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Synthesis and biological evaluation of new camptothecin derivatives obtained by modification of position 20

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ABSTRACT

The preparation and biological evaluation of a novel series of dimeric camptothecin derivatives are described. All the new compounds showed a significant ability to inhibit human tumor cell growth with IC_{50} values ranging from 0.03 to 12.2 μM. The interference with the activity of the nuclear enzymes topoisomerases has been demonstrated, highlighting the poison effect of one of the obtained byproducts toward topoisomerase I. A moderate antiangiogenic activity has been demonstrated for one of the obtained compounds. Moreover, the effects of four new compounds on caspases activity and ROS generation have been studied on transgenic mouse cell.

1. Introduction

Camptothecin (**1**, CPT), a pentacyclic alkaloid[1] isolated in 1958 from *Camptotheca acuminata*,[2] acts as a selective poison of the nuclear enzyme topoisomerase I by forming a ternary complex with topoisomerase I and DNA.[3] Therapeutic applications of CPT have been hindered by its poor aqueous solubility, and by severe toxicities identified in early clinical studies performed in the 1970's.[4] The toxicity is partially due to the instability of lactone ring (E), that results in the formation of the inactive but toxic carboxylate species.[5] Since then, academic and industrial research groups have focussed their attention on the synthesis of the alkaloids of the camptothecin family and their analogues as promising agents for the treatment of human cancers.[6] The compounds which have a 20-OH substituted groups can increase the stability of the drugs–DNA–Topo I ternary complex,[7] decrease the toxicity and inhibit the opening of the lactone ring.[8]

In the frame of our ongoing project devoted to the synthesis of bivalent compounds using natural products as building blocks,[9] we planned the formation of dimeric compounds of CPT with the aim to modulate the activity, to improve the solubility and to increase the stability of the lactone ring. We selected the use of polyamide spacers taking into consideration the possibility to improve the interaction with the biological targets.[10]

2. Results and discussion

2.1. Chemistry

We decided to synthesize the dimeric compounds according to the approach described in Scheme 1.

The presence of hydroxyl group at C-20 campthotecin position offered the opportunity for the direct introduction of a spacer moiety. We selected the use of a urethane function for the junction of the spacer to the CPT scaffold. Reaction of CPT (**1**) with triphosgene (bis(trichloromethyl) carbonate) and subsequently with *t*-butyl-*N*-(2-aminoethyl)carbamate[11] afforded unsuccessful results. Derivate **2**[12] was prepared by an alternative method using PNP-chloroformate. Subsequent reaction with *N*-Boc monoprotected ethylendiamine afforded compound **3**. The protective group was removed by treatment with HCl (dioxane saturated solution) to give **4** that we used for the preparation of several dimers by condensation with appropriate dicarboxylic acids. The reaction in the presence of DCC and DMAP gave the products **5–8** accompanied by the 1,3-dioxoazacyclo derivatives **9–12**. All the obtained compounds, but **5** and **9**, appeared sufficiently stable is solution[13] and showed a better solubility (0.5–0.8 mg/mL, MeOH/H_2O v/v 8:2) if compared with campthotecin (CH_3Cl/MeOH v/v 1:1).[14]

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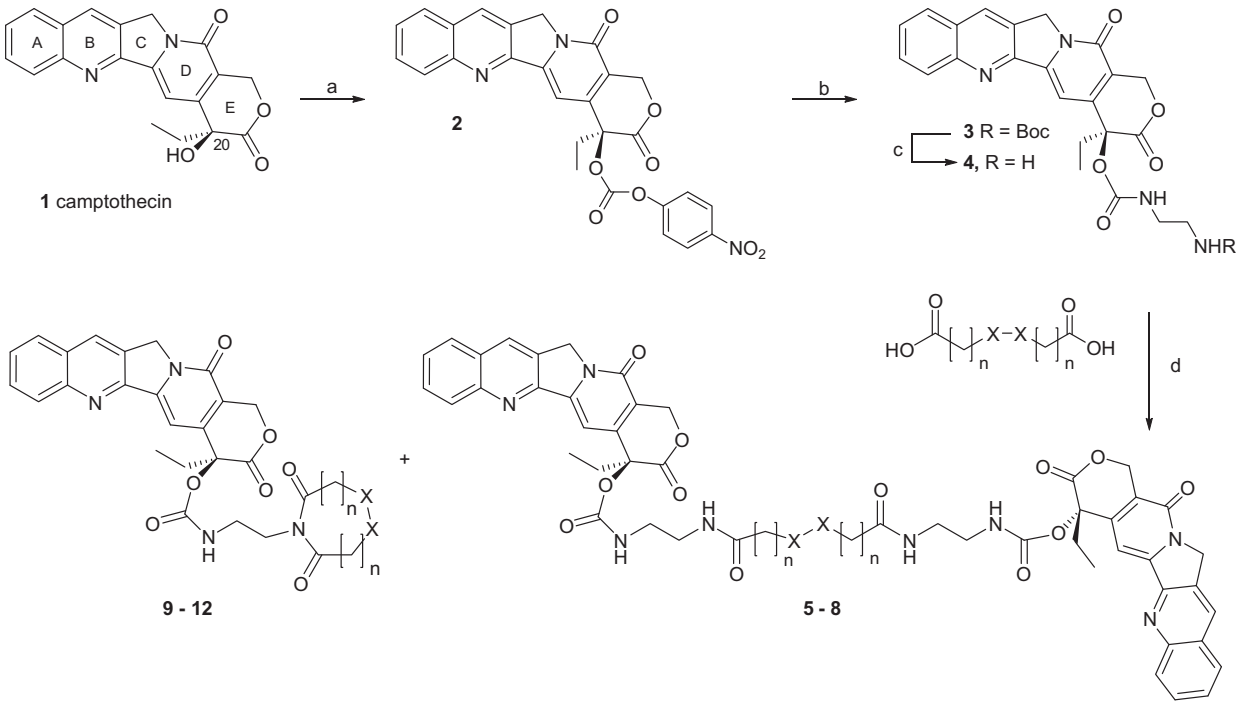

Compound	5 and 9	6 and 10	7 and 11	8 and 12
n	1	2	3	3
X	S	S	S	CH_2

Scheme 1. Reagents and conditions: (a) PNP-chloroformate, DMAP, CH_2Cl_2, 0 °C, rt; (b) 1-(*tert*-butyloxycarbonyl)ethyldiamine (**13**), DMF; (c) HCl/dioxane; (d) dicarboxylic acid, DCC, DMAP, CH_2Cl_2.

2.2. Antiproliferative activity

The ability of new derivatives and their byproducts to inhibit cell growth was evaluated by means of an in vitro assay performed on three human tumor cell lines, HeLa (cervix adenocarcinoma), HL-60 (promyelocytic leukemia), and A-431 (epithelial carcinoma).[15] The IC_{50} values were in the range 0.03–12 μM after 72 h incubation as reported in Table 1.

The antiproliferative activity of the new compounds was evaluated also toward 11 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, and Craf/Cmyc). Notably, the transgenic cell lines display epigenetic plasticity and express several tumor stem cell markers providing a mechanistic insight into lung cancer as recently reported by us.[16] The murine cell lines were more resistant, with IC_{50} values typically in the region of 1 μM. A comparison between the compounds that include one unit of camptothecin (**10–12**) and the ones with two units (**6–8**), evidenced for these latter a higher antiproliferative activity. Moreover, inside each series of derivatives the sulfur propyl seems to be the most convenient spacer. Indeed, it is possible to evidence in both classes a similar behavior where **7** > **8** > **6** and **11** > **12** > **10**. In particular, the dimeric derivative **7**, is the most active in all the tested cell lines.

2.3. Interaction with DNA

The noticeable antiproliferative activity shown by the new camptothecin derivatives, prompted us to study their mechanism of action. In the first place, we investigated the ability of compounds to form a molecular complex with DNA by linear dichroism (LD) experiments.[17] The obtained results showed that the DNA spectrum is not significantly modified by the addition of the examined compounds at different [drug]/[DNA] ratios (data not shown), according to the behavior of camptothecin.

2.4. Effect on DNA topoisomerases

DNA topoisomerases have been shown to be the molecular target of many anticancer drugs: camptothecin, in particular, acts

Table 1
Compound concentration (μM) that causes 50% reduction in cell number with respect to the control culture; data are means of at least three independent experiments with triplicate samples each ± SD

	Compound IC_{50}						
Cell lines	CPT	**6**	**7**	**8**	**10**	**11**	**12**
A-431	0.011 ± 0.002	0.15 ± 0.06	0.03 ± 0.01	0.08 ± 0.01	2.97 ± 0.78	1.21 ± 0.04	1.88 ± 0.39
HeLa	0.0089 ± 0.0016	0.7 ± 0.38	0.17 ± 0.07	0.30 ± 0.05	11.96 ± 0.37	0.57 ± 0.17	8.3 ± 0.29
HL-60	0.017 ± 0.0028	0.92 ± 0.06	0.29 ± 0.09	0.42 ± 0.07	8.1 ± 1	1.55 ± 0.26	12.25 ± 0.17

as topoisomerase I poison, that is, stabilizing the covalent ternary complex with enzyme in the cutting phase, and preventing the rejoining of the filament.[18,19]

In order to investigate the ability of new compounds (**6**–**8** and **10**–**12**) to interact with the nuclear enzyme, we performed topoisomerase I relaxation assay on supercoiled plasmid pBR322 DNA. The results did not show any inhibitory effects on topoisomerase I activity (data not shown). To study the influence of new compounds on the formation of the cleavable complex between DNA and topoisomerase I a further test was performed.[15] The obtained results indicated only for the monomer **12** the ability to stabilize the formation of the cleavable complex, as demonstrated by the appearance of the 'nicked' band, according to the reference drug CPT (Fig. 1).

To further investigate the ability of all compounds to interfere with the enzymatic activity of DNA topoisomerase II, a relaxation assay was performed.

The appearance of supercoiled DNA with a concurrent decrease of the relaxed one demonstrated a detectable inhibition on the relaxation activity only for **6** and **10**. In details, compounds **6** and **10** showed a dose dependent effect at concentration ranging from 12.5 to 100 μM (Figs. 2 and 3). In particular, the inhibition at 50 μM and 100 μM for compound **6** and **10**, respectively, are similar to that obtained by incubating the enzyme in the presence of 8 μM amsacrine (*m*-AMSA), taken as referenced drug.[20]

On the other hand, the new compounds appeared unable to induce the formation of the cleavable complex in the presence of topoisomerase II, indicating that they are not a poison for the enzyme (data not shown). Overall these results suggest that the compounds **6** and **10** act as catalytic inhibitors of topoisomerase II.

2.5. Effects on the induction of apoptosis

In light of the induction of cell death, we investigate the activation of caspases (a family of cysteine proteases) to assess the ability of test compounds to trigger the apoptotic pathway. Upon activation, these enzymes cleave specific substrates and thereby mediate many of the typical biochemical and morphological changes in apoptotic cells, such as cell shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing. Hence, the detection of activated caspases can be used as a biochemical marker for apoptosis. We selected compounds **7**, **8**, **10**, and **12** to investigate their effects on caspase activity. We used a cell-permeable caspase substrate, (Asp)2-rhodamine 110. Upon caspase cleavage, the nonfluorescent substrate is converted to rhodamine 110 leading to an increase in fluorescence.[21] The new compounds appear different from the starting CPT. In Figure 4 we report the result we obtained in the case of compound **7**. In the case of cmyc/craf cell line the mean rhodamine 110 fluorescence was 13-fold significantly increased (**7**, 10 μmol/L). On the same cell line compound **8** induced an eightfold increasing of the rhodamine 110 fluorescence. In the case compounds **10** and **12** we did not observe any relevant activity.

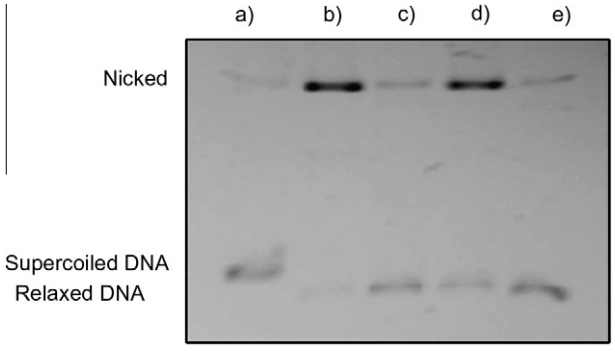

Figure 1. Effect of compound **12** on the stabilization of covalent DNA–topoisomerase I complex: (a) supercoiled pBR322 DNA; (b) supercoiled DNA incubated with topoisomerase I in the presence of compound (100 μM); (c) supercoiled DNA incubated with topoisomerase I alone; (d) supercoiled DNA incubated with topoisomerase I in the presence of CPT (100 μM); (e) supercoiled DNA incubated with topoisomerase I in the presence of solvent.

Compounds **7**, **8**, **10**, and **12** were additionally investigated on ROS (reactive oxygen species) generation in the transgenic mouse cells on the base that ROS are connected with induction of apoptosis. The influence of the new compounds on ROS generation deviates from the behavior of CPT. Compound **7** showed a significant increase of ROS production in the majority of the cell lines with exception of A2B1 (10-fold increasing in cmyc/craf, Fig. 5).[20] Compound **8** resembled the behavior of compound **7** in all the cell lines apart from βD10, γA7, γB8 and γD12. Compounds **10** and **12** showed a reduced efficacy.

2.6. Anti-angiogenic activity

We considered that the new compounds are characterized by modulated biological activity in comparison with CPT. As recent publications describe the antiangiogenic activity of topotecan[22] and irinotecan,[23] we evaluated our new compounds for anti-angiogenic activity. Compounds **7**, **10**, and **11** were assessed for effects on organotypic capillary-like vessel formation using co-culture of primary human endothelial cells (EC) and vascular smooth muscle cells (vSMC) according to a procedure developed by our laboratory.[24] CPT (**1**, Fig. 6) showed a potent inhibitory effect on capillary-like network formation at the second lowest concentration tested (25 nM). Compound **7** had an intermediate inhibitory effect and reduced the total tube length by 50% at concentrations exceeding 125 nM. However, image analysis revealed that the morphology of the endothelial cells is not affected; instead, there were overall fewer tubules formed. Compounds **10** and **11** had no effect in the concentration range tested (2–200 nM, in Fig. 6 is represented the behavior of compound **11**).

3. Conclusions

The convenient synthesis of four new dimeric compounds of CPT is reported with three of them stable enough to be considered for the biological evaluation on cancer cells. The condensation reaction induces the formation of the corresponding cyclic imides that have been considered as well for the biological evaluation. In particular we achieved an improvement in the solubility due to the polyamide character of the linker. Three different human cancer cells have been used for the evaluation and the results moved us toward the evaluation on eleven different murine stem cell lines. The obtained results showed a general dissimilar activity with respect to CPT. The dimeric derivative **7**, is the most active in all the tested cell lines. The interference with the activity of the nuclear enzymes topoisomerases has been demonstrated, highlighting the poison effect of compound **12** toward topoisomerase I. Compounds **6** and **10** act as catalytic inhibitors of topoisomerase II. The effects of compounds **7**, **8**, **10**, and **12** on caspases activity and ROS generation evidenced an interesting activity of compound **7**. The observed general diversity of the biological activity drove us to study the influence of the obtained compounds on angiogenesis. Compound **7** showed a moderate antiangiogenic activity. In conclusion, the interesting behavior of dimeric derivative **7** allows us to consider this scaffold as a promising lead worth to be further developed in order to obtain more precise structure–activity relationships. We are convinced that the use of natural products as building blocks for the creation of new entities is a noteworthy

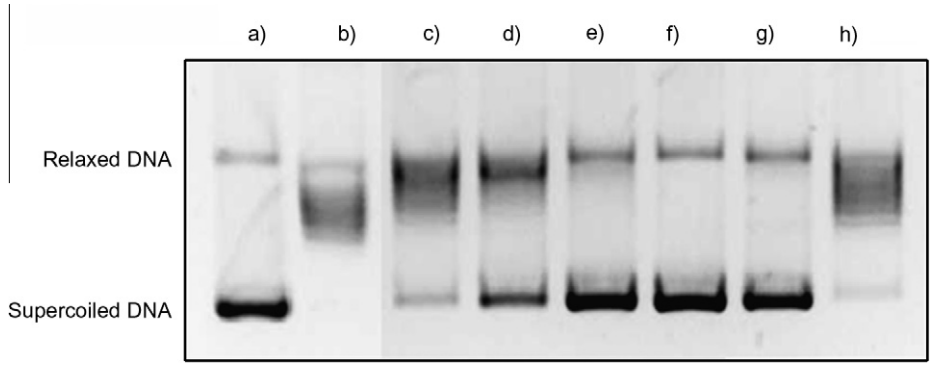

Figure 2. Effect of compound **6** on relaxation of supercoiled pBR322 DNA of topoisomerase II: (a) supercoiled pBR322; (b) same as lane (a) in the presence of 1 U topoisomerase II; (c–f) same as lane (b) in the presence of 12.5, 25, 50, and 100 μM test compound, respectively; (g) same as lane (b) in the presence of 8 μM *m*-AMSA; (h) same as lane (b) in the presence of solvent alone.

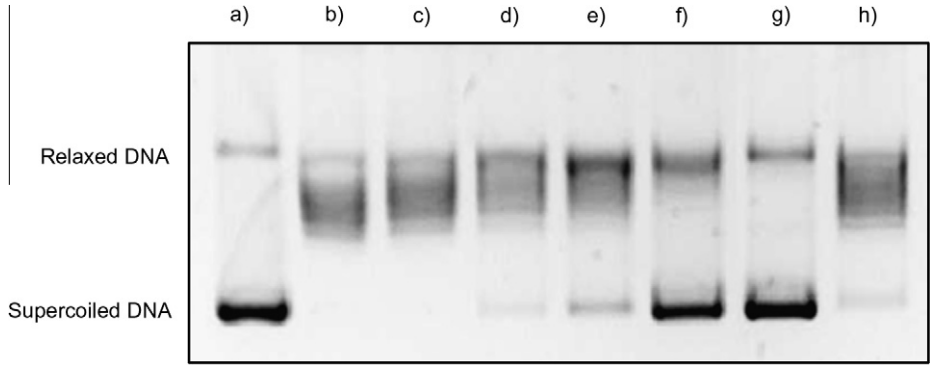

Figure 3. Effect of compound **10** on relaxation of supercoiled pBR322 DNA of topoisomerase II: (a) supercoiled pBR322; (b) same as lane (a) in the presence of 1 U topoisomerase II; (c–f) same as lane (b) in the presence of 12.5, 25, 50, and 100 μM test compound, respectively; (g) same as lane (b) in the presence of 8 μM *m*-AMSA; (h) same as lane (b) in the presence of solvent alone.

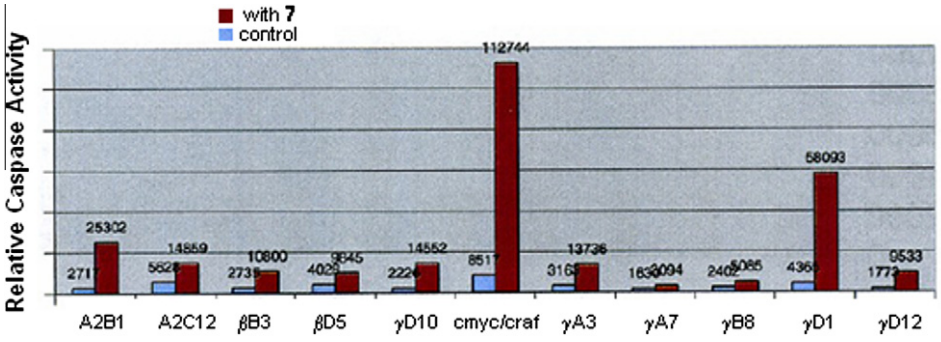

Figure 4. Effects of **7** on caspase activity. Cells were pretreated with **7** (10 μmol/L) for 2 h. The caspase substrate (Asp)2-rhodamine 110 was added at 10 μmol/L for additional 2 h.

strategy to improve the exploration of the chemical space for the sake of finding new biological activities.[25]

4. Experimental section

4.1. General

Thin-layer chromatography (TLC) was performed on Merck precoated 60F254 plates. Reactions were monitored by TLC on silica gel, with detection by UV light (254 nm) or by charring with sulfuric acid. Flash chromatography was performed using silica gel (240–400 mesh, Merck). ^{1}H NMR spectra were recorded with Brucker 200, 300, and 400 MHz spectrometers using chloroform-*d* ($CDCl_3$) and methanol-d_4 (CD_3OD). Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane (TMS) as internal standard. EI mass spectra were recorded at an ionizing voltage of 6 Kev on a VG 70-70 EQ. ESI mass spectra were recorded on FT-ICR APEX^{II} (Bruker Daltonics). All reactions were carried out in dry solvents.

4.2. Preparation of (2)

(*p*-Nitrophenyl camptothecin carbonate): camptothecin (**1**) (228 mg, 0.65 mmol) and PNP-chloroformate (460 mg, 2.29 mmol) were dissolved in CH_2Cl_2 (30 mL) at 0 °C, followed by the addition of DMAP (444 mg, 3.60 mmol). The resulting clear solution was

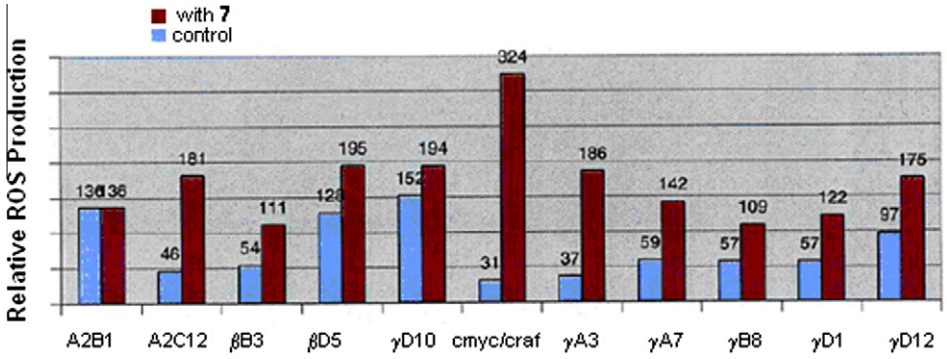

Figure 5. Effects of **7** (10 μmol/L) on ROS production. ROS production was measured by flow cytometry with DCFH-DA.

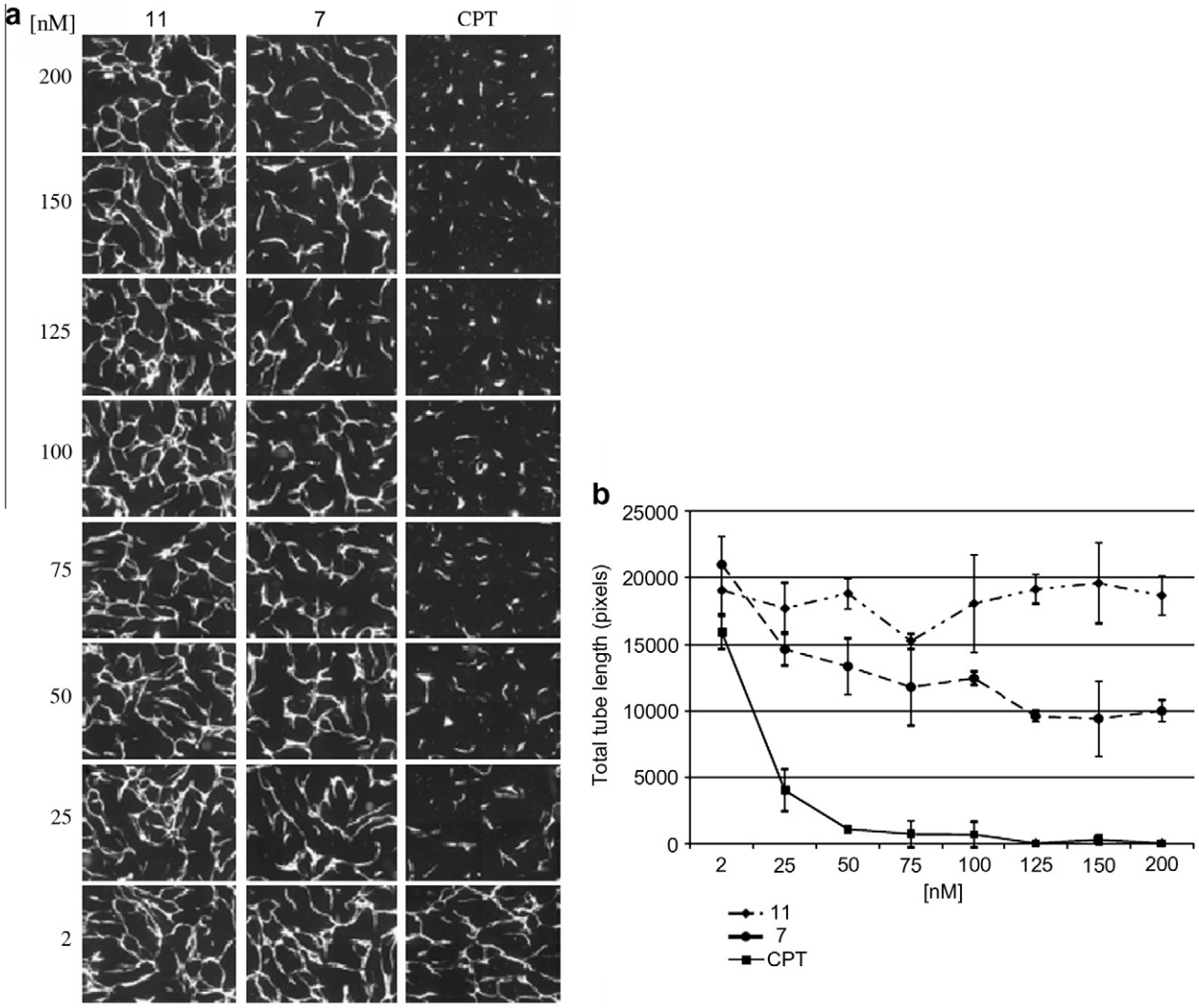

Figure 6. Anti-angiogenesis assay—(a) SMC and fluorescent HUVEC were seeded in a 96-well plate and treated with dilution series for the compounds **11**, **7**, **10** and camptothecin (CPT) in the concentration range 2–200 nM.

stirred at room temperature for 1 h. The reaction was monitored by TLC (CH_2Cl_2/MeOH 60:1). After completion, the mixture was diluted with 50 mL of CH_2Cl_2 and washed with 10 mL HCl 0.1 N. The organic layer was dried over Na_2SO_4, concentrated under reduced pressure. The remaining residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH 80:1), affording compound **2** in the form of yellow powder (292 mg; yield 87%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 8.44 (1H, s), 8.24 (1H, d, J = 7.5 Hz), 8.20 (2H, d, J = 10.0 Hz); 7.99 (1H, d, J = 7.5 Hz), 7.85 (1H, t, J = 7.5 Hz); 7.69 (1H, t, J = 7.5 Hz), 7.40 (2H, d, J = 10 Hz), 7.36 (1H, s), 5.70 (1H, d, J = 16.0 Hz); 5.40 (1H, d, J = 16.0 Hz), 5.31 (2H, s), 1.99–2.44 (2H, m), 1.06 (3H, t, J = 6.5 Hz). Anal. Calcd for $C_{27}H_{19}N_3O_8$: C, 63.18; H, 3.73; N, 8.18. Found: C, 63.21; H, 3.79; N, 8.11; ESIMS: 536.20 (MNa^+); $[\alpha]_D^{25}$ = +20.57 (c 1, $CHCl_3$).

4.3. Preparation of (3)

p-Nitrophenyl camptothecin carbonate (**2**) (70 mg, 0.14 mmol) was dissolved in DMF (7 mL), and 1-(*tert*-butyloxycarbonyl)ethyldiamine (**13**) (43 mg, 0.27 mmol) was added at room temperature. The mixture was stirred for 10 min, and the DMF was removed under reduced pressure. AcOEt was added to the crude residue and then extracted with water. The organic layer was dried over sodium sulfate and concentrated under reduced pressure to give **3** (68 mg, yield 90%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 8.18 (1H, d, J = 7.8 Hz), 8.08 (1H, s), 8.83 (2H, s), 7.80 (1H, t, J = 7.8 Hz), 7.65 (1H, d, J = 7.8 Hz), 7.53 (1H, t, J = 7.8 Hz), 5.5 (1H, br s), 5.19 (2H, s), 5.06 (2H, s), 3.86 (2H, m), 3.47–3.55 (2H, m), 2.36–2.51 (2H, m), 1.47 (9H, s), 1.05 (3H, t, J = 7.3 Hz). Anal. Calcd for $C_{28}H_{30}N_4O_7$: C, 62.91; H, 5.66; N, 10.48. Found: C, 62.87; H, 5.62; N, 10.45; ESIMS: 536.20 (MNa^+); $[\alpha]_D^{25}$ = +24.5 (c 1, $CHCl_3$).

4.4. Preparation of (4)

Compound (**3**) (452 mg, 0.85 mmol) was dissolved in dioxane (10 mL). A freshly prepared saturated solution of HCl in dioxane (8 mL) was added portion wise at 0 °C for 1 h. The reaction was monitored by TLC (CH_2Cl_2/MeOH v/v 10:1). After completion, the reaction mixture was worked-up with saturated solution of $NaHCO_3$ obtaining a basic pH and then was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, concentrated under reduced pressure to afford compound **3** (365 mg; quantitative yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 8.42–7.19 (6H, m), 5.23 (2H, br s), 5.10 (2H, br s), 5.02 (2H, br s), 3.84 (2H, m), 3.11 (2H, m), 2.35 (2H, m), 1.1 (3H, t, J = 7.1 Hz). Anal. Calcd for $C_{23}H_{22}N_4O_5$: C, 63.59; H, 5.10; N, 12.90. Found: C, 63.57; H, 5.07; N, 12.92; ESIMS: 457.20 (MNa^+); $[\alpha]_D^{25}$ = +21.57 (c 1, $CHCl_3$).

4.5. General procedure for preparation of compounds (5–12)

DMAP (0.57 mmol), (**4**) (1.14 mmol) and DCC (1.70 mmol) were added to a solution of diacid (0.57 mmol) in CH_2Cl_2 dry at room temperature. The mixture was stirred for 13 h then concentrated under reduced pressure. The crude residue was filtered on Celite, the organic layer was concentrated and the crude residue was purified by flash chromatography (silica gel, CH_2Cl_2/MeOH 40:1) to give the dimeric compounds (yields: 42–43%).

4.5.1. Compound (5)

^{1}H NMR (400 MHz, DMSO-d_6, 80 °C): δ 8.56 (2H, s), 8.18 (2H, m), 8.11 (2H, m), 7.84 (2H, m), 7.74 (2H, m), 7.45 (2H, s), 6.72 (4H, br s), 5.25 (4H, m), 4.86 (4H, s), 3.40 (8H, m), 2.43 (4H, m), 2.14 (4H, m), 0.95 (6H, m); ^{13}C NMR (100.6 MHz, DMSO-d_6, 80 °C): δ 172.4 (2C); 168.2 (2C); 167.6 (2C); 156.6 (C); 153.5 (2C) 148.1 (2C); 145.7 (2C); 144.0 (2C); 131.6 (2C); 131.4 (2C); 130.2 (2C); 129.7 (2C); 129.6 (2C); 129.0 (2C); 128.0 (2C); 127.5 (2C); 97.2 (2C); 88.6 (2C) 55.5 (2C); 50.5 (2C); 42.4 (2C); 36.4 (2C); 31.2 (2C); 30.9 (2C); 7.40 (2C). Anal. Calcd for $C_{50}H_{46}N_8O_{12}S_2$: C, 59.16; H, 4.57; N, 11.04. Found: C, 59.14; H, 4.55; N, 11.06; HR-ESIMS. Anal. Calcd for $C_{50}H_{46}N_8O_{12}S_2Na^+$, 1037.2569. Found 1037.2581; $[\alpha]_D^{25}$ = +24.5 (c 1, $CHCl_3$).

4.5.2. Compound (6)

^{1}H NMR (400 MHz, CD_3OD-d_4, 50 °C): δ 8.27 (2H, s), 7.81 (2H, m), 7.79 (2H, m), 7.76 (2H, m), 7.74 (2H, m), 7.28 (2H, s), 5.32 (2H, m), 5.19 (2H, m), 5.06 (4H, m), 3.73 (4H, m), 3.54 (4H, m), 3.02–2.66 (8H, m), 2.44 (4H, m), 1.0 (6H, m); ^{13}C NMR (100.6 MHz, CD_3OD-d_4, 50 °C): δ 171.4 (2C), 167.2 (2C), 167.9 (2C), 156.57 (2C), 153.5 (2C), 148.5 (2C), 145.5 (2C), 143.9 (2C), 132.0 (2C), 131.3 (2C), 130.2 (2C), 130.1 (2C), 129.4 (2C), 128.9 (2C), 128.1 (2C), 127.5 (2C), 96.1 (2C), 81.6 (2C), 55.6 (2C), 51.6 (2C), 42.9 (2C), 39.1 (2C), 36.4 (2C), 36.5 (2C), 34.8 (2C), 8.6 (2C). Anal. Calcd for $C_{52}H_{50}N_8O_{12}S_2$: C, 59.87; H, 4.83; N, 10.74; S, 6.15. Found: C, 59.83; H, 4.85; N, 10.76; S, 6.11; HR-ESIMS. Anal. Calcd for $C_{52}H_{50}N_8O_{12}S_2Na^+$, 1065.2882. Found 1065.2908; $[\alpha]_D^{25}$ = +20.9 (c 1, $CHCl_3$).

4.5.3. Compound (7)

^{1}H NMR (400 MHz, CD_3OD-d_4, 50 °C): δ 8.48 (2H, s), 8.07 (2H, m), 7.94 (2H, m), 7.70 (4H, m), 7.43 (2H, s), 5.16 (2H, m), 5.05 (6H, m), 3.78 (4H, m), 3.50 (4H, m), 2.50–2.05 (16H, m), 1.01 (6H, m); ^{13}C NMR (100.6 MHz, CD_3OD-d_4, 50 °C): δ 173.6 (2C); 169.2 (2C); 167.9 (2C); 157.4 (2C); 153.5 (2C); 148.1 (2C); 145.9 (2C); 144.7 (2C); 131.7 (2C); 131.4 (2C); 131.1 (2C); 130.7 (2C); 130.1 (2C); 129.4 (2C); 128.9 (2C); 128.5 (2C); 99.7 (2C); 89.6 (2C); 55.47 (2C); 51.52 (2C); 42.56 (2C); 37.70 (2C); 34.84 (2C); 33.84 (2C); 32.76 (2C); 30.95 (2C); 7.40 (2C). Anal. Calcd for $C_{54}H_{54}N_8O_{12}S_2$: C, 60.55; H, 5.08; N, 10.46; S, 5.99. Found: C, 60.51; H, 5.06; N, 10.44; S, 6.01; ESIMS: 1093.70 (MNa^+); $[\alpha]_D^{25}$ = +20.7 (c 1, $CHCl_3$).

4.5.4. Compound (8)

^{1}H NMR (400 MHz, DMSO-d_6, 80 °C): δ 8.65 (2H, s), 8.19 (2H, m, J = 7.2 Hz), 8.09 (2H, m, J = 7.3 Hz), 7.85 (2H, m, J = 7.3 Hz), 7.77 (2H, m, J = 7.2 Hz), 7.46 (2H, s), 6.82 (br s, 4H), 5.28 (4H, m), 4.89 (4H, m), 3.62 (4H, m), 3.34 (4H, m), 2.20 (4H, m), 1.92–1.25 (16H, m), 0.98 (6H, m); ^{13}C NMR (100 MHz, DMSO-d_6, 80 °C): δ 174.0 (2C), 172.8 (2C), 165.5 (2C), 155.3 (2C), 151.4 (2C), 148.1 (2C), 146.1 (2C), 144.3 (2C), 130.7 (2C), 130.4 (2C), 130.2 (2C), 130.1 (2C), 129.7 (2C), 129.4 (2C), 127.8 (2C), 127.4 (2C), 99.3 (2C), 88.9 (2C), 57.2 (2C), 50.3 (2C), 40.8 (2C), 37.5 (2C), 36.6 (1C), 33.6 (2C), 32.0 (1C), 29.9 (1C), 29.6 (1C), 29.2 (1C), 28.7 (1C), 28.5 (1C), 25.1 (1C); 7.5 (2C). Anal. Calcd for $C_{56}H_{58}N_8O_{12}$: C, 64.98; H, 5.65; N, 10.83. Found: C, 64.93; H, 5.01; N, 10.40; ESIMS 1057.70 (MNa^+); $[\alpha]_D^{25}$ = +20.4 (c 1, $CHCl_3$).

4.5.5. Compound (9)

^{1}H NMR (400 MHz, DMSO-d_6, 80 °C): δ 8.71 (1H, s), 8.33 (1H, m), 8.13 (1H, m), 8.03 (br s, 1H), 8.00 (1H, m), 7.75 (1H, m), 7.53 (1H, s), 5.47 (1H, m), 5.34 (3H, m), 3.39 (4H; m), 2.38 (2H, m), 2.01–1.10 (4H, m), 0.98 (3H, t, J = 7.1 Hz); ^{13}C NMR (100.6 MHz, DMSO-d_6, 80 °C): δ 176.2 (1C); 174.8 (1C); 172.2 (2C); 163.6 (1C); 156.7 (1C), 153.2 (1C); 152.3 (1C); 148.1 (1C); 131.6 (1C); 130.2 (1C); 129.6 (1C); 128.7 (1C); 127.9 (1C); 127.7 (1C); 127.5 (1C); 125.9 (1C); 115.7 (1C); 88.7 (1C); 50.4 (2C); 42.3 (1C); 39.1 (1C); 33.2 (1C); 32.2 (1C); 25.3 (1C); 7.3 (1C). Anal. Calcd for $C_{27}H_{24}N_4O_7S_2$: C, 55.85; H, 4.17; N, 9.65; S, 11.04. Found: C, 55.83; H, 4.14; N, 9.67; S, 11.06; HR-ESIMS. Anal. Calcd for $C_{27}H_{24}N_4O_7S_2Na^+$, 603.0984. Found 603.0978; $[\alpha]_D^{25}$ = +27.7 (c 1, $CHCl_3$).

4.5.6. Compound (10)

^{1}H NMR (400 MHz, CD_3OD-d_4, 50 °C): δ 8.64 (1H, s), 8.22 (1H, d, J = 8.3 Hz), 8.07 (1H, d, J = 8.4 Hz), 8.03 (1H, s), 7.87 (1H, t, J = 7.1 Hz), 7.72 (1H, t, J = 7.2 Hz), 5.16 (1H, m), 5.05 (3H, m), 3.83 (2H, m), 3.47 (2H, m), 3.04–2.77 (6H, m), 2.43 (4H, m), 1.02 (3H, t, J = 7.3 Hz); ^{13}C NMR (100.6 MHz, CD_3OD-d_4, 50 °C): δ 171.4 (1C), 167.2 (1C), 167.9 (2C), 156.5 (1C), 153.5 (1C), 148.5 (1C), 145.5 (1C), 143.9 (1C), 132.0 (1C), 131.3 (1C), 130.2 (1C), 130.1 (1C), 129.3 (1C), 128.9 (1C), 128.1 (1C), 127.4 (1C), 96.1 (1C), 81.6 (1C), 55.5 (1C), 51.5 (1C), 42.9 (1C), 39.1 (1C), 36.3 (2C), 36.5 (2C), 34.8 (1C), 8.6 (1C). Anal. Calcd for $C_{29}H_{28}N_4O_7S_2$: C, 57.22; H, 4.69; N, 9.20; S, 10.54. Found: C, 57.23; H, 4.67; N, 9.23; S, 10.57; ESIMS: 631.13 (MNa^+); $[\alpha]_D^{25}$ = +26.6 (c 1, $CHCl_3$).

4.5.7. Compound (11)

^{1}H NMR (400 MHz, CD_3OD-d_4, 50 °C): δ 8.67 (1H, s); 8.25 (1H, d, J = 8.3 Hz); 8.09 (1H, d, J = 8.2 Hz), 7.96 (1H, s); 7.90 (1H, t, J = 7.4 Hz); 7.74 (1H, t, J = 7.2 Hz); 5.48 (1H, m); 5.37 (3H, m);

3.79 (2H, m); 3.48 (2H, m); 2.95–2.44 (12H, m); 2.19 (2H, m); 1.08 (3H, t, J = 7.2 Hz); ^{13}C NMR (100.6 MHz, CD_3OD-d_4, 50 °C): δ 175.8 (1C), 174.5 (1C), 173.3 (2C), 164.0 (1C), 156.4 (1C), 154.1 (1C), 151.3 (1C), 147.8 (1C), 133.6 (1C), 132.5 (1C), 131.6 (1C), 131.0 (1C), 130.8 (1C), 130.3 (1C), 130.1 (1C), 125.8 (1C), 100.1 (1C), 89.5 (1C), 61.4 (1C), 52.9 (1C), 42.5 (1C), 39.1 (1C), 36.9 (2C), 36.2 (2C), 36.1 (2C), 35.0 (1C), 8.65 (1C). Anal. Calcd for $C_{31}H_{32}N_4O_7S_2$: C, 58.47; H, 5.07; N, 8.80; S, 10.07. Found: C, 58.42; H, 5.05; N, 8.82; S, 10.05; ESIMS: 659.16 (MNa^+); $[\alpha]_D^{25}$ = +28.7 (c 1, $CHCl_3$).

4.5.8. Compound (12)

^{1}H NMR (400 MHz, CD_3OD-d_4, 50 °C): δ 8.65 (1H, s); 8.25 (1H, d, J = 8.3 Hz); 8.24 (1H, d, J = 8.2 Hz); 7.93 (1H, s); 7.89 (1H, t, J = 7.5 Hz); 7.73 (1H, t, J = 7.4); 5.52 (1H, m); 5.39 (3H, m); 3.73 (2H, m); 3.50 (2H, m); 2.38–2.16 (12H, m); 1.85–1.10 (6H, m); 0.94 (3H, t, J = 7.2 Hz); ^{13}C NMR (100.6 MHz, CD_3OD-d_4, 50 °C): δ 174.6 (1C), 173.1 (1C), 172.6 (2C), 162.5 (1C), 156.1 (1C), 153.4 (1C), 149.5 (1C), 133.9 (1C), 133.4 (1C), 132.6 (1C), 132.0 (1C), 131.0 (1C), 130.4 (1C), 130.1 (1C), 122.7 (1C), 100.6 (1C), 88.8 (1C), 60.7 (1C), 52.8 (1C), 39.2 (1C), 37.5 (1C), 34.6 (1C), 35.6 (1C), 31.5 (1C), 30.9 (2C), 30.3 (1C), 30.1 (1C), 27.6 (1C), 26.8 (1C), 25.5 (1C), 9.1 (1C). Anal. Calcd for $C_{33}H_{36}N_4O_7$: C, 65.99; H, 6.04; N, 9.33. Found: C, 65.95; H, 6.02; N, 9.34; ESIMS: 623.25 (MNa^+); $[\alpha]_D^{25}$ = +25.8 (c 1, $CHCl_3$).

4.6. Preparation of (13)

A solution of di-*tert*-butyl dicarbonate (1.21 g, 5.55 mmol) in dichloromethane (80 mL) was added dropwise at 0 °C to a solution of ethylenediamine (2 g, 33.3 mmol) in dichloromethane (10 mL) over 1 h with vigorous stirring. The reaction mixture was stirred at room temperature for 19 h. After concentration to an oily residue, the reaction mixture was dissolved in aqueous sodium carbonate (60 mL) and extracted with dichloromethane (2 × 60 mL). The organic layer was dried (anhydrous Na_2SO_4) and the solvent evaporated under reduced pressure to yield **13** (1.44 g, 100%) as a colorless viscous liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.89 (br s, 1H), 3.18 (q, J = 5.9 Hz, 2H), 2.80 (t, J = 6.1 Hz, 2H), 1.60 (br s, 2H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.2 (1C), 79.2 (1C), 43.3 (1C), 41.8 (1C), 28.4 (3C).

4.7. Inhibition growth assay

HL-60 (human myeloid leukemic cells) and HeLa (human cervix adenocarcinoma cells) were grown in RPMI 1640 (Sigma Chemical Co.) supplemented with 15% heat-inactivated fetal calf serum (Biological Industries) and in Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal calf serum (Biological Industries), respectively. A-431 (skin carcinoma squamous cell) were grown in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% fetal bovine serum (Seromed). 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma Chemical Co.) were added to the media. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air. HL-60 cells (4×10^4) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agents were added to the complete medium and incubated for a further 72 h. HeLa and A-431 (4×10^4) cells were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of fresh medium, and various concentrations of the test agents were added. The cells were then incubated in standard conditions for a further 72 h. A Trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as IC_{50} values, that is, the concentration of the test agent inducing 50% reduction in cell number compared with control cultures. A2B1, A2C12, B3, βD5, βD10, c-myc-c-raf, γA3, γA7, γB8, γD1, and γD12 cells were harvested and plated in 96-well flat-bottomed microplates at a density of 103 cells/well. Assays were performed in quintuplicates. Cells were allowed to attach for 24 h. The drugs were prepared in medium at different concentrations 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® AQueous One Solution Reagent (Promega Corporation, Madison, WI, USA) were added to each well and the plates were incubated for 1 h at 37 °C. The CellTiter 96® AQueous One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphe-nyl)-2-(4-sulfophenyl)-2*H*-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 (VictorTM 1420 Multilabel Counter, Perkin–Elmer Instruments, Shelton, USA). Cell cytotoxicity was expressed as the percentage of the controls.

4.8. Linear flow dichroism

LD measurements were performed on a Jasco J500A circular dichroism spectropolarimeter converted for LD and equipped with an IBM PC and a Jasco J interface. Linear dichroism is defined as:

$$LD(\lambda) = A_{\parallel}(\lambda) - A_{\perp}(\lambda)$$

where $A_{\parallel}$ and $A_{\perp}$ correspond to the absorbances of the sample when polarized light is oriented parallel or perpendicular to the flow direction, respectively. The orientation is produced by a device designed by Wada and Kozawa[51] at a shear gradient of 500–700 rpm and each spectrum was accumulated four times.

A solution of salmon testes DNA (1.9×10^{-3} M) in ETN buffer (containing 10 mM TRIS, 10 mM NaCl, and 1 mM EDTA, pH 7) was used. Spectra were recorded at 25 °C at different [drug]/[DNA] ratios.

4.9. Topoisomerases-mediated DNA relaxation

Supercoiled pBR322 plasmid DNA (0.25 μg, Fermentas Life Sciences) was incubated with 1 U topoisomerase II (human recombinant topoisomerase II α, USB) or 2 U topoisomerase I (calf thymus topoisomerase I, USB) and the test compounds as indicated for 60 min at 37 °C in 20 μl of reaction buffer. Reactions were stopped by adding 4 μl of stop buffer (5% sodium dodecyl sulfate (SDS), 0.125% bromophenol blue, and 25% glycerol) and 50 μg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel at room temperature. The gels were stained with ethidium bromide 1 μg/mL in TAE buffer (0.04 M Tris–acetate and 0.001 M EDTA), transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.10. Topoisomerase II-mediated DNA cleavage

Reaction mixtures (20 μl) containing 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL bovine serum albumin (BSA), 1 mM ATP, 0.25 μg pBR322 plasmid DNA, 10 U topoisomerase II (human recombinant topoisomerase II α, USB), and test compounds were incubated for 60 min at 37 °C. Reactions were stopped by adding 4 μl of stop buffer (5% SDS, 0.125% bromophenol blue, and 25% glycerol) and 50 μg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel containing ethidium bromide 0.5 μg/mL at room temperature in TBE buffer (0.09 M Tris–borate and 0.002 M EDTA).

4.11. Topoisomerase I-mediated DNA cleavage

Reaction mixtures (20 μl) containing 35 mM Tris–HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% bovine serum albumin (BSA), 10 ng pBR322 plasmid DNA, 5 U topoisomerase I (calf thymus topoisomerase I, USB), and test compounds were incubated for 60 min at 37 °C. Reactions were stopped by adding 4 μl of stop buffer (5% SDS, 0.125% bromophenol blue, and 25% glycerol) and 0.6 μl of proteinase K 2 mg/mL (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel containing ethidium bromide 0.5 μg/mL (Sigma) at room temperature in TBE buffer (0.09 M Tris–borate and 0.002 M EDTA).

4.12. Anti-angiogenesis assays

Cell culture: Human umbilical vein endothelial cells (HUVEC) and pulmonary artery smooth muscle cells (Pa-vSMC) were purchased from Lonza (C2517A, CC2581). To simplify imaging, early passage HUVEC cells were infected with retrovirus carrying a RFP-expressing construct. Cells were maintained in culture in the supplier's recommended complete medium (EGM-2 and SmGM-2, respectively) at 37 °C, 5% CO_2. The growth medium was changed every third day and cells were passaged prior to reaching confluence. For both HUVEC and Pa-vSMC the passage number was 5. *Co-culture tube formation assay*: PaSMC and HUVEC were seeded together, centrifuged briefly at 200 g to achieve an even distribution of cells and cultured in EGM-2 for 72 h to allow network formation. Cell numbers and culture volume were as follows (96-well plate per well): 5×10^4 PaSMC and 10×10^3 HUVEC in 200 μl EGM-2. Compounds were arrayed on a 96-well plate as 200× stocks in dilution series in the concentration range 0.4–40 μM with two replicates per concentration. Final concentrations of compounds in co-cultures were 2–200 nM. To ensure even distribution of compounds in each well co-cultures were seeded in 100 μl EGM-2 and were allowed to attach to the bottom of the well for 3 h. Then 1 μl of compound (200×) were mixed with 100 μl EGM-2 on a separate 96-well plate before the 100 μl EGM-2/2× compound mix were transferred to the seeded co-cultures to reach 1× concentration and a final volume of 200 μl per well. Cell cultures were imaged after 72 h. *Imaging and image analysis*: For quantitative analysis of the co-cultures, a BD Pathway 855 bioimaging system (BD Biosciences, San Jose, CA) was used for automated high-throughput-imaging. Statistical analysis of acquired images was done with BD Image Data Explorer software. Images demonstrating network formation were acquired as 2×2 montages with a 10× objective. Filter used: dsTomato HUVEC cells: excitation filter 548/20 and emission filter 570LP. Exposure time: 0.014 s. Background subtraction, noise reduction (rolling ball 25×25), and image thresholding were performed using the AttoVision v1.6.1 software supplied by BD Biosciences. Statistics on tube branch lengths per region of interest were obtained using the 'Tube Formation' image analysis module of AttoVision v1.6.1 and size criteria for an object to be included in the analysis was 5000 pixels.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.069. These data include MOL files and InChiKeys of the most important compounds described in this article.

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