

## RESEARCH COMMUNICATION

# Norcantharidin Anti-Angiogenesis Activity Possibly through an Endothelial Cell Pathway in Human Colorectal Cancer

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### Abstract

The present study was based on the unexpected discovery that norcantharidin exerted anti-angiogenesis activity when effects on growth of human colon cancer were studied. The aim was to further verify this finding and explore possible mechanisms using a tumor xenograft model in nude mice. We confirmed that norcantharidin (5 or 15 mg/kg) could inhibit angiogenesis of human colon cancer in vivo. In vitro, crossing river assay, cell adhesion assay and tube formation assay indicated that NCTD could reduce the migration, adhesion and vascular network tube formation ability of HUVECs. At the same time, the expression levels of VEGF and VEGFR-2 proteins which play important roles in angiogenesis were reduced as examined by western blotting analysis. Taken together, the results firstly showed NCTD could inhibit angiogenesis of human colon cancer in vivo, probably associated with effects on migration, adhesion and vascular network tube formation of HUVECs and expression levels of VEGF and VEGFR-2 proteins.

**Keywords:** Human colorectal cancer - norcantharidin - angiogenesis - vascular endothelial growth factor

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### Introduction

Norcantharidin (NCTD) is a demethylated analogue of cantharidin which is an active ingredient of Chinese medicine-Mylabris. It has been used as an anticancer drug for the treatment of colon cancer, primary hepatoma, carcinomas of esophagus and breast cancer, leukopenia in China for many years (Wang, 1989; Fang et al., 1993). Previous studies have shown that NCTD could suppress the invasion and metastasis of colorectal adenocarcinoma CT26 cells in vitro and in vivo models. In addition, NCTD could mediate a Fas-dependent apoptotic cell death in colon cancer cells such as CT-26, HT-29 (Chen et al., 2008; Peng et al., 2009). However, there is an unexpected discovery that micro-vessels was less in the group treated with NCTD than the control group when we studied the effect of NCTD on HCT116 cell xenografts in nude mice. Hence, we suppose whether NCTD could inhibit angiogenesis of human colorectal cancer, which maybe a new mechanism for anticancer effect of NCTD.

Angiogenesis is a hallmark of tumor in order to supply the tumor with its metabolic requirements (Hanahan and Weinberg, 2000; Heath and Bicknell, 2009). Mechanistically, angiogenesis is a proliferation of blood vessel networks from the pre-existing vasculature (Pang and Poon, 2006) and penetrates into cancerous tissue, plays an essential role in carcinogenesis, cancer

progression, and metastasis (Carmeliet, 2005; Kitadai, 2010; Svagzdys et al., 2009). This complex process involves several key steps such as activation, proliferation, migration and adhesion of endothelial cells, assembly of endothelial cells into new capillary tubes, followed by synthesis of a new basement membrane and surrounding extracellular matrix, and finally maturation of vessels (Des Guetz et al., 2006). Therefore, endothelial cells play important roles in angiogenesis. Any of these steps can be a potential target to inhibit angiogenesis and, hence, to treat cancer and other angiogenesis-dependent disease (Quesada et al., 2006).

Herein, basing on the exiting facts that NCTD affects angiogenesis of colon cancer, in this study, we designed these series of experiments to further confirm this phenomenon and to explore the possible mechanism. Our results proved that NCTD could inhibit angiogenesis of human colorectal cancer in vivo and vitro, which was associated with inhibition of proliferation, migration, adhesion and tube-formation. Also, NCTD down-regulated the expression of VEGF/VEGFR-2 level in endothelial cells.

### Materials and Methods

#### Drugs

NCTD was purchased from Ronghe Medical

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Technology Development Limited Company (Shanghai, China) and dissolved in hot-water(70°C) at the concentration of 1 M. Then it was diluted to the desired concentration (7.5/15/30 μM) with RPMI 1640.

#### Cell lines and cell Culture

There are several human colorectal cancer cell lines, of which HCT-116 is widely used in laboratory cancer research (Mohr and Illmer, 2005). The HCT116 human colon cancer cell line and human umbilical vein endothelial cells (HUVECs) were purchased from Shanghai Institute of Biochemistry and Cell Biology (China). HCT116s was cultured in RPMI 1640 and HUVECs in DMEM (GIBCO Company) supplemented with 10% fetal bovine serum, 100 U penicillin, 0.1 μg streptomycin, 200 mmol/L HEPES and 2 mmol/L L-glutamine, which were maintained in a human atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C.

#### Adhesion assay

At first, 96-well plates were coated with collagen I (5 μg/cm<sup>2</sup>). HUVECs exposed to different concentrations of NCTD for 24 hours were seeded at a density of 1x 10<sup>3</sup>/well and then incubated for 20 minutes. Three duplicate wells were set up for each group. And then, non-adherent cells were washed away with PBS and 20 μl MTT (Sigma-Aldrich, St. Louis, MO) solution was added to each well respectively, the plates were further incubated for 4 hours. The formed crystals were dissolved in 200 μl dimethyl sulfoxide. The absorbance was measured with a microplate reader (Bio-Rad, Hercules, CA) at 490 nm. The rate of adhering was calculated as this: rate of adhering = [(A of treated cells – A of background) / (A of control cells – A of background)] x 100%.

#### Migration assay

HUVECs (1x10<sup>5</sup>) exposed to different concentrations of NCTD for 24 hours were plated in 24-well plates coated with collagen I (5 μg/cm<sup>2</sup>). Three duplicate wells were set up for each group. And then the plate was incubated for 24 hours until cells grew to confluence. The monolayer was wounded by scratching with a sterile pipette tip lengthwise along the chamber. After wounding, cells were washed twice with PBS and cultured at 37°C for 24 hours. Images were captured twice at 0 and 24 hours after cell wounding. The width of the wound area was measured by Image J to determine cell migration distance. Relative Migration rate = (Distance t = 0 h – Distance t = 24 h) / Distance t = 0 h x 100%.

#### Tube-formation assay

HUVECs (1x10<sup>5</sup>) exposed to different concentrations of NCTD for 24 hours were plated in 96-well plates coated with 50 μl Matrigel (BD Biosciences) and incubated for 8 hours. Tube formation was inspected and photographed using the Olympus digital camera.

#### Western Blot Analysis

At the end of NCTD treatment, cells were rinsed twice with ice-cold PBS and then lysed with ice-cold lysis buffer (50 mM of Tris-HCl, pH7.5, 150 mM of NaCl, 0.5% NP-40, 1 mM of EDTA, 0.2 mM of PMSF,

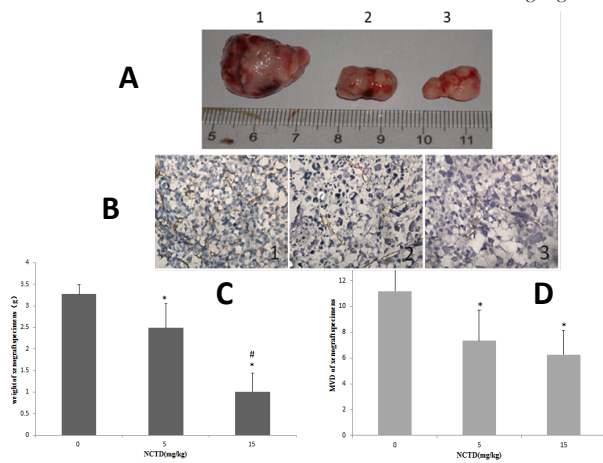
100 μl/ml of proteinase inhibitor Aprotinin) for 30 minutes. The cell lysates were centrifuged at 12,000 g for 5 minutes at 4°C, and the supernatant which contained total protein was collected and stored at -80°C until use. The protein concentration was determined by using a microbicinchoninic acid protein assay (BCA, Byotime, China) and equal amounts of protein were mixed with SDS sample buffer (Byotime, China) and boiled for 5 minutes. The samples were loaded onto SDS-PAGE for electrophoresis. The separated proteins were transblotted onto PVDF membrane (Millipore), and then the membrane was blocked in 5% non-fat dried milk for 2 hours, rinsed with TBST (TBS containing 0.01% Tween 20) and then incubated with antibody to human VEGF and VEGF-2 (R&D Systems) overnight at room temperature. The next day, excess antibody was removed by washing the membranes in TBST for 3 x 10 minutes and membranes were incubated 2 hours with HRP-conjugated secondary antibodies at room temperature. After being washed in TBST as above, bands were visualized by an enhanced chemiluminescence (ECL, Millipore) system and exposed to radiography film (Koda).

#### Animal models and immunohistochemistry

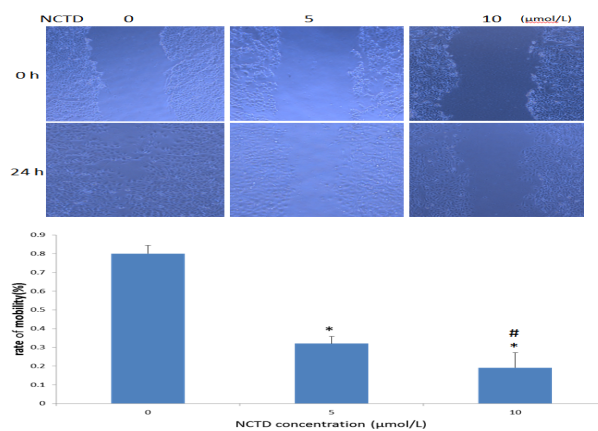
All animal experiments were approved by the local animal ethics committee. All experiments were performed in accordance with the official recommendations of the Chinese Community Guidelines. Six-week-old male Balb/c nu/nu mice were purchased from SINO-BRITISH SIPPR/BK LAB. ANIMAL LTD., CO (Shanghai, China). Human colon cancer HCT116 cells were used to establish the xenografts, which were resuspended at a density of 1 x 10<sup>7</sup>/ml. The suspension (0.1ml/10 g body weight) was injected subcutaneously into the nude mice. After 10 days, tumor nodules were palpable. Then the mice were randomly assigned three groups: one control group (n = 12), injected intraperitoneally with 0.9% NaCl twice a week; two NCTD groups (n=12), injected intraperitoneally with NCTD 5 or 15mg/kg twice a week respectively. The treatments were kept for 30 days. At the end, mice were sacrificed by cervical decapitation and the tumors were removed, weighed and fixed in 10% neutral buffered formalin and paraffin-embedded. Paraffin-embedded specimens were cut into serial 5-μm sections. Antigen retrieval was performed by microwaving in citrate buffer. The immunohistochemistry was conducted with monoclonal rabbit antibodies to the endothelium marker CD31 (1:50 dilution, Cell Signaling Technology, USA) and biotinylated anti-rat secondary antibody (RD). DAB chromogen was used subsequently and all of the sections were counter-stained with hematoxylin, dehydrated and mounted at last. Images were obtained by using an Olympus microscope, equipped with a camera system, and images were captured by using the Olympus microscope software package.

#### Statistics analysis

All data were described as mean ± SEM. Statistics analysis were performed using software from SPSS for Windows 15.0 (SPSS Inc., Chicago, IL, USA). To analyze the data statistically, unpaired Student's t-test were used.



**Figure 1. Effect of NCTD on tumor growth and micro-vessel in vivo.** \*VS 0 mg/kg group,  $p < 0.05$ ; #VS 5 mg/kg group,  $p < 0.05$ ; 1: control group; 2: NCTD(5mg/kg); 3: NCTD(15mg/kg)



**Figure 2. The Effects of NCTD on Migration Capacity of HUVECs Using Crossing River Assay (x40).** \*: VS 0 μmol/L group,  $p < 0.001$ , #: VS 5 μmol/L group,  $p < 0.05$

Calculated levels of significance were  $p < 0.05$ .

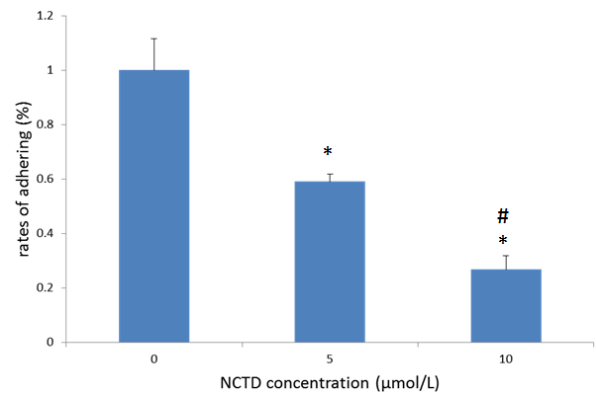
## Results

### Xenograft tumor growth and micro-vessels

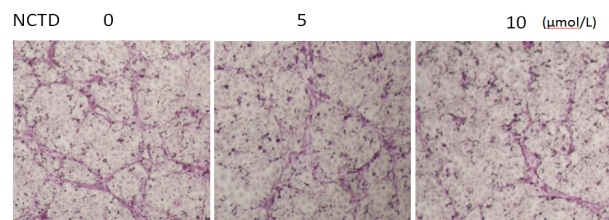
To investigate the effect of NCTD on xenograft tumor growth and micro-vessels, we performed an experiment in Balb/c nude mice bearing HCT116 tumors treated with intraperitoneal injection of NCTD. The results demonstrated that tumor size of control group was larger than NCTD intervention group at a dose of 5mg/kg or 15 mg/kg (Figure 1A), and the tumor weight was as shown in Figure 1C. The immunohistochemical staining of CD31 antigen showed that there were micro-vessels around tumor cells in the tumor xenograft (the brown parts in Figure 1B). A significant reduction of the average micro-vessels area in tumor treated with NCTD was shown in Figure 1D. Collectively, these findings support the role of NCTD in inhibiting tumor vessels.

### Migration, adhesion and tube formation of HUVECs

Cell migration and adhesion are two key steps in both angiogenesis and tumor progression. To detect the effect of NCTD on migration and adhesion of HUVECs, we used wound healing assay and adhesion assay as



**Figure 3. The Effects of NCTD on Adhesive Ability of HUVECs.** \*VS 0 μmol/L group,  $p < 0.01$ ; #VS 5 μmol/L group,  $p < 0.05$



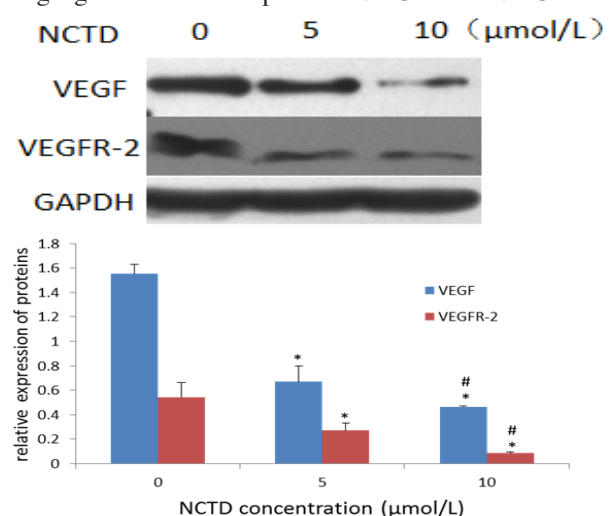
**Figure 4. The Effects of NCTD on Network Tube Formation Ability of HUVECs in Vitro (x40)**

described above. The results were shown in Figure 2 and 3 respectively. There were significant differences between the control and two NCTD-treated groups.

The formation of network tubes by endothelial cells is the final events during angiogenesis. Tube-formation assay was used to observe the effect of NCTD on tube formation of HUVECs. The results were shown in Figure 4. In vitro, HUVECs plated on matrigel formed networks in control group. However, after being treated with NCTD at two concentrations without affecting their viability, the networks reduced significantly.

### Expression of VEGF and VEGFR-2

Since the results above, the expression of the angiogenesis-related protein VEGF and VEGFR-2



**Figure 5. The Effects of NCTD on Expression Levels of VEGF, VEGFR-2 Proteins in HUVECs Detected by Western Blotting.** \*: VS 0 μmol/L group,  $p < 0.01$  #: VS 5 μmol/L group,  $p < 0.05$



in HUVECs after 48 hours exposure to different concentrations of NCTD with western blot analysis were further examined. The results (see Figure 5) showed that 5 and 10  $\mu\text{M}$  NCTD could significantly inhibit VEGF and VEGFR-2 protein expression ( $P < 0.05$ ). The level in control group was almost two and four times more than the 5 and 10  $\mu\text{M}$  treated groups respectively.

## Discussion

Based on the previous literature, there were some reports about the cytotoxicity of NCTD on HUVECs, and the inhibition of angiogenesis of breast cancer and gallbladder cancer in Chinese. However, most investigations performed which were focused on the cytotoxicity on cancer cells was not comprehensive. In our study, we newly discovered that NCTD could inhibit tumor growth, and angiogenesis of human colorectal cancer in nude mice model as shown in Figure 1. Because intratumoral vasculature density is believed to be associated directly with cancer cell entrance into the systemic blood circulation, with the ability of cancer cells to invade locally normal anatomic structures, and the establishment of blood-borne metastases in distant organs (De Vita et al., 2004), microvessel density (MVD) is most commonly used to quantify intratumoral and peritumoral angiogenesis in cancer (Pang and Poon, 2006; Rodrigo et al., 2009). Oftenly, MV is marked by pan-endothelial immunohistochemical staining, mainly with Factor VIII related antigen (F. VIII Ag or von Willebrand's factor), CD31 or CD34, and rarely CD105 (Des Guetz et al., 2006). So MVD was used to reflect angiogenesis through immunohistochemical staining with CD31. The decrease of MVD reflects less angiogenesis and vessels which possibly leads to tumor necrosis and growth inhibition (Graziano and Cascinu, 2003). It's well known that angiogenesis is important in carcinogenesis, cancer progression, and metastasis (Carmeliet, 2005; Svagzdys et al., 2009; Kitadai, 2010). It was for sure that anti-angiogenesis plays an important role in anti-tumor effect of NCTD.

But how did NCTD perform the effect of anti-angiogenesis? It was confirmed that endothelial cells were the main players in angiogenesis which is regulated by many proangiogenic and anti-angiogenic factors (Tanigawa et al., 1997). Enlightened by many other papers, we firstly thought of endothelial cells pathway. Herein, we made a hypothesis routinely that NCTD might affect the proliferation, migration, adhesion and tube formation abilities of endothelial cells and some protein factors in which. In the pre-experiment, we studied the cytotoxicity of NCTD on HUVECs in vitro and found that NCTD had strong cytotoxic effects on HUVECs ( $\text{IC}_{50}=37.41\mu\text{M}$ ). And in this study, we observed that NCTD at the concentration of 10  $\mu\text{M}$  suppressed migration, adhesion and tube formation of HUVECs, more obviously than 5  $\mu\text{M}$ , compared with the control group (Figure 2, 3 and 4).

In addition, tumor angiogenesis is the result of imbalance of a variety of proangiogenic and anti-angiogenic factors (Kerbel, 2008). The vascular endothelial growth factor family VEGF-A (often VEGF only),

VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) are the most studied angiogenic pathways (Folkman, 1995; Veikkola and Alitalo, 1999). Among these factors, VEGF is the most important angiogenesis stimulating factor and a negative regulator of the function of pericytes and maturation of blood vessels (Carmeliet, 2005; Greenberg et al., 2008). VEGF can maintain survival as well as induce proliferation and migration of endothelial cells, recruit bone marrow derived hematopoietic progenitor or stem cells, and increase vascular permeability (Jain et al., 2006). However, the biologic activities of VEGF must be mediated by two tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1, Flt-1) and VEGFR-2 (KDR) (Ferrara, 2005; Veikkola and Alitalo, 1999). Numerous studies have demonstrated that VEGF/VEGFR binding encourages receptor dimerization, leads to receptor autophosphorylation, and subsequently activates downstream angiogenic and growth pathways like cellular proliferation, vascular differentiation, altered vascular permeability, and migration (Gille et al., 2001). And then, VEGF signaling in angiogenesis is mainly mediated through VEGFR-2 (Shibuya and Claesson-Welsh, 2006), the role of VEGFR-1 in angiogenesis remains to be defined (Cao, 2009). The activation of VEGFR-2 on endothelial cells results in their proliferation, migration, and increased survival and promotes vascular permeability. So VEGF/VEGFR-2 is recognized as the most important pathway in angiogenesis. We detected the VEGF and VEGFR-2 protein level with western blot analysis. The results shown in Figure 5 revealed that NCTD inhibits the expression of VEGF and VEGFR-2 protein which was dose-dependent.

In summary, the results of the present study proved that NCTD may exert anti-angiogenic activity. That's probably because NCTD had an effect on migration, adhesion, tube formation and stimulatory factors of endothelial cells. This might be one aspect of anti-tumor mechanisms. Inhibition of angiogenesis has become a pathophysiological protective mechanism against cancer (Karamysheva, 2008). The potential anti-angiogenic effect of NCTD observed in the present study may enrich the pharmacological mechanism of NCTD and contribute to expand the clinical application.

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