid gave rise to homodimers with an improved water solubility (1.2–1.5 mg/ml as hydrochloride salts). Heterodimer **20** was obtained by a two-step condensation reaction. First, deacetylthiocolchicine was reacted with 4,4′-dithiodibutyric **6** acid to give the monoamide thiocolchicine derivative that was subsequently reacted with podophyllotoxin, affording **20** (Fig. 3) in an overall 56% yield. The heterodimer **21** [20] (Fig. 3) was prepared by disulfide exchange between the corresponding Boc-protected derivatives of **14** and **18**, according to the method we have previously reported [19], but unfortunately the product of Boc-group removal proved too unstable for characterisation.

Table 1
Tubulin (3 mg/ml) was polymerized in the absence or presence of 10 μM solution of selected compounds and ratio of polymerized/unpolymerized tubulin reported.

	P/U (mean ± SEM)
Control	1.82 ± 0.06
1	0.87 ± 0.14*
2	0.80 ± 0.11*
12	0.74 ± 0.17*
13	0.98 ± 0.04*
14	1.13 ± 0.23*
15	1.83 ± 0.45
16	0.75 ± 0.16*
17	1.51 ± 0.21
18	1.29 ± 0.29
19	1.20 ± 0.10
20	1.36 ± 0.29*

*$P < 0.05$ *vs.* control, according to ANOVA, Dunnet post-hoc.

2.2. Biological evaluation

2.2.1. Cytotoxicity by MTS assay

All of the compounds displayed moderate cytotoxicity across a broad panel of cell lines: A549 (human lung carcinoma), Caco-2 (Human Caucasian colon adenocarcinoma), HepG2 (Human Caucasian hepatocyte carcinoma), KLN205 (Mouse squamous cell carcinoma) and eleven transformed mouse cell lines from lung tumors of double c-myc and c-raf transgenic mice (A2C12, γB8, γD12, βD10, γA7, γA3, B3, βD5, A2B1, γD1, Craf/Cmyc). Notably, the latter cell lines display epigenetic plasticity and express several tumour stem cell markers providing a mechanistic insight into lung cancer as recently reported by us [21]. In the human cell lines the IC_{50} values were in the range 0.5–1 μM after 24 h incubation, whilst the murine cell lines were more resistant, with IC_{50} values typically in the region of 10 μM.

2.2.2. Tubulin assembly assay

To get an insight into the potential biological activity of the dimers we investigated their ability to affect tubulin polymerization *in vitro*. In order to assess the effect of the compounds on tubulin assembly, bovine tubulin (purified from brain) was mixed with a standard solution of each sample in the absence of GTP.

Table 2
The novel thiocolchicine–podophyllotoxin conjugates were grouped on the grounds of the severity of their anti-microtubule action in A549 cells.

Group	Effect (severity of the action)	Compounds
I	mild effect	**15**, **19**
II	50% of the cells become roundish	**14**, **16**, **17**
III	network completely disappears	**1**, **2**, **12**, **13**, **18**, **20**

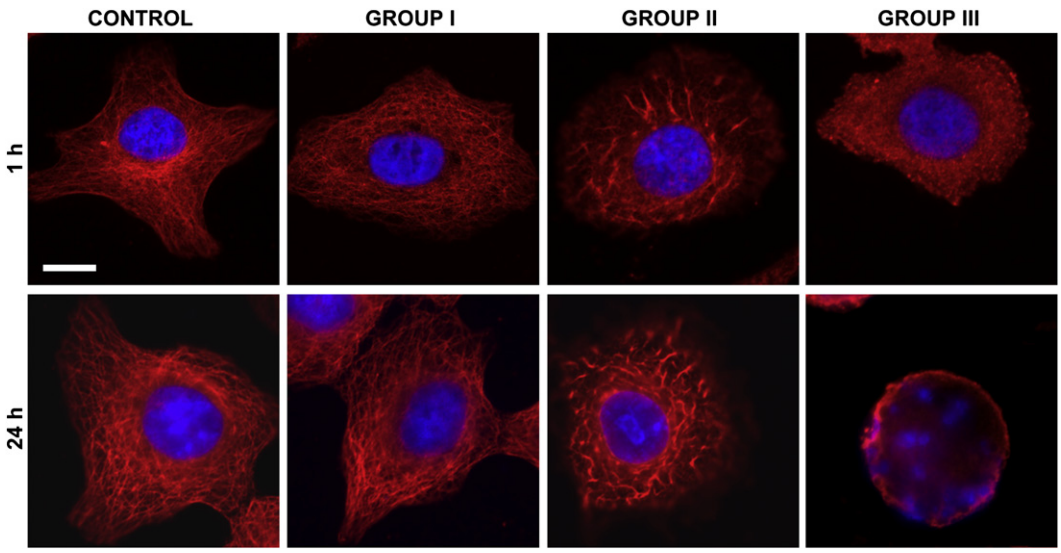


Fig. 4. Microtubule organization in human lung carcinoma cell line A549 exposed for 1 h and 24 h to solvent vehicle alone (control) or 0.5 μM selected compounds belonging to group I, group II (compound **16**), and group III, as revealed by immunofluorescence localization of α-tubulin (red) and nuclei staining (blue). Bar: 10 μm.

The solutions were then incubated at 37 °C to allow slow binding drugs to bind to the tubulin, and after 15 min GTP was added to initiate microtubule assembly. After 30 min the polymerized and the unpolymerized fractions were separated by centrifugation and quantified by densitometry. Reported in Table 1 are the ratios of polymerized/unpolymerized tubulin obtained in the presence of the different compounds.

It can be seen that the best inhibitors of tubulin polymerization are the homodimers of thiocolchicine, with **12**, **13** and **16** showing comparable levels of activity to the parent compound, while **14** shows slightly reduced activity. Compounds **17**, **18**, **19** and **20** incorporating podophyllotoxin, either as homodimers or as heterodimers with thiocolchicine, are all rather less active than either parent monomer. Interestingly, dimers built up with the same moieties connected by different spacers showed different effects on tubulin polymerisation. Amino substitution on the linker (compounds **14**, **15**, **18**), whilst improving aqueous solubility, did not improve the activity, and may even have contributed to a decrease in the ability to inhibit tubulin polymerization.

2.2.3. Immunofluorescence analysis

In order to confirm the anti-microtubulule effect in cells we investigated microtubule structure and distribution in human lung carcinoma cell line A549 exposed to the dimers by indirect immunofluorescence using anti α-tubulin antibodies.

A549 cells were incubated for 1 h and 24 h in the presence of the different compounds (final concentration 0.5 μM). We put the compounds into three categories as a function of the severity of action on microtubule organization in A549 cells (Table 2), and Fig. 4 shows representative microtubule organization for each category.

In control cells, we observed a widespread network of long microtubules other than the typical accumulation of microtubules at one side of the nucleus in the region called the microtubule organizing center (MTOC). Group I contains compounds **15** and **19** that exert a mild effect: the treated cells show a well organized network but a lack of a perinuclear region enriched in microtubules. Group II contains compounds **14**, **16** and **17** that induce severe effects on microtubule arrangement: about 50% of the cells become roundish, whereas the cells showing a conventional morphology lack microtubules at the cell periphery and display heavy fragmentation of the microtubule network in the perinuclear region. Particularly noteworthy is compound **16** that induces the peculiar fragmentation and reorganization of microtubules into short bundles. Group III includes the moieties thiocolchicine and podophyllotoxin, along with **12**, **13**, **18** and **20**. These compounds induce the most severe effect on microtubular organization in A549 cells: the network completely disappears at early time points; almost all of the cells become roundish following a treatment of 24 h and they show evident apoptosis, as indicated by the fragmented nuclei.

2.2.4. In vitro activity with L-glutathione

The biological data highlighted compounds **12**, **13** and **16** as the most active. Compounds **12** and **16** differ only in the fact that the disulfide bond of **12** is replaced by a two-carbon chain in **16**, yet there was a noticeable difference in their *in vivo* activity; it is possible that their rather different behavior in the modification of the cytoskeleton network (with **16** in Group II and **12** in Group III, Fig. 3) could be due to cleavage of the disulfide bond in **12**, something that clearly cannot occur in **16**. Disulfide cleavage could be the consequence of the high levels of glutathione (GSH) present in tumour cells. To determine whether **12** could undergo disulfide cleavage, we evaluated the action of glutathione on compound **12** in aqueous solution. After an incubation of 3 h we detected the organic species in solution by high resolution ESI-MS. We confirmed the effective reductive cleavage of the disulfide bond by detection of the disulfide derivative of glutathione (GSH) with sulfahydrylthiocolchicine (Thio–CO–CH_2–SH).

3. Conclusions

In this paper the preparation of homo- and heterodimers of podophyllotoxin and thiocolchicine is described, along with their biological evaluation in a number of cellular assays. Thiocolchicine homodimers exhibited a much greater ability to disrupt tubulin polymerization *in vitro* than dimers containing podophyllotoxin, but this was not directly reflected in their *in vivo* activity, with compounds based on both parent molecules (particularly compounds **12**, **13**, **18** and **20**) acting as potent disruptors of the microtubule network in A549 cells. The nature of the linker unit can have an important influence on the biological activity of the dimer.

The presence of two amino groups on the spacer improves the water solubility but reduces the biological efficacy, while the potential cleavage of disulfide-linked dimers by the high levels of glutathione in tumour cells may result in these compounds acting in their monomeric form. In future research we will be examining the potential of these hybrid compounds to overcome drug resistance frequently encountered with chemotherapeutic agents.

4. Material and methods

4.1. N-Boc-L-allylglycine

L-Allylglycine (250 mg; 2.17 mmol) was dissolved in 10 mL of a mixture dioxane/water 1:1. $(Boc)_2O$ (980 mg; 2.39 mmol) was added and the reaction mixture was stirred for 18 h. The organic component of the solvent of the reaction was removed *in vacuo* and the aqueous residue was acidified with HCl until pH 4. The solution was extracted with Et_2O and the combined organic phases were dried over sodium sulfate. After evaporation of the solvent, *N*-Boc-L-allylglycine was obtained as a colorless oil (444 mg; 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (br s, 1H), 5.75–5.70 (m, 1H), 5.14–5.19 (m, 3H), 4.43–4.39 (m, 1H), 2.59–2.55 (m, 2H), 1.44 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) detected signals δ 176.0, 155.4, 132.1, 119.1, 80.1, 52.7, 36.3, 28.1. Anal. Calcd. for $C_{10}H_{17}NO_4$: C, 55.80; H, 7.96; N, 6.51; found: 55.83; H, 7.92; N, 6.52.

4.2. (2S)-Benzyl 2-(tert-butoxycarbonylamino)pent-4-enoate 10

Benzyl chloroformate (348 μL; 2.47 mmol) was added at 0 °C to a solution of *N*-Boc-L-allylglycine (444 mg; 2.06 mmol), triethanolamine (355 μg; 2.38 mmol) and DMAP (25 mg; 0.20 mmol) in CH_2Cl_2 (6 mL). After 2 h the reaction mixture was washed with $NaHCO_3$ (5%) and HCl (1 M). The aqueous layers were extracted with CH_2Cl_2, and the combined organic phases were dried over sodium sulfate and the solvent removed *in vacuo*. The crude residue was submitted to column column chromatography (EtOAc/hexane 1:10) to give 540 mg of the desired product as a colorless oil (86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33–7.39 (m, 5H), 5.61–5.71 (m, 1H), 5.00–5.25 (m, 5H), 4.37–4.49 (m, 1H), 2.43–2.61 (m, 2H), 1.44 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) detected signals δ 171.9, 155.2, 135.4, 132.2, 128.5, 128.4, 128.3, 119.1, 79.9, 67.0, 53.0, 36.7, 28.3. ESI positive MS: Anal. Calcd. for $C_{17}H_{23}NO_4Na$: 328.1519; found 328.1518.

4.3. (2S,7S)-Dibenzyl 2,7-bis(tert-butoxycarbonylamino) oct-4-enoate 11

Compound **10** (100 mg; 0.32 mmol) was dissolved in dry toluene (6 mL) under an inert atmosphere (N_2). Grubbs' catalyst (II generation) (14 mg; 0.016 mmol) was added and the mixture was heated to 55 °C for 16 h. The solvent was removed *in vacuo* and the residue was submitted to column column chromatography (EtOAc/hexane 1:10) to give 60 mg of the desired product as a colorless oil (yield: 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29–7.39 (m, 10H), 5.25–5.34 (m, 2H), 5.04–5.24 (m, 6H), 4.31–4.40 (m, 2H), 2.32–2.51 (m, 4H), 1.43 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) detected signals δ 171.7, 155.1, 135.4, 128.6, 128.4, 79.9, 67.0, 53.1, 35.4, 28.3; ESI positive MS: Anal. Calcd. for $C_{32}H_{42}N_2O_8Na$ 605.2833; found 605.2825.

4.4. (2S,7S)-2,7-Bis(tert-butoxycarbonylamino)octanoic acid 9

Compound **11** (174 mg; 0.30 mmol) was dissolved in EtOAc (10 mL) under an inert atmosphere (N_2). Pd/C (50 mg, 5% w/w) was added and the reaction mixture was stirred under one atmosphere of H_2 for 4 h. The reaction mixture was filtered through celite to remove the catalyst and the solvent was removed *in vacuo* to give compound **9** (118 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (br s, 2H), 5.17–5.41 (m, 2H), 4.31–4.40 (m, 2H), 1.74–1.80 (m, 4H), 1.60–1.73 (m, 4H), 1.42 (s, 18H); ^{13}C NMR (100 MHz, $CDCl_3$) detected signals δ 176.9, 155.8, 80.3, 53.2, 32.1, 28.3, 27.8. ESI positive MS: Anal. Calcd. for $C_{18}H_{32}N_2O_8Na$ 427.2051; found 427.2046.

4.5. General procedure for the preparation of compounds 12–19

Method A: A mixture of dicarboxylic acid (0.20 mmol), DMAP (0.20 mmol), DCC (0.60 mmol) and deacetylthiocolchicine or podophyllotoxin (0.40 mmol) in CH_2Cl_2 (10 mL) was stirred at room temperature (19–60 h). The mixture was submitted to column chromatography. *Method B*: A mixture of dicarboxylic acid (0.20 mmol), DMAP (0.20 mmol), DCC (0.60 mmol) and deacetylthiocolchicine or podophyllotoxin (0.40 mmol) was stirred at 50 °C for 4 h in THF (3 mL). The mixture was submitted to column chromatography.

4.6. Acetamide 2,2′-dithiobis[N-(N-deacetylthiocolchicinyl)] 12

[19] Method A. Deacetylthiocolchicine and dithiodiglycolic acid **4**. Column chromatography (CH_2Cl_2/EtOH 27/1) afforded compound **12** as a yellow amorphous solid in 67% yield; $[\alpha]_D$: + 0.64 ($CHCl_3$, $c = 1$). Anal. Calcd. for $C_{44}H_{48}N_2O_{10}S_4$: C, 59.17; H, 5.42; N, 3.14; found: 59.21; H, 5.44; N, 3.11.

4.7. Propanamide 3,3′-dithiobis[N-(N-deacetylthiocolchicinyl)] 13

[19] Method A. Deacetylthiocolchicine and 3,3′-dithiodipropionic acid 5. Column chromatography (CH_2Cl_2/MeOH 20/1) afforded compound **13** as a yellow amorphous solid in 50% yield; $[\alpha]_D$: - 0.18 ($CHCl_3$, $c = 1$); Anal. Calcd. for $C_{46}H_{52}N_2O_{10}S_4$: C, 59.98; H, 5.69; N, 3.04; found: C, 59.93; H, 5.72; N, 3.02.

4.8. 2-Aminopropanamide 3,3′-dithiobis[N-(N-deacetylthiocolchicinyl)] 14

Condensation of deacetylthiocolchicine and (Boc–Cys–OH)$_2$ **8** according Method B afforded the corresponding Boc-protected dimer (82.7 mg; 0.07 mmol). The crude product was dissolved in dioxane (5 mL) and treated with a saturated solution of HCl in dioxane (5 mL) at −10 °C. The solution was stirred at 0 °C for 1.5 h, before the solvent was removed *in vacuo* and the resulting yellow–orange solid was dissolved in CH_2Cl_2 and washed with a saturated solution of $NaHCO_3$. Removal of the solvent *in vacuo* gave compound **14** as a yellow amorphous solid (63.7 mg; 95% yield). R_f: 0.18 (CH_2Cl_2/MeOH 10/1). $[\alpha]_D$: - 1.5 ($CHCl_3$, $c = 0.4$). ^{1}H NMR ($CDCl_3$, 300 MHz, detected signals) 7.30 (2H, d, $J = 6$), 7.20 (2H, d, $J = 6$), 6.80 (2H, s), 4.50–4.40 (2H, m) 3.88 (6H, s), 3.85 (6H, s), 3.62 (6H, s), 2.45 (6H, s), 2.40–2.25 (2H, m) 1.82–1.71 (4H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) 182.0, 171.7, 157.3, 154.1, 151.1, 143.8, 138.7, 136.0, 131.1, 126.6, 126.1, 125.2, 108.2, 61.7, 60.0, 56.0, 54.2, 38.4, 36.5, 30.6, 15,1; Anal. Calcd. for $C_{46}H_{54}N_4O_{10}S_4$: C, 58.08; H, 5.72; N, 5.89; found: C, 58.07; H, 5.70; N, 5.88. Data for the Boc-protected dimer: R_f: 0.22 (CH_2Cl_2/AcOEt 1.5/1); α_D: – 6.00 ($CHCl_3$, $c = 1$). ^{1}H NMR ($CDCl_3$, 300 MHz, detected signals) 8.60–8.50 (2H, m), 7.60 (2H, s), 7.3–7.25 (2H, d, $J = 11$), 7.07–7.02 (2H, d, $J = 11$), 6.55 (2H, s), 4.80–4.71 (4H, m) 3.93 (6H, s), 3.90 (6H, s), 3.60 (6H, s), 2.40 (6H, s), 1.40 (18H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) detected signals δ 182.3, 169.3, 158.1, 156.6, 154.8, 151.1, 141.8, 138.0, 135.0, 131.5, 128.5, 126.0, 125.5, 107.4 79.0, 61.5, 60.0, 56.1, 54.0, 38.3, 36.7, 30.6, 28.1, 15,1. Anal. Calcd. for $C_{56}H_{70}N_4O_{14}S_4$: C, 58.42; H, 6.13; N, 4.87; found: C, 58.45; H, 6.10; N, 4.90.

4.9. 2,7-Diaminooctanediamide N-(N-deacetylthiocolchicinyl) **15**

Condensation of deacetylthiocolchicine and (2*S*,7*S*)-2,7-bis(*tert*-butoxycarbonylamino)-octanedioic acid **9** according to Method A afforded the corresponding Boc-protected dimer (130 mg; 0.12 mmol). The crude product was dissolved in dioxane (5 mL) and treated with a solution of HCl in dioxane (saturated, 5 mL) at −10 °C. The solution was then stirred at r.t. for 1 h. Removal of the solvent *in vacuo* gave a yellow–orange amorphous solid that was redissolved in CH_2Cl_2 (10 mL) and was washed with a saturated solution of $NaHCO_3$. The organic phase was dried with sodium sulfate and the solvent removed *in vacuo* to afford a yellow solid that was purified by column chromatography (CH_2Cl_2/MeOH 5/1) to give **15** as a yellow amorphous solid (111 mg; 99% yield). $[\alpha]_D$: – 3.2 ($CHCl_3$, $c = 0.2$). ^{1}H NMR (400 MHz, $CDCl_3$): 8.78 (d, $J = 7.6$, 2H), 7.63 (s, 2H), 7.34 (d, $J = 10.4$, 2H), 7.10 (d, $J = 10.8$, 2H), 6.53 (s, 2H), 4.68–4.80 (m, 2H), 3.94 (s, 6H), 3.90 (s, 6H), 3.68 (s, 6H), 2.46–2.55 (m, 2H), 2.42 (s, 6H), 2.20–2.39 (m, 8H), 1.74–2.15 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) detected signals δ 182.1, 173.7, 158.3, 153.7, 152.8, 151.1, 141.6, 139.1, 134.9, 134.6, 129.1, 127.0, 125.6, 107.5, 61.7, 61.4, 56.1, 54.0, 52.3, 36.3, 33.7, 30.1, 24.2, 15.1. ESI positive MS: Anal. Calcd. for $C_{48}H_{58}N_4O_{10}S_2Na$: 937.3487; found: 937.3465. Data for the Boc-protected dimer: ^{1}H NMR (400 MHz, $CDCl_3$): 8.60 (d, $J = 7.6$, 2H), 7.85 (s, 2H), 7.32 (d, $J = 10.4$, 2H), 7.08 (d, $J = 10.4$, 2H), 6.52 (s, 2H), 5.39 (d, $J = 7.2$, 2H), 4.79 (dt, $J = 10.8$, 7.4 Hz, 2H), 4.48 (dd, $J = 6.4$, 4.8 Hz, 2H), 3.94 (s, 6H), 3.89 (s, 6H), 3.69 (s, 6H), 2.46–2.54 (m, 2H), 2.41 (s, 6H), 2.14–2.40 (m, 8H), 1.86–2.07 (m, 6H), 1.37 (s, 18H).^{13}C NMR (100 MHz, $CDCl_3$) detected signals δ 181.9, 171.2, 158.5, 155.3, 153.6, 152.7, 151.1, 141.6, 138.9, 134.7, 129.7, 126.8, 125.8, 107.5, 79.0, 61.7, 61.3, 56.1, 53.4, 52.3, 36.2, 32.5, 30.2, 28.3, 23.6, 15.1. ESI positive MS: Anal. Calcd. for $C_{58}H_{74}N_4O_{14}S_2Na$: 1137.4535; found: 1137.4496.

4.10. Diadipamide N-(N-deacetylthiocolchicinyl) **16**

Method A. Deacetylthiocolchicine and adipic acid 7. Column chromatography (CH_2Cl_2/MeOH 40/1) afforded compound **16** as a yellow amorphous solid in 45% yield. $[\alpha]_D$: – 1.2 ($CHCl_3$, $c = 0.3$). ^{1}H NMR (300 MHz, $CDCl_3$): 8.85 (d, $J = 6$, 2H), 7.71 (s, 2H), 7.35 (d, $J = 10$, 2H), 7.12 (d, $J = 10$, 2H), 6.57 (s, 2H), 4.66 (ddd, $J = 12$, 6, 6, 2H), 3.92 (s, 6H), 3.90 (s, 6H), 3.65 (s, 6H), 2.60–2.65 (m, 2H), 2.45 (s, 6H), 1.70–2.40 (m, 14H).^{13}C NMR (75 MHz, $CDCl_3$) detected signals δ 181.9, 173.1, 158.0, 153.7, 152.7, 151.0, 141.0, 139.2, 134.9, 134.7, 128.6, 127.0, 125.8, 107.4, 61.8, 61.4, 56.1, 53.0, 37.0, 35.1, 30.1, 25.8, 15.1; Anal. Calcd. for $C_{46}H_{52}N_2O_{10}S_2$: C, 64.47; H, 6.12; N, 3.27. Found: C, 64.51; H, 6.13; N, 3.29.

4.11. Dipodophyllotoxin-4-yl 2,2′-dithiobisacetate **17**

[19] Method A. Podophyllotoxin **2** and dithiodiglycolic acid **4**. Column chromatography (CH_2Cl_2/MeOH 20/1) afforded compound **17** as a white amorphous solid in 50% yield. R_f: 0.53 (CH_2Cl_2/MeOH 20/1); $[\alpha]_D$: – 0.53 ($CHCl_3$, $c = 1$). Anal. Calcd. for $C_{48}H_{46}O_{18}S_2$: C, 59.13; H, 4.76. Found: C, 59.16; H, 4.78.

4.12. Dipodophyllotoxin-4-yl 3,3′-bisthio-2-amino propanoate **18**

Condensation of podophyllotoxin **2** and (Boc–Cys–OH)$_2$ **8** according Method B afforded the corresponding Boc-protected dimer (149.3 mg; 0.12 mmol). The crude product was dissolved in CH_2Cl_2 (10 mL), TFA (2.22 g; 1.5 mL; 19.4 mmol) was added at −15 °C and the reaction mixture was stirred for 48 h. The organic phase was washed with a saturated solution of $NaHCO_3$ at −20 °C. The temperature was allowed to rise to r.t. over 1 h. The organic phase was dried over sodium sulfate, the solvent was removed *in vacuo* and the residue purified by column chromatography (CH_2Cl_2/MeOH 15/1) to afford the product **18** as a white amorphous solid in 21% yield. R_f: 0.17 (CH_2Cl_2/MeOH 15/1). $[\alpha]_D$: -1.3 ($CHCl_3$, $c = 0.25$). ^{1}H NMR ($CDCl_3$, 400 MHz, detected signals) 6.80 (2H, s), 6.60 (2H, s), 6.45 (4H, s), 6.05 (4H, s), 6.03–5.98 (m, 2H), 4.75–4.65 (m, 2H) 4.63 (s, 2H), 4.42–4.38 (m, 2H), 4.22–4.19 (m, 2H) 3.70 (s, 12H), 3.35–3.15 (m, 2H), 2.90–2.80 (m, 4H); ^{13}C NMR ($CDCl_3$, 100 MHz) detected signals δ 174.3, 172.3, 154.1(2C), 149.6, 148.5, 139.1, 135.5, 133.0, 128.0, 110.5, 109.5(2C), 108.0, 102.4, 76.0, 72.0, 61.5, 57.0, 46.1, 44.0, 39.3, 35.7; Anal. Calcd. for $C_{50}H_{52}N_2O_{18}S_2$: C, 58.13; H, 5.07; N, 2.71; found: C, 58.15; H, 5.09; N, 2.68. Data for the Boc-protected dimer: R_f: 0.45 (CH_2Cl_2/AcOEt 4/1);; $[\alpha]_D$: – 4.79 ($CHCl_3$, $c = 1$). ^{1}H NMR ($CDCl_3$, 400 MHz, detected signals) 6.80 (s, 2H), 6.60 (s, 2H), 6.40 (s, 4H), 6.00 (s, 4H), 5.92–5.89 (m, 2H), 4.71 (s, 2H) 4.63 (s, 2H), 4.42–4.39 (m, 2H), 4.21–4.18 (m, 2H), 3.80 (s, 6H), 3.75 (s, 6H), 3.70 (s, 6H), 3.22–3.18 (m, 4H), 2.82–2.79 (m, 4H), 1.50 (s, 18H); ^{13}C NMR ($CDCl_3$, 100 MHz) detected signals δ 174.3, 172.3, 154.1(2C), 149.6, 148.8, 139.1, 135.5, 133.0, 128.0, 110.5, 109.5(2C), 108.0, 102.4, 78.0(2C), 76.0, 72.0, 61.5, 57.0, 46.1, 44.0, 39.3, 35.7, 30.6, 29.1. Anal. Calcd. for $C_{60}H_{68}N_2O_{22}S_2$: C, 58.43; H, 5.56; N, 2.27; found: 58.45; H, 5.59; N, 2.30.

4.13. Dipodophyllotoxin-4-yl adipate **19**

Method A: podophyllotoxin **2** and adipic acid 7. Column chromatography (CH_2Cl_2/AcOEt 50/1) afforded compound **19** as a white amorphous solid in 44% yield; $[\alpha]_D$: – 0.7 ($CHCl_3$, $c = 0.4$). ^{1}H NMR (400 MHz, $CDCl_3$): 6.74 (s, 2H), 6.53 (s, 2H), 6.38 (s, 4H), 5.95 (s, 4H), 5.86 (d, $J = 8.8$, 2H), 4.59 (d, $J = 4.4$, 2H), 4.33 (dd, $J = 9.2$, 6.8 Hz, 2H), 4.13 (t, $J = 9.8$, 2H), 3.78 (s, 6H), 3.74 (s, 12H), 2.89–2.97 (m, 2H), 2.75–2.87 (m, 2H), 2.42–2.51 (m, 4H), 1.71–1.78 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) detected signals δ 173.6, 173.5, 152.6, 148.1, 147.5, 137.1, 134.8, 132.4, 128.2, 109.7, 108.1, 106.9, 101.5, 73.7, 71.3, 60.7, 56.1, 45.5, 43.7, 38.6, 33.8, 24.2. ESI positive MS: Anal. Calcd. for $C_{50}H_{50}O_{18}Na$: 961.2889; found: 961.2864

4.14. Podophyllotox-4-yl 4-[N-(N-deacetylthiocolchicinyl)-4-oxobutyl disulfanyl butanoate **20**

A solution of 4,4′-dithiodibutyric acid **6** (198 mg; 0.83 mmol), DMAP (38 mg; 0.31 mmol), DCC (194 mg; 0.94 mmol) and deacetylthiocolchicine (234 mg; 0.63 mmol) was stirred at room temperature for 2 h before being filtered through celite. The solvent was removed *in vacuo* and the residue was dissolved in CH_2Cl_2 and washed with a solution of ammonium hydroxide. The organic phase was dried over sodium sulfate and the solvent was removed *in vacuo* to give the corresponding crude monomer (265 mg, 65% yield). The monomer (178 mg, 0.30 mmol), DMAP (26 mg, 0.21 mmol), DCC (123 mg, 0.60 mmol) and podophyllotoxin **2** (124 mg, 0.30 mmol) were dissolved in CH_2Cl_2 (10 mL). After 7 h the solvent was removed *in vacuo* and the residue purified by column chromatography (CH_2Cl_2/MeOH 5/1) to give **20** (253 mg, 86% yield) as a yellow amorphous solid. R_f: 0.59 (CH_2Cl_2/MeOH 30/1). $[\alpha]_D$: – 0.9 ($CHCl_3$, $c = 0.5$). ^{1}H NMR ($CDCl_3$, 300 MHz, detected signals): 8.52 (s, 1H, Thio), 7.25 (d, $J = 9.4$, 1H, Thio), 7.17 (s, 1H, Thio), 7.03 (d, $J = 9.4$, 1H, Thio), 6.75 (s, 1H, Pod), 6.53 (s, 1H, Thio), 6.51 (s, 2H, Pod), 6.38 (s, 2H, Pod), 5.98 (d, $J = 7.5$, 2H, Pod), 5.88 (d, $J = 9.4$, 1H, Pod), 4.68–4.56 (2H, m, Thio + Pod), 4.36 (dd, $J = 6.9$, 9.7, 1H, Pod), 4.21 (dd, $J = 3.2$, 6.5, 1H, Pod), 3.91 (s, 3H, Thio), 3.89 (s, 3H, Thio), 3.81 (s, 3H, Pod), 3.75 (s, 6H, Pod), 3.63 (s, 3H, Thio), 2.97–2.94 (m, 1H, Pod), 2.85–2.75 (m, 1H, Pod), 2.76–2.49 (m, 2H, Thio), 2.42 (s, 3H, Thio), 2.41–1.86 (m, 14H, Thio+ linker). ^{13}C NMR (100 MHz, $CDCl_3$) detected signals δ 182.6 (1C, Thio), 173.5 (1C, Podo), 170.0 (1C, Linker), 168.5 (1C, Linker), 158.3 (1C, Thio), 153.5 (1C, Thio), 152.1 (2C, Podo), 151.9 (1C, Thio), 151.0 (1C, Thio), 148.4

(1C, Podo), 147.7 (1C, Podo), 141.7 (1C, Thio), 138.5 (1C, Thio), 137.4 (1C, Podo), 135.1 (1C, Thio), 133.9 (1C, Podo), 133.2 (1C, Thio), 130.4 (1C, Podo), 128.1 (1C, Thio),127.3 (1C, Podo), 126.1 (1C, Thio), 126.0 (1C, Thio), 109.2 (1C, Podo), 108.3 (2C, Podo), 107.5 (1C, Thio), 107.0 (1C, Podo), 101.1 (1C, Podo), 74.0 (1C, Podo), 71.0 (1C, Podo), 61.4 (1C, Thio), 61.2 (1C, Thio), 60.1 (1C, Podo), 56.0 (1C, Thio), 55.9 (2C, Podo), 52.1 (1C, Thio), 45.5 (1C, Podo), 44.3 (1C, Podo),42.1 (1C, Thio), 38.1 (1C, Podo), 36.5 (1C, Linker), 34.3 (2C, Linker), 34.0 (2C, Linker), 33.6 (1C, Linker), 30.1 (1C, Thio), 15.0 (1C, Thio). Anal. Calcd. for $C_{50}H_{55}NO_{14}S_3$: C, 60.65; H, 5.60; N, 1.41; found: C, 60.68; H, 5.62; N, 1.43.

4.15. In vitro activity with L-glutathione

L-Glutathione (0.009 mmol) in water was added to a solution of **12** (0.009 mmol) in acetone (5 mL) at room temperature. After 6 h the reaction mixture was analyzed by HRESI-MS. GS-SG and Thio–CO–CH_2–S–S–G were detected. HRESI positive MS: GS-SG Anal. Calcd. for $C_{20}H_{32}N_6O_{12}S_2Na$: 635.1415; found: 635.1417 ESI positive MS: Thio–CO–CH_2–S–S–G Anal. Calcd. for $C_{32}H_{40}N_4O_{11}S_3Na$: 775.1748; found: 775.1745.

4.16. Immunofluorescence analyses

Microtubule organization in cell was revealed by indirect immunofluorescence (IF) analyses. Human lung carcinoma cell line A549 (CCL-185; American Type Culture Collection, Rockville, MD, U.S.A.) was grown in minimal essential medium with Earle's (E-MEM), supplemented with 10% fetal bovine serum (Hyclone Europe, Oud-Beijerland, Holland), 2 mM L-glutamine, 100 U/ml penicillin and non-essential amino acids. Cells were maintained at 37 °C in a humidified atmosphere at 5% CO_2. Experiments were carried out with cells plated on glass coverslips at a density of 1.5×10^4 cells/cm^2 and grown for 24 h in control medium following an incubation of 24 h in the presence of 0.5 μM of the drug or solvent vehicle alone (DMSO). At the end of the treatments, cells were fixed and stained as previously described. [22] Briefly, A549 cells were fixed and permeabilized for 10 min with methanol at −20 °C, washed with PBS and blocked in PBS + 1% bovine serum albumin (BSA) for 15 min at room temperature. To localize tubulin, the cells were incubated with monoclonal anti α-tubulin antibody (clone B-5-1-2, Sigma–Aldrich), 1:500 in PBS for 1 h at 37 °C. As secondary antibodies we used goat anti-mouse Alexa Fluor™ 594 (Molecular Probes), 1:1000 in PBS + 5% BSA for 45 min at 37 °C. Nuclei staining was performed by incubation with DAPI (0.25 μg/ml in PBS) for 15 min at room temperature. The coverslips were mounted in Mowiol® (Calbiochem)–DABCO (Sigma–Aldrich) and examined with a Zeiss Axiovert 200 microscope equipped with a 63 × Neofluor lens. Images were acquired with an Axiocam camera (Zeiss) and PC running Axiovision software (Zeiss).

4.17. Tubulin assembly assay

Tubulin was purified from bovine brain purchased from a local slaughterhouse, conserved before use in ice-cold Pipes buffer (100 mM K-Pipes pH 6.9, 2 mM EGTA, 1 mM $MgCl_2$) and used as soon as possible. Pure tubulin was obtained by two cycles of polymerization–depolymerization in a high-molarity buffer (Castoldi and Popov, 2003) and protein concentration was determined by MicroBCA assay kit (Pierce). Stock solutions of the drugs were prepared by dissolving the powers at a concentration of 5 mM in DMSO. To assess their effects on tubulin assembly, bovine tubulin (3 mg/ml) was mixed with different compounds (final concentration 10 μM) or an equal volume of the solvent (final 0.2% DMSO) at room temperature in an assembly buffer minus GTP (100 mM K-Pipes pH 6.9, 2 mM EGTA, 1 mM $MgCl_2$, 10% glycerol). The reaction mixtures were incubated at 37 °C for 15 min to allow slow binding drugs to bind to the tubulin. The reaction mixtures were then chilled on ice, GTP (final concentration, 1 mM) was added, and they were incubated for 30 min at 37 °C. At the end of polymerization, unpolymerized and polymerized fractions of tubulin were separated by centrifugation at 16500 × *g* for 30 min at 30 °C. The collected microtubules were resuspended in SDS-PAGE sample buffer (2% w/v SDS, 10% v/v glycerol, 5% v/v b-mercaptoethanol, 0.001% w/v bromophenol blue, and 62.5 mM Tris, pH 6.8) and the unpolymerized tubulin was diluted 3:1 with 4X SDS-PAGE sample buffer. Equal proportions of each fraction were resolved by a 7.5% SDS-gel and stained with Coomassie blue. Densitometric analyses of stained gels was performed by using the ImageMaster VDS Software (Pharmacia Biotech), and the obtained data were elaborated using SigmaPlot 8.0 program (Systat Software Inc., Point Richmond, CA, USA). At least three independent experiments were performed with each compound. Differences between the effects of the different compounds were evaluated by ANOVA followed by Dunnet's test.

4.18. Cytotoxicity by MTS assay

Cells were harvested and plated in 96-well flat-bottomed microplates at a density of 10^3 cells/well. Assays were performed in quintuplicates. Cells were allowed to attach for 24 h. The drugs were prepared in medium at different concentrations (0.1 μM, 1 μM and 10 μM) and were added to the plates at a volume of 100 μl/well. After 24 (or 96) h incubation 20 μl of the CellTiter 96® AQ_{ueous} One Solution Reagent (Promega Corporation, Madison, WI, USA) were added to each well and the plates were incubated for one hour at 37 °C. The CellTiter 96® AQ_{ueous} One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). PES has a high chemical stability, which allows it to form a stable solution with MTS. The absorbance was read at 490 nm on a plate spectrophotometer (Victor$^{3\text{TM}}$ 1420 Multilabel Counter, Perkin Elmer Instruments, Shelton, USA). Cell cytotoxicity was expressed as the percentage of the controls.

Acknowledgments

This research has been developed under the umbrella of CM 0602 COST Action "*Inhibitors of Angiogenesis*: *design*, *synthesis and biological exploitation*".

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[20] Data for compound **21**: ^{1}H NMR (400 MHz, Acetone-d_6): 8.60–8.50 (m, 3H, NH), 7.7.27–7.13 (m, 3H, Thio), 6.78 (s, 1H, Podo), 6.53 (s, 1H, Thio), 6.47 (s, 1H, Podo), 6.38 (s, 2H, Podo), 6.05 (d, $J = 8.5$, 2H, Podo), 5.92 (d, $J = 8.3$, 1H, Podo), 4.71 (d, $J = 7.4$, 1H, Podo), 4.63–4.51 (m, 3H, Thio+Linker), 4.43 (dd, $J = 12.1$, 8.2, 1H, Podo), 4.253 (t, $J = 10.7$, 1H, Podo), 3.94 (s, 3H, Thio), 3.89 (s, 3H, Thio), 3.68 (s, 9H, Thio+Podo), 3.59 (s, 3H,Podo), 3.30–3.20 (m, 2H, CH_2Linker), 3.05–2.93 (m, 2H, Podo) 2.71–2.62 (m, 2H, CH_2Linker), 2.50–2.41 (m, 2H, Thio), 2.45 (s, 3H, Thio), 2.09–1.75 (m, 2H, Thio), 1.50 (s, 9H, Boc), 1.43 (s, 9H, Boc).^{13}C NMR (100 MHz, Acetone-d_6): 182.2 (1C, Thio), 174.8 (1C, Podo), 171.0 (1C, Linker), 169.1 (1C, Linker), 161.3 (2C, Boc), 158.2 (1C, Thio), 157.9 (1C, Boc), 153.8 (1C, Thio), 152.7 (2C, Podo), 152.2 (1C, Thio), 151.1 (1C, Thio), 148.5 (1C, Podo), 148.0 (1C, Podo), 141.6 (1C, Thio), 138.7 (1C, Thio), 137.6 (1C, Podo), 135.5 (1C, Thio), 134.5 (1C, Podo), 134.1 (1C, Thio), 131.1 (1C, Podo), 128.7 (1C, Thio), 128.3 (1C, Podo), 127.1 (1C, Thio), 125.9 (1C, Thio), 109.2 (1C, Podo), 108.3 (2C, Podo), 107.7 (1C, Podo), 107.3 (1C, Thio), 101.6 (1C, Podo), 79.1(2C, Boc), 74.1 (1C, Podo), 70.8 (1C, Podo), 64.9 (1C, Thio), 60.7 (1C, Thio), 60.3 (1C, Podo), 59.6 (1C, Thio), 55.5 (2C, Podo), 53.9 (1C, Linker), 53.8 (1C, Thio), 52.1 (1C, Thio), 44.6 (1C, Podo), 43.7 (1C, Podo), 41.3 (1C, Thio), 38.6 (1C, Podo), 35.7 (1C, Linker), 34.6 (1C, Linker), 29.8 (1C, Thio), 29.0 (3C, Boc), 28.4 (3C, Boc), 15.1 (1C, Thio); Anal. Calcd. for $C_{58}H_{69}N_3O_{18}S_3$: C, 58.42; H, 5.83; N, 3.52. Found: C, 58.44; H, 5.82; N, 3.53
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