Norcantharidin inhibits DNA replication and induces apoptosis with the cleavage of initiation protein Cdc6 in HL-60 cells

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Norcantharidin (NCTD), a demethylated form of cantharidin, is currently used as an anti-cancer drug in China. However, the exact anti-cancer mechanism of NCTD on human cancer cells remains poorly understood. In the present study, NCTD inhibited proliferation and DNA replication effectively in HL-60 cells. DNA replication-initiation protein Cdc6 was cleaved after 12 h treatment with NCTD. This cleavage generated a truncated Cdc6 fragment with a relative molecular weight of 49 kDa and elongated treatment with NCTD resulted in a complete loss of Cdc6. In addition, we found that Cdc6 was present in both non-chromatin- and chromatin-bound fractions in the untreated HL-60 cells, and NCTD treatment led to the cleavage of Cdc6 in both fractions. NCTD-induced cleavage of Cdc6 was prevented by pre-treatment with caspase-3 inhibitor, suggesting the involvement of caspase-3 activity in the process. Furthermore, NCTD treatment resulted in apoptotic changes including granular nuclear morphology, DNA laddering and sub-G_1 arrest in HL-60 cells. In conclusion, our study reveals that NCTD can inhibit DNA replication, and induce apoptosis and caspase-3-dependent cleavage of Cdc6. The anti-cancer effect of NCTD may be closely associated with the dysfunction of Cdc6 and our report is the first to put forward this point of view. *Anti-Cancer Drugs* **17:307–314**

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Introduction

Mylabris, the dried body of the blister beetle, has been used as a traditional Chinese medicine for over 2000 years. Its active constituent, cantharidin, possesses anti-tumor activity, but its clinical application is limited by the severe toxicity to the mucous membrane of gastrointestinal and urinary tracts [1]. Norcantharidin (NCTD), the demethylated form of cantharidin, has less toxicity and is easier to synthesize. In previous reports, NCTD could inhibit the proliferation of some cancer cells and had anti-tumor activity against transplanted hepatoma in a mouse model. Its mechanisms included inducing apoptosis, disturbing the cell cycle, and blocking tumor invasion and metastasis [2–5]. In addition, NCTD was proven to be a strong inhibitor of protein phosphatase types 1 and 2A (PP2A) [6,7]. PP2A dephosphorylates myriad substrates *in vitro* and is involved in the regulation of nearly all cellular activities. All these findings suggest that NCTD is a potential anti-tumor agent. However, the exact mechanism responsible for its anti-proliferation effect has not been thoroughly elucidated.

Initiation of DNA replication in eukaryotic cells requires sequential assembly of multiple proteins. Those proteins assembled before the initiation of DNA synthesis are origin recognition complex (ORC), Cdc6 and minichromosome maintenance (MCM) complex, and together they constitute a pre-replication complex (pre-RC) [8]. Cdc6 is essential for the formation and maintenance of pre-RCs, and acts as the rate-limiting step in the initiation of DNA replication. The central role of Cdc6 is to link ORC with MCM proteins to form pre-RCs at the origins of DNA replication [9]. In early G_1, Cdc6 is the first to be recruited by ORC and this binding of Cdc6 to ORC is a precondition for the consequent loading of MCM proteins onto chromatin [10]. Once MCM proteins are recruited to pre-RCs, DNA is licensed for replication. Cdc6 was proven to be an effective target to disturb DNA replication. Depletion of Cdc6 could prevent replication initiation and result in reductional mitosis in which cells randomly segregated their unreplicated chromosomes [11]. Silencing of the *cdc6* gene by antisense oligodeoxynucleotides and small-interfering RNA methods could effectively inhibit DNA replication in cultured human cells. In addition, silencing of the *cdc6* gene resulted in apoptosis in cancer cells rather than in normal cells [12].

In addition to regulating DNA replication, Cdc6 plays an important role in regulating cell death. Many apoptotic

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stimuli could lead to the destruction of Cdc6 [13–15]. Human Cdc6 was rapidly destroyed during the early stages of programmed cell death induced by the DNA-damaging drug adozelesin or by tumor necrosis factor (TNF)-α and cycloheximide [14]. Yim *et al.* reported that Cdc6 underwent caspase-3-mediated cleavage in the early stages of apoptosis induced by etoposide, paclitaxel, ginsenoside Rh2 or TNF-related apoptosis-inducing ligand (TRAIL) in HeLa cells and SK-HEP-1 cells [15]. Accordingly, it is indicated that Cdc6 proteins may be attractive targets for the development of effective anti-cancer drugs. Cdc6 was regulated by protein phosphorylation. In human cells, Cdc6 was phosphorylated and exported out of the nucleus at the G_1/S transition. PP2A could dephosphorylate Cdc6 through a subunit PR48 to control the initiation of DNA replication [16].

Taken together, we asked whether NCTD exerts its anti-cancer effect through regulating replication-initiation proteins such as Cdc6. In the present study, the HL-60 cell line was used to investigate the effects of NCTD on DNA replication and apoptosis. Cdc6 protein in NCTD-treated HL-60 cells was also analyzed by Western blotting. Our observations suggest that NCTD can inhibit DNA replication and induce apoptosis in HL-60 cells, and destruction of Cdc6 may contribute to the anti-cancer effect of NCTD.

Materials and methods

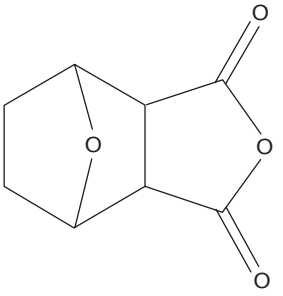
Cell line and regents

HL-60 cells were cultured in RPMI 1640 with 10% heat-inactivated FCS at 37°C in 5% CO_2. NCTD was a kind gift of Beijing Double-Crane Pharmaceutical (Beijing, China). The chemical structure of NCTD is shown in Fig. 1. Monoclonal Cdc6 antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, California, USA). Proteasome inhibitor MG132 and caspase-3 inhibitor Ac-DEVD-CHO were obtained from Sigma (St Louis, Missouri, USA) and BD Biosciences (San Jose, California, USA), respectively (stock solutions were made in DMSO)

Cell growth inhibition test

The MTT (Sigma) test was performed to evaluate the in-vitro cytotoxicity induced by NCTD on HL-60 cells. Briefly, HL-60 cells were plated in a 96-well plate (2×10^4 cells/well) and cultured in fresh medium with various concentrations of NCTD added. After incubation for 68 h, MTT was added to each well (100 μg/well) and incubated for an additional 4 h. The produced insoluble formazan was dissolved with 200 μl DMSO and the optical density was measured using an ELISA reader (Thermo Labsystems, Espoo, Finland) at wavelengths of 570 and 630 nm. Experiments were performed in triplicate. From these results, the percentage of live cells in each well could be estimated and plotted against the drug concentrations as dose–response curves from which the IC_{50} was derived.

Fig. 1



Chemical structure of NCTD.

DNA replication assay

DNA replication was measured by the incorporation of [^{3}H]thymidine in DNA. Proliferating HL-60 cells were seeded onto 96-well plates (2×10^4 cells/well) and treated with various concentrations of NCTD for 12 h. [^{3}H]Thymidine (1 μCi/well) was added 4 h before the end. The cells were then harvested onto glass fiber filters with an automatic harvester. Filters were dried and radioactivity was quantified with a liquid scintillation counter (Beckman, Fullerton, California, USA). The results are shown as counts per minute (c.p.m.). Experiments were performed in triplicate.

Flow cytometry

Aliquots of 1×10^5 cells were centrifuged at 1500 r.p.m. for 5 min, and the pellets were washed twice with PBS and fixed with 70% ethanol for 4 h. Cells were washed twice with PBS, resuspended in 1 ml solution (containing 3.4 mmol/l sodium citrate, 20 μg/ml propidium iodide and 100 μg/ml RNase A) and stored in the dark for 1 h. Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, California, USA).

Detection of apoptosis

Apoptotic nuclear morphology was assessed by Hoechst 33258 staining (Sigma). HL-60 cells were treated with 50 μmol/l NCTD for the indicated time. After collection, the cells were fixed with 3.7% paraformaldehyde for 2 h at room temperature, and then washed and stained with Hoechst 33258 (5 ng/ml) for 10 min at 37°C. Apoptotic nuclear morphology was observed with a fluorescence microscope (Nikon, Tokyo, Japan).

Isolation and analysis of apoptotic DNA fragments were performed as described [17]. After harvesting, the cell

samples were washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10 s with lysis buffer (1% NP-40, 20 mmol/l EDTA and 50 mmol/l Tris–HCl, pH 7.5; 10 µl/10^6 cells). After centrifugation for 5 min at 1600 *g*, the supernatant was collected and the extraction was repeated with the same amount of lysis buffer. The supernatants were brought to 1% SDS and treated for 2 h with RNase A (final concentration of 5 µg/µl) at 56°C, followed by digestion with proteinase K (final concentration of 2.5 µg/µl) for at least 2 h at 37°C. After addition of 0.5 volumes 10 mol/l ammonium acetate, the DNA was precipitated with 2.5 volumes ethanol, dissolved in gel loading buffer and separated by electrophoresis in 1.5% agarose gels.

Preparation of proteins and Western blotting

To prepare total proteins, cells were harvested and suspended in ice-cold lysis buffer containing 50 mmol/l Tris–HCl (pH 7.5), 150 mmol/l NaCl, 1% NP-40, 1 mmol/l PMSF and 10 U/ml aprotinin for 20 min, and then centrifuged at 12 000 r.p.m. for 10 min at 4°C. Supernatants were stored at −80°C before use.

The chromatin/nuclear matrix fraction assay was used as described [18]. In brief, HL-60 cells were washed 3 times with ice-cold PBS and then suspended with modified CSK buffer (10 mmol/l PIPES, pH 6.8, 100 mmol/l NaCl, 300 mmol/l sucrose, 1 mmol/l $MgCl_2$, 1 mmol/l EGTA, 1 mmol/l DTT, 1 mmol/l PMSF, 10 U/ml aprotinin, 20 µg/ml leupeptin, 5 mmol/l NaF and 0.5% Triton X-100). The cells were incubated on ice for 10 min. After low-speed centrifugation (3000 r.p.m., 3 min at 4°C), the supernatants were carefully removed and used as the non-chromatin-bound fraction (supernatant cytoplasmic fraction). The pellets were extracted twice more with ice-cold modified CSK buffer for 10 min on ice, resolved in ice-cold modified CSK buffer and used as the chromatin-bound fraction. Subsequently, the non-chromatin-bound fraction and chromatin-bound fraction were analyzed by Western blotting.

For the Western blotting analysis described in this paper, proteins were separated by SDS–PAGE, immunoblotted with primary antibodies (antibodies to Cdc6 and GAPDH) and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000). Proteins were then visualized using an enhanced chemiluminescence detection kit (Cell Signaling Technology, Beverly, Massachusetts, USA). GAPDH was used as a loading control.

Statistical analysis

Results are expressed as means ± SD from at least three experiments. Differences were considered significant at $P < 0.05$. All statistical analyses were carried out with SPSS 10.0 software.

Results

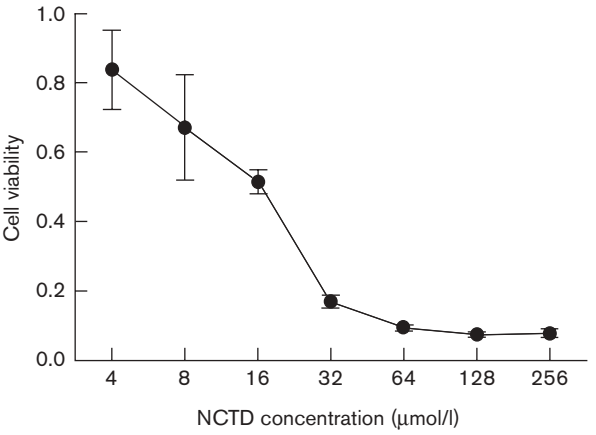
Cytotoxicity of NCTD

NCTD strongly inhibited the proliferation of HL-60 cells in a dose-dependent manner, with IC_{50} values of approximately 50 µmol/l (Fig. 2).

[^{3}H]Thymidine incorporation assay

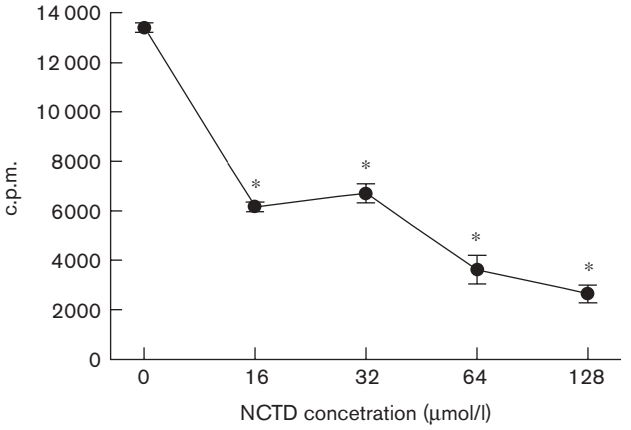
As shown in Fig. 3, a low dose of NCTD (16 µmol/l) significantly reduced the amount of [^{3}H]thymidine incorporated into DNA (to about one-third of the control) and increasing concentrations resulted in more obvious incorporation inhibition. Our results showed that

Fig. 2



Cytotoxicity of NCTD on HL-60 cells. HL-60 cells were cultured with various concentrations of NCTD (0–256 µmol/l) for 72 h and cytotoxicity was determined by the MTT assay. Data from three separate experiments are expressed as the means ± SD.

Fig. 3



Inhibition of DNA syntheses in HL-60 cells by NCTD. HL-60 cells were treated with various concentrations of NCTD for 12 h and the incorporation of [^{3}H]thymidine was assayed by liquid scintillation counting (the IC_{50} at 12 h is 200 µmol/l as confirmed by the MTT assay). *Significant change compared with untreated control.

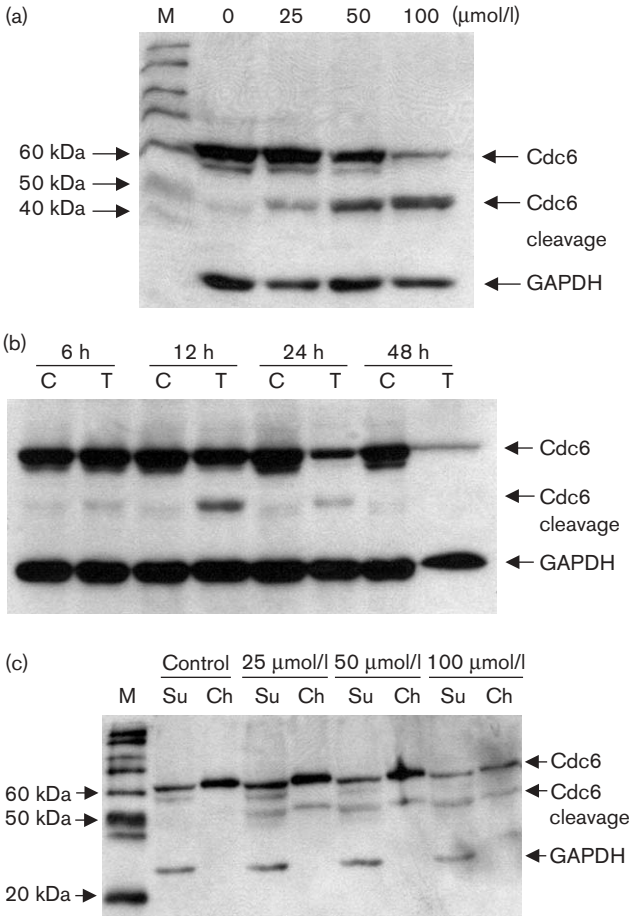
NCTD could inhibit DNA replication in HL-60 cells in a dose-dependent manner.

NCTD induced cleavage and degradation of pre-RC protein Cdc6

To determine whether NCTD could alter the biochemical properties or the relative amount of the proteins required for the initiation of DNA replication, we analyzed the proteins extracted from drug-treated HL-60 cells by Western blotting. As shown in Fig. 4(a), the 62-kDa Cdc6 was cleaved into a smaller fragment with a relative molecular weight of 49 kDa, as confirmed by the appearance of a faster migrating band in Western blotting. It was proved that the overexpression of Cdc6 could result in re-replication and suppression of Cdc6 could disturb DNA replication [11,12]. Thus, we presumed that cleavage of Cdc6 is likely to underlie the inhibition of DNA replication observed in NCTD-treated cells. To further confirm the cleavage effect of NCTD on Cdc6, HL-60 cells were treated with 50 μmol/l NCTD for 6, 12, 24 and 48 h, and Cdc6 protein was analyzed by Western blotting. NCTD cleaved Cdc6 after 6 h treatment, and the elongated treatment with NCTD resulted in degradation and complete loss of Cdc6. The intact Cdc6 and the cleaved fragment disappeared after 48 h treatment with NCTD (Fig. 4b).

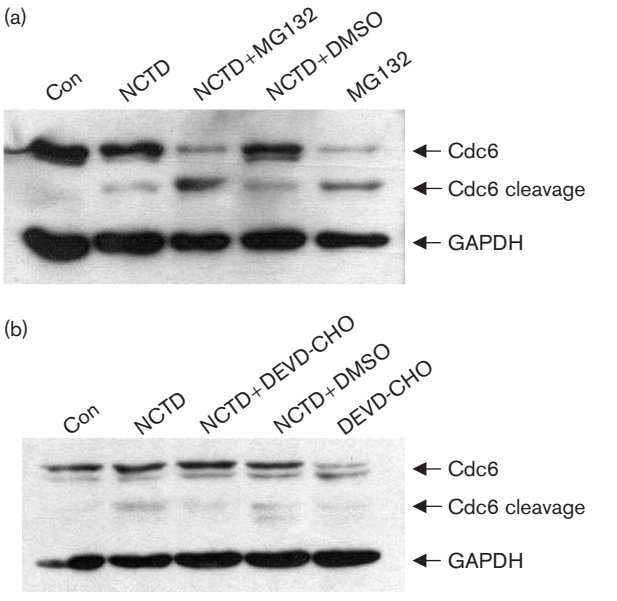
Cdc6 can be found in two fractions in mammalian cells – one readily extracted from chromatin and the other bound to chromatin. To determine whether NCTD induced cleavage in one or both fractions of Cdc6, the chromatin-bound proteins were separated from the non-chromatin-bound proteins as described in Materials and methods. Cdc6 levels in each fraction were analyzed by Western blotting. As shown in Fig. 4(c), Cdc6 could be found in both fractions in the control group with most of the population bound to chromatin, and NCTD treatment resulted in the cleavage of Cdc6 in both non-chromatin- and chromatin-bound fractions.

Fig. 4



NCTD induced cleavage of Cdc6. GAPDH was used as a loading control. (a) HL-60 cells were untreated or treated with various concentrations of NCTD for 12 h and Cdc6 protein was analyzed by Western blotting; M: marker. (b) HL-60 cells were untreated (C) or treated (T) with 50 μmol/l NCTD for 6, 12, 24 and 48 h, and Cdc6 protein was analyzed by Western blotting. (c) HL-60 cells were untreated (control) or treated with 25, 50 and 100 μmol/l NCTD for 12 h; the chromatin-bound fractions (Ch) were isolated from the non-chromatin-bound fraction (Su) and the Cdc6 protein was analyzed by Western blotting; M: marker.

Fig. 5



Caspase-3-dependent cleavage of Cdc6 induced by NCTD. GAPDH was used as a loading control. (a) HL-60 cells were untreated (con) or treated with 50 μmol/l NCTD in the presence or absence of 10 μmol/l MG132 for 12 h; DMSO was used as a solvent control. At the end of the treatment, the Cdc6 protein was analyzed by Western blotting. (b) HL-60 cells were exposed to Ac-DEVD-CHO (100 μmol/l) as indicated for 2 h followed by treatment with or without NCTD for an additional 12 h. DMSO was used as a solvent control. The Cdc6 protein was analyzed by Western blotting at the end of treatment.

NCTD-induced cleavage of Cdc6 is mediated by a caspase-3-like protease

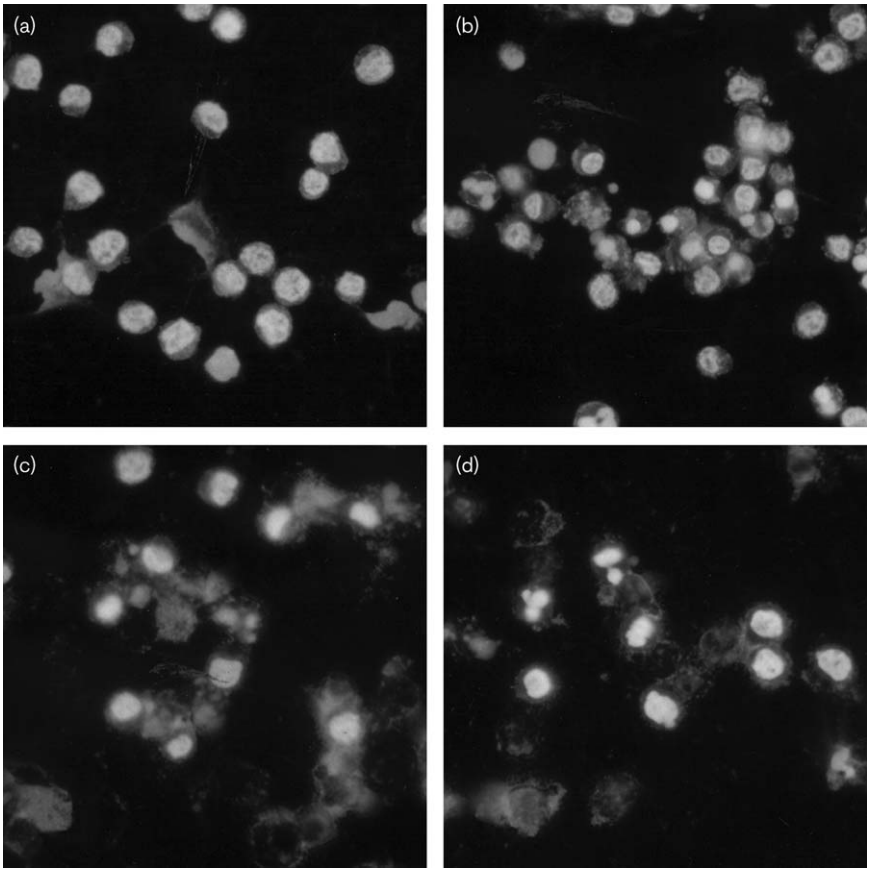
There are two possible pathways suggested to degrade Cdc6 – ubiquitin-mediated proteasome proteolysis and caspase-3-mediated cleavage of Cdc6. To test whether cleavage and degradation of Cdc6 by NCTD were mediated by the proteasome, HL-60 cells were treated with NCTD (50 μmol/l) in the presence of a potent inhibitor of proteasome MG132 (10 μmol/l). After 12 h treatment, cells were collected and Cdc6 was analyzed by Western blotting. Unexpectedly, MG132 did not inhibit NCTD-induced cleavage of Cdc6. On the contrary, the combination of NCTD with MG132 led to more obvious cleavage of Cdc6 than that produced by NCTD treatment alone. Furthermore, MG132 alone could induce Cdc6 cleavage, whereas the addition of DMSO alone, as a solvent control, had no effect (Fig. 5a). It seems that the proteasome pathway is not involved in NCTD-induced cleavage of Cdc6.

We then investigated whether NCTD cleaved Cdc6 through the caspase-3 pathway. The effect of Ac-DEVD-CHO (a caspase-3 special inhibitor) on the cleavage of Cdc6 in HL-60 cells treated by NCTD was studied. As shown in Fig. 5(b), exposure of HL-60 cells to Ac-DEVD-CHO (100 μmol/l) 2 h before the addition of NCTD prevented NCTD from inducing Cdc6 cleavage.

Degradation of Cdc6 induced by NCTD is associated with apoptosis

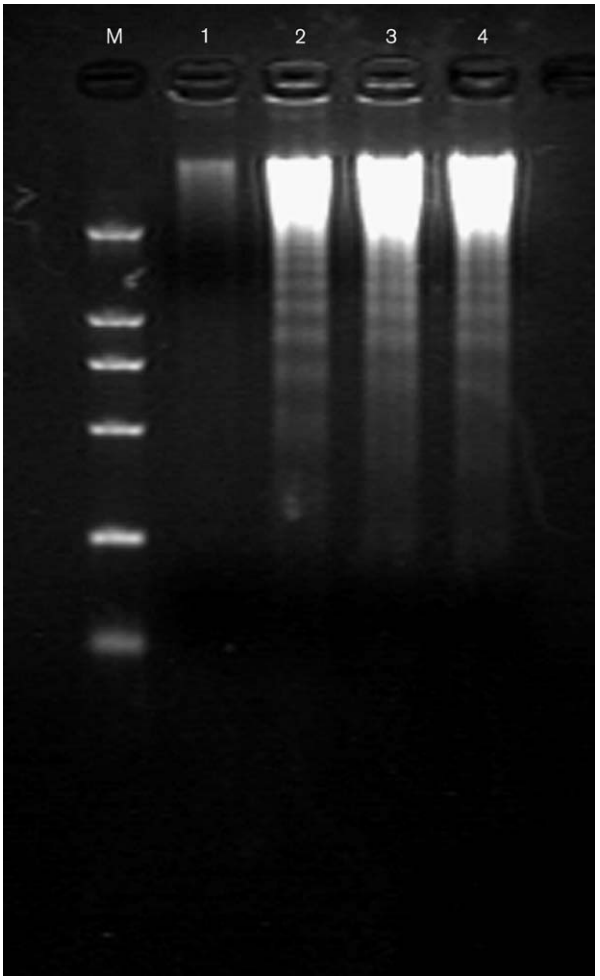
Caspase-3 plays a central role in apoptosis and many apoptotic stimuli were demonstrated to cleave Cdc6 [11–13]. Thus, we asked whether the degradation of Cdc6 induced by NCTD was associated with apoptosis. We performed the Hoechst 33258 staining assay to observe nuclear morphology and DNA agarose gel electrophoresis to determine DNA fragmentation. Cell cycle distribution was also analyzed by flow cytometry. In the control group, HL-60 cells were round in shape and stained homogeneously. After 12 h treatment with NCTD (50 μmol/l), blebbing nuclei and granular apoptotic bodies appeared (Fig. 6). DNA laddering (a hallmark of apoptosis) was observed in HL-60 cells after NCTD treatment (Fig. 7). Cell cycle distribution analysis showed the accumulation of cells in the sub-G_1 phase after 12 h NCTD treatment and numbers 10-fold higher than the control group after

Fig. 6



Cellular morphology of NCTD-treated HL-60 cells. HL-60 Cells were untreated (a) or treated with NCTD for 12, 24 and 48 h (b–d, respectively). Cells were fixed and stained with Hoechst 33258. Morphological changes were observed by fluorescent microscopy (original magnification × 200).

Fig. 7



Agarose gel electrophoresis of DNA extracted from HL-60 cells. M: DNA marker; 1: control group; 2–4: groups treated with 50 μmol/l NCTD for 12, 24 and 48 h, respectively.

48 h NCTD treatment (Fig. 8). These results indicated that NCTD induced apoptosis in HL-60 cells.

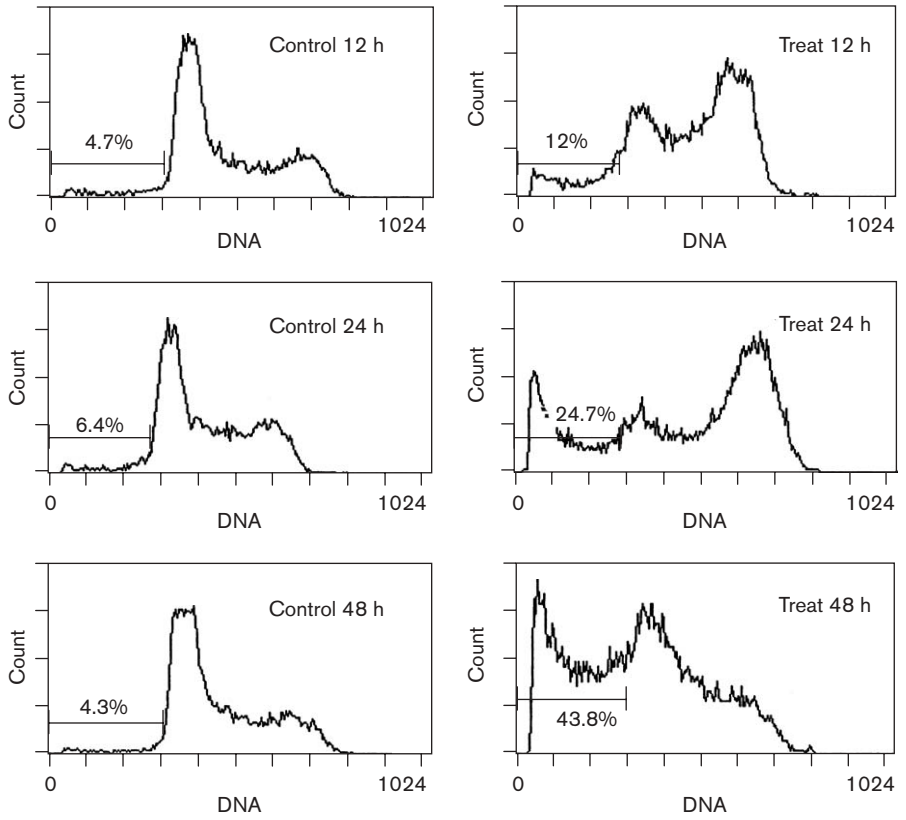
Discussion

First, our study has verified the inhibitory effect of NCTD on DNA replication in HL-60 cells (Fig. 3). Initiation of DNA replication is a tightly controlled process, depending on the coordinated assembly and function of pre-RCs at chromosomal origin sequences. Limiting the availability of pre-RCs has been suggested to be an effective pathway to prevent DNA replication [19]. Cdc6, which is essential for the formation and maintenance of pre-RCs, has been proved to be an effective target for disturbing DNA replication [9,11,12]. In the present study, we have found that Cdc6 was cleaved into a small fragment after NCTD treatment for 12 h, as confirmed by the presence of a faster migrating band of about 49 kDa in Western blotting (Fig. 4a). Furthermore, the intact Cdc6 and the cleaved fragment disappeared after 48 h treatment with NCTD (Fig. 4b). The function of Cdc6 depends on its binding to discrete chromosomal locations [20]. Our study has shown that NCTD could induce cleavage of Cdc6 in both non-chromatin and chromatin fractions, and 100 μmol/l NCTD could dramatically reduce the protein level of Cdc6 in the chromatin-bound fraction (Fig. 4c). Therefore, it is presumed that the decrease of chromatin-bound Cdc6 can exert an inhibitory effect on DNA replication. Although expressed in a constrained manner in cancer cells, a subset of initiation proteins for DNA replication (such as Cdc6, MCM proteins and Cdc45) was not expressed in non-proliferating normal cells [12,21,22]. Thus, downregulating the expression of these proteins could effectively abate DNA replication in cancer cells, leaving most normal cells unaffected. Therefore, Cdc6 may be an attractive target for the development of effective anti-cancer drugs with few side-effects.

In synchronized U2OS cells and HeLa cells, Cdc6 was absent in G_1 phase cells and could be stabilized by the inhibition of the proteasome [23]. However, in this study, the proteasome inhibitor MG132 (10 μmol/l) did not inhibit the cleavage of Cdc6 induced by NCTD. On the contrary, the combination of NCTD with MG132 led to more obvious cleavage of Cdc6 than that produced by NCTD treatment alone and MG132 alone could induce Cdc6 cleavage (Fig. 5a). There is a difference between our results and those from previous studies, which is possibly due to the different concentration of MG132 used in the experiment. Different cell lines involved in the study may also affect the results. The ubiquitin–proteasome pathway is the principal mechanism for the degradation of short-lived proteins in eukaryotic cells, especially the proteins regulating cell cycle and cell death. Proteasome inhibition will influence the proteins to different degrees and in different ways with different results. Recently, it has been revealed that inhibition of the proteasome could activate caspase-3 and induce Bcl-2 cleavage [24]. We propose that the proteasome pathway was not involved in NCTD-induced cleavage of Cdc6. The inhibition of the proteasome and NCTD treatment may be two parallel methods to activate caspase-3 to cleave Cdc6.

There are also detailed reports about the cleavage of Cdc6 induced by caspase-3, including characterization of the cleavage sites $DEMD^{287}$ and $SEVD^{442}$ [25]. Thus, we examined the possibility of caspase-3-mediated cleavage of Cdc6 after NCTD treatment. NCTD-induced cleavage of Cdc6 was inhibited after pre-treatment with Ac-DEVD-CHO (Fig. 5b). These observations indicate that caspase-3-like protease contributes to NCTD-induced cleavage of Cdc6.

Fig. 8



Effect of NCTD on cell cycle progression. HL-60 cells were untreated or treated with 50 μmol/l NCTD for 12, 24 and 48 h. After treatment, cells were fixed and stained with propidium iodide, and cell cycle distribution was examined by flow cytometry.

Growing evidence has shown that an important feature of apoptosis is the disruption of the link between DNA synthesis and cyclin-dependent kinase (CDK) activity resulting in the unscheduled activation of CDKs in the presence of unreplicated or partially replicated chromosomes [26]. A previous study reported that disturbing the function of Cdc6 led to apoptosis. In both p53-positive and -negative cancer cells, inhibiting the function of Cdc6 by selected antisense oligodeoxynucleotides and small-interfering RNA not only inhibited DNA replication, but also induced apoptosis of those cells [12]. Caspase-3-mediated cleavage of Cdc6 generated an N-terminal truncated Cdc6 fragment (p49-tCdc6). This truncation of Cdc6 resulted in nuclear retention of p49-tCdc6, which could promote apoptosis. Moreover, ectopic expression of p49-tCdc6 not only promoted apoptosis in etoposide-treated HeLa cells, but also induced apoptosis in untreated cells [15]. Our report also demonstrated the relationship between the disruption of DNA replication and apoptosis after NCTD treatment. NCTD treatment led to the cleavage of Cdc6 and produced a 49-kDa fragment. Apoptotic changes were also detected, which included blebbing nuclei, granular apoptotic bodies, DNA ladders and sub-G_1 phase accumulation. Our results suggest that destruction of Cdc6 may contribute to NCTD-induced apoptosis.

In conclusion, we have demonstrated that NCTD could inhibit DNA replication and induce apoptosis in HL-60 cells. Cdc6 was cleaved in a caspase-3-dependent manner after NCTD treatment. This cleavage produced a protein fragment of about 49 kDa, similar to the previous reported results of the destruction of Cdc6 induced by apoptotic stimuli, and was obvious in both chromatin- and non-chromatin-bound fractions of Cdc6. This is the first time that it has been revealed that NCTD could induce the cleavage of replication-initiation protein Cdc6. Although the role of destruction of Cdc6 in NCTD-treated cells awaits further investigation, the anti-cancer effect of NCTD may be closely associated with the dysfunction of Cdc6.

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