Norcantharidin induces anoikis through Jun-N-terminal kinase activation in CT26 colorectal cancer cells

Yu-Jen Chen[a,*], Cheng-Deng Kuo[b,*], Yin-Meng Tsai[c], Chih-Chia Yu[c], Guang-Sheng Wang[d] and Hui-Fen Liao[b,c]

Norcantharidin (NCTD), a chemically modified form of cantharidin, is a potential anticancer drug. This study investigated the effect of NCTD on anoikis in CT26 colorectal adenocarcinoma cells. NCTD treatment of CT26 cells showed a dose-dependent and time-dependent decrease in viability and cell proliferation. Growth inhibition was accompanied by cell cycle arrest in the S and G_2/M phases. Mitogen-activated protein kinase expression, assayed by Western blot, was unchanged except for Jun-N-terminal kinase (JNK). At 24 h of treatment with 0–20 μmol/l NCTD, JNK expression increased at 24 h, but then decreased at 48 h; in contrast, the phosphorylated JNK levels markedly increased. JNK inhibitor (SP600125) in the culture effectively blocked NCTD-induced cytotoxicity and detachment of cells. CT26 cells treated with NCTD not only displayed inhibited cell adhesion and down-expression of integrin β1, but also changed from being shuttle-shaped to round, the latter cells being more susceptible to anoikis-mediated apoptosis. Flow cytometric assay of the DNA content in NCTD-treated CT26 cells at 24 and 48 h showed a marked increase in the sub-G_1 level, indicating that NCTD induced apoptosis. NCTD inhibited the viability of CT26 cancer cells preferentially over normal bone marrow and mononuclear cells. NCTD inhibits CT26 cancer cells by blocking proliferation and inducing anoikis-mediated apoptosis, a process that might be regulated by JNK activation.
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[a]Department of Radiation Oncology, Mackay Memorial Hospital, [b]Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, and [c]Department of Biochemical Science and Technology, National Chiayi University, Chiayi, Taiwan and [d]Wan-Hui Pharmaceutical group, Beijing, China

Correspondence to Dr Hui-Fen Liao, PhD, Department of Biochemical Science and Technology, National Chiayi University, 300 University Road, Chiayi 600, Taiwan
Tel: +886 5 2717779; fax: +886 5 2717780;
e-mail: liaohf@seed.net.tw

*Yu-Jen Chen and Cheng-Deng Kuo contributed equally to this work.

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Introduction

Norcantharidin (NCTD), a demethylated form of cantharidin derived from blister beetles (*Mylabris phalerata* Pall.), is currently being used as an anticancer drug in China. Cantharidin is a potent serine/threonine protein phosphatase 1 and 2A inhibitor [1] and has antitumor properties [2]; however, it also causes urinary organ toxicities [3]. NCTD is easier to synthesize and might be less toxic, while retaining anticancer activity [4]. Oral cancer KB cells are more sensitive to NCTD-induced cytotoxicity than normal keratinocytes [5], suggesting a preferential anticancer cell effect. NCTD has been shown to inhibit the proliferation of many tumor cells *in vitro*, such as cells from hepatoma [6], leukemia [7], and gallbladder carcinoma [8]. In in-vivo animal models, it has caused decreased tumor growth and prolonged survival [9]. The antitumor effects of NCTD can include interruption of DNA synthesis, retardation of progression through the cell cycle, induction of apoptosis via *p53* gene expression, and Bcl-2 regulation [10]. Other possible mechanisms remain to be investigated. Specifically, we are interested in the effects of NCTD on anoikis and mitogen-activated protein kinases (MAPKs).

Anoikis, that is, apoptosis resulting from loss of cell-matrix interactions with resulting deprivation of adhesion-derived signals, might act as a physiological barrier to the proliferation and metastasis of tumor cells [11,12]. Most tumor cells constitutively express adhesion molecules (such as integrins). Anoikis-resistant phenotypes have dysregulated intracellular signaling, such as the Rho kinase-dependent pathway [13]. Restoring the expression of tumor-suppressor genes such as phosphatase and tensin homolog (*PTEN*) and $p16^{INK4a}$ can induce anoikis in such cells [14,15]. In an earlier study, we demonstrated that NCTD inhibits metastasis, apparently by the down-expression of adhesion molecules and a resulting decrease in cell adhesion, although the actual mechanisms involved are still unclear [16].

MAPKs are proline-directed serine/threonine kinases, which respond to chemical and physical stress by connecting cell-surface receptor responses to the activity of regulatory proteins [17]. Three members of the MAPK family are known: two members, Jun-N-terminal kinase (JNK) and P38, are activated by a broad number of stress-related stimuli [18], whereas a third, extracellular

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signal-regulated kinase (ERK), is largely activated by growth factor signals [19]. MAPK activation is followed by phosphorylation of a variety of cytosolic substrates, which are then translocated to the nucleus where they activate transcription factors such as AP-1 and Ets-1 [19]. P38 and JNK mediate apoptotic signals, whereas ERK promotes cell growth, differentiation, and proliferation [20]. The MAPK signaling pathway, which plays an important role in regulating the growth and survival of tumor cells, is an attractive pathway for anticancer therapies [21].

This study was designed to examine the antitumor effect of NCTD exerted through anoikis-mediated apoptosis in murine colorectal adenocarcinoma CT26 cells, including an assessment of the expression of MAPKs in NCTD-treated cells.

Materials and methods

Materials and cells

NCTD [exo-7-oxabicylo-(2.2.1) heptane-2,3-dicarboxylic anhydride] was kindly provided by Professor Guang-Sheng Wang (Beijing, China). CT26 cells, that is, N-nitroso-*N*-methyl urethane-induced mouse undifferentiated colorectal carcinoma cells of BALB/c origin, were purchased from the American Type Culture Collection (Manassas, Virginia, USA) and were cultured in RPMI-1640 medium (Gibco, Grand Island, New York, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, Utah, USA) at 37°C in a humidified 5% CO_2 incubator. The cells were passaged every 2–3 days with Tris–EDTA–glucose solution (0.25% trypsin, 0.1% EDTA, and 0.05% glucose in Hanks' balanced salt solution) and maintained in exponential growth. MAPK inhibitors [SP600125 (JNK), PD98059 (ERK), and SB203580 (P38)] and antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), [integrin β1, JNK, ERK, P38, and phosphorylated-JNK (p-JNK)] were purchased from Biosource (Camarillo, California, USA). Wright's stain, Trypan blue dye, MTT, and other chemical reagents were purchased from Sigma (St Louis, Missouri, USA). Fluorescein isocyanate-conjugated anti-bromodeoxyuridine (BrdU) and propidium iodide (PI) were purchased from Becton Dickinson (Lincoln Park, New Jersey, USA).

Cell viability

CT26 cells (1×10^5 cells/ml) were cultured in 3.5-cm dishes for 12 h; they were then treated with NCTD (at concentrations ranging from 0 to 100 μmol/l) in each well for 12, 24, and 48 h. At the end of the incubation period, the culture dishes were scraped with a rubber policeman, to collect all adhesive and nonadhesive cells. Cell viability was measured by Trypan blue dye exclusion. To assess the influence of MAPK in NCTD-treated CT26 cells, MAPK inhibitors (10 μmol/l SP600125, 10 μmol/l PD98059, and 10 μmol/l SB203580) were added to NCTD-treated CT26 cells and cocultured for 24 and 48 h. The cells were collected; cell viability (%) was measured by Trypan blue dye exclusion, and cell morphology observed under a microscope at a magnification of $\times 100$. To compare the viability of normal and cancer cells treated with NCTD, normal actively dividing bone marrow (BM) cells and nondividing mononuclear cells (MNCs) were used. Nonadherent BM cells, which are enriched with hematopoietic stem cells, were eluted from the femurs of mice and allowed to adhere for 90 min, after which cells at a concentration of 1×10^5 cells/ml were collected for cell-viability assay. MNCs were isolated from human peripheral blood by centrifugation on a density gradient (Ficoll-Hypaque, 1.077 g/ml, Amersham Pharmacia Biotech, Aylesbury, UK). Viability of cells in a concentration of 1×10^5 cells/ml was assessed as above. The effects of NCTD on both tumor and normal cells were assessed by comparing the results of the Trypan blue dye test, to calculate the rate of inhibition of cell growth. IC_{50} values and 95% confidence intervals (CIs) were calculated by GraphPad Prism 4 software (GraphPad Software, San Diego, California, USA).

Analysis of cell proliferation by bromodeoxyuridine labeling

BrdU incorporation analysis was modified as described by Kikuchi *et al.* [22]. In brief, cells were treated with NCTD (50 μmol/l) for 4, 6, 8, and 12 h; they were then harvested with fluorescein isocyanate-conjugated anti-BrdU (10 μmol/l) for 1 h to allow BrdU incorporation into newly synthesized DNA. The cells were then washed with 10% FCS/phosphate-buffered saline (PBS), and fixed with ice-cold 70% ethanol for at least 30 min. Cells were collected and washed with 10% FCS/PBS and analyzed on a FACScan flow cytometer (Becton Dickinson).

Cell cycle analysis by flow cytometry

Cells were treated with NCTD (50 μmol/l) for 0, 4, 6, 8, and 12 h, washed with 10% FCS/PBS, and fixed with ice-cold 70% ethanol for at least 30 min. Cells were collected, washed with 10% FCS/PBS, and incubated with 0.1% Triton X-100, 0.2 mg/ml RNase A (Sigma), and 10 μg/ml PI for 30 min at 37°C in the dark. To determine DNA indices, data acquisition and cell cycle analysis were performed on a FACScan flow cytometer with the accompanying CellQuest software (Becton Dickinson).

Adhesion assay

For the adhesion assay, 10^4 cells/well were cultured in a 96-well microplate (coated with 10 mg/ml fibronectin) for 12 h and then incubated with different concentrations of NCTD (0–100 μmol/l) or/and MAPK inhibitors (10 μmol/l SP600125, 10 μmol/l PD98059, and 10 μmol/l SB203580) for a further 48 h. The plate was then washed twice and nonadhering cells were aspirated. MTT reagent was

added to each well and incubated for 2 h at 37°C to allow conversion of tetrazolium salts to a colored formazan product. The optical density was measured spectrophotometrically at a wavelength of 550 nm to calculate the percentage of adherent cells. The morphology of cells after treatment with NCTD and MAPK inhibitors was photographed with a microscope at a magnification of × 400.

Morphological analysis of detached cells after norcantharidin treatment

Cells were treated with NCTD (50 μmol/l) for 48 h; nonadherent cells were collected, and their morphology examined using Wright's and PI stains. For the former, cells were cytocentrifuged onto a microscope slide using a Cytospin (Shandon Southern Instrument, Sewickly, Pennsylvania, USA), stained with Wright's stain, and observed under an inverted microscope (Olympus, Tokyo, Japan) at a magnification of × 1000. For PI staining, detached cells were fixed with 70% ethanol for 30 min, washed with PBS, stained with PI (10 μg/ml), and photographed under a fluorescence microscope at a magnification of × 400.

Western blot analysis

For Western blot analysis, 10^7 CT26 cells were cultured in 10-cm dishes with NCTD at various concentrations (0, 12.5, 25, 50, and 100 μmol/l) for 48 h, after which they were scraped from the culture dish and disrupted with ×2 concentrated electrophoresis sample buffer (1 mol/l Tris, pH 6.8, 5% SDS, 40% glycerol, 0.005% bromophenol blue, and 8% β-mercaptoethanol). The samples were centrifuged for 5 min and the supernatant subjected to gel electrophoresis using 10% (w/v) SDS–polyacrylamide gels. The protein samples were then blotted on a polyvinylidene fluoride membrane. Primary antibodies (anti-JNK, anti-ERK, anti-P38, anti-p-JNK, and integrin β1) were used at a dilution of 1 : 1 000 and detected using horseradish peroxidase-conjugated antimouse immunoglobulin (1 : 10 000-fold dilution) followed by enhanced chemiluminescence using ECL kits (Amersham Pharmacia Biotech). Anti-GAPDH antibody was used as an internal control. Relative protein levels were determined by densitometry using ImageJ software (Version 1.36b; National Institutes of Health, Bethesda, Maryland, USA). The mean values were normalized to the internal GAPDH control and were calculated from at least three independent experiments.

Apoptosis assay

For the apoptosis assay, cells were treated with NCTD (50 μmol/l) for 0, 16, 24, and 48 h, washed with 10% FCS/PBS, and fixed with ice-cold 70% ethanol for at least 30 min. They were stained with 10 μg/ml PI for 30 min at 37°C in the dark, and DNA sub-G_1 phase was assessed by flow cytometry (Becton Dickinson).

Data analysis

Results are expressed as the mean ± standard error (SE) from at least three experiments. For flow cytometric analysis of BrdU uptake, cell cycle, and apoptosis, the acquisition was at least 10 000 events for each group. Statistical comparisons were performed using Student's *t*-test or analysis of variance as appropriate. Differences were considered significant at a P of < 0.05. All statistical analyses were carried out using GraphPad Prism 4 (GraphPad) and SigmaPlot (Version 9.0; Systat Software Inc., San Jose, California, USA) software.

Results

Norcantharidin inhibition of CT26 cells

The numbers of viable cells were markedly decreased by NCTD in a dose-dependent and time-dependent manner (Fig. 1). The calculated IC_{50} concentration was 20.13 μmol/l (95% CI: 16.40–23.86) for treatment for 24 h and 20.09 μmol/l (95% CI: 17.68–20.50) for 48 h.

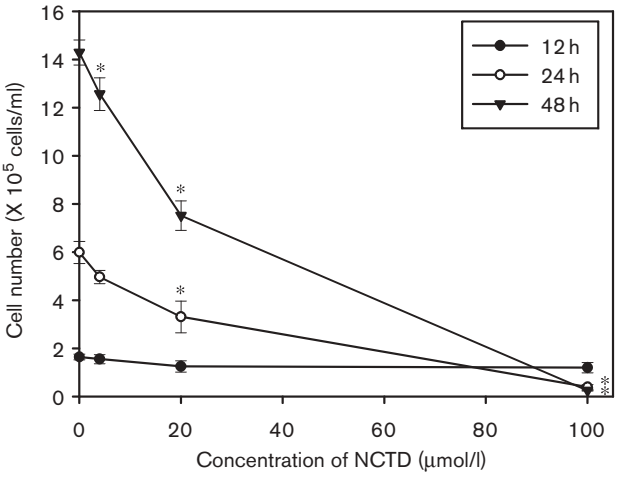
Inhibition of proliferation of CT26 cells

BrdU incorporation in NCTD-treated cells decreased significantly in a time-dependent manner, indicating that NCTD inhibited cell proliferation (Fig. 2). After 12 h of treatment, the number of BrdU-positive cells had decreased to 48.0%.

Cell cycle arrest

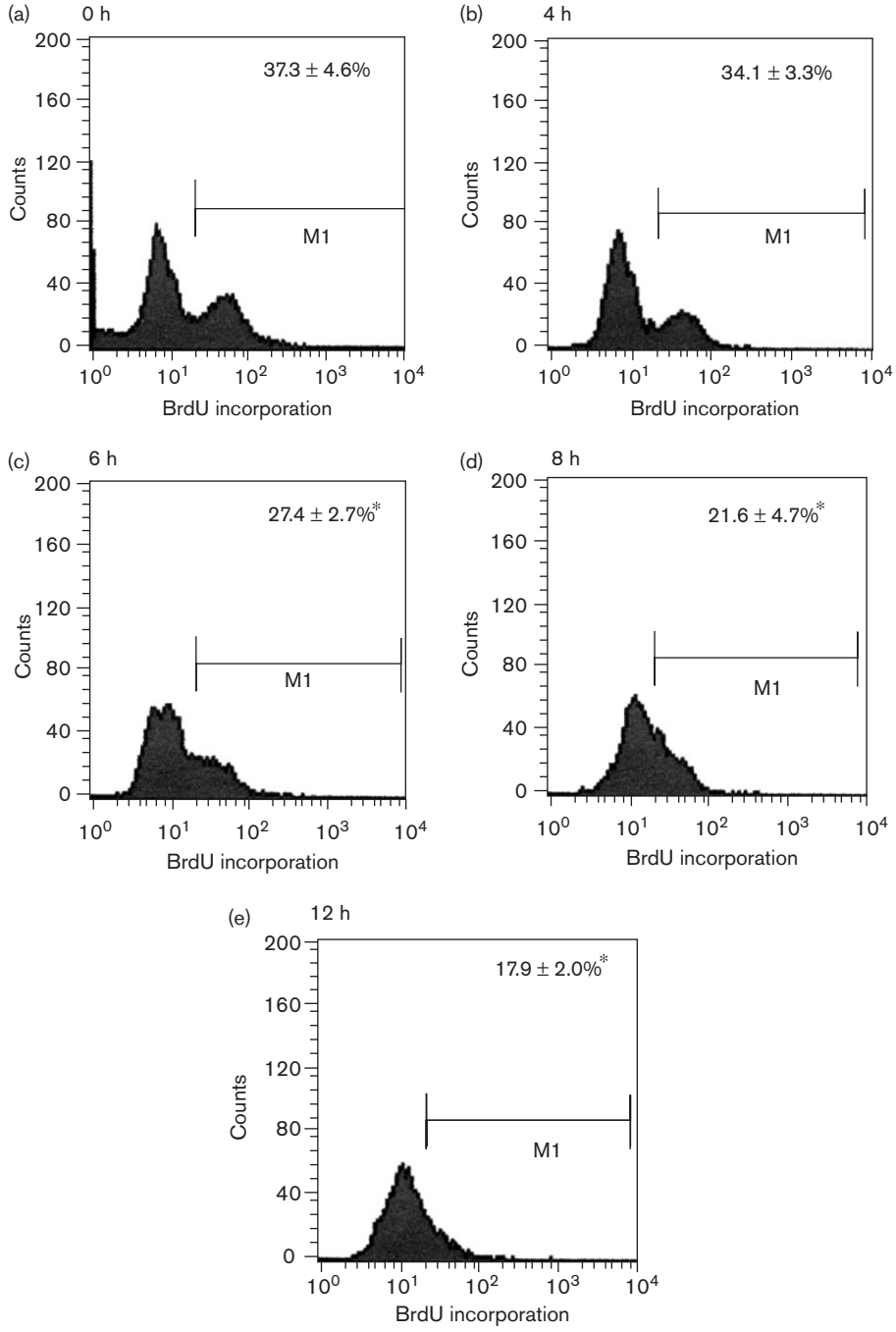
NCTD treatment for 4 and 6 h resulted in a slight cell cycle arrest in the S phase as demonstrated by flow

Fig. 1



Cytotoxicity assay of norcantharidin (NCTD)-treated CT26 colorectal cancer cells. Cells were cultured with NCTD (0–100 μmol/l) for 12, 24, and 48 h, and cell viability was measured by Trypan blue dye exclusion. Data from at least three separate experiments are expressed as mean ± SE. *Significant decrease in viable cells compared with untreated control cells.

Fig. 2



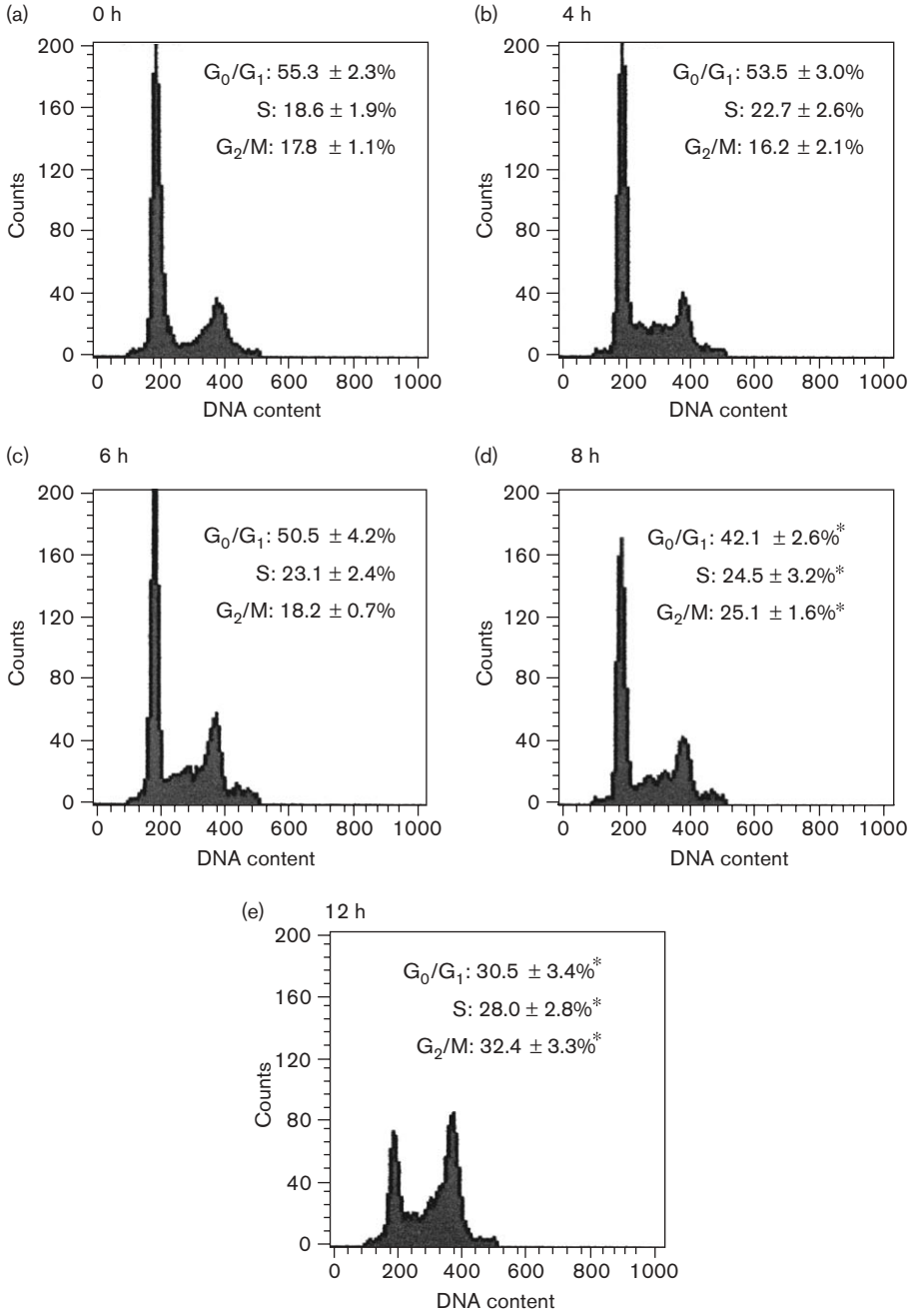
Cell proliferation assay of norcantharidin (NCTD)-treated CT26 colorectal cancer cells. (a–e) Cells were treated with NCTD (50 μmol/l) for 0, 4, 6, 8, and 12 h, respectively; harvested with fluorescein isocyanate-conjugated anti-bromodeoxyuridine (BrdU, 10 μmol/l) for 1 h; and then collected, fixed, and assayed by flow cytometry. Data from at least three separate experiments are expressed as mean ± SE. *Significant decrease in BrdU uptake compared with untreated control cells.

cytometry (Fig. 3). With longer treatment (8 and 12 h), there was marked arrest in the S and G_2/M phases. Compared with control cells at 0 h, the numbers of cells arrested in the S and G_2/M phases at 12 h were 1.5-fold and 1.8-fold higher, respectively.

Mitogen-activated protein kinase expression on norcantharidin-treated CT26 cells

Untreated CT26 cells expressed P38, ERK, and JNK at 24 and 48 h as shown by Western blot. At an NCTD concentration of 100 μmol/l, the level of these MAPKs

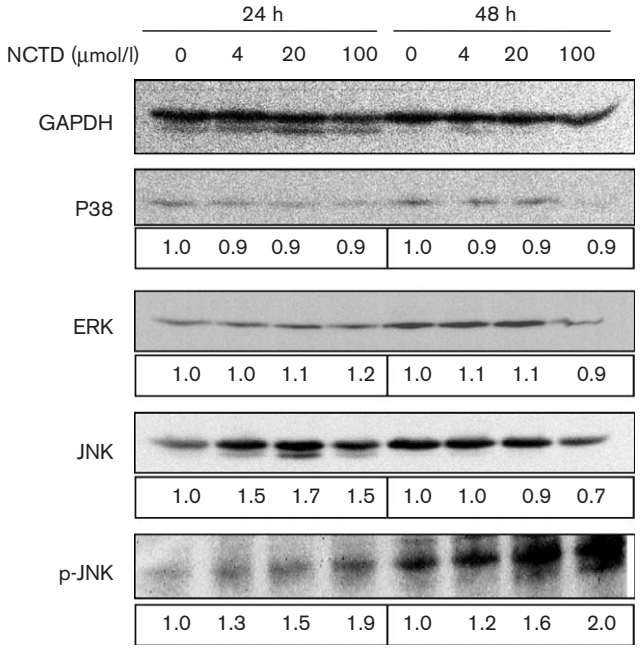
Fig. 3



Cell cycle analysis of norcantharidin (NCTD)-treated CT26 cells. (a–e) Cells were treated with NCTD (50 μmol/l) for 0, 4, 6, 8, and 12 h, respectively; then washed with 10% fetal calf serum/phosphate-buffered saline (FCS/PBS); and fixed with ice-cold 70% ethanol for at least 30 min. Cells were collected, washed with 10% FCS/PBS, and incubated with 0.1% Triton X-100, 0.2 mg/ml RNase A and 10 μg/ml propidium iodide for 30 min at 37°C in the dark. Cell cycle analysis was performed by flow cytometry. Data from at least three separate experiments are expressed as mean ± SE. *Significant cell cycle arrest compared with untreated control cells.

decreased (Fig. 4), suggesting that severe cytotoxicity resulted in the down-expression of these proteins. We therefore decreased the NCTD concentration in subsequent experiments to yield only moderate cytotoxicity. At NCTD concentrations of 0–20 μmol/l, there were no obvious changes in P38 and ERK at either 24 or 48 h. JNK expression, however, was increased at 24 h but slightly decreased at 48 h. Further assessment of p-JNK showed that it markedly increased in a concentration-dependent and time-dependent manner. CT26 cells had almost no

Fig. 4



Expression of mitogen-activated protein kinases in norcantharidin (NCTD)-treated CT26 cells by Western blot analysis. Cells were cultured with NCTD at various concentrations (0–100 μmol/l) for 24 and 48 h. Aliquots of lysed cell extracts were subjected to Western blot analysis and probed with antibodies specific for P38, extracellular signal-regulated kinase (ERK), Jun-N-terminal kinase (JNK), and phosphorylated JNK (p-JNK). Identical results were obtained in three independent experiments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Relative protein levels were determined by densitometry using ImageJ software.

p-JNK expression when cultured for 24 h in the absence of NCTD. NCTD, however, stimulated a high expression of p-JNK, especially at 48 h.

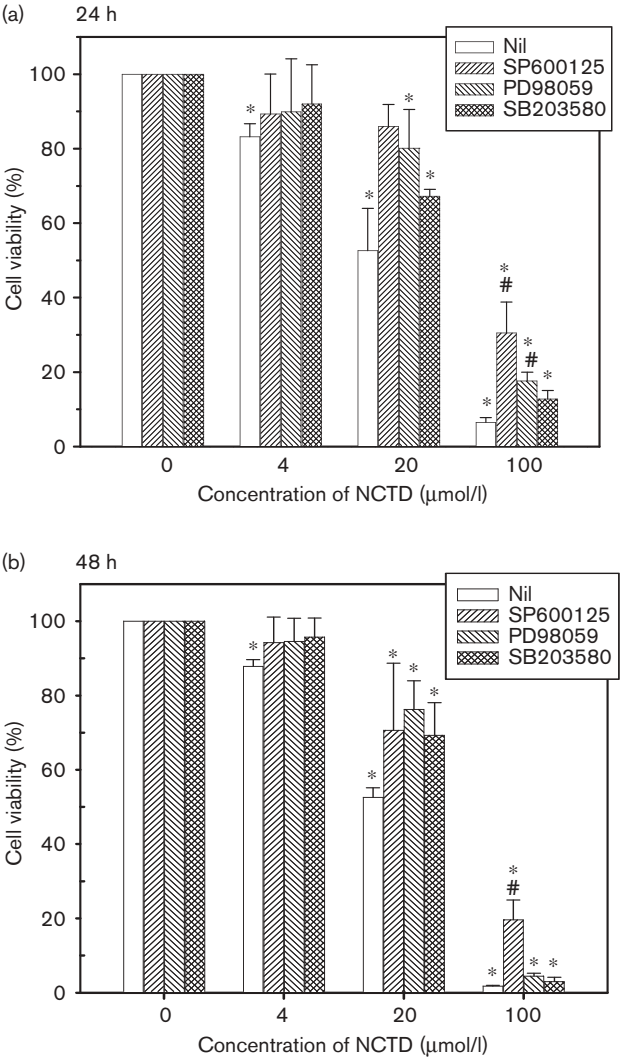
Effect of mitogen-activated protein kinase inhibitors on norcantharidin-treated CT26 cells

To clarify the exact role of MAPKs in NCTD-treated CT26 cells, MAPK inhibitors (SP600125, PD98059, and SB203580) were used. The cell viability of NCTD-treated CT26 cells, with and without the addition of MAPK inhibitors, is shown in Fig. 5. After cotreatment of CT26 cells for 24 (Fig. 5a) and 48 h (Fig. 5b) with NCTD (0–100 μmol/l) and MAPK inhibitors (10 μmol/l SP600125, 10 μmol/l PD98059, and 10 μmol/l SB203580), cell viability markedly decreased. At an NCTD concentration of 100 μmol/l, cell viability was less than 20% at 24 and less than 10% at 48 h, with the exception of cells treated with SP600125 (a JNK inhibitor).

Effect of norcantharidin on cell adhesion

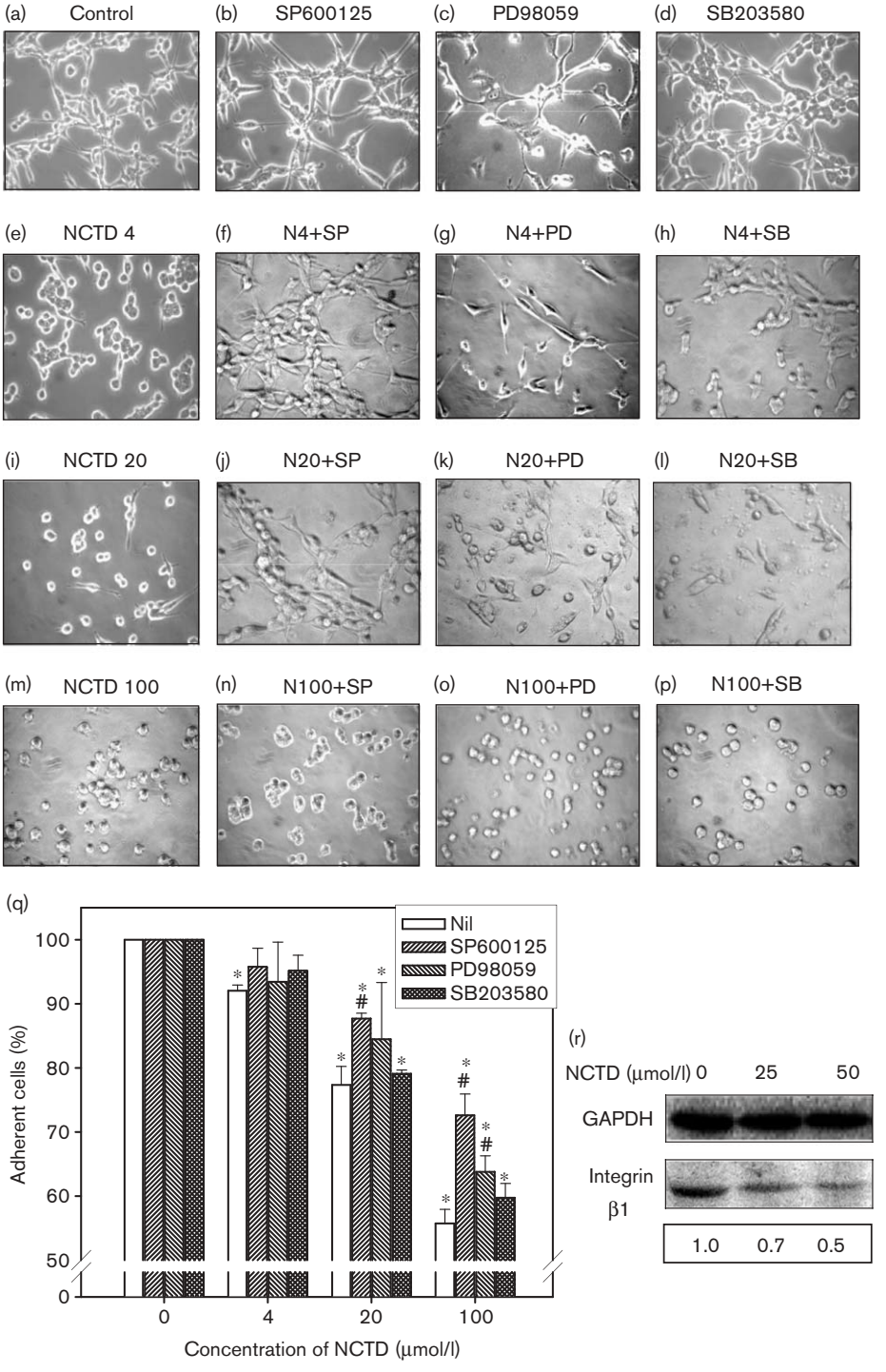
The adhesion assay showed a concentration-dependent effect of NCTD, with the cells becoming markedly less adherent and changing from a spindle-shaped (Fig. 6a) to a more rounded form (Fig. 6e, i, and m). Morphological observation of cells treated with MAPK inhibitors, especially SP600125 (10 μmol/l), showed that JNK antagonized the NCTD-induced effect on cell adhesion. When the NCTD concentration was 100 μmol/l, the addition of JNK inhibitor increased cell adherence to about 30.3% (Fig. 6q). In addition, the expression of the

Fig. 5



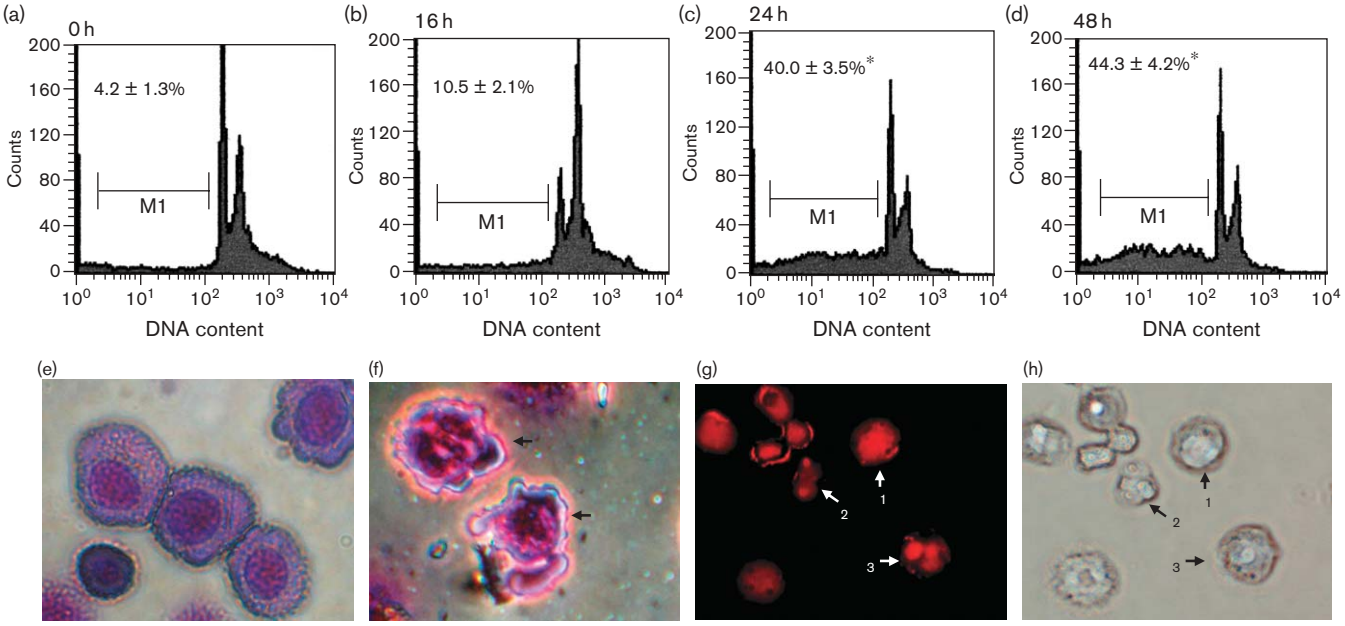
Cell viability (%) assay when mitogen-activated protein kinase (MAPK) inhibitors were added to norcantharidin (NCTD)-treated CT26 colorectal cancer cells. Cells were cultured for 12 h, then pretreated with NCTD (0–100 μmol/l) in each well for 2 h, and further cultured with or without MAPK inhibitors (SP600125, PD98059, and SB203580, all at concentrations of 10 μmol/l). After 24 and 48 h, cells were collected, and cell viability (%) was determined by Trypan blue dye exclusion. (a) Cell viability values (%) with NCTD and MAPK inhibitor treatment for 24 h are shown. (b) Cell viability values (%) with NCTD and MAPK inhibitor treatment for 48 h are shown. Data from three separate experiments are expressed as mean ± SE. *Significant change compared with untreated control cells. #Significant change compared with cells treated with NCTD at the same concentration but without an inhibitor (nil group).

Fig. 6



Adhesion assay assessing norcantharidin (NCTD) effects on CT26 cells. Cells were seeded in a 96-well plate for 12 h; then treated with various concentrations of NCTD (0–100 μmol/l) and/or 10 μmol/l mitogen-activated protein kinase (MAPK) inhibitor PD98059 (extracellular signal-regulated kinase), SP600125 (Jun-N-terminal kinase), and SB203580 (P38); and cultured for a further 48 h. (a–p) Morphological observation of CT26 cells. Cells were treated with NCTD and MAPK inhibitors for 48 h, and photographed under a microscope at a magnification of ×400. (q) Percentage of adherent cells (%). (r) Expression of integrin β1 by Western blot analysis and the relative protein levels were determined by densitometry using ImageJ software. Cells were treated with NCTD and MAPK inhibitors for 48 h, and then the nonadherent cells were removed, the plate was washed twice with phosphate-buffered saline, and the adherent cells were assayed by the MTT method. Data are shown as mean ± SE of at least three determinations. *Significant change compared with untreated control cells. #Significant change compared with cells treated with NCTD at the same concentration but without an inhibitor (nil group).

Fig. 7



Apoptosis-inducing effect in norcantharidin (NCTD)-treated CT26 cells. (a–d) Cells were treated with NCTD (50 μmol/l) for 0, 16, 24, and 48 h, respectively; then fixed with ice-cold 70% ethanol for at least 30 min; and stained with 10 μg/ml propidium iodide (PI) for 30 min at 37°C in the dark. In the apoptotic assay, analysis of the DNA sub-G_1 phase was performed by flow cytometry. Data from at least three separate experiments are expressed as mean ± SE. *Significant change compared with untreated control cells. Wright's stain analysis of untreated CT26 cells (e) and detached cells (f) treated with NCTD (50 μmol/l) for 48 h, photographed under a microscope at a magnification of × 1000. (g) PI staining of detached cells treated with NCTD for 48 h, and then photographed under a fluorescence microscope and (h) inverted microscope at a magnification of × 400. (arrow: apoptotic bodies).

adhesion molecule integrin β1 was markedly decreased by NCTD treatment (Fig. 6r).

Apoptosis and morphology assay of detached cells

For the apoptosis assay, sub-G_1 phases of NCTD-treated CT26 cells were determined by flow cytometry. NCTD treatment for 16 h only slightly increased cells in the sub-G_1 phase, but markedly induced arrest in the G_2/M phase (Fig. 7a and b, cf. Fig. 3). Apoptosis was obviously increased after NCTD treatment for 24 h (Fig. 7c). After 48 h culture with NCTD, cells in the sub-G_1 phase increased 10.5-fold compared with control cells (Fig. 7d). Most detached cells treated with NCTD (50 μmol/l) contained apoptotic bodies as shown on both Wright's stain (Fig. 7e and f) and PI stain (Fig. 7g and h), with shrinkage of nuclei and fragmentation of DNA.

Norcantharidin effect on the viability of CT26 cells and normal mononuclear cells and bone marrow cells

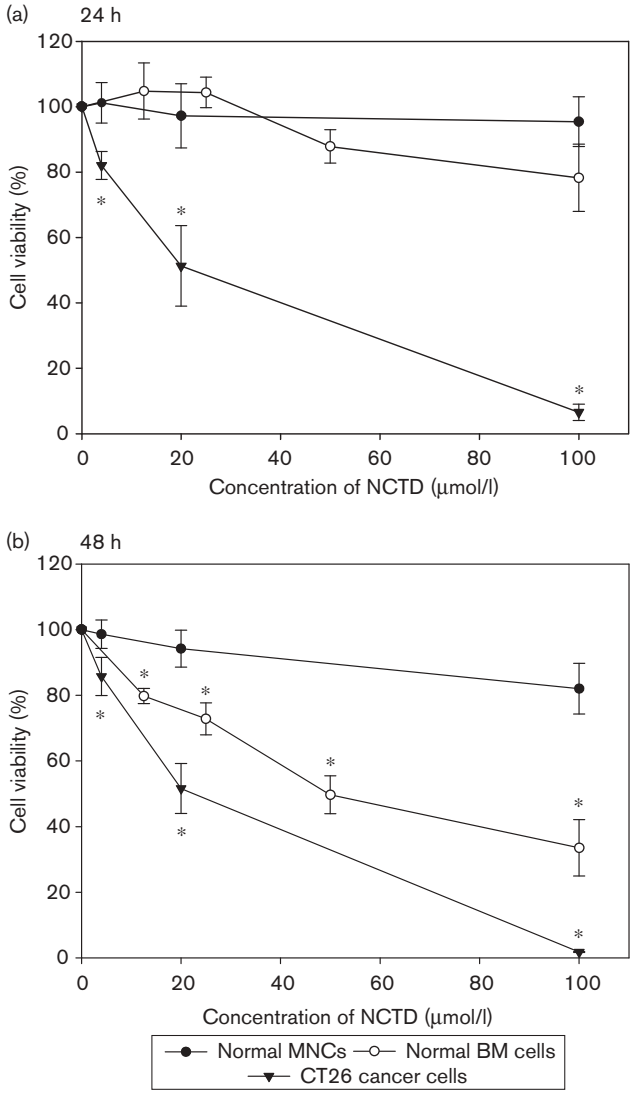
NCTD (0–100 μmol/l) treatment markedly decreased the viability of CT26 cells (Fig. 8). After 24 h, however, NCTD, at concentrations that were toxic to CT26 cells, did not inhibit the growth of BM cells and MNCs. At 48 h, NCTD only moderately reduced the number of viable BM cells, but still had no cytotoxic effect on MNCs. No morphological changes typical of apoptosis were observed in BM cells and MNCs treated with NCTD (data not shown). These results indicate that NCTD is toxic to CT26 colorectal cancer cells in concentrations that have little or no effect on normal BM cells or MNCs.

Discussion

In the current study, we found that NCTD decreased the cell viability, proliferation, and adhesion of colorectal adenocarcinoma CT26 cells. The induction by NCTD of apoptosis in detached cells, specifically anoikis, might be regulated by JNK/MAPK. This is the first demonstration that NCTD-induced apoptosis of CT26 cells might be mediated by anoikis.

Anoikis was first discovered in epithelial and endothelial cells where the loss of cell–matrix contact resulted in this particular form of apoptosis [23]. In neoplastic cells, alteration of cell-adhesion molecules or regulators of apoptosis can lead to resistance to anoikis and facilitate cell growth that is independent of normal cell anchoring [24]. Anoikis *in vivo* might prevent detached cells from reattaching to new matrices and growing dysplastically

Fig. 8



Viability of mononuclear cells (MNCs), bone marrow (BM), and CT26 cells at the same concentration (1×10^5 cells/ml) after norcantharidin (NCTD) treatment. Human MNCs were isolated from peripheral blood by centrifugation on a density gradient (1.077 g/m). Murine BM cells were eluted from the femurs of mice, allowed to adhere for 90 min, and the nonadherent BM cells collected for cell viability assay. Aliquots of 1×10^5 cells/ml MNCs, BM, and CT26 cells were treated with NCTD (0–100 μmol/l) for (a) 24 and (b) 48 h, and viability was assessed by a Trypan blue dye exclusion test. Data from at least three separate experiments are expressed as mean ± SE. *Significant change compared with untreated control cells.

[25]. In our study, NCTD treatment of CT26 cells markedly decreased the percentage of adherent cells, and the cells became round (Fig. 6). The shape change has been explored in a culture system, in which round cells were more susceptible to apoptosis, whereas cells forced to spread out survived and proliferated [26]. The expression of adhesion molecules (such as integrin β1) was also markedly decreased, demonstrating that NCTD resulted in loss of tumor cell adhesion, thus increasing the possibility of anoikis. This is an effect that might be important to prevent tumor invasion and metastasis.

The anti-invasive activity of NCTD might be the result of a decrease in the ratio of matrix metalloproteinase 2 to its tissue inhibitor [8]. The exact anticancer mechanism of NCTD, however, remains poorly understood. We previously demonstrated that NCTD inhibited tumor invasion and metastasis in both in-vitro and in-vivo models [16]. NCTD not only decreases the activity of matrix metalloproteinase 2 and 9, but also eliminates cell adhesion via the down-expression of several cadherin–catenin adhesion molecules, including desmoglein, N-cadherin, α-catenin, and β-catenin [16]. Taken together, these studies suggest that NCTD can block tumor growth and progression by antiproliferative, anoikis-inducing, and antimetastatic effects.

NCTD has been shown to inhibit cell proliferation and DNA replication in several cancer cells, such as human leukemic HL-60 cells [7] and human gallbladder carcinoma GBC-SD cells [8]. NCTD-induced apoptosis might be caspase-dependent and effected through the mitochondrial pathway rather than by death receptor (Fas/FasL) signaling [27]. We demonstrated that NCTD not only decreased cell viability in a dose-dependent and time-dependent manner, but that it also reduced cell proliferation with cell cycle arrest in the S and G_2/M phases after 12 h of treatment. Longer treatment increased the number of cells in the sub-G_1 phase, indicating that apoptosis was being induced. We further demonstrated an increase in JNK expression in NCTD-treated cells, with a sharp increase in p-JNK at 48 h, an effect that was effectively blocked when JNK was inhibited by SP600125. This result was similar to that in a report on hepatoma HepG2 cells, demonstrating that NCTD treatment was associated with increased levels of phosphorylated forms and kinase activity of ERK and JNK. Modulation of the downstream transcription factors NF-κB and AP-1 was thought to be involved in NCTD-induced apoptosis [28].

JNK was originally identified by its ability to phosphorylate c-Jun, a component of the AP-1 transcription factor, which activates transcription in response to a plethora of extracellular stimuli [29]. JNK is important in a variety of physiological and pathological processes, including cell proliferation, survival, and death, and DNA repair and metabolism [17]. Givant-Horwitz *et al.* [30] indicated that expression and phosphorylation of all three MAPK members (JNK, P38, and ERK) is associated with better outcome and survival in patients with ovarian carcinoma. JNK phosphorylates and activates p53, which might lead to such cellular responses as cell cycle arrest and apoptosis [31]. She *et al.* [32] reported that JNKs are involved in resveratrol-induced p53-dependent

transcription activity and induction of apoptosis. Another study indicated that activation of JNK is modulated by intracellular reactive oxygen species, which serve as a regulator of anoikis [33]. Therefore, it seems that JNK might be an important factor in NCTD-mediated cytotoxicity, cell cycle arrest, and the cellular detachment that leads to tumor cell anoikis.

In conclusion, NCTD inhibits CT26 colorectal cancer cell growth and proliferation, causing arrest in the S and G_2/M phases of the cell cycle; modulates JNK/p-JNK expression; and decreases cell adhesion, leading to anoikis-mediated apoptosis in cells. The effect on JNK expression might change the state of the transcription of certain genes in NCTD-treated cells. The cytotoxic effects of NCTD are much more pronounced in CT26 cells than in normal nondividing MNCs and actively dividing BM cells, suggesting selective antitumor cell effects. NCTD is thus worth investigating further as a potential chemotherapeutic agent, particularly if this differential cytotoxic effect is found in other cell types and, most importantly, *in vitro*.

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