In vitro Cytotoxicity and Apoptotic Effect of Chloromethyl-2-dihydroxyphosphinyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline on HL-60 Cells

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The chloromethyl-2-dihydroxyphosphinyl-6,7-dimethoxy-1,2,3,4-tetrahydro- isoquinoline (CDDT) is a newly synthesized derivative from 1,2,3,4-Tetra- hydroisoquinoline (THIQ). The THIQs include potent cytotoxic agents that display a range of antitumor activities, antimicrobial activity, and other biological properties. In this study, we investigated the effect of CDDT on the cytotoxicity, induction of apoptosis in human promyelocytic leukemia cells (HL-60 cells). CDDT showed a significant cytotoxic activity in HL-60 cells (IC_{50} = approximately 37 ㎍/㎖) at a 24 hr incubation. Treatment of HL-60 cells with CDDT displayed several features of apoptosis, including formation of DNA ladders in agarose gel electrophoresis, morphological changes of HL-60 cells with DAPI stain. Here we observed that CDDT caused activation of caspase-3, caspase-8, and caspase-9. The most efficacious time on the activation of caspases-3 was achieved at 12 hr. Further molecular analysis demonstrated that CDDT led to cleavage of poly(ADP-ribose) polymerase (PARP), increase of hypodiploid (Sub-G1) population in the flow cytometric analysis. In conclusion, these above results indicate that CDDT dramatically suppresses HL-60 cell growth by activation of caspase-3 with caspase-8, -9 activity. These data may support a pivotal mechanism for the use of CDDT in the prevention and treatment of leukemia.

Key words : Apoptosis, CDDT, caspase-8, caspase-9, caspase-3

Introduction

Chloromethyl-2-dihydroxyphosphinyl-6,7-dimethoxy-1,2,3,4-tetrahydroiso- quinoline (CDDT) was newly synthesized from 1,2,3,4-Tetrahydroisoquinoline, a kind of isoquinoline derivatives. The isoquinoline (IQ) and tetrahydro- isoquinoline (THIQ) alkaloids, which are widely distributed in plant and animal kingdoms, have received much attention because of their important biological activities (i.e., calcium antagonistic, cardiovascular, b-adrenergic receptor antagonism, antibacterial, antiplasmodial, antitumor, and antibacterial activity)[1-5]. Specifically, THIQs are strong complex I inhibitor of mitochondrial respirationafter their transformation into N-methyl-isoquinoline ions[6]. However, the potential mechanisms underlying the anti-tumor function is not well known. In recent years, we discovered that CDDT has not effective of inducing apoptosis and cytotoxicity in the various solid tumor cells (data not shown). After tested on solid tumor cells, we used human promyelocytic leukemia cells (HL-60) to investigate the molecular mechanism of apoptosis.

HL-60 cells have provided a valid model for studying the mechanisms and relationships involved in the induction of differentiation and apoptosis in response to anti-leukemic or general anti-tumoral agents[7].

The growth of malignant tumors generally results from deregulated pro-liferation or an inefficiencyof cells to undergo apoptotic cell death[8,9]. But, anticancer drugs suppressproliferation and cause apoptosis in sensitive tumor cells[10,11]. Accordingly, it has been shown that most chemotherapeutic drugs used for undergoing apoptotic pathway through their cytotoxic effect[12].

Apoptosis, or programmed cell death, is an intensively controlled process that is related with activation of a series of dramatic molecular events. It is characterized by cell shrinkage,

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blebbing of the plasma membrane, and chromatin condensation that are compatible with DNA cleavage in ladders revealed by electrophoresis[13-15]. There are two independent pathways based on effective initiator events[16-18]. One pathway is activated by ligation of death receptors such as Fas (also called Apo-1 or CD95) and tumor-necrosis factor receptor (TNFR)1. In these cases, caspase-8 is added tothe ligated receptors via FADD(Fas-associated death domain), an adaptor molecule, and lead to its proteolytic activation. The activated caspase-8 either directly activates caspase-3 or it produces the cleavage of the BH-3-only Bcl-2 family member Bid to createtBid, which subsequently induces cytochrome c release[19,20]. The other pathway is the mitochondrial pathway which responds to anticancer drugs and various kindsof environmental stresses[21]. In the mitochondria initiated pathway, mitochondria undergoing permeability transition flow out of apoptogenic proteins such as cytochrome c or apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space into the cytosol. Released cytochrome c can activate caspase-9, and its activated form in turn cleaves and activates caspase-3[22]. Especially, specific substrates for caspase-3 such as poly (ADP-ribose) polymerase (PARP) are cleaved, and lead a cellular mechanism into apoptosis[23].

In this study, we investigated the cellular mechanisms of cell death using the HL-60 cells. We found that various caspases were involved in this induction of apoptosis.These results will be a help to understand the mechanism of CDDT effect in the cellular mechanism.

Materials and Method

1. Drug

Chloromethyl-2-dihydroxyphosphinyl-6,7-dimethoxy-1,2,3,4-tetra- hydroisoquinoline (CDDT) was synthesized by Dr, Chai-Ho, Lee, Department of Chemistry, Wonkwang University (Iksan, Korea). CDDT was dissolved in PBS at a concentration 10 mg/ml and stored at -20℃ before use. The structure of CDDT is illustrated in Fig. 1.

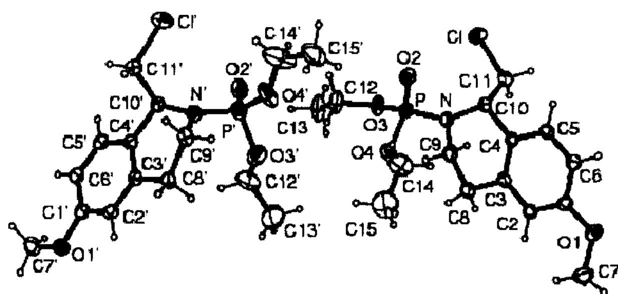


Fig. 1. An ORTEP drawing of CDDT with atomic numbering scheme.

2. Cell culture

HL-60 cells were purchased from Korea Cell Line Bank (KCLB, Seoul, Korea) and cultured in suspension at 37℃ in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics in a humidified atmosphere containing 5% CO_2 in air. The pH of the medium was adjusted to 7.2~7.4 with 2g/L of sodium bicarbonate. The cells were passaged twice a week.

3. Materials

Methanol and DAPI were purchased from CARLO ERBA REAGENTI and Roche, respectively. MTT and PI were obtained from Sigma. RPMI 1640 and Fetal bovine serum were obtained from Gibco. The Wizard Genomic DNA Purification Kit from Promega was used for DNA ladder formation analysis. Mouse monoclonal anti-human anti-PARP was purchased from Cell Signaling Technology. Mouse monoclonal anti-Bid and anti-cytochrome c were obtained from BD Pharmingen (San Diego, CA), and rabbit polyclonal anti-human anti-caspase-3, anti-Bcl-2 and anti-Bax were bought from Santa Cruz (Santa Cruz, CA).

4. MTT assay

In the primary study, the cytotoxic effect of CDDT was determined by MTT assay. MTT was dissolved in phosphate buffered saline (PBS) at a concentration of 5 mg/ml. MTT is introduced into cells and reduced in a mitochondria-dependent reaction to yield a formazan product. The ability of cells to reduce MTT is considered to be an indication of mitochondrial integrity and activity[24]. To assess the cytotoxicity of CDDT, cells were treated with CDDT at concentration of 0,20, 40, 80, 100, 120, 140, 160, 180, and 200 ㎍/㎖. After cells were seeded at $1x10^5$ cells/well in 500 ㎕ of medium in 24-well plates, those were incubated for 24 hr and 125 ㎕ of MTT were added to each well, and plates were gently shaken and incubated at 37℃ for 4 hr. The resulting blue formazan crystals were dissolved with the same volume (625 ㎕) of the solubilization solution containing (10% SDS /0.01 N HCl) added to each well. The results were read on a 96-well plate by enzyme-linked immunosorbent assay (ELISA) reader (575 nm).

5. DAPI staining

The change of cell morphology was analyzed to investigate the cytotoxic and apoptotic effect of CDDT on HL-60 cells. Cells were treated with CDDT in a concentrations-dependent manner for 24 hr, and collected by centrifugation. The pellet was resuspended and stained with the methanol solution containing 1 ㎍/㎖ DAPI for 15 min at

37℃. Cells were viewed by a fluorescence microscope at the magnification of x400, after centrifugation of the stained cell using the cytospin.

6. DNA fragmentation assay

Genomic DNA was obtained using the Wizard Genomic DNA Purification Kit of Promega and DNA fragmentation was assayed by agarose gel electrophoresis. The cells were treated with CDDT at various concentrations. After exposure to CDDT, the cells were immediately incubated at 37℃ for 24 hr, and collected by centrifugation. After cells were washed once in ice-cold phosphate buffered saline (PBS), DNA was isolatedaccording to the procedure provided by Wizard Genomic DNA Purification Kit of Promega. The DNA sample was loaded on a 1% agarose gel containing ethidium bromide (0.5 μg/mℓ). Electrophoresis was carried out in TAE buffer. Gels were examined and photographed under UV light.

7. Caspase activity

To investigate the caspase-3activity, CDDT-treated cells were incubated with appropriate dose- and time-dependent manner. After measurement of caspase-3 activity, cells cultured at indicated concentrations for verifying the caspase-8, and -9 activities during 12 hr. Untreated and CDDT-treated cells ($2x10^6$) were harvested, washed once in ice-cold PBS and gently resuspended in cold lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 0.5% Igepal and 150 mM NaCl) and incubated on ice for 30 min. After centrifugation the lysed cells at 14,000 rpm for 20 min at 4℃, cell lysate (30 μg of total protein for caspase-3, 150 μgfor caspase-8, 9) were mixed with fluorogenic substrates, respectively, 20 μM Ac-DEVD-AMC (specific for caspase-3), Ac-IETD-AMC (specific for caspase-8), Ac-LEHD-AMC (specific for capase-9) inreaction buffer [10% glycerol, 2 mM DTT, 20 mM HEPES, pH 7.5] on ice and incubated for 2 hr at 37℃in the dark. The release of fluorocrome AMC (7-Amino-4-methylcoumarin) was measured at 360 nm excitation and 460 nm emission using a fluorescence spectrophotometer (F-2500, HITACHI, Japan).

8. Flow cytometric Analysis of Apoptosis by PI staining

To determine apoptosis, cells were stained with propidium iodide (PI). HL-60 cells ($1x10^6$) were incubated in a 6 well plate with different concentrations of CDDT for 12 hr. Cells were collected, washed twice in ice-cold PBS and then fixed with 70 % ethanol at 4℃for 15 min. Cellular DNA was stained with PI staining solution (25 μg/ml PI, 100 μg/ml RNase, 0.05% NP-40 in 1 ml of PBS). Stained cells were incubated for 15 min at 37℃ in the dark. Analytic flow cytometric measurements were performed using a FACS vantage flow cytometer (Becton Dicknson), and the fluorescence was detected through a 564-606 nm band-pass filter. Tenthousand cells were analyzed in each sample using CELLQuest software (Becton Dickinson).

9.Western blot analysis

After treatment with the concentration-dependent manner of CDDT for 12 hr, HL-60 cells were collected and lysed in ice-chilled 120 μℓ lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 0.5% Igepal and 150 mM NaCl) with 1% fresh protease inhibitor cocktail for 30 min. Cell lysates were prepared by centrifugation at 14,000 rpm for 20 min at 4℃, and protein concentration was determined by Bradford assay kit (Bio-rad). Equal amounts of protein were boiled in equal volume of SDS-PAGE sample loading buffer for 5 min at 100℃, and electrophoresed on SDS-polyacrylamide gel with Tris-glycine running buffer for 1 hr. After separation, proteins were transferredonto nitrocellulose membrane (Schleicher & Schuell) for 3 hr at 40V. The membrane was blocked with 5% skim milk in Tirs-buffered saline (TBS) for 1 hr at room temperature. The blocked membrane was subsequently probed with mouse monoclonal anti-human anti-PARP, anti-Bid, and rabbit polyclonal anti-human anti-caspase-3, anti-Bax, and anti-Bcl-2 at 4℃ for overnight. These primary antibodies were used at a dilution of 1:1000 in TBS containing 5% skim milk. After washing the membrane in TBS for 15 min 2 times, blots were detected using either anti-mouse or anti-rabbit (Zymed) peroxidase-conjugated secondary immunoglobulin G antibodies diluted 1:2000 in TBS containing 5% skim milk at 4℃ for 1 hr and rinsed in TBS again. The transferredproteins were visualized with SuperSignal West Pico Chemiluminescent (PIERCE).

Results

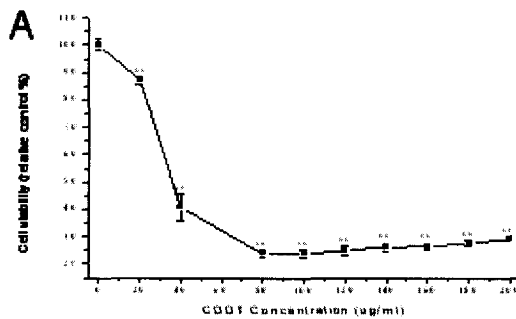
1. Cytotoxic and apoptotic effect on HL-60 cells

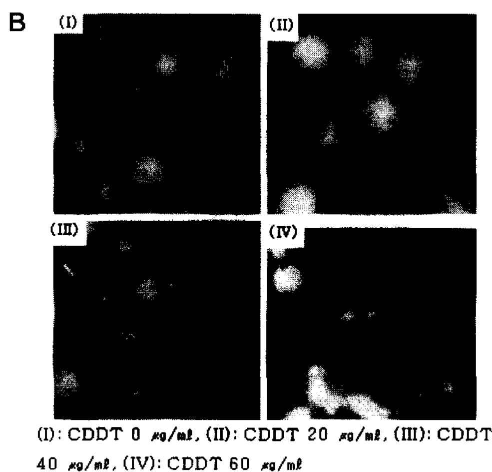
The cells were incubated with different concentrations of CDDT for 24 hr. MTT assay was carried out in CDDT free media as the control. As shown in Fig. 2A, viabilities of cells incubated with CDDT were significantly decreased as compared with the control value, respectively. The result of MTT assay clearly showed that the cytotoxic effect of CDDT on HL-60 cells was increased in a concentration-dependent manner (Table 1).To investigate morphological changes, cells were observed by a fluorescence microscope after DAPI staining. Fig. 2B. shows that the morphological change of the cells were induced by CDDT. The rounding, cytoplasmic blebbing, and

irregularities in shape were observed in the cells treated with CDDT at various concentrations for 24 hr. It was thought that the CDDT induced morphological changes of cells indicated an apparent apoptotic cell death pattern. In order to determine the pattern of cell death, HL-60 ($2x10^5$/ml) cells were incubated with indicated concentrations of CDDT for 24 hr. The endonucleolytic DNA cleavages were analyzed by agarose gel electrophoresis. DNA fragmentation, reflecting the endonuclease activity which was characteristic of apoptosis, was shown from 40 μg/mℓ CDDT treatment and respond to a dose-dependent manner in Fig. 2C.

Table 1. Cytotoxic effect of CDDT on HL-60 cells for 24 hr, as measured by MTT assay. Values are represented of four or more independent experiments.

CDDT concentration(μg/ml)	cell viability %
Un-treated (Control)	100 ± 2.1
20	87.1 ± 1.3
40	40.7 ± 4.8
80	23.7 ± 1.3
100	23.8 ± 1.5
120	24.9 ± 1.5
140	26.2 ± 1.4
160	26.3 ± 1.2
180	27.7 ± 0.8
200	29.1 ± 0.7





(I): CDDT 0 μg/mℓ, (II): CDDT 20 μg/mℓ, (III): CDDT 40 μg/mℓ, (IV): CDDT 60 μg/mℓ

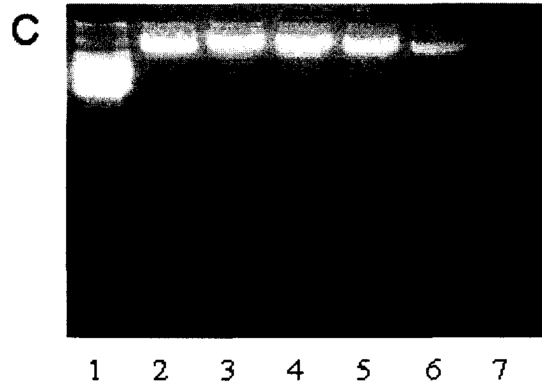


Fig. 2. Apoptosis-inducing effect of CDDT. (A) Cytotoxic effect of CDDT on HL-60. Cells were incubated with CDDT at various concentrations prior to the determination of cellularviability through MTT assay. Results are presented as mean ± standard error. Asterisk (*) represents p⟨0.01 compared to the control. (B) Characterization of CDDT - induced cell death (arrows) in HL-60. Cells were cultured without CDDT (control) and with various concentrations for 24 hr in each case. Cells were stained with DAPI. (C) DNA ladder formation following exposure of HL-60 cells to CDDT for 24 hr. Genomic DNA was extracted and analyzed by electrophoresis on 1% agarose gels electrophoresis; molecular-weight markers (lane1), control cells (lane2), cell treated with 20, 30, 40, 50, 60 μg/ml (lane3-7).

2. CDDT stimulated caspase-3, -8, and -9 activities in dose-dependent manner

To examine the involvement of aspartate-specific cysteinyl protease (caspases) in apoptotic process, we tested to know whether treated-cells exhibit the activation of upstream caspase-8, -9 and of the downstream caspase-3 (Table 2). CDDT markedly induced a time- and concentration-dependent activation of caspase-3. This protease activity was maximal at 12 hr in Fig. 3(A-D). In particular, we estimated caspase-8 activity because it shows the apical rolein the death receptor (extrinsic) pathway and measured caspase-9 activity, as it serves as the apical caspase of the intrinsic pathway. To determine whether activation of caspase-8 and -9 plays a pivotal role in CDDT-induced apoptosis, HL-60 cells were incubated with CDDT for 12 hr and were analyzed fluorimetrically. A dose-dependent increase in the activities of caspase-8, and -9 was observed in CDDT-treated HL-60 cells (Fig. 3 E, F).

Table 2. Caspase-3, -8, and -9 activations in HL-60 cells. Each experiment were carried out four or more times.

CDDT concentration(μg/ml)	Caspase-3 activity (%)			
	3 hr	6 hr	12 hr	24 hr
Un-treated (Control)	100 ± 8.3	100 ± 5.4	100 ± 6.4	100 ± 6.4
20	123.3 ± 7.4	279.3 ± 1.5	259 ± 3.6	130.2 ± 6.7
40	347.6 ± 4.9	632.3 ± 1.8	1469.1 ± 4.3	430.5 ± 7.1
60	2338.2 ± 2.4	3189.8 ± 1.7	3477.5 ± 3.9	2633.5 ± 9.6
80	2921.8 ± 5.3	3695.4 ± 1.9	4020.1 ±4.7	3573.8 ± 4.4
100	3281.2 ± 4.7	3576.1 ± 4.2	3898.8 ± 4.8	3720.9 ± 6.5
150	3989 ± 5.1	3452 ± 1.6	3454.6 ± 3.6	2529.1 ± 6.8

CDDT concentration(㎍/ml)	12 hr	
	Caspase-8 activity (%)	Caspase-9 activity (%)
Un-treated (Control)	100 ± 4.6	100 ± 15.7
20	107.6 ± 2.9	130.6 ± 6.8
40	124.6 ± 11.7	183.9 ± 1
80	213.9 ± 13.2	362.7 ± 13.4
100	202.7 ± 5.9	337.7 ± 10

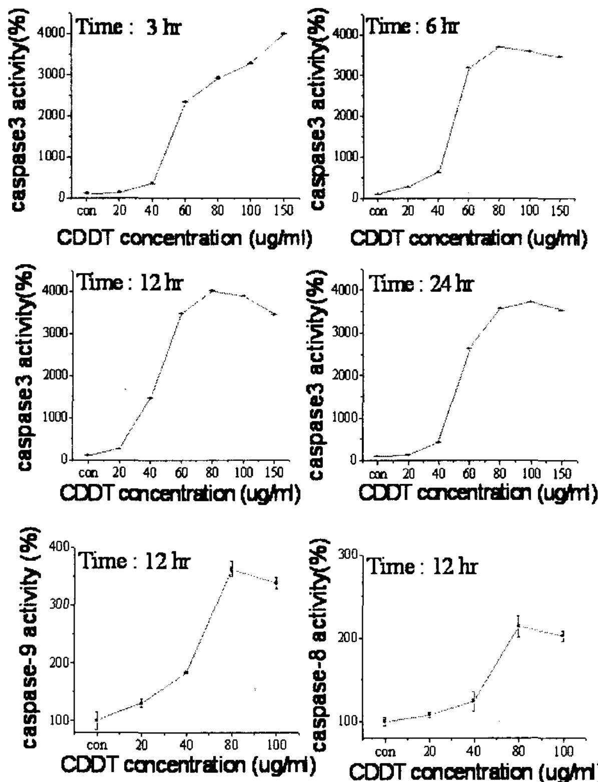


Fig. 3. Time- and dose-dependent activation of caspase-3 and dose-dependent activation of caspase-8, and -9 at 12 hr. Cytosolic extracts were prepared and assayed for caspase-3, -8, and -9 activities. (A-D)Cells were treated with indicated concentrations CDDT in a time-dependence. The activation was measured as the increase in caspase-3 activities (%) with respect to control cells (100%). In (E, F), cells were incubated with increasing concentration of CDDT for 12 hr. Values were the mean±standard error of four experiments.

3. Treatment with CDDT causes Degradation of PARP, an endogenous substrate of caspase-3

Activation of caspase-3 degrades numerousproteins, one of which is PARP (116 kDa). Cleaved PARP (85 kDa) fragment is a typical evidence of apoptotic process[25]. Accordingly, we first carried out an experiment inthe effects of CDDT on cleavage of caspase-3 by Western blot analysis in HL-60 cells. CDDT-treated cells revealed to decreaseof the 35-kDa pro-enzyme caspase-3 (Fig. 4). In a second step, we assessed the degradation of the DNA-repair enzyme PARP, the known substrate of caspase-3. As shown in Fig. 4, incubation with increasing dose of CDDT resulted in marked degradation of PARP, a typically 85-kDa band, which was almost complete degraded at 80 and 100 ㎍/㎖ concentrations of CDDT. These data shows that caspase-3 cleaves PARP in dose-dependent manner at same concentrations.

Fig. 4. Procaspase-3 and PARP cleavage in HL-60 cells in response to CDDT treatment. Cytosolic extracts (40 ㎍/㎖of protein for caspase-3 and PARP, respectively) from HL-60 cells treated with various concentrations for 12 hr were resolved by SDS-PAGE as described in "Material and methods" and probed for caspase-3 and PARP. These experiments were repeated three times with similar results, and typical data are presentedhere.

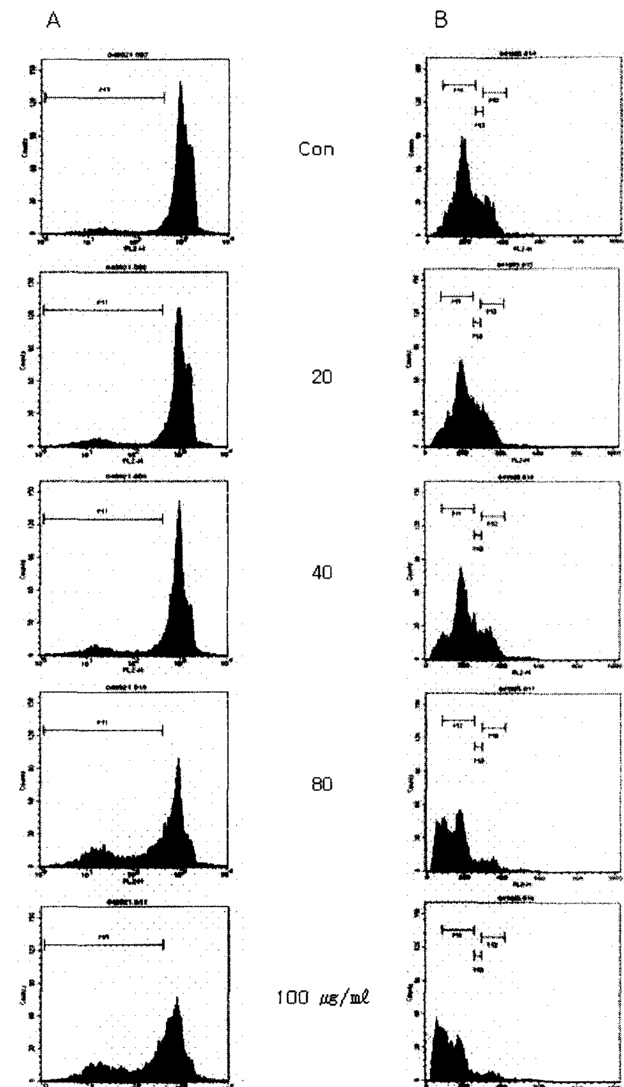


Fig. 5. Determination of cell cycle arrest and hypodiploid cells (apoptotic cells) by flow cytometry. HL-60 cells were cultured with increasing concentrations of CDDT for 12 hr. After incubation, cells were stained with PI and analyzed by flow cytometry. Data represent the result from one of three similar experiments. (A) M1 displays quantity of hypodiploid cells and (B) shows each phases of cell cycle (M1 = G1, M2 = S, M3 = G2/M).

4. Cell cycle was arrested and apoptotic cells were increased in HL-60 cells

We examined DNA contents of HL-60 cells treated with CDDT. Cell cycle arrest and apoptotic cells were confirmed by flow cytometric analysis of the DNA-stained cells. Flow cytometric analysis of treated HL-60 cells was informedthe increase of hypodiploid apoptotic cells in a concentration-dependent manner (Fig. 5A. Table 3.), and occurs the decrease of the peak of the cell cycle on the S and G2/M phase (Fig. 5B Table 3.) after 12 hr incubation, respectively. These results suggested a possibility that CDDT-induced apoptosis happened at S and G2/M phase of cell cycle.

Table 3.Increase of apoptotic cells and arrested cell cycle by CDDT treatment

CDDT concentration(㎍/ml)	Apoptotic cells (%)	Cell cycle arrest (%)		
		G1	S	G2/M
Un-treated (Control)	6.56	59.34	15.34	10.98
20	9.79	57.20	16.70	11.53
40	13.59	55.46	15.35	8.80
80	38.32	44.79	4.62	2.29
100	44.69	37.11	2.90	1.39

Discussion

The study presented here demonstrates that CDDT, a newly synthesized derivative from TIQ, have cytotoxic effect and induces apoptosis in human myeloid leukemia HL-60 cells. Apoptosis, cellular suicide or programmed cell death, is triggered by the activation of an intracellular pathway[26]. Apoptosis displays some features including cell shrinkage, formation of cytoplasmic vacuoles, nuclear and plasma membrane blebbing, chromatin condensation, and formation of apoptotic bodies[27]. After HL-60 cells were treated with CDDT for 24 hr, MTT assay showed that CDDT exert a cytotoxic effect in a concentration-dependent manner. The fluorescencemicroscopic observations by DAPI stain demonstrated the apoptotic characters. We have also noted DNA fragment ladder formation, a characteristic gel electrophoretic band pattern associated with apoptosis. DNA ladder, the biochemical hallmark of apoptosis, informs degradation of DNA by endogenous DNase, which cleaves the internucleosomal DNA into fragmented products of 180200 base pairs[28].

Recently, it has been demonstrated that caspases play a critical role in the induction of apoptosis. According to their signaling pathways with specific substrates and target proteins, caspases are classified into 'apoptotic initiator', such as caspase-8, and 'apoptotic effector' including caspase-3[29-31].Using a fluorogenic substrate, we showed that CDDT increases caspase-3 activity in a time- and dose-dependent manner. The most effective induction of apoptosis was viewed on 80 and 100 ㎍/㎖ at 12 hr and the maximal degradation of the DNA repair enzyme PARP, which precedes the onset of apoptosis, was detected at the same time and dose. During the apoptotic process, Caspase-3 is a major component of the caspase cascade, and DNA fragmentation is mediated by caspase-activated DNase (CAD, also known as DFF, DNA fragmentation factor) which is an essential protease in the process of apoptosis[16].

Consequently, we compared the caspase-3 activation, which is able to reflect the action of DNase, with these results of a flow cytometric analysis for DNA contents. It was shown that CDDT-treated cells were notably increased apoptotic DNA matters and were markedly blocked onthe S and G2/M phase of the cell cycle at 80 and 100 ㎍/㎖ in contrast with control. These data are similar to the measurement of caspase-3 activation and the western blot analysis. In this induction of apoptosis, the activation of caspase-8, and -9 were also appeared.

In our model, a remarkable change of caspase-3 activation was revealed with the activation of caspase-9, the essential caspase of the intrinsic pathway of apoptosis (markedly increased from 40 ㎍/㎖ by dose-dependence at 12 hr but didn't at 6 hr). On the other hand, the activation of caspase-8, the apical caspase of the extrinsic pathway, was markedly activated by higher (80 ㎍/㎖) concentration than caspase-9 at 12 hr.

In conclusion, we report here for the first time that CDDT induces apoptosis in HL-60 cells involved with caspase-3, -9, -8 activation and PARP cleavage leading to DNA fragmentation. But, additional research is needed to determine the factors implicated with extrinsic and intrinsic pathway.

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