Inhibitory effect of norcantharidin, a derivative compound from blister beetles, on tumor invasion and metastasis in CT26 colorectal adenocarcinoma cells

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Norcantharidin (NCTD), a potential anti-cancer drug, is the demethylated analog of cantharidin isolated from blister beetles. The present study investigated the effect of NCTD on tumor invasion and metastasis. A cytotoxicity assay of NCTD in CT26 colorectal adenocarcinoma cells showed a dose- and time-dependent decrease in cell viability. NCTD (50 μM)-treated CT26 cells not only showed an inhibited cell invasion of 65.6%, but also decreased the activity of matrix metalloproteinase-2 and -9. NCTD decreased the adhesive ability of CT26 cells in a dose-dependent manner. At a concentration of 100 μM, NCTD showed a down-expression of several cadherin–catenin adhesion molecules, including Desmoglein, N-cadherin, and α- and β-catenin, while there were no obvious changes in E-cadherin and γ-catenin. Intraperitoneal injection of NCTD (2 mg/kg/day) in BALB/c mice reduced both the pulmonary metastatic capacity of CT26 cells and prolonged the survival day of the mice. These results demonstrated that it was effective in blocking both tumor invasion and metastasis. *Anti-Cancer Drugs* 16:293–299 © 2005 Lippincott Williams & Wilkins.

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We would like to dedicate this work to the memory of our advisor, Professor Sheng-Yuan Wang.

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Introduction

Mylabris, a species of blister beetle (*Mylabris phalerata* Pall.), has been used as a traditional remedy in Chinese medicine for thousand years for the elimination of swelling. Cantharidin, an active constituent in mylabris, has anti-tumor properties [1], but causes severe side-effects in the urinary system [2]. Norcantharidin (NCTD), the demethylated form of cantharidin, is easier to synthesize and less toxic [3]. NCTD effectively inhibited the growth of hepatoma and leukemic cells, with a high LD_{50} of 12.5 mg/kg [4–6]. NCTD decreased tumor growth and prolonged survival in human HepG2 cell-transplanted nude mice [6]. The anti-tumor effect of NCTD may go through interrupting DNA synthesis, retarding progression of the cell cycle, and inducing apoptosis via p53 gene expression and Bcl-2 regulation [7–9].

Metastasis is the spread of cancer cells from a primary lesion to distant sites and is the major cause of treatment failure as well as cancer mortality. The metastasis process includes: (i) detachment from the primary tumor, (ii) invasion of the extracellular membrane and entry into vessels, and (iii) adherence to subendothelial basement membranes and entry into organ parenchyma [10,11]. There are two major protein families involved in the process of tumor invasion and metastasis, including matrix metalloproteinases (MMPs) and adhesion molecules.

MMP activity in a primary tumor correlates progression of metastasis. More than 20 MMPs have been characterized in humans and other animals. MMP-2 and -9 have a gelatinase activity which is capable of proteolytically cleaving plasminogen to form angiostatin that mediates angiogenesis, tumor invasion and metastasis [12].

Cellular adhesion molecules mediate cellular adhesion with other cells and matrix components in the processes of metastasis formation [13]. Shibanuma *et al.* reported that the dysfunction of cadherin–catenin-mediated cell–cell adhesion increases the potential for local invasion and distant metastasis [14]. Caffeic acid phenethyl ester and curcumin, plant-derived phenolics, were effective in

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preventing colorectal cancer development and decreased expression of β-catenin [15].

Taken together, it indicates that modulation of MMP activity and/or adhesion molecule expression may suppress the development of metastasis.

This study was designed to examine the effect of NCTD on tumor cell invasion and metastasis using murine colorectal adenocarcinoma CT26 cells for *in vitro* and *in vivo* experiments. The expression of MMP activity and cadherin–catenin adhesion molecules in NCTD-treated CT26 cells was also assessed.

Materials and methods

Materials

NCTD was kindly provided by Dr Wang Guang-Sheng (Peking Fourth Pharmaceutical Works, Beijing, China). The chemical structures of cantharidin and NCTD are shown in Figure 1.

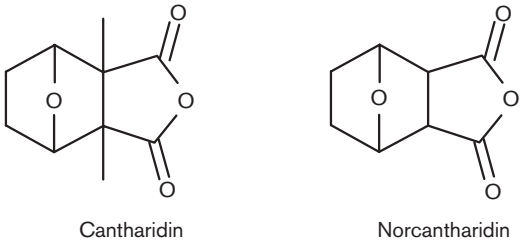
Cell and cell culture

CT26 cells, *N*-nitroso-*N*-methyl urethane-induced mouse undifferentiated colorectal carcinoma cells of BALB/c origin, purchased from ATCC (Manassas, VA), were used for both the *in vitro* and *in vivo* experiments. The cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT) at 37°C in a humidified 5% CO_2 incubator, passaged every 2–3 days with TEG solution (0.25% trypsin, 0.1% EDTA and 0.05% glucose in Hanks' balanced salt solution) and maintained at exponential growth.

Tumor cell viability

CT26 cells (1×10^5 cells/ml) were cultured in dishes for 12 h, and then incubated with different concentrations of NCTD (0–200 μM) for a further 24, 48 and 72 h. At the end of incubation, culture dishes were scraped by a rubber policemen to collect all adhesive and non-adhesive cells. Cell viability was measured by Trypan blue dye exclusion and observed under a microscope at a magnification of × 100.

Fig. 1



Cantharidin Norcantharidin

Chemical structure of cantharidin (2,3-dimethyl-7-oxabicylo-[2.2.1] heptane-2,3-dicarboxylic anhydride) and norcantharidin (exo-7-oxabicylo-[2.2.1] heptane-2,3-dicarboxylic anhydride).

Matrigel invasion assay

Assays of cell invasion properties were performed using a modified Boyden chamber with polyethylene terephthalate filter inserts coated with a Matrigel matrix in 24-well plates containing 8-mm pores. In brief, 10^5 tumor cells were suspended in a serum-free medium with 0.5% BSA. Then, they were plated into the upper chamber followed by filling the lower chamber with the same medium with or without NCTD (0–50 μM). Cells were incubated for 24 h, and then non-invading cells were gently removed and stained with the solution in a Chemicon cell invasion assay kit (ECM550; Chemicon, Temecula, CA). Cells on the upper side of the filter were carefully removed and those cells invading the lower side were counted by microscopic examination. For the purposes of quantitation, 10% acetic acid (100 μl/well) was used to dissolve stained cells, which were transferred to 96-well plates for spectrophotometric measurement at a wavelength of 560 nm.

Detection of MMPs by gelatin zymography analysis

Because MMP-2 and -9 proteins possess gelatinase activity, their activity can be assessed by the degree of gelatin degradation shown on zymography. Analysis of gelatinolytic activity was performed using 10% (w/v) polyacrylamide gels impregnated with 0.1% (w/v) gelatin. Total protein of 50 μg/well in conditioned media harvested from explant cultures after 48 h of NCTD treatment was mixed with 10% (v/v) glycerol, 2% (w/v) SDS, 0.0025% (w/v) bromophenol blue and 0.5 M Tris (pH 6.8), and subjected to SDS–PAGE. Gels were washed twice in 2% (v/v) Triton X-100 for 30 min at room temperature to remove the SDS. They were then equilibrated with developing buffer (50 mM Tris–HCl, 0.2 M NaCl, 5 M $CaCl_2$; pH 7.2) for 30 min at room temperature and incubated overnight with the same buffer at 37°C. Gels were stained with 0.1% (w/v) Coomassie brilliant blue G-250, and destained in 5% acetic acid and 10% methanol in water to view zones of gelatinase activity [16].

Adhesion assay

The adhesion assay was modified from Mototsugu *et al.* [17]. Briefly, cells were cultured in a 96-well microplate (1×10^4/well in 100 μl medium) for 12 h and then incubated with different concentrations of NCTD (0–200 μM) for a further 24 h. At the end of incubation, the plate was washed twice and non-adhering cells were aspirated. MTT (Sigma, St Louis, MO) was added to each well and incubated for 2 h at 37°C, as indicated by the conversion of tetrazolium salts to a colored formazan product. Then, we took the optical density which could be measured spectrophotometrically at the wavelength of

550 nm to calculate the percent of adherent cells. Morphology of cells with and without NCTD treatment were observed and photographed under a microscope at a magnification of $\times 400$.

Western blot analysis of adhesion molecules

For Western blot analysis of endogenous catenin family expression, 10^7 CT26 cells were cultured with NCTD at various concentrations (0, 12.5, 25, 50 and 100 μM) for 48 h. Then, we scraped cells from the culture dish, disrupted cells with 2 times concentrated electrophoresis sample buffer (1 M Tris, pH 6.8, 5% SDS, 40% glycerol, 0.005% bromophenol blue and 8% β-mercaptoethanol), centrifuged the samples for 5 min to pellet insoluble material and subjected it to gel electrophoresis using 10% (w/v) SDS–polyacrylamide gels. Protein samples were then blotted to PVDF membranes. Primary antibodies (E-, M-, N-, R-cadherin, α-, β-, γ-catenin, pp120 and Desmoglein; BD Biosciences, Mountain View, CA) were used at dilution and detected using horseradish peroxidase-conjugated anti-mouse IgG (1:10,000-fold dilution) followed by enhanced chemiluminescence (ECL; Amersham Pharmacia, Wikstrams, Sweden). β-Actin was used as an internal control.

Pulmonary metastasis assay

BALB/c male mice (6–8 weeks old), obtained from the National Laboratory Animal Center (Taipei, Taiwan), were maintained in a germ-free environment, and allowed free access to food and water. All animal experiments were performed according to our institutional and NIH guidelines for the care and use of research animals. CT26 tumor cells were prepared as a single-cell suspension in sterile PBS and injected i.v. with 2×10^5 cells in the lateral tail vein of the mice in order to establish pulmonary metastases. On days 1–5 following the tumor challenge, the animals were treated with NCTD (2 mg/kg/day), 5-fluorouracil (10 mg/kg/day) or vehicle i.p. The number of mice was seven to 10 per group. The body weight was recorded every other day. After 14 days, when the control group became moribund, the mice were sacrificed using a high dose of pentobarbital sodium (250 mg/kg, i.p.). Lung tissues were dissected for histopathological assay and the metastatic lung nodules were counted by an investigator blinded to the treatment received. For survival analysis, another group of mice were treated as described above, but were not sacrificed. All experiments were performed in accordance with regulations in the NIH *Guide for the Care and Use of Laboratory Animals* (DHHS publ. NIH 85-23, revised 1996).

Histopathological assay of lung tissue

After pulmonary metastasis assay, the mice were sacrificed and the lung tissues were dissected. Lung tissues were prepared by formalin-fixed, paraffin-embedded biopsies. Tissue section slides were made, then washed 3 times in PBS for 10 min each and stained with a standard hematoxylin & eosin (H&E) protocol. Slides were observed under a microscope at a magnification of $\times 100$.

Statistical analysis

Results are expressed as the mean ± SE or SD from at least three experiments. Statistical comparisons were based on Student's *t*-test or analysis of variance. Differences were considered significant at $p < 0.05$. All statistical analyses were carried out using SigmaStat software (Jandel Scientific, San Rafael, CA).

Results

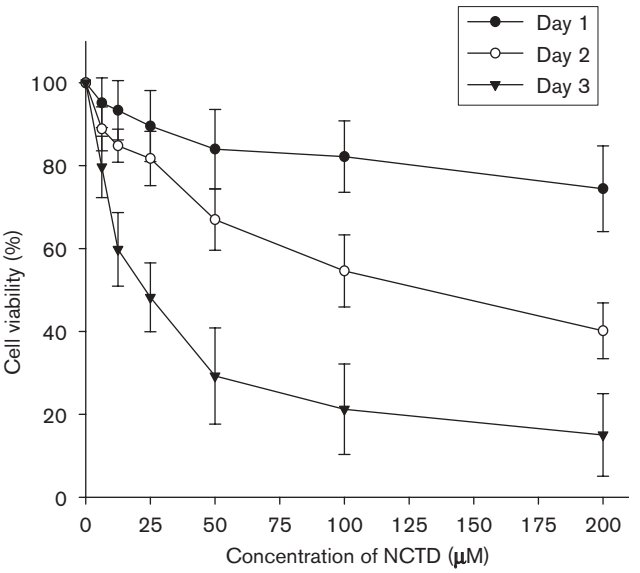
Cytotoxicity of NCTD

The cytotoxic effect of various concentrations of NCTD (0–200 μM) on colorectal cancer CT26 cells by Trypan blue dye exclusion is shown in Figure 2. The viability of CT26 colorectal cancer cells was decreased by NCTD in a dose- and time-dependent manner. NCTD-treated cells were detached from the Petri dish on day 1. However, cell viability was 74.4% when the concentration of NCTD was up to 200 μM. The 2- and 3-day cultures of NCTD-treated cells markedly increased their cytotoxicity with the IC_{50} being about 76 and 32 μM, respectively.

Inhibitory effect of NCTD against tumor cell invasion

The ability of tumor cells to invade was performed by a filter chamber (8-mm pores) coated with Matrigel matrix.

Fig. 2



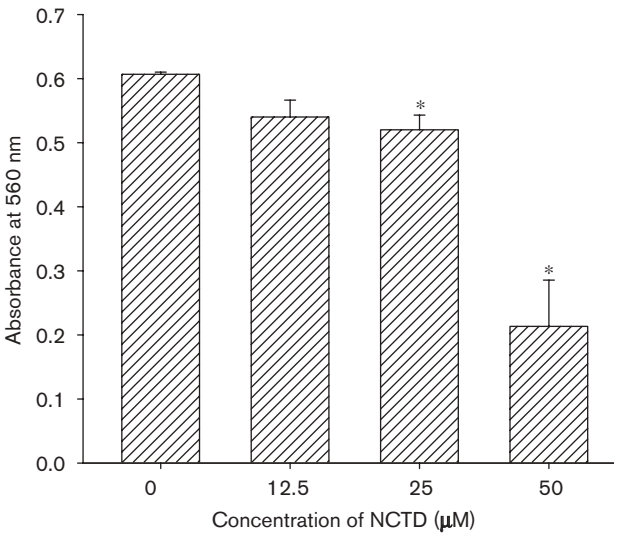
Cytotoxicity of NCTD on CT26 colorectal cancer cells. CT26 cells were cultured for 12 h and various concentrations of NCTD (0–200 μM) were added to each well. After 24, 48 and 72 h of further culture, all cells including adhesion and non-adhesion were collected, and cytotoxicity was determined by Trypan blue dye exclusion. Data from three separate experiments are expressed as mean ± SD. *Significant change compared with untreated control.

Those cells who managed to invade to the lower side were counted by microscopic examination. Figure 3 shows dose-dependent inhibition of CT26 colorectal cancer cell invasion by NCTD. NCTD (50 μM) inhibited invasion by 65.6%, while the cell viability only decreased about 15% at the same concentration (Fig. 2). Therefore, the invasion of CT26 cells was inhibited by NCTD, which may not be mainly due to the decrease in viability.

Inhibitory effect of NCTD on MMP activity

As shown in Figure 4, MMP-9 and -2 are found at 92 and 72 kDa, respectively, on SDS–PAGE. At a concentration of 12.5 μM, NCTD markedly inhibited CT26 cells in terms of their activity of MMP-9. When the concentration of NCTD was up to 50 μM, both MMP-2 and -9 almost lost their activities, indicating that the inhibitory effect of NCTD occurs by down-regulating their expression in CT26 cells.

Fig. 3



Inhibitory effect of NCTD on CT26 cell invasion *in vitro*. CT26 cells were seeded on an ECM layer with various concentrations of NCTD (0–50 μM) and absorbance by colorimetry at 560 nm was used for cell number estimation. Data are shown as mean ± SD of at least three determinations. *Significant change compared with untreated control.

Effect of NCTD on cell adhesion

NCTD-treated cells markedly decreased their adhesive ability in a dose-dependent manner (Fig. 5a). Compared to cytotoxicity, after NCTD treatment, the degree of decrease in cell viability is less than that in cell adhesion. When the NCTD concentration was up to 200 μM, only 44.8% of cells showed adherence. Moreover, NCTD treatment influenced the morphology of tumor cells, changing from shuttle shaped (untreated) to a rounded form (treated with 25 μM of NCTD) (Fig. 5b and c).

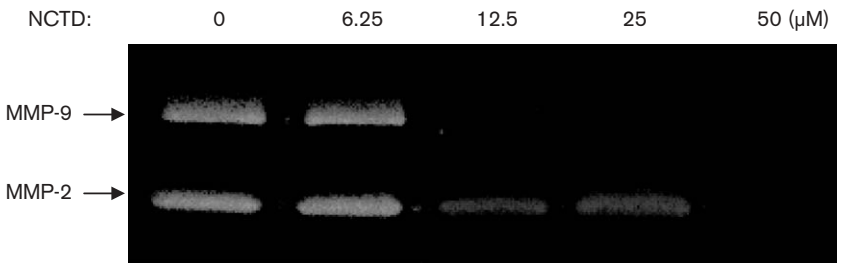
Effect of NCTD on the expression of cadherin–catenin adhesion molecules

As shown in Figure 6, mouse CT26 colorectal cancer cells were able to express several cadherin–catenin adhesion molecules. After 48 h of culture, the expression of adhesion molecules was reduced in NCTD (100 μM)-treated CT26 cells, including Desmoglein, N-cadherin, and α- and β-catenin. However, E-cadherin and γ-catenin showed no obvious changes in their expression in NCTD-treated CT26 cells, even at concentrations of NCTD up to 100 μM.

In vivo assay of NCTD

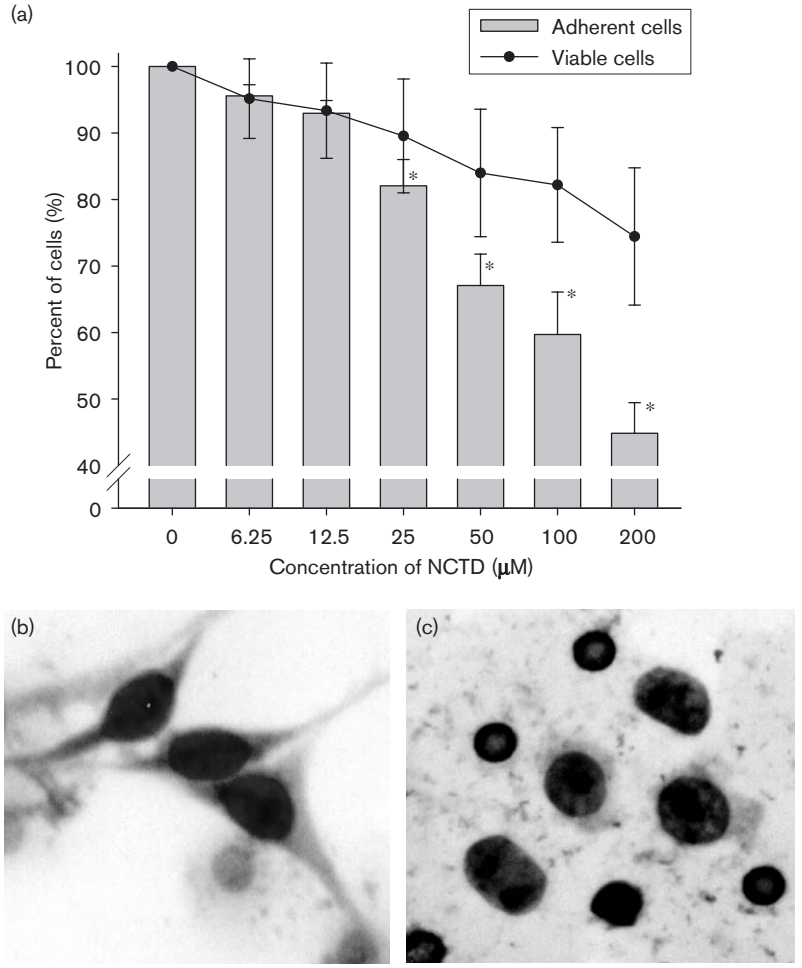
After injection with 10^5 murine CT26 colorectal adenocarcinoma cells, all untreated animals developed multiple lung metastases (Fig. 7a). As shown in Figure 7(b), daily i.p. injection of NCTD at a dose of 2 mg/kg/day decreased tumor colonization in the lung, and did so more effectively than 5-FU (10 mg/kg/day). Moreover, CT26-bearing mice treated with NCTD or 5-FU survived longer than untreated controls (Fig. 7c). The average survival times in control, 5-FU and NCTD groups were 31.7 ± 2.8, 37.4 ± 3.6 and 44.9 ± 3.7 days, respectively.

Fig. 4



Detection of MMP activity in conditioned media from the culture of CT26 cells with or without NCTD treatment. Conditioned medium harvested from NCTD (0–50 μM)-treated CT26 cells, and the gelatinase activity of MMP-2 (72 kDa) and MMP-9 (92 kDa) were determined by gelatin zymography.

Fig. 5



Adhesion assay of NCTD on CT26 cells. Cells were cultured for 12 h and then various concentrations of NCTD (0–200 μM) were added to each well. After 24 h of further culturing, all cells were collected and cell viability was determined. For adherent assay, only the adherent cells were collected, washed twice with PBS and determined by the MTT assay. (a) Percent of adherent and viable cells. (b) Morphological observation of untreated CT26 cells under a microscope (× 400). (c) Morphological observation of NCTD (50 μg/ml)-treated CT26 cells under a microscope (× 400). Data are shown as mean ± SD of at least three determinations. *Significant change compared with untreated control.

There were no obvious changes in body weight of all mice during the experimental period (Fig. 7d).

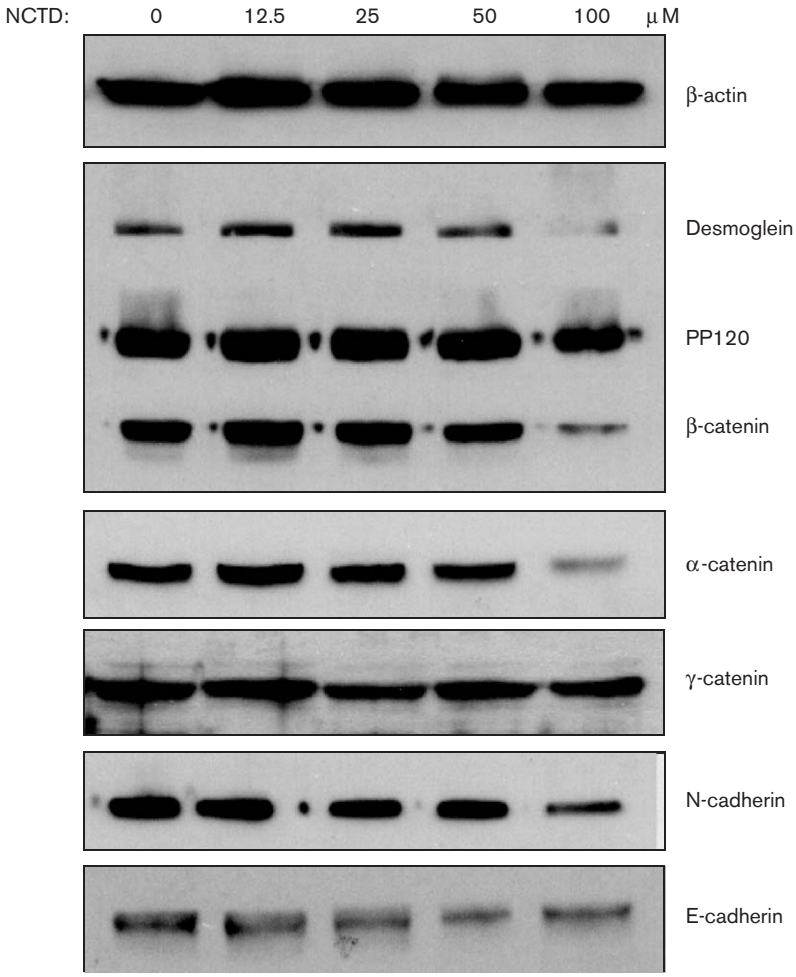
Discussion

Our report is the first one demonstrating the inhibitory effects of NCTD on the viability, invasion, MMP-2 and -9 activities, and expression of adhesion molecules in murine colorectal adenocarcinoma CT26 cells *in vitro*. Moreover, it suppresses pulmonary metastasis and prolongs survival in BALB/c mice implanted with CT26 cells.

MMPs, which can cause proteolytic defects of the basement membrane, are considered to play an important role in tumor cell invasion and subsequent spread from the site of origin [12]. MMP-2 and -9, two members of this protein family, possess gelatinase activity and are key points for extracellular matrix (ECM) degradation mediating tumor cell invasion [18]. Our results showed that NCTD inhibited MMP-2 and -9 activity in CT26 cells, which may partially contribute to the inhibition of tumor cell invasion and metastasis. To elucidate which factor is critical to the anti-metastasis effect, we are going to block MMP expression and activity by using siRNA or inhibitors in further studies.

E-cadherin is the main cell–cell and cell–matrix adhesion molecule that is associated with three kinds of cytoplasmic proteins, including α-catenin, β-catenin and plakoglobin, and the formation of the cadherin–catenins adhesion complex in adherence junctions [14]. Aberrant expression of E-cadherin is correlated with lymph node

Fig. 6



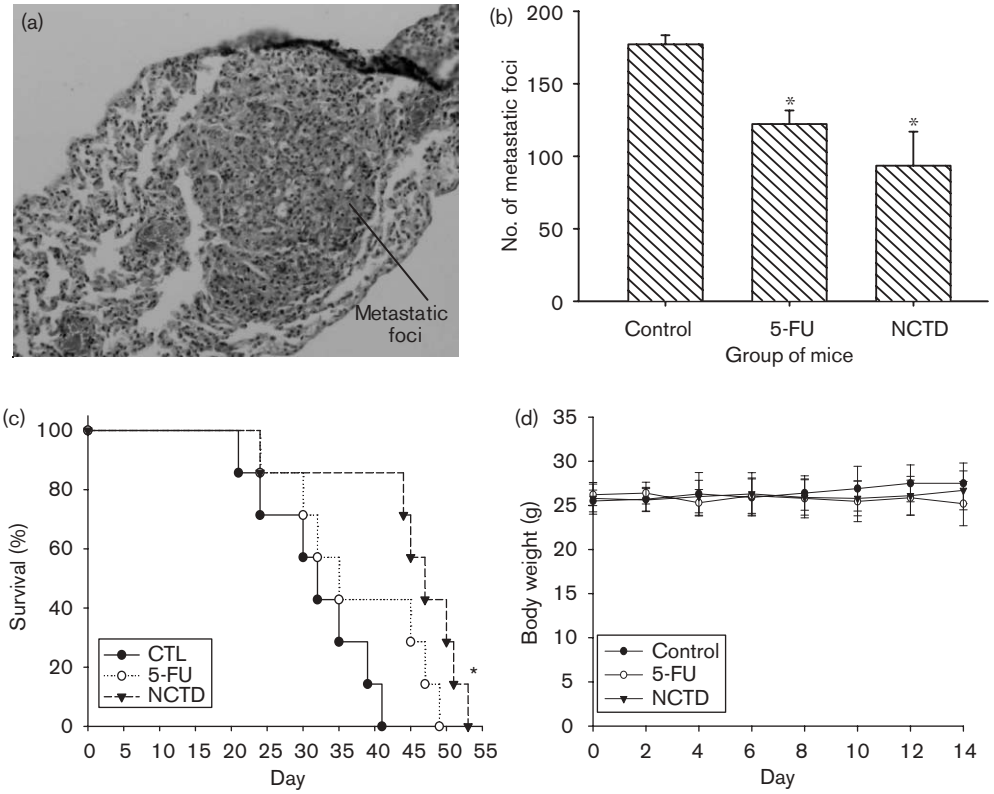
Expression of adhesion molecules in the NCTD-treated CT26 cells by Western blot analysis. Cells were cultured with NCTD at various concentrations (0–100 μM) for 48 h. Aliquots of cell lysates were subjected to Western blot analysis, and probed with antibodies specific for E-, M-, N-, R-cadherin, α-, β-, γ-catenin, pp120 and Desmoglein. Identical results were obtained in three independent experiments and β-actin was used as an internal control.

metastasis and cell types, while aberrant expression of γ-catenin is related to deep myometrial invasion in endometrial carcinoma patients [19]. Our results showed that NCTD decreased the expression of α- and β-catenin in CT26 cells, suggesting the anti-invasive and anti-metastasis effect of NCTD may involve the regulation of these cell adhesion molecules.

Since the NCTD might be a potent anti-cancer drug, we have searched in MEDLINE from 1966 for items related to the pharmacokinetic data of NCTD. No paper has been found for the determination of NCTD by HPLC or LC-MS-MS. It suggests that detailed pharmacokinetic study of NCTD is necessary in the future to optimize the dosage schedule.

Our previous study showed the anti-metastatic effect of several natural products that have anti-tumor activity, such as caffeic acid phenethyl ester [20] and tetrandrine [21]. These *in vitro* and *in vivo* experiments demonstrated that NCTD was more effective in inhibiting tumor cell invasion, reducing MMP activity and eliminating the number of pulmonary metastatic foci with low cytotoxicity. In addition, the anti-invasive and anti-metastatic activity of NCTD on CT26 cells was also accompanied with a decrease in cell adhesion. NCTD may be a safe chemotherapeutic agent in our experimental model because of the lack of any body weight loss. Whether the anti-metastatic activity of NCTD is related to cell adhesion will be further investigated.

Fig. 7



Inhibitory effect of NCTD on pulmonary metastasis. BALB/c mice after i.v. injection of 2×10^5 CT26 colorectal cancer cells were treated daily with NCTD (2 mg/kg, i.p.), 5-FU (10 mg/kg, i.p.) or vehicle for 5 consecutive days ($n = 7–10$ per group). (a) Lung tissue section was stained with H & E stain and photographs were taken under a microscope at a magnification of $\times 100$. (b) Number of pulmonary metastatic foci. (c) Survival rate of mice. Data are shown as mean ± SE of at least three determinations. (d) Changes in body weight. *Significant change compared with control group by one-way ANOVA followed by Student–Newman–Keuls method.

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