Anticancer activity and mechanisms of norcantharidin-Nd3II on hepatoma

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Norcantharidin (NCTD), a demethylated form of cantharidin, is currently used as an anticancer drug in China, but five newly synthesized derivatives have not been tested for antitumor efficacy. In this study, we investigated the in-vitro and in-vivo activity of five derivatives on Bel-7402, HeLa and PC-3M1E8 cell lines on a sulfarhodamine B assay. All of the derivatives showed significant antiproliferative activity, hence we elected to study further one of them, NCTD-Nd3II, in an in-vivo mouse model, and to examine its effects on cell cycle and protein expression. NCTD-Nd3II inhibited H22 tumors in mice in a dose-dependent manner with low toxicity. Flow cytometry results showed that apoptosis and G_2/M cell cycle arrest contributed to the cytotoxic and cytostatic effects of NCTD-Nd3II. Further studies showed that Bax and p21 protein expression was upregulated, whereas cyclin B1, Cdc-2 and Bcl-2 protein expression was downregulated. Our findings show that NCTD-Nd3II might be a promising chemotherapeutic agent for hepatomas.
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Introduction

Hepatoma (hepatocellular carcinoma) is the fifth most common malignancy in the world and causes more than 1 million deaths each year [1]. Of the estimated 350 000 new cases per year in Asia, one third occur in China [2] and the incidence in China has been increasing in recent years. Unfortunately, no effective chemotherapy for this disease exists at present.

Cantharidin, a 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid derivative [3], inhibits protein phosphatases type 2A [4,5] and some tumor cell lines, such as HeLa cells, murine ascites hepatoma or reticular cell sarcoma, but not murine erythroid leukemia cells *in vitro*, S180 *in vivo* and Walker tumor in rats [6]. Clinical trials indicated that cantharidin had effects on patients with primary hepatoma, but that the application was limited by its severe toxicity for mucous membranes, mainly in the gastrointestinal tract, ureter and kidney [6].

A series of bioactive analogues have been synthesized in an attempt to increase the utility and to reduce the toxicity of cantharidin. Norcantharidin (NCTD), the demethylated analogue of cantharidin, seemed to cause the least nephrotoxic and inflammatory side effects. NCTD inhibits the proliferation of a variety of human tumor cell lines [6–8]; in one study, it inhibited the growth of transplanted human hepatocellular carcinoma and prolonged host survival [9]. NCTD can stimulate bone marrow and increase peripheral leuokocytes significantly [10], which makes it useful in combination chemotherapy, as most chemotherapeutic agents suppress bone marrow.

NCTD has been used to treat cancer in China since 1984 [6], but its clinical effects are limited by its short half-life. Recent research has examined dosage reformations, but no attempts have been made to evaluate the effectiveness of NCTD derivatives. NCTD-La, NCTD-Gd2, NCTD-Nd2, NCTD-Nd3I and NCTD-Nd3II are derivatives of NCTD which have been synthesized by combining the parent compound with lanthanon. We examined the effects of these derivatives on several human cancer cell lines and an in-vivo mouse hepatoma model.

Materials and methods

Compounds preparation

NCTD, NCTD-La, NCTD-Gd2, NCTD-Nd2, NCTD-Nd3I and NCTD-Nd3II were synthesized by our laboratory with more than 95% purity [11]. They were dissolved in phosphate-buffered saline (PBS) to a concentration of 10 mmol/l as stock solution and were further diluted in PBS before use.

Cell culture

All human cell lines, including human cervical carcinoma (HeLa), human hepatoma (Bel-7402) and prostate cancer

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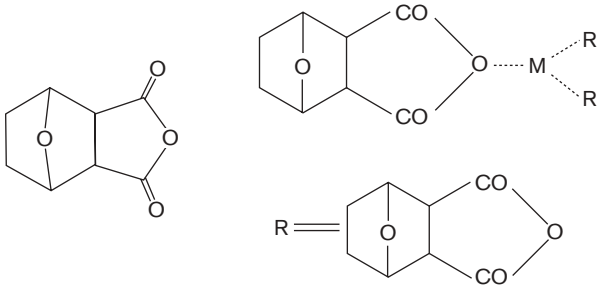


a: NCTD b: NCTD derivatives

M=La ---- NCTD-La
M=Gd ---- NCTD-Gd2
M=Nd ---- NCTD-Nd2, NCTD-Nd3I, NCTD-ND3II

(PC-3M-1E8), were purchased from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA). All cells were incubated in a humidified atmosphere of 37°C, with 5% CO_2 and 95% air. Cells were grown in RPMI-1640 medium (Gibco RBL, Grand Island, New York, USA) and supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Sulforhodamine B assay

HeLa, Bel-7402 and PC-3M-1E8 cells were harvested and resuspended to a final concentration of 10^4 cells/ml in fresh medium with 10% fetal calf serum. After 18–24 h, NCTD work dilution was added to reach designated final concentrations and cells were continued to be incubated for 48 h. After 48 h incubation, the upper layer of the solution was removed, 100 μl of 10% trichloroacetic acid was added and plates were fixed at 4°C for 1 h. Plates were air-dried and then stained for 10 min with 100 μl of 4% sulforhodamine B (SRB) dissolved in 1% acetic acid. At the end of the staining period, SRB was removed and plates were quickly rinsed with 1% acetic acid five times to remove unbound dye. After air drying, bound dye was solubilized with 100 μl of 10 mmol/l unbuffered Tris base (pH 10.5). The absorbent values were determined at 540 nm by the FLUOstar OPTIMA, microplate reader (BMG LABTECH, Offenburg, Germany) [12].

Anticancer evaluation on implanted mouse H22

Male CD-1 (ICR) mice (Beijing Vital Laboratory Animal Technology, Beijing, China), weighing 20–22 g, were used for implantation of hepatoma H22 (subcutaneously). Ascitic fluid (0.2 ml of 1 : 6 dilution) from tumor-bearing mice 7 days after tumor inoculation was injected (subcutaneously) into the armpit region of 40 mice. Four groups of 10 mice each were treated (intraperitoneally), 24 h after tumor inoculation, with either NCTD or NCTD derivatives once a day for 10 days. Normal saline was used as a negative control. The change in body weight was calculated by subtracting pretreatment weight from posttreatment weight. The tumor inhibition rate was calculated as $(1 - T/C) \times 100$, where T is the mean tumor weight of the treated group and C is the mean tumor weight of the negative control group [13].

Flow cytometry analysis

Cell cycle was determined according to the method described by Nicoletti *et al.* [14]. In summary, 2×10^6 cells treated with NCTD-Nd3II were harvested and washed twice with PBS. The cells were then fixed in 70% cooled ethanol at 4°C, until the beginning of the next procedure. The cells were centrifuged and ethanol was removed. Then the cells were incubated in PBS containing 10 μg/ml RNase A at 37°C for approximately 30 min. Before examining, 50 μg/ml propidium iodide was added to the cells. DNA content and cell cycle distribution were determined using a BECScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) equipped with an argon-ion laser set to excite at 488 nm. The percentage of apoptosis and analysis of the cell cycle distribution were performed using CellQuest and the Modfit software (Becton Dickinson).

Western blot

Bel-7402 cells were treated with NCTD-Nd3II in designed concentrations for 48 h. After washing twice with cold PBS, equal amounts of cells were lysed with lysis buffer containing 62.5 mmol/l Tris–HCl (pH 6.8), 4 mol/l urea, 2% sodium dodecyl sulfate, 10% glycerol, 0.4% Bromophenol and 5% β-mercaptoethanol. Equal amounts of protein from control cells and treated cells were subject to electrophoresis in a 12.5% sodium dodecyl sulfate–acrylamide gel followed by electroblot transfer to Hybond C membranes (Amersham, Arlington Height, Illinois, USA). For immunodetection, membranes were blocked with 3% Albumin Fraction V (ABS) in Tris-Buffered Saline Tween-20 (TBST) before incubation with mouse monoclonal anticyclin B, anti-Bcl-2, anti-Bax, anti-p21, anti-β-actin or rabbit monoclonal anti-cdc2 (Cell Signaling Technology, Beverly, Massachusetts, USA). Primary antibodies were detected using either a goat antimouse or goat antirabbit horseradish peroxidase-

Table 1 IC_{50} values of NCTD derivatives on different cell lines

Derivative	Cell line IC_{50} (μmol/l) (mean ± SD)		
	HeLa	PC-3M 1E8	Bel-7402
NCTD	48.9 ± 3.4	44.5 ± 1.9	53.4 ± 2.5
NCTD-La	11.4 ± 1.3***	12.2 ± 0.2***	15.7 ± 0.1***
NCTD-Gd2	16.8 ± 0.3***	14.0 ± 0.9***	14.8 ± 1.0***
NCTD-Nd2	15.5 ± 1.8***	14.9 ± 0.3***	15.6 ± 0.1***
NCTD-Nd3I	11.3 ± 0.7***	20.6 ± 0.1***	15.7 ± 2.3***
NCTD-Nd3II	16.6 ± 0.7***	18.8 ± 0.6***	19.0 ± 0.6***

Student's-*t* test, ***$P < 0.001$ compared with NCTD.
IC_{50} is the mean ± SD of three independent experiments.
NCTD, norcantharidin.

conjugated secondary antibody (Jackson Immuno Research, Baltimore, Pennsylvania, USA). The reactive band was identified using an enhanced chemiluminescent substrate to horseradish peroxidase (Amersham) and analyzed with the Image Acquisition and Analysis System (UVP, Upland, California, USA).

Statistics

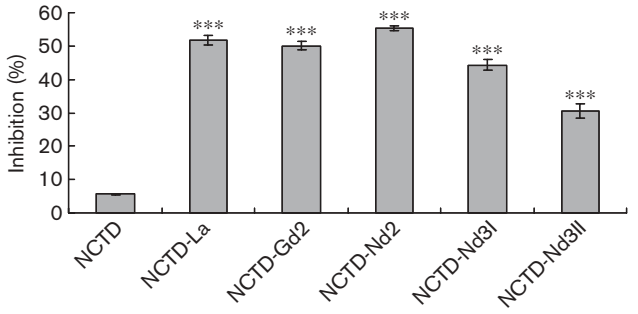
Student's *t*-test was used and $P < 0.05$ was considered significant.

Results

Antiproliferative activity of norcantharidin derivatives

In all cell lines, the inhibitory concentration of NCTD derivatives was less than half that of NCTD (Table 1). The IC_{50} values of NCTD-La and NCTD-Nd3I on HeLa cells were around 10 μmol/l, and the values for the other compounds were slightly higher, i.e. around 15 μmol/l. For PC-3 M1E8 cells, the IC_{50} values of NCTD-La, NCTD-Gd2 and NCTD-Nd2 were around 13 μmol/l, whereas the values for the other two derivatives were around 20 μmol/l. The derivatives IC_{50} values were significantly different from NCTD.

Fig. 1



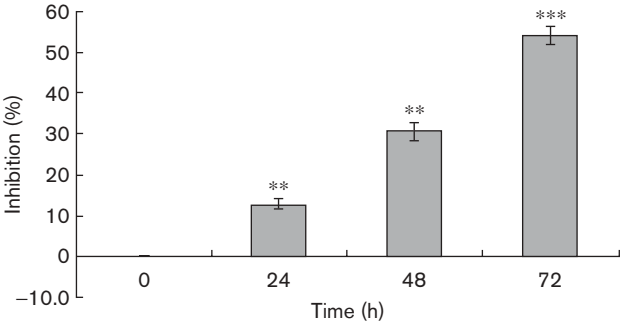
Antiproliferation effect on Bel-7402 cells treated with 12.5 μmol/l NCTD and derivatives for 48 h, evaluated by SRB assay. The inhibition is the mean ± SD of three independent experiments. Student's-*t* test, ***$P<0.001$ compared with NCTD. NCTD, norcantharidin; SRB, sulforhodamine B.

Table 2 Effect of different concentration of NCTD-Nd3II on the Bel7402 cell line (48 h)

NCTD-Nd3 II concentration (μmol/l)	OD value (mean ± SD)	Inhibition (%)
0	0.67 ± 0.20	0
6.25	0.66 ± 0.02	1.1
12.5	0.47 ± 0.01**	30.6
25	0.22 ± 0.01**	68.0
50	0.15 ± 0.02***	78.0

Student's-*t* test, **$P<0.01$, ***$P<0.001$ compared with control (0 concentration). OD values are the mean ± SD of three independent experiments. NCTD, norcantharidin; OD, optical density.

Fig. 2



Time-dependent effect of 12.5 μmol/l NCTD-Nd3II on the Bel-7402 cell line. The inhibition is the mean ± SD of three independent experiments. Student's-*t* test, **$P<0.01$, ***$P<0.001$ compared with 0 h. NCTD, norcantharidin.

Table 3 Effect of NCTD and NCTD-Nd3II on growth of H22 tumors

Agent	Body weight (g) (mean ± SD)		Tumor weight (g) (mean ± SD)	Tumor growth inhibition (%)
	Pretreatment	Posttreatment		
Control	25.40 ± 1.00	37.16 ± 3.13	1.43 ± 0.49	0
NCTD 2 mg/kg	24.62 ± 0.98	34.69 ± 1.97	0.90 ± 0.50*	37.5
NCTD-Nd3II 2 mg/kg	25.13 ± 0.96	37.59 ± 2.16	0.84 ± 0.39**	41.4
NCTD-Nd3II 4 mg/kg	25.01 ± 1.48	36.20 ± 4.74	0.64 ± 0.35***,Δ	55.6

NCTD, norcantharidin.
Student's *t*-test, *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with control; $^{\Delta}P<0.05$ compared with 2 mg/kg NCTD.

NCTD had less of an inhibitory effect on Bel-7402 cells than did the derivatives (Fig. 1), although NCTD-Nd3II had the lowest inhibitory effect of any derivative.

Inhibition observed with NCTD-Nd3II was concentration- (Table 2) and time-dependent (Fig. 2).

Inhibition of tumor growth by norcantharidin-Nd3II

Among the five NCTD derivatives, only NCTD-Nd3II shows an antitumor effect *in vivo*. Table 3 shows the effect of NCTD-Nd3II on the growth of H22 tumors.

Both NCTD-Nd3II and NCTD significantly suppressed the growth of H22 after 10 days of treatment (Table 3). At the end of the experiment, all the mice were alive. Body weight loss was significantly higher in the NCTD-treated group; while the NCTD-Nd3II-treated group also lost weight, the difference was not significantly different from control.

Effect of norcantharidin-Nd3II on cell cycle and apoptosis in Bel-7402 cells

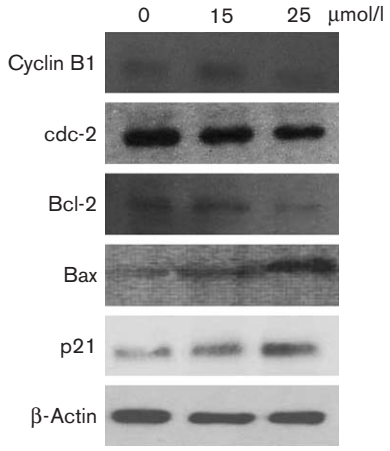
On the basis of results of the anticancer evaluation in the in-vivo mouse model, we elected to explore NCTD-

Table 4 Effect of NCTD-Nd3II on cell cycle and apoptosis on Bel-7402 cell line after 48 h

NCTD-Nd3 II concentration (μmol/l)	Cell cycle (%)			Apoptosis (%) (mean ± SD)
	G_1 (mean ± SD)	S (mean ± SD)	G_2 + M (mean ± SD)	
0	60.0 ± 11.8	30.2 ± 5.1	9.9 ± 6.7	3.7 ± 1.2
10	55.6 ± 7.9	29.71 ± 4.0	14.7 ± 7.2	11.2 ± 5.5
15	47.2 ± 5.0	29.8 ± 4.2	23.1 ± 0.7	14.4 ± 6.6*
20	45.8 ± 9.8**	28.6 ± 3.4	25.6 ± 7.3**	14.0 ± 6.7*
25	34.4 ± 3.6**	30.9 ± 4.2	34.8 ± 4.2***	20.5 ± 7.0***

NCTD, norcantharidin.
Student's *t*-test, *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with control (concentration 0).
Cell cycle (%) and apoptosis (%) are the mean ± SD of three independent experiments.

Fig. 3



Regulation of apoptosis and cell cycle-related protein expression on Bel-7402 cells treated with NCTD-Nd3II. Cellular lysate protein (40 μg/lane) was loaded on a 12.5% sodium dodecyl sulfate–polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose membranes. Immunoblots were probed with antibodies specific for cyclin B1, cdc-2, Bcl-2, Bax and p21. Lysates were from Bel-7402 cells treated with 0, 15 and 25 μmol/l NCTD-Nd3II for 48 h, respectively. NCTD, norcantharidin, SDS, sodium dodecyl sulfate.

Nd3II in our flow cytometry analysis. NCTD-Nd3II caused significant G_2/M arrest with a concomitant decrease of the cell population in G_1 phase (Table 4).

In addition, NCTD-Nd3II induced apoptosis in a significantly higher percentage of cells than did control (Table 4). The percentage of apoptotic cells was approximately five times higher in cells treated with 25 μmol/l than in control cells.

Regulation of apoptosis and cell cycle-related protein expression

To determine the relationship between NCTD-Nd3II-induced mitotic arrest and apoptosis, we initially examined the status of apoptosis-related proteins Bcl-2, Bax and p21 in Bel-7402 cells after treatment with NCTD-Nd3II. After 48 h of treatment, NCTD-Nd3II increased the expression of the proapoptotic proteins Bax and p21, and decreased the expression of the antiapoptotic protein Bcl-2 in a dose-dependent manner (Fig. 3).

NCTD-Nd3II also decreased the expression of cdc-2. As B-type cyclins are positive regulators of cdc-2 kinase, and as they function in meiosis and mitosis, we investigated the association between drug-induced G_2/M and alterations in cyclin B1 expression. NCTD-Nd3II treatment resulted in a greatly decreased accumulation of cyclin B1 protein in a concentration-dependent manner.

Discussion

We found that the in-vitro inhibitory effects of all the NCTD derivatives we studied were about three times higher than those of NCTD at the same concentrations. Although compounds that are effective *in vitro* might have less or no effect *in vivo*, in-vitro cytotoxicity against cancer cell lines from a particular organ generally predicts cytotoxicity against corresponding tumors in animals and humans. In in-vivo experiments, NCTD-Nd3II inhibited hepatoma H22 growth in mice more than the other derivatives did. The inhibitory effect was dose-dependent and did not appear to cause body weight loss. This finding suggests that NCTD-Nd3II might inhibit hepatoma growth in humans with low toxicity. NCTD-Nd3II induced inhibition in a dose- and time-dependent manner on Bel-7402 cells, which prompted us to further study the mechanism of NCTD-Nd3II at cellular and molecular levels. Flow cytometric analysis showed that Bel-7402 cells treated with different concentrations of NCTD-Nd3II for 48 h accumulated in the G_2/M phase of the cell cycle in a dose-dependent manner and that apoptosis increased.

Cell cycle dysregulation is a hallmark of tumor cells. Regulation of proteins that mediate critical events in the cell cycle can be a useful method for treating tumors [15]. The cell cycle is mediated by a highly conserved family of protein kinases, the cyclin-dependent kinases (cdks) [15–17]. Activation of a cdk requires binding to a specific regulatory subunit, termed a cyclin. Together, these cyclin/cdk complexes are the cell cycle regulators. B-type cyclins, which also associates with cdc-2, control entry into mitosis. The cyclin B/cdc-2 complex was originally defined as the maturation-promoting factor or M phase-promoting factor [18,19]. Regulation and the degradation of cyclin B/cdc-2 complexes at multiple levels ensure the tight regulation of the timing of mitotic entry and cell division. Without the synthesis of cyclin B before the G_2/M transition, cdc-2 remains inactive, and the cell does not enter mitosis. The cell cycle will arrest at the G_2 phase. If cyclin B is not degraded by ubiquitin [20], cells will not exit the mitosis. In this study, we found that

NCTD-Nd3II arrested Bel-7402 cells in the G_2/M phase, suggesting that NCTD-Nd3II might affect the cyclin B1/cdc-2 complex. Western blot analysis demonstrated that the expression of cyclin B1 and cdc-2 was decreased by NCTD-Nd3II, which suggests that NCTD-Nd3II arrests the cells at the G_2 phase and prevents them from entering the mitosis. We also found that NCTD-Nd3 II increased the expression of p21, which could arrest cell cycle progression at the G_2 phase by suppressing most cdk activity and binding to the proliferating cell nuclear antigen to inhibit the synthesis of DNA directly [21,22]. This finding implies that the upregulation of p21 can contribute to G_2/M arrest on Bel-7402 cells treated with NCTD-Nd3II.

Apoptosis is a genetically regulated biological process with two major pathways: the death receptor-induced extrinsic pathway and the mitochondria apoptosome-mediated apoptotic intrinsic pathway [23]. Bcl-2 family proteins have a central role in controlling the mitochondrial pathway. In humans, more than 20 members of this family have been identified including proteins that suppress apoptosis (Bcl-2, BCLX1, MCL1, BCLG, BCLW and A1) and proteins that promote apoptosis (BAX, BAK, BOK, BAD, BIK, BIMA and BID) [22,24]. The proapoptotic proteins and antiapoptotic proteins of the Bcl-2 family might turn apoptosis on and off heterodimers from among these proteins [25,26]. The heterodimerization results in mutual neutralization of the bound pro- and antiapoptotic proteins. Therefore, the balance between the expression levels of the protein units (e.g. Bcl-2 and Bax) is critical for cell survival or death. In Bel-7402 cells treated with NCTD-Nd3II, the level of Bax is increased and that of Bcl-2 is decreased concomitantly, suggesting that NCTD-Nd3II induced apoptosis through upregulation of Bax and downregulation of Bcl-2.

In conclusion, NCTD-Nd3II possesses antihepatoma activity both *in vitro* and *in vivo*. It exerts its antiproliferative activity via apoptosis and G_2/M cell cycle arrest and by regulating cyclin B1/cdc-2, p21 and Bcl-2/Bax. These results suggest that NCTD-Nd3II may have clinical applications in treating hepatoma.

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