

Inhibitory effect of norcantharidin on melanoma tumor growth and vasculogenic mimicry by suppressing MMP-2 expression

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Abstract. A form of microcirculation called vasculogenic mimicry (VM), which constitutes a novel approach for tumor blood supply in certain highly aggressive malignant tumors, was recently reported to contribute to tumor metastasis and poor prognosis in melanoma patients. Development of strategies to target tumor VM may be significant to reduce the recurrence and metastasis of melanoma. Norcantharidin (NCTD) has been shown to inhibit tumor growth and VM of human gallbladder carcinomas. Besides, NCTD could induce melanoma cell apoptosis. However, whether NCTD can inhibit the growth and VM formation of melanoma has not been evaluated. The present study aims to investigate the anti-VM activity of NCTD as a VM inhibitor for melanoma and its potential mechanisms. The anti-VM activity of NCTD was determined in human melanoma A375 cells and xenografts *in vitro* and *in vivo*. The findings indicate that NCTD inhibits tumor growth and VM formation of melanoma both *in vitro* and *in vivo* by suppressing matrix metalloproteinase-2 expression. The results suggest that NCTD is a potential therapeutic agent targeting VM in melanoma.

Introduction

Melanoma is the least common but most serious form of skin cancer (1). Rich blood supply affects the growth and metastasis of melanoma (2). A unique form of microcirculation called vasculogenic mimicry (VM), which is composed strictly of tumor cells without endothelial cells and efficiently supplies blood to tumor cells, has been reported (3). VM is associated with poor prognosis for patients with certain

aggressive malignant tumors, including melanoma (4), hepatocellular carcinoma (5) and breast cancer (6). It was reported that the expression and secretion of matrix metalloproteinase (MMP)-2 is important in VM formation (7). The sole application of angiogenic inhibitors proved to be ineffective on VM due to the different molecular mechanisms that exist in endothelium-dependent angiogenesis and VM (8,9). Thus, it is important to develop new angiogenic inhibitors that target tumor VM or to combine anti-VM drugs with conventional chemotherapies. Traditional Chinese medicines are reported to have multifunctional antitumor activities (10).

Norcantharidin (NCTD), a demethylated analog of cantharidin, is a 7-oxabicyclo heptane-2,3-dicarboxylic acid derivative isolated from natural blister beetles that has antitumor properties in a variety of tumors such as primary hepatocellular (11) and bladder cancer (12), and the mechanism of NCTD against bladder cancer may be attributable to its anti-VM activity. NCTD also induces cell apoptosis in melanoma *in vitro* (13). However, whether NCTD can inhibit the tumor growth of melanoma *in vivo* and its underlying mechanisms remain unclear.

The current study aims to investigate the antitumor activity of NCTD as a VM inhibitor for human melanoma and its underlying mechanisms. The results indicate that NCTD inhibits tumor growth and VM of human melanoma by suppressing MMP-2 expression *in vitro* and *in vivo*. Thus, the present study reveals that NCTD may be a potential anti-VM agent for human melanoma.

Materials and methods

Cell culture. The A375 human melanoma cell line was obtained from the Cell Resource Center (Beijing, China) and was maintained in RPMI 1640 medium (Beijing Neuronbc Laboratories, Co. Ltd., Beijing, China) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) in an incubator with high-efficiency particulate arrestance class 100 filter (Forma™ Series II; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO₂.

Invasion assays. Transwell membranes (8-μm pore size; Costar; Corning, Life Sciences, Cambridge, UK) were coated with Matrigel (1 mg/ml; BD Biosciences, Franklin Lakes, NJ, USA)

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prior to cell passage. A375 cells (1×10^5) were seeded into the upper wells in RPMI 1640 medium supplemented with 0.2% FBS. Cells were untreated (control group) or treated with 28 $\mu\text{g/ml}$ (1/2 half maximal inhibitory concentration) NCTD (NCTD group). NCTD was purchased from Jiangsu Kangxi Pharmaceutical Works (Jiangsu, China) in fresh culture medium. Lower wells contained RPMI 1640 medium supplemented with 20% FBS. After 24 h of incubation at 37°C, non-invading cells were removed from the upper surface of the membrane, and the cells that invaded each membrane were stained with a crystal violet solution and counted as described previously (7).

Three-dimensional (3-D) cultures. Matrigel was thawed at 4°C, and 20 μl was quickly added to each well of a 96-well plate and allowed to solidify for 40 min at 37°C in a humidified 5% CO₂ incubator. Tumor cells were seeded in complete RPMI 1640 medium onto the plate and incubated with or without NCTD (28 $\mu\text{g/ml}$) at 37°C for 12 and 24 h, respectively. Vasculogenic-like structure formation was filmed under an inverted phase-contrast light microscope.

Tumor xenografts. Mice (4–6 week-old BALB/c nu/nu male mice; Shanghai Laboratory Animal Center, Shanghai, China) weighing 18–24 g were randomly divided into the control group and the NCTD group (n=10/group). The mice were housed according to the official recommendations of the Chinese Community Guidelines (14). Tumors were established by inoculation of 1×10^6 A375 cells suspended in 100 μl normal saline into the right back of the mice by subcutaneous injection. After the tumors had grown to a size of $\sim 100 \text{ mm}^3$, NCTD was administered by intraperitoneal injection at 28 mg/kg (1/5 half maximal lethal dose) in 0.1 ml normal saline for 13 consecutive days. Control animals were administered 0.1 ml normal saline as a vehicle control. The maximum diameter (a) and the minimum diameter (b) were measured with calipers every 2 days. The tumor volume (V) was calculated by the following formula: $V = ab^2 \times 0.5$. The tumor weight was also evaluated once the mice were sacrificed. All animal procedures were performed according to an approved protocol by the Animal Ethical Committee of the Tianjin International Joint Academy of Biotechnology and Medicine (Tianjin, China).

Immunohistochemical analysis. Xenografted tumors from the mice were excised, fixed in 4% paraformaldehyde for 24 h, and then embedded in paraffin for histological studies. Paraffin-embedded tissues were sectioned into slices of 4- μm thickness for histological studies. Dewaxed and rehydrated tissue sections were subjected to antigen retrieval processes. Upon blocking, the sections were incubated overnight at 4°C with anti-cluster of differentiation (CD) 31 antibody (dilution 1:50; catalogue number JC70; Neomarkers, Fremont, CA, USA) or anti-MMP-2 antibody (dilution 1:200; catalogue number ab37150; Abcam, Cambridge, UK). Negative controls were prepared using PBS instead of the primary antibodies. Upon washing with PBS, the sections were incubated with a goat anti-mouse EnVision kit (Genentech, South San Francisco, CA, USA) for 40 min at 37°C, and then incubated with 3,3'-diaminobenzidine chromogen for 10 min. The slides for CD31-periodic acid-Schiff (PAS; Beijing Zhongshan Jinqiao Biotechnology Co. Ltd., Beijing, China) double staining were

Table I. Concentration and activity state of MMP-2 in serum-free conditioned medium from melanoma cells cultured on Matrigel.

Serum-free conditioned medium	MMP-2 (ng/ml)	MMP-2 activity (ng/ml)
Control	367.82 \pm 14.8	28.09 \pm 5.64
NCTD	43.15 \pm 5.67 ^a	2.29 \pm 0.18 ^a

^aP<0.05 compared with the control group. NCTD, norcantharidin; MMP, matrix metalloproteinase.

then exposed to a 0.5% periodic acid solution for 15 min and subsequently incubated in Schiff solution for 20 min in a dark chamber. Subsequently, the slides were washed with distilled water for 3 min and counterstained with hematoxylin. Multiplication of intensity and percentage scores was utilized to determine the staining index result.

Zymography assays and MMP-2 protein concentration determination. Gelatin zymography was used to examine the levels of MMP-2 activity in A375 cells that were either untreated (control group) or treated with 28 $\mu\text{g/ml}$ NCTD for 48 h. The culture media were collected and subjected to 10% SDS-PAGE using 0.01% w/v gelatin. The gel was washed twice in 2.5% (w/v) Triton X-100 solution and incubated overnight at 37°C in developing buffer [50 mmol/l Tris/HCl (pH 7.4), 10 mmol/l CaCl₂, 5 mmol/l ZnCl₂ and 0.05% Brij™ 35 Surfact-Amps™ Detergent Solution (Thermo Fisher Scientific, Inc.)]. The gels were subsequently stained with Coomassie Brilliant Blue R250 and destained until the wash remained clear and cleared zones associated with MMP activity were apparent. The concentrations of exogenous pro-MMP-2 and active MMP-2 in cell culture supernatants were assessed using the MMP-2 Biotrak Activity Assay (GE Healthcare Life Sciences, Chalfont, UK) according to the manufacturer's guidelines.

Statistical analysis. All data were reported as the mean \pm standard error of the mean and evaluated using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Student-Newman-Keuls *t* tests were used to evaluate differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

NCTD inhibits the invasion of A375 cells *in vitro*. Matrigel-coated transwell plates were used to assess the effects of NCTD on the ability of A375 melanoma cells to invade a basement membrane matrix. The results revealed that NCTD significantly reduced the number of cells that invaded through the Matrigel matrix after 24 h of treatment (P= 0.005) (Fig. 1A).

NCTD inhibits tube formation by A375 cells *in vitro*. The vasculogenic-like network formation ability of melanoma cells was assessed *in vitro* by seeding the cells onto Matrigel-coated plates and then observing the cells under an inverted phase-contrast light microscope. As shown in Fig. 1B, the formation of VM

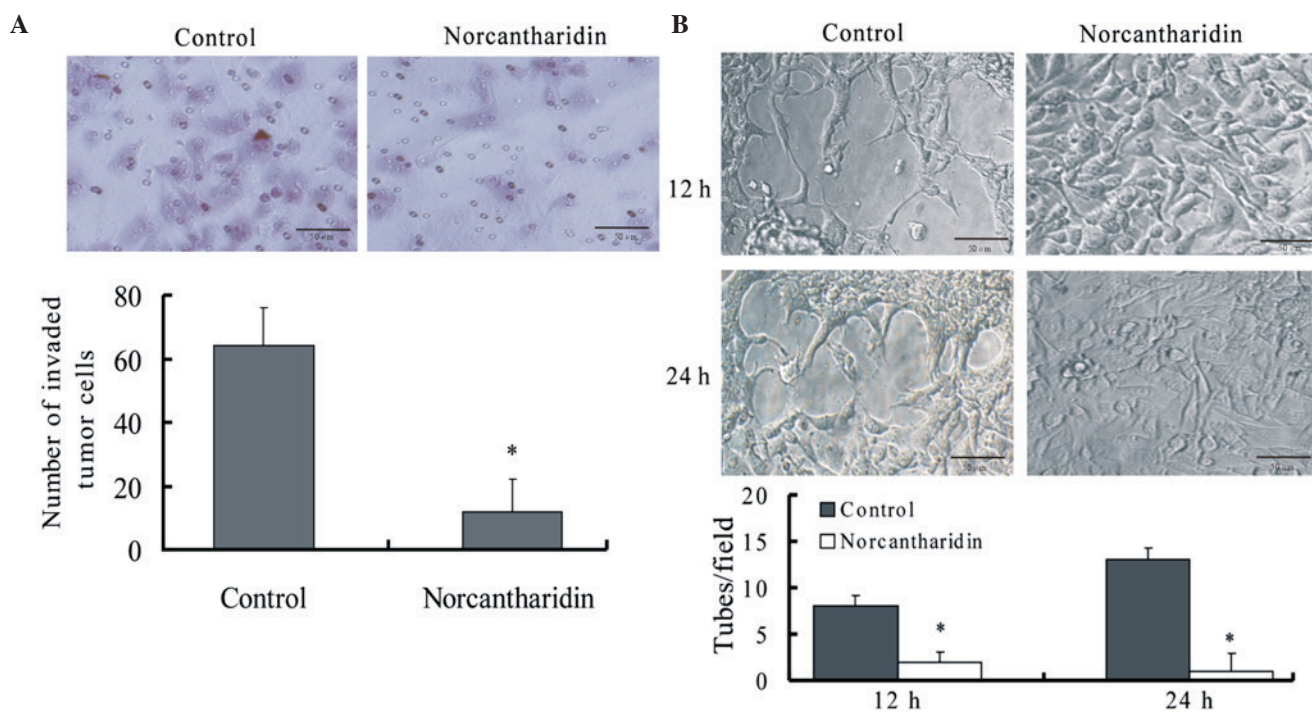


Figure 1. NCTD inhibits the invasion and capillary-like tube formation abilities of A375 melanoma cells *in vitro*. (A) Inhibitory effect of NCTD on the invasive ability of A375 melanoma cells *in vitro*. Upper panel, representative images of cells from the transwell invasion assay. Magnification, x200. Lower panel, quantitative analysis indicating that fewer melanoma cells invaded through the Matrigel matrix following NCTD treatment compared with those in the control group. (B) NCTD decreases the capillary-like tube formation ability of melanoma cells *in vitro*. Upper panel, NCTD impaired the vessel-like networks formed by A375 melanoma cells cultured on Matrigel at 12 and 24 h. Magnification, x400. Lower panel, quantitative analysis indicating that less capillary-like tubes were formed in cells subjected to NCTD treatment compared with those in the control group. * $P < 0.05$ compared with the control group ($n = 3$). NCTD, norcantharidin.

networks by A375 melanoma cells was disrupted by the addition of NCTD for 12 or 24 h.

NCTD inhibits melanoma growth and VM formation in mice. To investigate the efficacy of NCTD in inhibiting melanoma growth, the tumor sizes of melanoma-bearing mice were measured once every 2 days throughout the experiment. At the end of the experiment, the volume and weight of the xenografts in the NCTD group decreased significantly with increased tumor inhibition in comparison with those of the control group (Fig. 2). CD31-PAS double staining was used to identify VM in the xenografts on day 21 of tumor inoculation. Microscopically, the xenografts in the control group exhibited tumor cell-lined channels containing red blood cells (Fig. 3) without any evidence of tumor necrosis. The channels consisted of tumor cells negative for CD31 and positive for PAS. By contrast, VM could hardly be observed in the tumor tissues treated with NCTD, while large areas of necrosis were easily detected, suggesting that NCTD inhibits the VM formation of melanoma xenografts *in vivo*.

NCTD downregulates the expression and activity of MMP-2. MMP-2 is a key player in VM formation (15). Thus, to explore the possible mechanisms of NCTD effects on tumor growth and VM of human melanoma *in vitro* and *in vivo*, in the present study, the expression and activity of MMP-2 protein from sections of melanoma xenografts and supernatants of 3-D culture samples were determined. The results indicated that MMP-2 expression in the *in vivo* xenografts of the NCTD

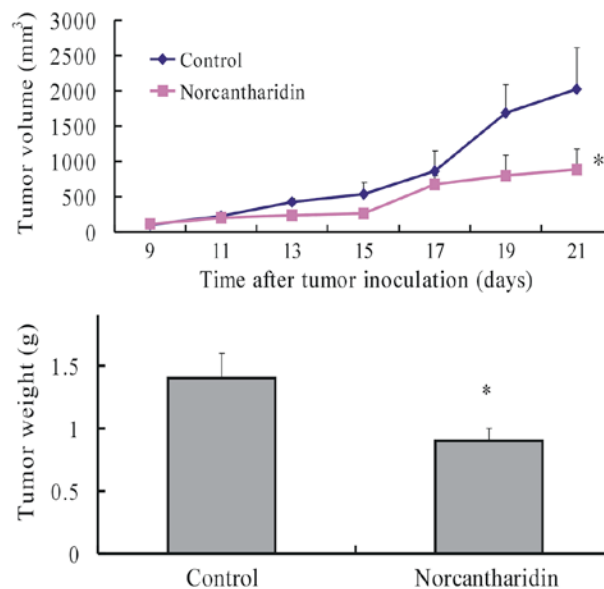


Figure 2. Norcantharidin inhibits A375 human melanoma tumor growth. Plot of tumor volume (upper panel) or tumor weight (lower panel) vs. the number of days post-tumor inoculation of melanoma-bearing mice in different groups. * $P < 0.05$ compared with the control group. Data are shown as the mean \pm standard error of the mean ($n = 10$ mice/group).

group was significantly lower than that of the control group (Fig. 4A). Furthermore, the addition of NCTD decreased both the expression and activity of MMP-2 in the 3-D culture samples (Fig. 4B and Table I).

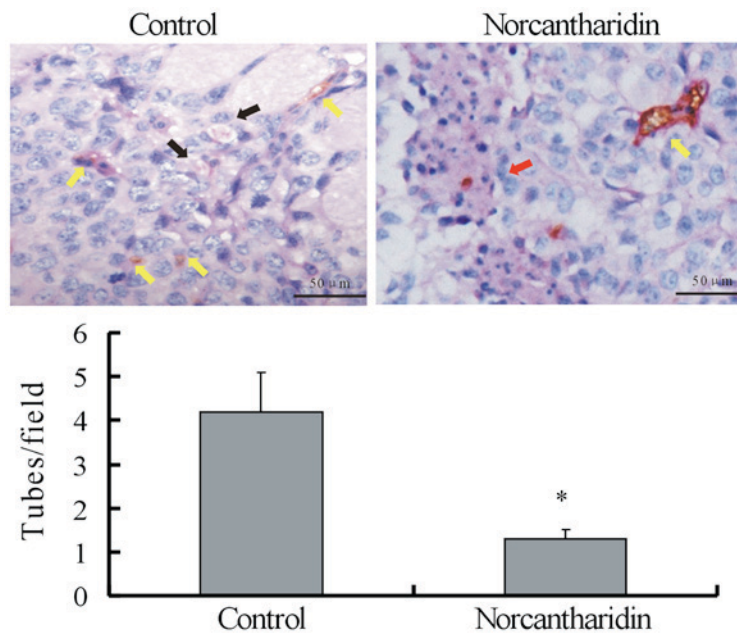


Figure 3. NCTD decreases the capillary-like tube formation ability of A375 melanoma xenografts *in vivo*. Upper panel, representative images of the presence of VM in tissue samples of A375 melanoma xenografts with or without treatment with NCTD. In the control group, CD31-periodic acid-Schiff staining was used for the morphological observations of VM formed by tumor cells without CD31-positively stained endothelial cells (black arrow). Red blood cells were present in the center of the channels. The yellow arrow points to CD31-positively stained endothelium-dependent vessels in the same field. In the NCTD-treated group, VM was not observed, while large areas of necrosis were easily noticed (red arrow). Yellow arrow, endothelium-dependent vessels. Magnification, x400. Lower panel, quantitative analysis indicating that less capillary-like tubes were formed upon NCTD treatment in the treated group compared with those in the control group. * $P < 0.05$ compared with the control group. NCTD, norcantharidin; CD, cluster of differentiation; VM, vasculogenic mimicry.

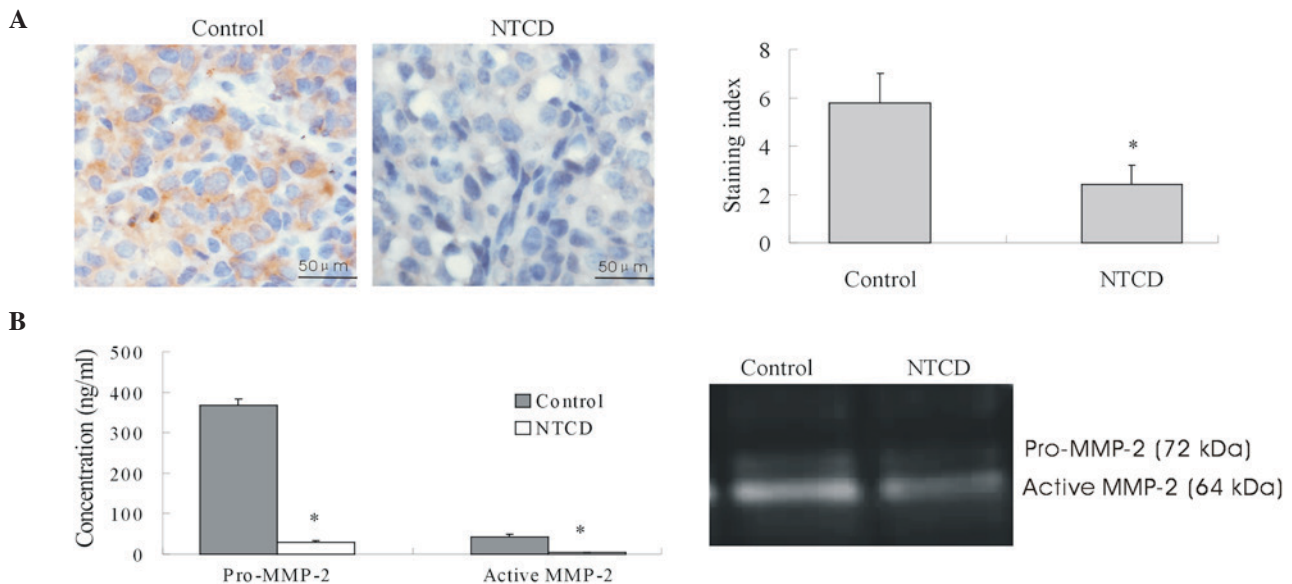


Figure 4. Expression and activity of MMP-2 protein from (A) sections of A375 melanoma xenografts and (B) serum-free conditioned medium from melanoma cells cultured on Matrigel. (A) Upper panel, representative images of MMP-2 expression in A375 melanoma xenografts. Positive staining of MMP-2 could be observed in the cytoplasm of tumor cells. The lower panel indicates the quantitative analysis of MMP-2 expression, and lower expression was observed in the NCTD group compared with that in the control group (* $P < 0.05$). (B) The concentration and activity of MMP-2 protein from supernatants of three-dimensional culture samples were measured either by MMP-2 Biotrak Activity Assay (left) or by zymography assay (right). Both the expression and activity of MMP-2 protein in the NCTD group were significantly lower than those in the control group (* $P < 0.05$). NCTD, norcantharidin; MMP, matrix metalloproteinase.

Discussion

The present study demonstrates that NCTD inhibits tumor growth and VM of melanoma by suppressing MMP-2 expression.

Widespread metastasis caused by increased cell motility and a rich blood supply of tumor cells is the main cause of

the poor prognosis of melanoma patients (16). Traditional anti-angiogenic drugs, including bevacizumab, sunitinib, angiostatin and endostatin, have yielded disappointing results on the management of melanoma, since VM exists as a particular microcirculation pattern, and the sole blockage of angiogenesis may not be effective (9,17-20).

Thus, the development of anti-VM drugs for the treatment of melanoma with VM is an urgent concern. NCTD is a demethylated and low-cytotoxic derivative of cantharidin (12). It has antitumor properties, hypotoxicity in a variety of tumor and apoptosis-promoting effects in melanoma *in vitro* (13). NCTD also inhibits VM formation in human gallbladder carcinomas (21). The present study further investigated the anti-VM activity of NCTD as a VM inhibitor for human melanoma. The results indicate that NCTD inhibits the growth and VM formation of melanoma both *in vitro* and *in vivo*, thus suggesting that NCTD may be a potential therapeutic agent targeting VM in melanoma.

We also sought to determine the possible mechanism of the inhibitory effects of NCTD on the growth and VM formation of melanoma. The MMP-2 protein is considered to play a key role in VM formation in melanoma via the cleavage of the laminin (Ln)-5 γ 2 chain into two segments (Ln-5 γ 2x and Ln-5 γ 2'), which results in VM formation (15). Zhang *et al* reported that NCTD inhibits tumor growth and VM of human gallbladder carcinomas by suppressing the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K)/MMP-2/Ln-5 γ 2 signaling pathway (12). Thus, in the current study, the effects of NCTD on MMP-2 protein expression and activity in melanoma were determined both *in vivo* and *in vitro*. The results demonstrate that NCTD not only inhibits VM formation of melanoma cells and xenografts, but also downregulates MMP-2 expression *in vitro* and *in vivo*. These results suggest that the PI3-K/MMP-2/Ln-5 γ 2 signaling pathway may also be the underlying molecular mechanism of the inhibitory effects of NCTD on the growth and VM formation of melanoma. Therefore, NCTD could be used as a potential anti-VM inhibitor in melanoma treatment.

In conclusion, the present study demonstrated that NCTD inhibits the growth and VM formation of melanoma by suppressing MMP-2 expression. NCTD may be used as a potential therapeutic agent targeting VM in melanoma. Further investigations are necessary to verify other molecular mechanisms of the inhibitory effects of NCTD on the VM formation of melanoma.

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