

The Camptothecin Derivative CPT-11 Inhibits Angiogenesis in a Dual-color Imageable Orthotopic Metastatic Nude Mouse Model of Human Colon Cancer

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Abstract. Recent studies have shown the expression of a stem cell marker protein, nestin, in nascent blood vessels in nestin-driven green fluorescent protein (ND-GFP) transgenic nude mice. In the present study, we visualized tumor angiogenesis and evaluated the antiangiogenic efficacy of CPT-11 in ND-GFP nude mice using dual-color fluorescence imaging. We orthotopically implanted ND-GFP nude mice with the human cancer cell line HCT-116 expressing red fluorescent protein (RFP). The mice were treated with CPT-11 at 40 mg/kg (10) on days 7, 10, 14. Tumor angiogenesis was imaged and visualized by dual-color fluorescence imaging on day 17, three days after the last CPT-11 treatment. Tumor volume and the mean nascent blood vessel density were determined and compared to the control mice. The growing tumor had high expressions of nestin in the nascent blood vessels. The nascent blood vessels showed co-localization of the endothelial-cell-specific marker CD-31 under immunohistochemical staining. The nascent blood vessels were highly visible and their density was determined. ND-GFP nude mice that were administered CPT-11 showed significant reduction in the mean nascent blood vessel density and tumor volume. The dual-color model of ND-GFP transgenic nude mice orthotopically implanted with HCT-116 expressing RFP proved to be effective in visualizing and quantitating tumor growth and tumor angiogenesis. The results showed that CPT-11 is an effective inhibitor of angiogenesis and provided strong implications for wider clinical application of CPT-11 for colon cancer.

Nascent blood vessels labeled with GFP can be observed in nestin-driven green fluorescent protein (ND-GFP) transgenic mice. Studies have shown the existence of GFP-labeled blood vessels in the interconnecting hair follicles on the skin. Amoh *et al.* (1) showed and confirmed the formation of new blood vessels from the ND-GFP labeled hair follicles that were transplanted into the skin of unlabeled nude mice suggesting that in the skin, hair follicles contain skin cells that can produce endothelial cells.

The ND-GFP is also capable of visualizing tumor angiogenesis as shown in many studies (2-5). The ND-GFP transgenic mice were orthotopically implanted with the murine melanoma cell line B16F10 expressing RFP. Using dual-color fluorescence imaging, progressive tumor angiogenesis expressing GFP was observed. Drug screening was applied to this model where doxorubicin was shown to simultaneously inhibit tumor growth and tumor angiogenesis (2).

The ND-GFP nude mouse was subsequently developed and showed expression of ND-GFP in the brain, spinal cord, heart, lung, esophagus, stomach, pancreas, blood vessels of glomeruli, blood vessels of skeletal muscle, testes, blood vessel network in the skin and hair follicles. Different human cancer cell lines including pancreatic cancer, lung cancer, and colon cancer cell lines, all expressing RFP were orthotopically implanted into the ND-GFP nude mice. Under dual-color fluorescence imaging the nascent blood vessels had high expressions of ND-GFP (3).

In the present study, the efficacy of CPT-11 as an angiogenic inhibitor was evaluated using the dual-color model. CPT-11 is a semi-synthetic derivative of camptothecin that has been used to treat colon cancer along with other gastrointestinal cancers. CPT-11 has been used in combination chemotherapy along with common chemotherapeutic drugs such as 5-FU and leucovorin for treating colon cancer. In this

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study, human colon cancer cell line HCT-116 expressing RFP was orthotopically transplanted into ND-GFP transgenic nude mice. The antiangiogenic and tumor-growth-suppressing activity of CPT-11 in colon cancer was visualized using dual-color fluorescence imaging.

Materials and Methods

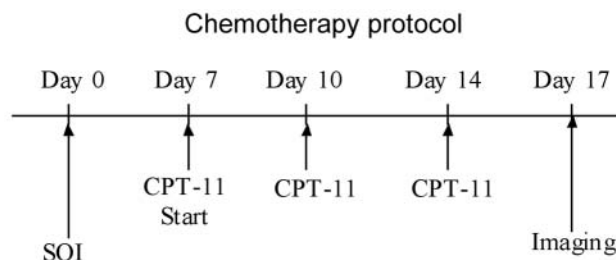
ND-GFP transgenic nude mice. ND-GFP transgenic C57/B6 mice (1, 6) carry GFP under the control of the nestin second-intron enhancer. In the present study, the ND-GFP gene was crossed into nude mice on the C57/B6 background to obtain ND-GFP nude mice (3).

RFP vector production. The RFP (DsRed-2) gene (Clontech, Palo Alto, CA, USA) was inserted in the retroviral-based mammalian expression vector pLNCX (Clontech) to form the pLNCX DsRed-2 vector (7). Production of retrovirus resulted from transfection of pLNCX DsRed-2 into PT67 packaging cells, which produce retroviral supernatants containing the DsRed-2 gene. Briefly, PT67 cells were grown as monolayers in DMEM supplemented with 10% FCS (Gemini Biological Products, Calabasas, CA, USA). Exponentially growing cells (in 10 cm dishes) were transfected with 10 µg expression vector using a LipofectAMINE Plus (Life Technologies, Grand Island, NY, USA) protocol. Transfected cells were replated 48 hours after transfection and 100 µg/mL G418 was added 7 hours after transfection. Two days later, the medium was changed to 200 µg/mL G418. After 25 days of drug selection, surviving colonies were visualized under fluorescence microscopy and RFP-positive colonies were isolated. Several clones were selected and expanded into cell lines after virus titering on the 3T3 cell line.

RFP gene transduction of tumor cell lines. For RFP gene transduction, 70% confluent HCT-116 human colon cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 or other culture media (Life Technologies) containing 10% fetal bovine serum (Gemini Biological Products) for 72 hours. Fresh medium was replenished at this time. Tumor cells were harvested with trypsin/EDTA and subcultured at a ratio of 1:15 in selective medium which contained 50 µg/mL G418. To select brightly fluorescent cells, the level of G418 was increased to 800 µg/mL in a stepwise manner. Clones expressing RFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, USA) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent.

Transplantation of tumor cells. ND-GFP transgenic nude mice, 6 to 8 weeks old, were used. The mice were anesthetized during the surgery with a ketamine mixture (10 µl of ketamine HCL, 7.6 µl of xylazine, 2.4 µl of acepromazine maleate and 10 µl of H₂O) via s.c. injection. Fifty microliters containing 2x10⁶ HCT-116-RFP cells per mouse were injected into the subcutaneous tissue in 6- to 8-week-old nude mice using a 0.5 mL 28 G latex-free syringe (TYCO Health Group LP, Mansfield, MA, USA). Tumor fragments (1 mm³), stably expressing RFP, were harvested from the resulting s.c. tumors in the nude mice, were implanted by surgical orthotopic implantation on the colon of the ND-GFP

Table I. CPT-11 treatment. The mice were treated with 40 mg/kg CPT-11 (i.p.) or NaCl solution (vehicle controls) at day 7, 10 and 14 after orthotopic implantation of the HCT-116 tumor. Tumor samples were excised and imaged at day 17 after implantation.



*CPT-11 was injected i.p. at 40 mg/kg

nude mice. After proper exposure of the colon through a lower-left abdominal incision, 8-0 surgical sutures were used to penetrate the tumor pieces and attach them under the serosa of the ascending colon (8). The incision in the abdominal wall was closed with a 6-0 surgical suture in one layer.

Measurement of length of nestin-positive nascent blood vessels and evaluation of antiangiogenic efficacy of CPT-11. The mice were treated with 40 mg/kg CPT-11 i.p. (Pharmacia & Upjohn Company, Kalamazoo, MI, USA) or NaCl solution (vehicle controls) at day 7, 10 and 14 after s.c. implantation of tumor cells (Table I). Tumor samples were excised under anesthesia at day 17 after implantation. Past studies showed that tumor angiogenesis in ND-GFP nude mice starts to appear by day 7 and the blood vessel density increases until at least 21 days after implantation (2). At the end of experiment, the mice were euthanized. The tumors visible with the naked eye were surgically removed. The tumors were measured in three dimensions with calipers. Tumor volume (mm³) was calculated with the formula $V = 0.5 \times \text{length} \times \text{width} \times \text{height}$. Tumor samples were divided into two parts, one for fluorescence microscopy and the other for frozen sections. Angiogenesis was quantified in the tumor mass by measuring the total length of ND-GFP-expressing nascent blood vessels under fluorescence microscopy in both CPT-11-treated and control mice. Tumors were flattened on a glass slide to enable the 3-dimensional vascular structure to be seen in a flattened field. The length was measured by eye with a ruler. Tumor vessel density at day 17 was calculated by the total length of nestin-positive nascent blood vessels divided by the tumor volume (mm³). Each experimental group consisted of three mice.

fluorescence imaging in live mice. The Olympus OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), was used for imaging in live mice (9). High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell^R (Olympus Biosystems).

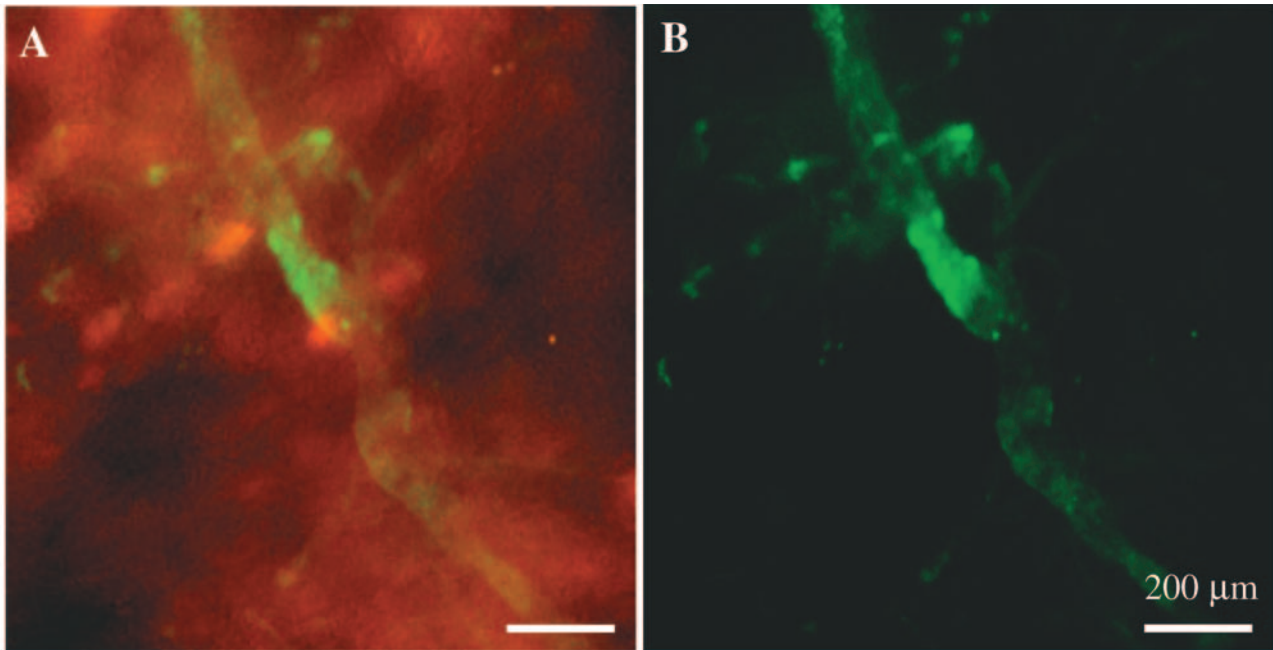


Figure 1. Angiogenesis of HCT-116 human colon cancer. Imaging was carried out at day 17 after surgical orthotopic implantation of RFP-expressing HCT-116 human colon cancer into the colon of ND-GFP transgenic nude mice. A) ND-GFP-expressing blood vessels are seen in the RFP-expressing tumor. B) Same as Figure A but showing only GFP fluorescence. Bars: 200 μ m.

Immunohistochemical staining with CD31 as an endothelial cell marker. Tissue was embedded in tissue-freezing embedding medium and frozen at -80°C overnight. Frozen sections 10 μ m thick were cut with a Leica CM1850 cryostat and were air-dried. Co-localization of ND-GFP fluorescence and CD31 in the frozen skin sections of the nestin-GFP transgenic mice were detected with the anti-rat immunoglobulin horseradish peroxidase detection kit (BD PharMingen, San Diego, CA, USA; CD31) following the manufacturer's instructions. The primary antibody used was CD31 monoclonal antibody (1:50). Substrate-chromogen 3,3'-diaminobenzidine staining was used for antigen staining. Anti-CD31 monoclonal antibody (CBL1337) was purchased from Chemicon (Temecula, CA, USA).

Statistical analysis. The experimental data are expressed as the mean \pm SD. Statistical analysis was performed using the two-tailed Student's *t*-test.

Results

Tumor angiogenesis visualized by ND-GFP. At day 17 after implantation of the RFP-expressing HCT-116 human colon cancer into the colon of ND-GFP transgenic nude mice, tumors were excised and flattened on a glass slide. The vascular structures were not disrupted by this process. This technique enabled imaging of angiogenesis throughout the tumor. Newly formed ND-GFP-expressing blood vessels were observed growing into the RFP-expressing tumor mass (Figure 1).

Immunohistochemical staining showed that CD31 co-localized in the blood vessels in the growing tumor. Frozen sections showing the ND-GFP blood vessels and RFP-expressing HCT-116 colon cancer under fluorescence microscopy were compared with sister sections stained for CD31, demonstrating co-localization of ND-GFP and CD31 (Figure 2).

Effects of CPT-11 on tumor growth and angiogenesis. Mice were given *i.p.* injections of 40 mg/kg of CPT-11 at days 7, 10 and 14 after implantation of the HCT-116 human colon cancer cells. This protocol was used in order to minimize CPT-11 toxicity. At day 17 after implantation, the number of ND-GFP-expressing blood vessels was significantly reduced in the CPT-11-treated animals compared to that in NaCl-injected control mice (Figure 3). Treatment with CPT-11 significantly decreased tumor volume as well as nascent blood vessel formation. Mean nascent blood vessel length per tumor volume was also decreased (Figures 4 and 5; $*p < 0.05$ versus NaCl solution-injected mice).

These results show the utility of the dual-color ND-GFP nude mouse-RFP colon cancer model to visualize and quantitate angiogenesis. The dual-color model described here should be very useful to screen and evaluate potential new angiogenesis inhibitors that inhibit colon cancer.

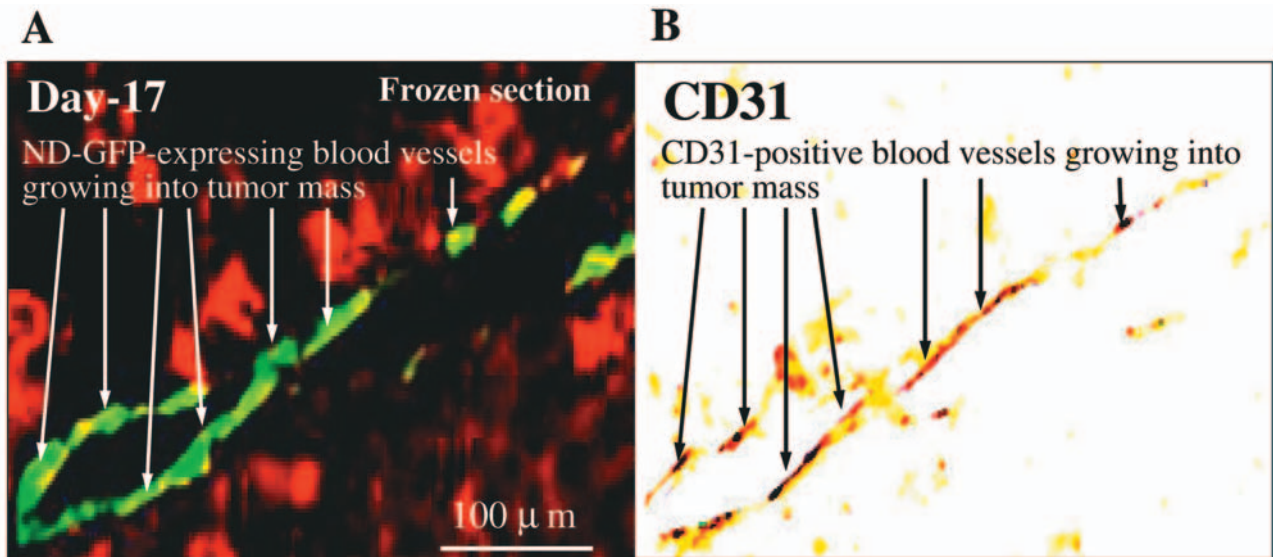


Figure 2. Immunohistochemical staining of CD31. A) ND-GFP is visualized in blood vessels in the HCT-116 tumor in a frozen section. B) Immunohistochemical staining in a sister frozen section shows CD31 co-localizing with ND-GFP in blood vessels in the growing tumor. Bar: 100 μ m.

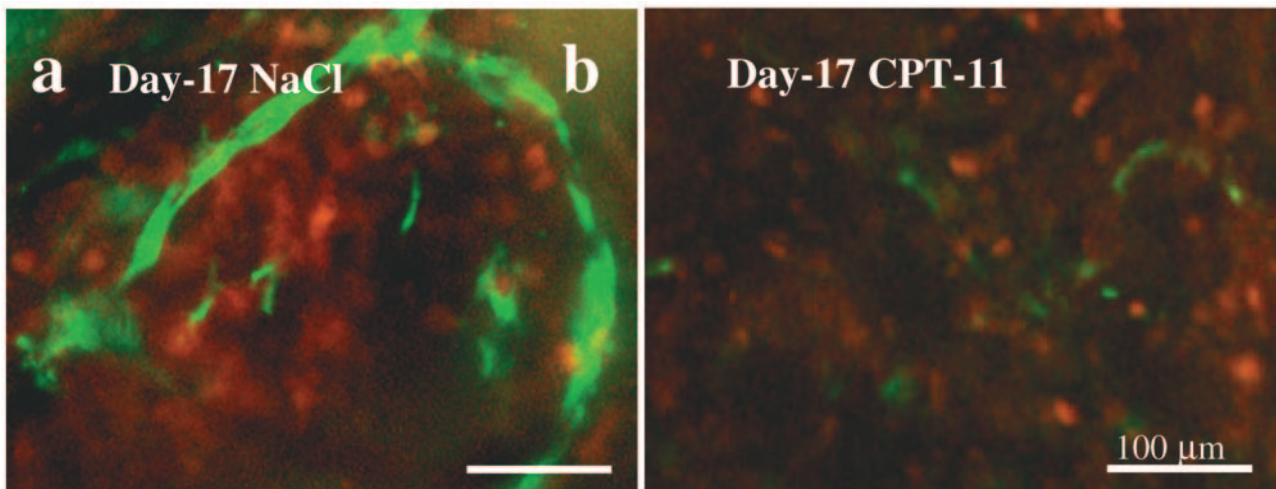


Figure 3. Efficacy of CPT-11 on angiogenesis of the HCT-116 human colon tumor in ND-GFP nude mice. A) Control HCT-116 human colon cancer cells in ND-GFP transgenic nude mice. On day 17 after tumor implantation, the ND-GFP-expressing nascent blood vessels are seen in the tumor mass. B) The mice were given i.p. injections of CPT-116 at day 7, 10, and 14 after tumor implantation. On day 17 after tumor implantation, the ND-GFP-expressing nascent blood vessels were decreased relative to the control.

Discussion

The formation of blood vessels plays a crucial role in tumor development. These blood vessels nourish the tumor with oxygen and nutrients essential for growth and eventually for metastatic activity. Previously, immunohistochemical staining of tumor tissue using various endothelial cell-specific antibodies was performed to determine angiogenesis. In our present study, we

visualized proliferating endothelial cells expressing ND-GFP simultaneously with the tumor expressing RFP using dual-color fluorescence imaging. The dual-color model has proven to be a powerful tool in visualizing the interaction of nascent tumor angiogenesis with the growing tumor.

Allegrini *et al.* (10) showed CPT-11 significantly inhibiting the growth of human colon tumor xenograft transplanted in mice. CPT-11 at 100-300 mg/kg/dose was

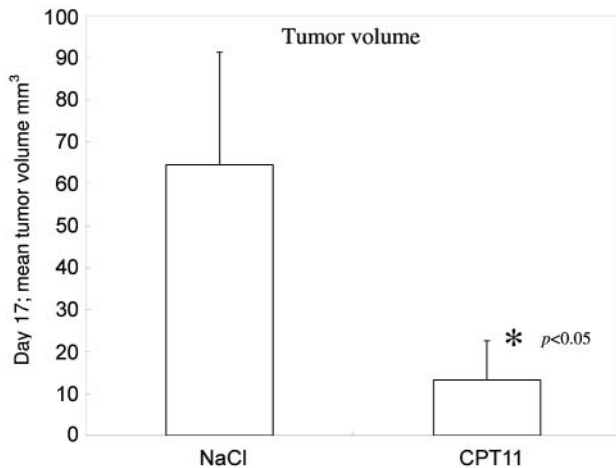


Figure 4. Efficacy of CPT-11 on tumor growth. At day 17 after tumor implantation, CPT-11 significantly decreased the mean tumor growth. * $p < 0.05$ vs. NaCl solution-injected mice. Bars: 100 μ m.

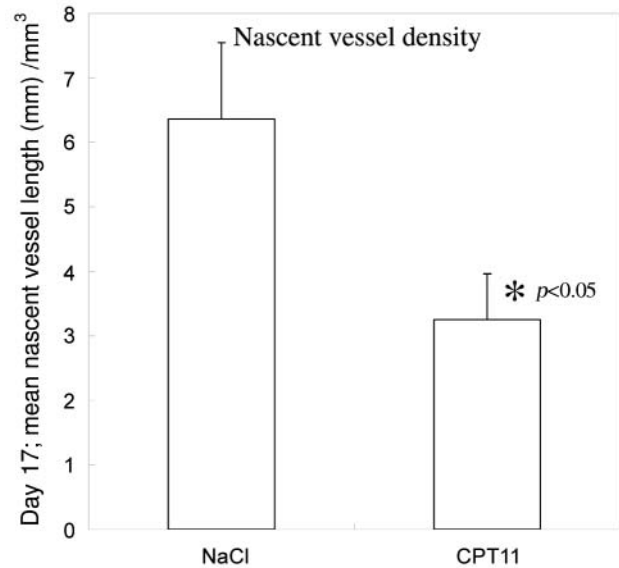


Figure 5. Efficacy of CPT-11 on angiogenesis. At day 17 after tumor implantation, CPT-11 significantly decreased the mean nascent blood vessel length per tumor area. * $p < 0.05$ vs. NaCl injected mice.

injected once per week for four consecutive weeks. Bellone *et al.* (11) mentioned in his investigation that results from his preliminary study on the reduction of vascular endothelial growth factors with different chemotherapeutic regimes has provided a rationale for exploring whether continuous or frequent administration of anti-neoplastic agents can allow it to also exhibit anti-angiogenic activity, a regimen known as metronomic therapy (12). In studies done by O'Leary *et al.* (13), CPT-11 exhibited anti-angiogenic activity. Angiogenesis was induced by basic fibroblast growth factor in the cornea. CPT-11 showed significant reduction of neoangiogenesis.

In the present study, we demonstrated the antiangiogenic effect of CPT-11 in an orthotopic metastatic human colon cancer cell line HCT-116 in ND-GFP nude mice. The ND-GFP transgenic nude mice and dual-color fluorescence imaging will allow investigators to simultaneously visualize *in vivo* the behavior of angiogenesis and tumor growth and progression. This model will allow rapid anti-angiogenic drug screening.

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