Effects of camptothecin on tumor cell proliferation and angiogenesis when coupled to a bombesin analog used as a targeted delivery vector

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The camptothecin–bombesin conjugate termed DC-51-43, as a novel targeted drug delivery system, was examined in over 10 human tumor cell lines and shows a potent antiproliferative activity. This conjugate has also demonstrated its antitumor activity in our previous experiments. In our present study, we evaluate this conjugate for its antiangiogenic activity by in-vitro and in-vivo experiments. The camptothecin–bombesin conjugate and free camptothecin show potent in-vitro inhibitory activities of cell adhesion to various extracellular matrix components and integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$, not $\beta_1/\alpha\beta_1$. This conjugate displays inhibitory activity to cell migration and invasion at concentrations of 10 μmol/l or above. This conjugate is also effective against in-vitro capillary-like tube formation of endothelial cells (at 40 μmol/l), and in-vivo angiogenesis as seen by blocking the spread of host mice endothelial cells into matrigel plugs. These experimental results support the fact that the camptothecin–bombesin conjugate has therapeutic activities against angiogenesis. By binding to bombesin receptor-expressing sites, this bombesin analog, consisting of 11 amino acids, is potentially a novel delivery vector for nonspecific cytotoxic agents. *Anti-Cancer Drugs* 18:341–348 © 2007 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2007, **18**:341–348

Keywords: angiogenesis, camptothecin–bombesin conjugate, prostate cancer

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Received 22 September 2006 **Accepted** 24 October 2006

Introduction

Camptothecin (CPT), originally extracted from the Chinese tree *Camptotheca accuminata*, is a topoisomerase I inhibitor binding directly to the topoisomerase I–DNA complex, resulting in DNA damage and eventually cell apoptosis [1–3]. CPT and its analogs already have shown their significant antiangiogenic [4–6] and antitumor activities against a wide spectrum of tumors such as pancreatic, lung, ovarian and breast carcinomas in in-vitro and in-vivo experiments [3,7–12]. The insolubility problems of CPT severely restrict its clinical application. To get around this difficulty, investigators have attempted to develop various kinds of water-soluble analogs or utilize other cellular uptake methods [3,4,13]. To improve cellular uptake, CPT delivery systems have been developed by coupling CPT to nonspecific vehicles such as polyethylene glycol [13–15]. Meanwhile, more interest was paid to using preferential site-specific delivery of antitumor agents, via ligand–receptor interactions to improve tumor-specific activity [7,16–18].

Site-specific delivery can reduce toxicity to normal tissues while improving agent bioactivity in specific targets. Short peptides, such as somatostatin, bombesin (BN), urotensin, trasportan and penetratin or their analogs are present, which could be used as cytotoxic agent-carrying vehicles. As for BN, it is a short peptide and can be easily synthesized. Various kinds of these BN analogs have been made and tested for their bioactivity in our laboratories. Some of them could be used for potential vectors (data not shown). Reportedly, there are four different subtypes of BN receptors, including the Neuromedin B receptor, gastrin-releasing peptide receptor (GRPR), and BN receptor subtype-3 and subtype-4 [19,20]. Except for the GRPR, all others are poorly studied in human tissues. As reported, human GRPR is expressed aberrantly in different types of cancers such as gastric, colon, prostate and breast [20,21]. The specific characteristics described above show that BN and its receptors could be used as a specific drug delivery system for cancer-targeted therapeutics via ligand–receptor interaction.

In our laboratories, BN, somatostatin and urotensin have been evaluated as carriers to bring chemical agents such as CPT, colchicine and methotrexate into cells or tissues, and these conjugates have shown potent, specific inhibitory activities by binding to the relevant receptors (data not shown). The aim of the current study is to test the in-vitro and in-vivo inhibitory activities against angiogenesis of CPT when conjugated to a potent BN analog. Through a series of experiments, the CPT–BN conjugate was found to have potent inhibitory activities in both in-vitro and in-vivo experiments.

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Materials and methods

Camptothecin–bombesin conjugate

CPT was coupled directly, via a normal peptide bond, to a BN 4–14 analog (NC-11-40R) to form a CPT–BN conjugate (Cpt-carbonyl-NMeAmEtGly-DSer-DTyr-Gln-Trp-Ala-Val-Bal-His-Phe-Nle-NH_2) [22], termed DC-51-43 or CPT-L2-BA3.

Cell culture

Human umbilical vein endothelial cells (HUVECs) from GlycoTech (Gaithersburg, Maryland, USA) were cultured in endothelial cell growth medium (Cambrex, East Rutherford, New Jersey, USA). The human prostate cancer PC-3 cells and all other tumor cells from American Type Culture Collection (Manassas, Virginia, USA) were cultured as previously described in detail [7,23–25].

Cell proliferation assay

This cell proliferation assay (Promega, Madison, Wisconsin, USA) is the same as described previously [19]. The absorbance was measured at 570 nm.

Flow cytometry

The DNA-prep reagents for analysis of flow cytometry were purchased from Beckman Coulter (Miami, Florida, USA). The experimental protocol was the same as described previously [7].

Cell adhesion

The reagents of cell adhesion to human collagen type I (ECM104) were from Chemicon (Temecula, California, USA). PC-3 cells (70–80% confluent) were washed once with phosphate-buffered saline and incubated for 18–24 h in serum-free medium in the presence or absence of the compounds. The treated cells were harvested and diluted in serum-free medium to a density of 1.5×10^5 cells/ml. One hundred microliters of cell suspension was added to each well of the prerehydrated strips. After being incubated for 45 min at 37°C, the strips were gently washed three times with phosphate-buffered saline containing Ca^{2+}/Mg^{2+}. One hundred microliters of 0.2% crystal violet was then added and the strips were kept at room temperature for 5 min. The strips were again washed three times with PBS. One hundred microliters of solubilization buffer was then added to each well and the strips were incubated at room temperature until the cell-bound stain was completely solubilized. The absorbance at 570 nm was recorded.

Integrin-mediated cell adhesion assays (Chemicon) were performed following the manufacturer's protocol and were similar to that described above. The cell density was 4×10^5 cells/ml and the incubation time was 2 h.

Cell migration and invasion assays

For cell migration/invasion assays, the relevant test kits (ECM510 for migration, ECM556 for invasion) were purchased from Chemicon. Generally, 150 µl of medium with 10% fetal bovine serum, in the presence of the tested compounds, was added to the lower chamber (feeder tray) of the migration plate assembly. After being starved for 18–24 h in serum-free medium, PC-3 cells at 80% confluencey were harvested and resuspended in serum-free medium. One hundred microliters of the cell suspension was added to the top migration chamber (insert). The entire migration plate was incubated at 37°C overnight. After the removal of the nonmigrant cells/remaining media from the top of the insert membrane, cells that had migrated to the bottom side were dislodged with cell detachment buffer, subsequently lysed and detected by green fluorescent CyQuant dye. Data were recorded with a fluorescence plate reader using a 485/535 nm filter set. Each concentration was carried out in triplicate.

For the migration assay, the cell density used was 4×10^4 cells/well in 100 µl. For the invasion assay, the top migration chamber had to be prerehydrated with 100 µl of serum-free media. PC-3 cells were added to each well at a concentration of 1×10^5 cells in 100 µl.

In-vivo capillary-like tube formation assay

The assay for the endothelial cell capillary-like tube formation (Chemicon) was performed as described in the manufacturer's protocol. Seventy-five microliters of 2% serum-containing medium, in the presence or absence of the compounds, was added to each well and then 75 µl of HUVECs (1×10^5 cells/ml) was added. Plates were incubated for 8 h at 37°C. The tube formation was inspected and photographed.

In-vivo Matrigel plug assay

The in-vitro matrigel plug assay was performed as described previously [26]. Matrigel was mixed with basic fibroblast growth factor (bFGF) (150 ng/ml), in the presence or absence of the tested compounds, and injected subcutaneously into the ventral area of C57B1/6N female mice (National Cancer Institute, Frederick, Maryland, USA). Three mice were used for each group. The matrigel plugs were removed 10 days later, fixed and dissected. The slides were stained with Massion's trichrome, which stains the endothelial cells/vessels dark red and the matrigel blue. Photographs were taken at ×40 magnification. Total areas occupied by endothelial cells of three random fields were calculated using MetaVue software from Universal Imaging (Downingtown, Pennsylvania, USA) and data among the three replicate mouse plugs in each group were averaged.

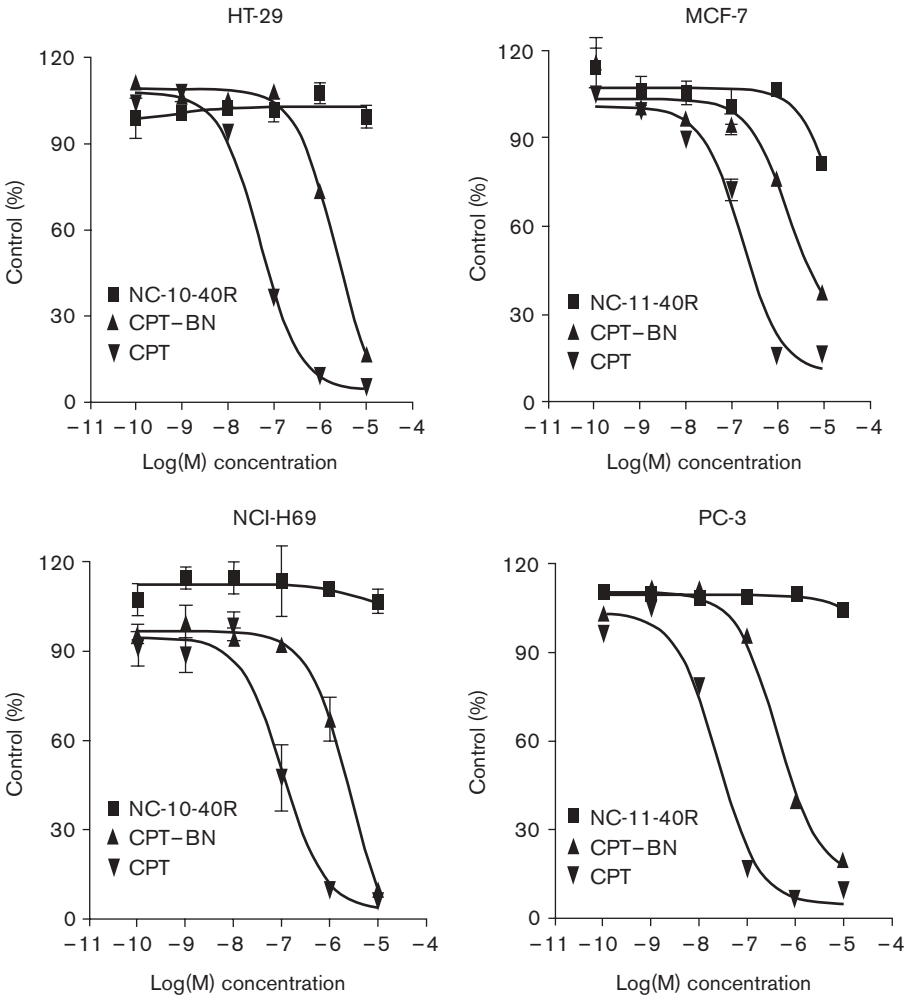
Results

Camptothecin–bombesin conjugate inhibits tumor cell proliferation and arrests cells at the G_1/G_0 phase

The CPT–BN conjugate DC-51-43, free CPT and free vector NC-11-40R were tested for their ability to inhibit cell growth in over 10 different human tumor cell lines including lung cancer, breast cancer, colon cancer, prostate cancer, small-cell lung cancer (SCLC), glioblastoma, gastric carcinoma, neuroblastoma and leukemia. CPT was a potent cytotoxin in all tested tumor cell lines, the IC_{50} values ranged from 1.12 to 219.40 nmol/l. Usually, in in-vitro experiments, CPT loses part of its cytotoxic activity after being conjugated to a peptide vector. IC_{50} values of the conjugate are almost 10-fold less than that of free CPT. For instance, IC_{50} values are 0.17 μmol/l (CPT) and 1.70 μmol/l (CPT–BN) to human breast cancer MCF-7 cells, 23.72 nmol/l (CPT) and 429.85 nmol/l (CPT–BN) to human prostate cancer PC-3 cells, and 0.11 μmol/l (CPT) and 2.71 μmol/l (CPT–BN) to SCLC NCI-H69 cells, respectively (Fig. 1). In most tested tumor cell lines, the CPT–BN conjugate showed the same trends as the free CPT, except for more difference in human colon cancer HT-29 (IC_{50}: 49 nmol/l to CPT, 2269 nmol/l to CPT–BN) (Fig. 1) and neuroblastoma SKNSH cells (29 nmol/l to CPT, 1610 nmol/l to CPT–BN). NC-11-40R (free BN peptide) had no or only weak antiproliferative activity, even at high concentrations (IC_{50} > 100 μmol/l) (Fig. 1).

Further results from the analysis of cell cycles demonstrated that the conjugate DC-51-43 at 1 μmol/l could significantly arrest cells at the G_1/G_0 phase. Free CPT arrested cells at a low concentration of 0.1 μmol/l, but the BN analog NC-11-40R itself did not arrest cells even at a

Fig. 1



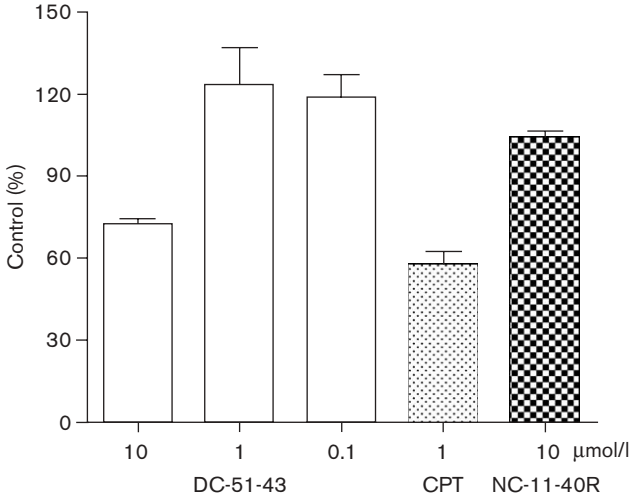
Cell proliferation assay. Four human cancer cell lines, including breast cancer cell MCF-7, small-cell lung cancer (SCLC) cell NCI-H69, colon cancer cell HT-29 and prostate cancer cell PC-3, were treated with free camptothecin (CPT), the camptothecin–bombesin (CPT–BN) conjugate DC-51-43 and the vector NC-11-40R as previously described [23].

Table 1 Effects of the CPT–BN conjugate DC-51-43 on cell cycles

Code	Name	Dose	G_1/G_0 phase (%)	S phase (%)	G_2/M phase (%)
DC-51-43	CPT–BN conjugate	1 μmol/l	67.35 ± 0.85	28.49 ± 3.50	4.16 ± 3.68
NC-11-40R	BN vector	1 μmol/l	42.63 ± 1.72	46.82 ± 1.53	10.55 ± 0.19
CPT	free CPT	0.1 μmol/l	75.94 ± 4.71	23.14 ± 4.26	0.91 ± 0.71
Control	DMSO	≤ 1%	41.56 ± 2.09	48.38 ± 2.07	9.49 ± 1.60

CPT–BN, camptothecin–bombesin; CPT, camptothecin; DMSO, dimethyl sulfoxide; BN, bombesin.

Fig. 2



Effects of the camptothecin–bombesin (CPT–BN) conjugate DC-51-43 on prostate PC-3 cell adhesion to collagen type I.

concentration of 1 μmol/l (Table 1). This supports the statement that DC-51-43's arresting activity is identical to its inhibition of cell growth. The cytotoxic activity depends on the CPT part of this CPT–BN conjugate, the BN peptide mainly working as a vector to target BN receptors. This has been demonstrated in our previous studies [7,22,27]. This conjugate has showed its antitumor activity in in-vivo experiments [22]. Our current studies are to assess its antiangiogenic ability, also by in-vitro and in-vivo experiments.

Camptothecin–bombesin conjugate inhibits cell adhesion, migration and invasion

Cell adhesion, migration and invasion are basic cellular actions related to tumor invasion, metastasis and angiogenesis. Human prostate cancer PC-3 cells have high specific affinity to BN receptors [28] and are highly invasive, thus, they were chosen as the in-vitro cell model. Pre-experiments showed that NC-11-40R had little or no inhibitory activity to cell adhesion, invasion or migration. Free CPT, however, strongly inhibited various extracellular matrix (ECM) components and integrins (cell surface receptors interacting with ECM components). For instance, CPT displayed significant inhibition to collagen I, collagen IV and vitronectin at the concentration of 1 μmol/l, to laminin and fibronectin at 2–10 μmol/l and to integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ at 1 μmol/l, but not to integrins $\beta_1/\alpha\beta_1$ at 1 μmol/l. Meanwhile, CPT demonstrated its ability against cell invasion and migration (data not shown). Herein, the conjugate DC-51-43 was tested to confirm its effects on these cell actions.

For cell adhesion, this CPT–BN conjugate was assayed for its ability to inhibit cells adhering to collagen type I. As shown in Fig. 2, DC-51-43 obviously inhibited human prostate cancer PC-3 cell adhesion at the concentration of 10 μmol/l. At the same concentration, NC-11-40R did not. Meanwhile, this conjugate displayed its inhibitory ability to cell adhesion to integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ at concentrations of 10 and 20 μmol/l, but not to $\beta_1/\alpha\beta_1$ (Fig. 3).

The results from both migration and invasion assays illustrated that the conjugate DC-51-43 could inhibit the highly invasive PC-3 cell's invasive and migration abilities at concentrations of 10 μmol/l or more (Fig. 4). No observations on this conjugate's inhibitory activities at lower concentration could be acquired.

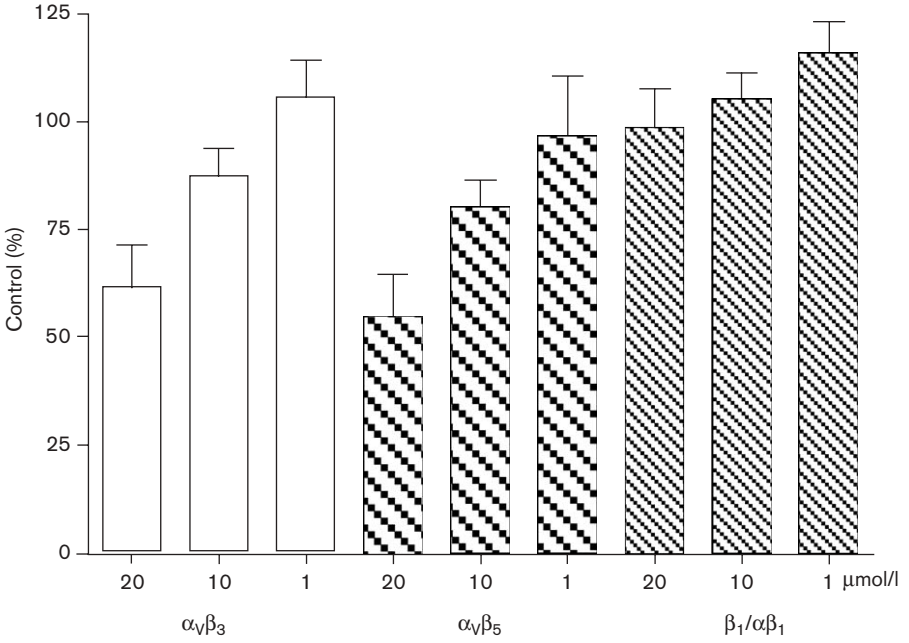
Camptothecin–bombesin conjugate inhibits capillary-like tube formation

Endothelial cells express a high density of BN receptors [29] and play a key role in angiogenesis and tumor metastasis. HUVECs plated on matrigel could differentiate and form capillary-like tube structures. This technique was used to assess the effects of compounds on in-vitro angiogenesis. The conjugate DC-51-43, at different concentrations, was tested for its ability to inhibit HUVEC differentiation *in vivo* in 96-well plates precovered with matrigel. Compared with controls, the conjugate inhibited tube-like formation at 40 μmol/l (Fig. 5), free CPT at 1 μmol/l, but the BN analog vector did not inhibit at any concentration (data not shown).

Inhibitory activity of camptothecin–bombesin conjugate on in-vivo angiogenesis

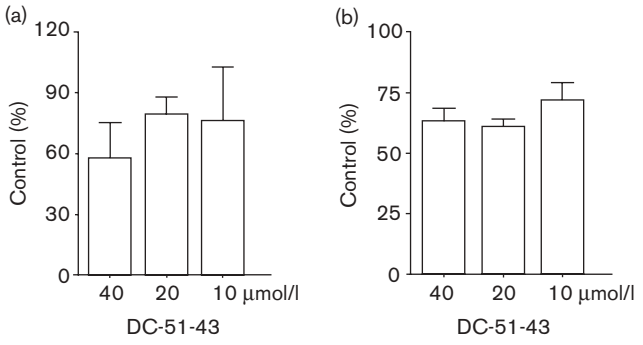
The conjugate DC-51-43 was mixed with cold liquid matrigel and bFGF, and injected subcutaneously into the mouse ventral area. Matrigel plugs were formed and taken out for further quantitative analysis of the DC-51-43's in-vivo antiangiogenic ability. The conjugate obviously inhibited the bFGF-induced endothelial cells of host mice from spreading into matrigel plugs (Fig. 6). The measurements of endothelial cells in the matrigel

Fig. 3



Effects of DC-51-43 on prostate PC-3 cell adhesion to integrins $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\beta_1/\alpha\beta_1$.

Fig. 4



Effects of the conjugate DC-51-43 on prostate PC-3 cell migration and invasion. (a) Cell invasion assay. (b) Cell migration assay.

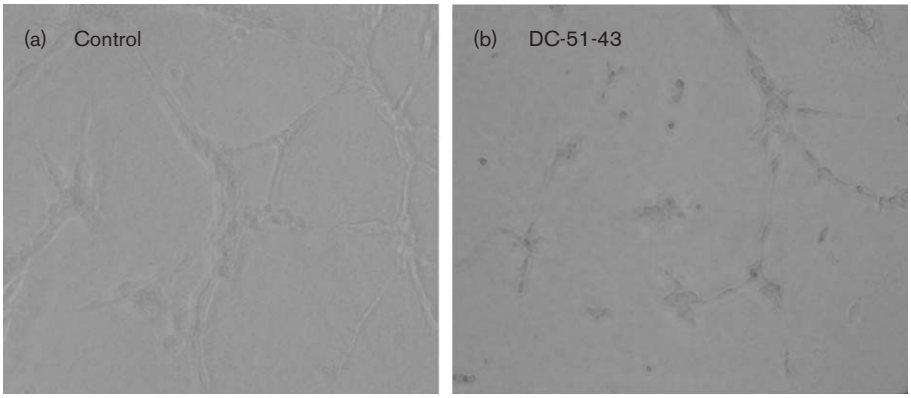
plugs confirmed the observation made under the microscope, and demonstrated that DC-51-43 at 10 and 20 μmol/l could result in a 76.86 and 82.54% reduction, respectively (Fig. 6). This supports DC-51-43's potential ability to block in-vivo angiogenesis.

Discussion

CPT and its analogs have potent antitumor activities [3,7,12]; however, their water-insoluble characteristics and toxic side effects limit their clinical application. To solve this problem, we coupled CPT to a short peptide BN analog vector consisting of 11 amino acids. This CPT–BN conjugate (DC-51-43) could join CPT to BN receptor-expressing target sites via ligand–receptor interaction and increase specific action and rapid internalization. CPT in the CPT–BN conjugate executes the cytotoxic function and the vector NC-11-40R acts as a CPT carrier to target BN receptor-expressing sites. This conjugate has been demonstrated to have high binding affinity to each of the BN receptors and have the same trends as the vector NC-11-40R [22]. Reportedly, BN receptors may be widely expressed in prostate carcinoma, breast carcinoma, gastrinoma, renal cell carcinoma, SCLC, colon cancer, glioblastoma and neuroblastoma [20,21,30,31]. Therefore, it is reasonable to assume that BN conjugates of CPT or other cytotoxic agents possibly could be specific tumor-targeted therapeutics. Usually, CPT will lose part of its in-vitro activity after being conjugated to a peptide vector (Fig. 1). DC-51-43 shows its potent antiproliferative activity in a variety of tumor cell lines. Furthermore, this conjugate was found to result in a significant arrest of cells at the G_0/G_1 phase of the cell cycle, supporting the reports that the conjugate's antiproliferative activity is to arrest the targeted cells, destroy cellular DNA and finally result in cell apoptosis.

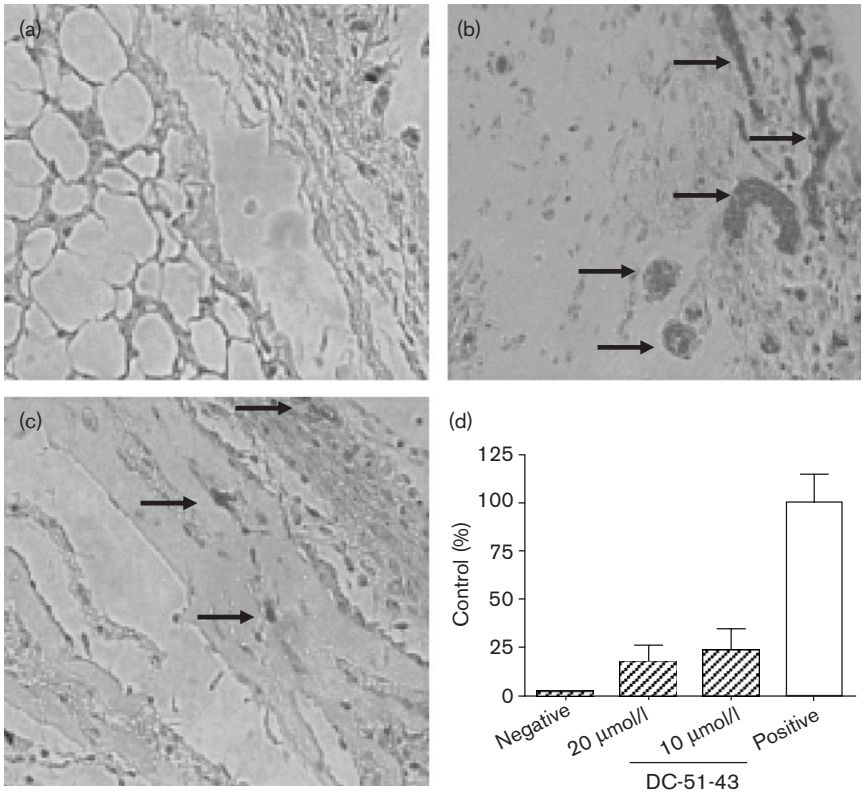
CPT and its analogs have been demonstrated to be antiangiogenic in in-vitro and in-vivo experiments.

Fig. 5



Effects of the conjugate DC-51-43 on in-vitro capillary-like tube formation of endothelial cells. (a) Control. (b) DC-51-43 (40 μmol/l).

Fig. 6



In vivo angiogenesis assay. Cold liquid matrigel, containing 150 ng/ml basic fibroblast growth factor, in the presence or absence of the conjugate DC-51-43 (10 μmol/l), was injected subcutaneously into the ventral area of female mice forming matrigel plugs into which the host endothelial cells could migrate. The matrigel plugs were removed, fixed and dissected for assessment of angiogenesis. (a) Negative control, (b) positive control, (c) DC-51-43 (10 μmol/l), (d) statistical graph. Arrows show endothelial cells.

In in-vitro studies, for instance, Nakashio *et al.* [4] reported that topotecan (a CPT analog) could inhibit VEGF-induced and bFGF-induced HUVEC migration. In other in-vivo studies, CPT analogs, 9-amino-20(*S*)-camptothecin, topotecan and CPT-11, reduced neoangiogenesis when tested both in the cornea of inbred

Swiss–Webster mice [5] and in the disc angiogenesis system, performed by implanting sponge discs subcutaneously in the rat dorsum [6]. Our series of experiments demonstrated that this conjugate and CPT itself could significantly block adhesion, invasion and migration of highly invasive PC-3 cells [32], and also that the conjugate and free CPT both inhibited the capillary-like tube formation of endothelial cells with highly expressed BN receptors [29] and the migration of endothelial cells into the matrigel plugs in mice. All these results suggested that CPT and its BN conjugate could inhibit angiogenesis.

In the previous studies, the conjugate DC-51-43 was found to inhibit potently growth of BN receptor-expressing prostate cancer PC-3 and SCLC NCI-H69 tumors grown in nude mice [22]. No loss of bodyweight was observed and no mice died, suggesting that the conjugate DC-51-43, at the tested dose and frequency of injection, had weak or no toxicity. Drug candidates could inhibit tumor growth either directly by binding to tumor cell surfaces, resulting in local cell apoptosis, and/or indirectly by targeting peritumoral vessels and blocking nutrients brought through the vessels to the tumor. No measurable BN receptors were found in the tumor tissue of pancreatic carcinomas; however, the receptors were detected clearly in peritumoral vessels surrounding exocrine pancreatic carcinomas [30,33]. In our past studies, the CPT–BN conjugate could inhibit pancreatic CFPAC tumor growth in nude mice. Tumor volume dramatically decreased [22]. This gives significant support that this conjugate might inhibit tumor growth directly and/or indirectly.

Taken together, this targeted drug delivery system is a very attractive approach that could reduce toxicity to normal tissues and increase the efficacy of drug treatment by allowing concentrated, localized effects on targeted sites. The CPT–BN conjugate clearly showed its inhibitory activities against angiogenesis and tumor growth. This system potentially could be developed to be a specific and effective cancer therapy by targeting BN receptor-expressing sites.

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