Molecular and Cellular Biochemistry **292:** 99–105, 2006.
DOI: 10.1007/s11010-006-9222-7

Induction of G2/M phase arrest and apoptosis by a novel enediyne derivative, THDA, in chronic myeloid leukemia (K562) cells

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Received 21 February 2006; accepted 1 May 2006

Abstract

We studied the effect of 2-(6-(2-thieanisyl)-3(Z)-hexen-1,5-diynyl)aniline(THDA), a newly developed anti-cancer agent, on cell proliferation, cell cycle progression, and induction of apoptosis in K562 cells. THDA was found to inhibit the growth of K562 cells in a time-and dose-dependent manner. Cell cycle analysis showed G2/M phase arrest and apoptosis in K562 cells following 24 h exposure to THDA. During the G2/M arrest, cyclin-dependent kinase inhibitors (CDKIs), p21 and p27 were increased in a time-dependent manner. Analysis of the cell cycle regulatory proteins demonstrated that THDA did not change the steady-state levels of cyclin B1, cyclin D3 and Cdc25C, but decreased the protein levels of Cdk1, Cdk2 and cyclin A. THDA also caused a marked increase in apoptosis, which was associated with activation of caspase-3 and proteolytic cleavage of poly (ADP-ribose) polymerase. These molecular alterations provide an insight into THDA-caused growth inhibition, G2/M arrest and apoptotic death of K562 cells. (Mol Cell Biochem **292:** 99–105, 2006)

Key words: THDA, G2/M arrest, apoptosis, K562 cells

Introduction

Cell cycle control is the major regulatory mechanism of cell growth [1–3]. Many cytotoxic agents and/or DNA damaging agents arrest the cell cycle at the G1, S or G2/M phase and then induce apoptotic cell death [2, 4, 5]. The cell cycle checkpoint may function to ensure the cells have time for DNA repair [3, 6]. In recent years, considerable advances have been made in understanding the roles of cyclins, cyclin-dependent kinases (Cdks), cyclin-dependent kinase inhibitors (CDKIs) in cell cycle progression. This process is regulated by the coordinated action of Cdks in association with their specific regulatory cyclin proteins [3]. G2 to M phase progression is regulated by a number of the Cdk/cyclin family, especially, activation of the Cdk1/cyclin B1 complex is required for transition from G2 to the M phase of the cell cycle [7, 8]. CDKIs including $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ also contribute to the regulation of cell cycle progression by controlling Cdk activity [9, 10]. Several studies have shown that various cytotoxic drugs can induce G2/M phase accumulation [11–13]. In addition to the cell cycle, apoptosis is an important mode of cell death that occurs in response to a variety of agents including ionizing radiation or anticancer chemotherapeutic drugs. Recently, several lines of evidence have suggested that the caspase family plays a crucial role in apoptosis. Caspase-3, a key component of the apoptotic machinery, has been shown to be activated in apoptotic cells and cleaves several cellular proteins including poly (ADP-ribose) polymerase

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(PARP) protein, the cleavage of which is a hallmark of apoptosis.

Families of molecules consisting of unique enediyne subdomain cores, which were derived from either natural isolation or synthetic routes, display manifold biological function [14–16]. In our most recent report, acyclic enediynes exhibited cytotoxicities toward cancer cells in low range of micromolar concentrations [17–19]. Recently, we synthesized 2-(6-aryl-3(Z)-hexen-1,5-diynyl)anilines derivatives, which were then submitted to the National Cancer Institute for an *in vitro* screen of 60 cell lines and found that 2-(6-(2-thieanisyl)-3(Z)-hexen-1, 5-diynyl)aniline (THDA) exhibited the highest cytotoxic activity in cancer cells. Therefore, this new investigation will be helpful in further elucidation of undiscovered biological properties of this novel antitumor enediyne.

To elucidate the mechanism of THDA, THDA was used together with K562 cells, a valid model for testing anti-leukemia as well as general anti-tumor agents. We found that treatment with THDA on K562 cells resulted in G2-growth arrest, presumably involving the concomitant reduction of Cdk1, Cdk2, and cyclin A, and marked up-regulation of p21 and p27. Furthermore, THDA induced apoptosis via activation of caspase-3, and PARP cleavage.

Materials and methods

Chemicals

RPMI 1640 medium, fetal calf serum (FCS), trypan blue, penicillin G and streptomycin were obtained from GIBCO BRL (Gaithersburg, MD). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), ribonuclease (RNase) and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, MD). Antibodies against p21, p27, cyclin A, B1, D3, Cdk1, Cdk2, PARP and Cdc25C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-mouse and anti-rabbit 1 g G peroxidase-conjugated secondary antibody were purchased from Pierce (Rockford, IL). Hybond ECL transfer membrane and ECL Western blotting detection kit were obtained from Amersham Life Science (Buckinghamshire, UK). The colorigenic synthetic peptide substrate, Ac-DEVD-pNA, and the Caspase inhibitor Z-VAD-FMK were purchased from Calbiochem-Novabiochem Co (La Jolla, CA).

Cell culture

Human leukemia K562 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and human purified lymphocytes preparation was obtained from blood as described previously [20]. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

Preparation of THDA

2-(6-(2-thieanisyl)-3(Z)-hexen-1,5-diynyl)aniline (THDA) (Fig. 1) was prepared from the coupling reaction between iodoaniline and trimethylsilylacetylene with palladium catalyst. After filtration and removal of solvent in vacuo, the residue was purified by column on silica gel to yield THDA. The structure of this compound has been verified by means of mass spectrometry and spectroscopic techniques [19]. THDA was dissolved in dimethyl sulfoxide (less than 0.01%) and made immediately prior to experiments.

Cell viability assay

The viability of cells was determined by MTT assay and the trypan blue dye exclusion assay was performed to confirm and verify cell viability. Cells, grown in 96-well microtiter plates (1×10^4 cells/well) for 24 h, were incubated with varying concentrations of THDA (diluted in DMSO at 0.01% final concentration) at 37 °C for 48 h. MTT solution was added to each well (1.2 mg/ml) and incubated for 4 h. The viable cell number was directly proportional to the production of formazan, which was then solubilized with DMSO, and estimated by measuring absorbance at 570 nm in an ELISA plate reader. For trypan blue dye exclusion assay, cells were seeded at density of 1×10^5 cells/well onto a 12-well plate for 24 h, then THDA were added to medium at various indicated times and concentrations. After incubation, cells exposed to 0.2 %trypan blue were counted in a hemocytometer.

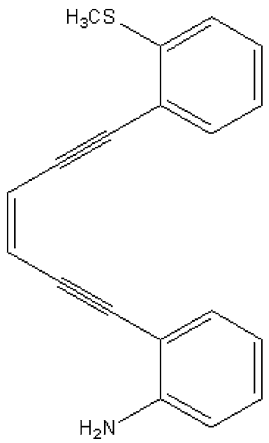

Fig. 1. Chemical structure of THDA.

Flow cytometry analysis

Cells (10^6 cells) were seeded in 6-cm dishes for 24 h. The medium was replaced with fresh complete medium containing desired concentrations of THDA or DMSO (control), and the dishes were incubated for 24 h at 37 °C. The cells were washed with PBS and fixed in 70% ethanol overnight at 4 °C. The cells were then treated with RNase A solution (500 unit/ml) and propidium iodide (50 μg/ml) for 30 min, and analyzed using a COULTER EPICS XL Flow Cytometer (Coulter Corp., Miami, FL, USA). The fractions of the cells in G0/G1, S, and G2/M phase were analyzed using cell cycle analysis software, Multicycle (Phoenix flow system, San Diego CA, USA).

Western blotting analysis

Cells were washed in PBS, suspended in lysis buffer containing 50 mM Tris (pH 7.5), 1% NP-40, 2 mM EDTA, 10 mM NaCl, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and placed on ice for 30 min. After centrifugation at 20,000$\times g$ for 30 min at 4 °C, the supernatant was collected. The protein concentration in the supernatant was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Whole lysate (50 μg) were resolved by 12% SDS-PAGE, transferred onto PVDF membranes (Roche) by electroblotting, and probed with anti-p21, -p27, anti-Cdk1,-Cdk2, anti-cyclin A,-cyclin B1,-cyclin D3, and anti-Cdc25C (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The blot was developed by enhanced chemiluminescence.

Assays of caspase-3 activity

After different treatments, cells (10^6 cells/ml) were collected and washed three times with PBS and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 18,000$\times g$ for 3 min, and clear lysates containing 50 μg of protein were incubated with 100 μM of enzyme-specific colorigenic substrates at 37 °C for 1 h. The activity of caspase-3 was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm.

Statistics

All data are expressed as the mean ± SD. Difference between the treated and the control was analyzed by *t*-test. A probability of $P < 0.05$ was considered significant.

Results

To verify the effect of THDA on cell growth, K562 cells were treated with increasing concentrations of THDA for 48 h, and cell survival was assessed by MTT assay. As shown in Fig. 2A, survival was inversely correlated with THDA concentration. Significant loss of viability was detected at 20, 50, 100 and 150 μM of THDA in a dose-and time-dependent manner (Fig. 2A, $P < 0.05$). Meanwhile, the parallel treatment of THDA to normal human lymphocytes showed much less strong effect on inhibition of viability (Fig. 2B), indicating that transformed (K562) cells are more susceptible to THDA.

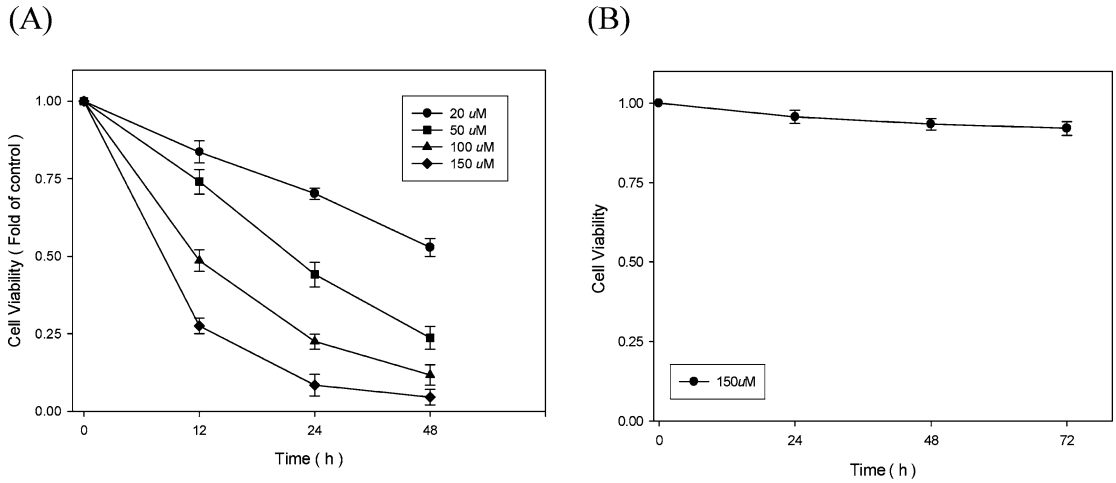

Fig. 2. Effect of THDA on cell viability of K562 cells and normal lymphocytes. MTT assay of (A) K562 cells, (B) normal lymphocytes. Cells were incubated with a series of indicated concentrations of THDA for 48 h, and cell viability were determined. The percentage of viable cells was calculated as a ratio of treated to control cells (treated with PBS). Data are mean ± SD of three independent experiments.

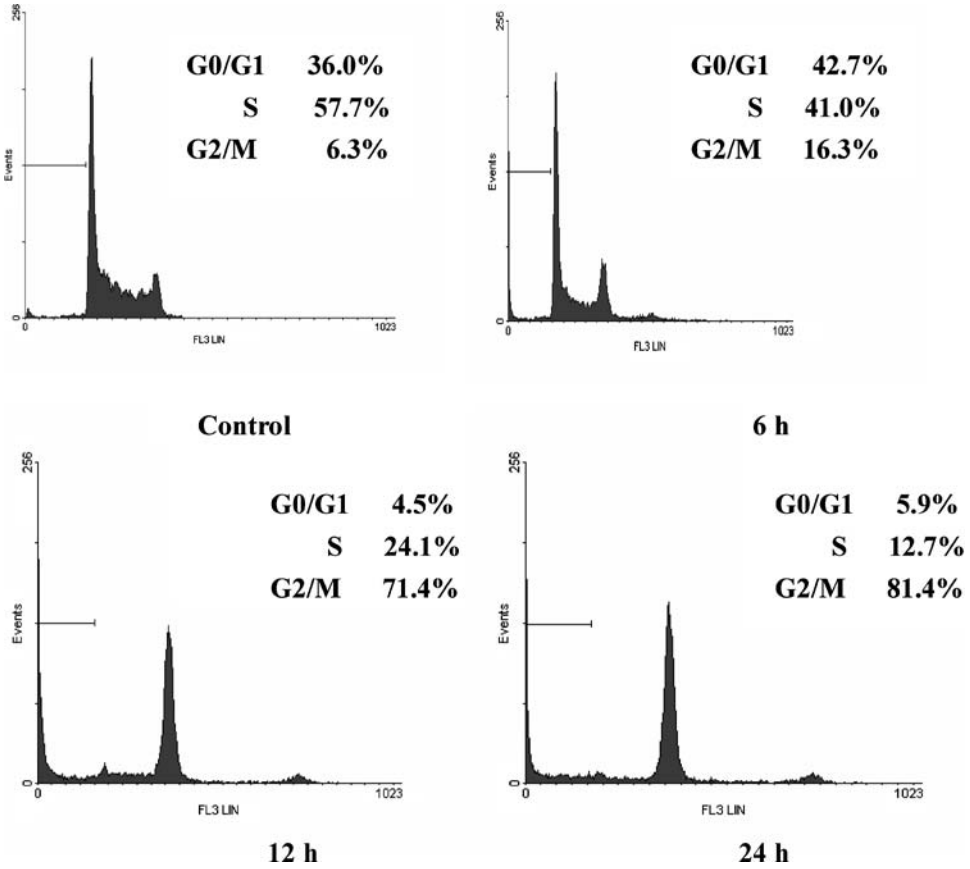

Fig. 3. Accumulation of G2/M phase of cell cycle in THDA-treated K562 cells. Cells (10^6 cells) were seeded in 6-cm dishes for 24 h, then the cells were cultured in the absence or in the presence of 50 μM of THDA for 6, 12, and 24 h. The cells were washed with PBS and fixed in 70% ethanol overnight at 4 °C. The cells were then treated with RNase A solution (500 unit/ml) and stained by propidium iodide (50 μg/ml) at 37 °C for 30 min before analysis. Flow cytometric determination of DNA content was analyzed by COULTER EPICS XL Flow Cytometer (Coulter Corp., Miami, FL, USA).

To determine if cell growth inhibition involved cell cycle changes, we examined cell cycle phase distribution by flow cytometry. When cells were treated with 50 μM of THDA for 24 h, a similar level of G2/M phase arrest was observed in a time-dependent patterns (Fig. 3). For example, 50 μM of THDA treatment for 24 h resulted in an increase in the percentage of cells in the G2/M phase from 16.3 to 81.4%. Concomitant with this increase in the percentage of cells in the G2/M phase was a significant decrease in the percentage of cells in the G0/G1 phase from 36.0 to 5.9%. These results suggested that THDA inhibited the cellular proliferation of K562 cells via G2/M phase arrest of the cell cycle.

Cyclin-dependent kinase inhibitors (CDKIs) play a key role in controlling cell cycle progression by negatively regulating the Cdks activities at an appropriate time in the cell cycle [10]. Since THDA induced G2/M arrest in K562 cells, we wanted to determine the levels of CDKIs, p21 and p27 protein in K562 cells exposed to 50 μM of THDA. The levels of p21 and p27 proteins were progressively increased in a time-dependent manner after treatment with THDA (Fig. 4). In vertebrate cells, the G2/M transition is triggered by regulation of cyclin A, B1, Cdk1, Cdk2, and Cdc25C which promote the breakdown of the nuclear membrane, chromatin condensation, and microtubule spindle formation [11]. Immunoblotting revealed that THDA treatment resulted in a significant reduction in the protein levels of Cdk1, Cdk2 and cyclin A, but not in cyclin B1 and Cdc25C in a time-dependent manner (Fig. 4).

Caspase-3 is an executioner caspase whose activation leads to the cleavage of key cellular proteins including DNA repair enzyme poly-(ADP-ribose) polymerase (PARP). The caspase-3 activity was started at 6 h and sustained for 24 h (Fig. 5A). In regard to PARP protein, which is a major substrate for executed caspases and a hallmark of apoptosis, Western blotting showed that the intact 116 kDa moiety of PARP was degraded, as evidenced by increased 89 kDa cleavage products in THDA-treated K562 cells (Fig. 5B). To determine whether activation of caspase-3 contributed to cell death by THDA, effect of Z-VAD-FMK, a pan-caspase inhibitor, on THDA-induced growth inhibition was determined.

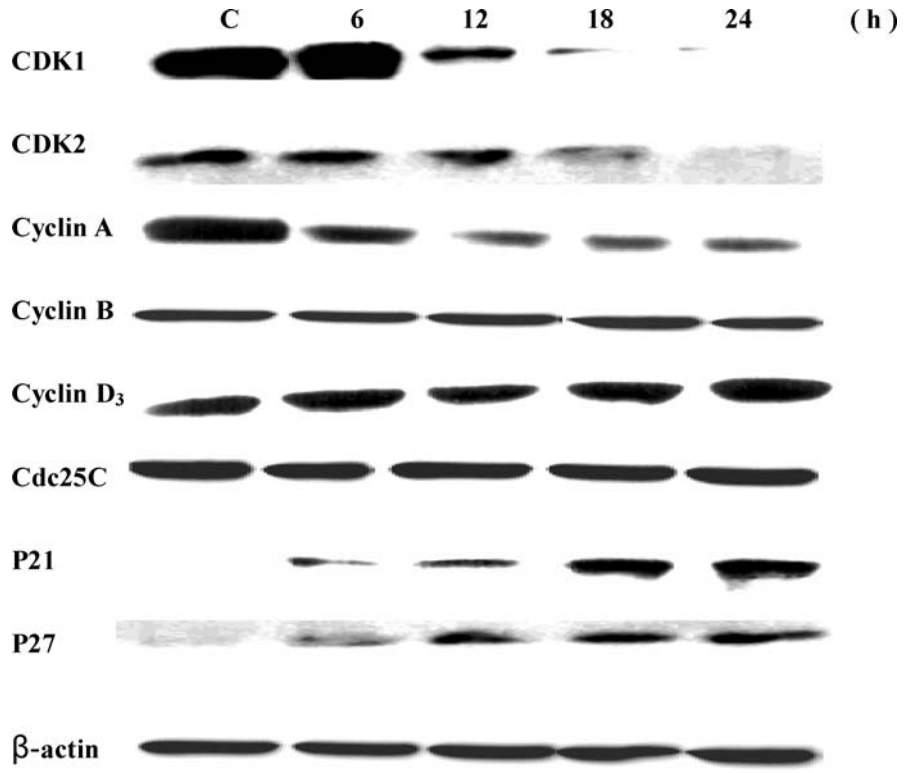

Fig. 4. Immunoblot analysis for the levels of cell cycle regulatory proteins. K562 cells were treated with 50 μM of THDA for the indicated times. Total cell lysates were prepared and 50 μg protein was subjected to SDS-PSGE followed by Western blot analysis and chemiluminescent detection. Each antigenic protein was detected by using the respective antibody against Cdk1, Cdk2, cyclin A, cyclin B1, cyclin D3, Cdc25C, p21, p27, or β-actin.

The growth inhibitory effect of THDA against K562 cells was also significantly attenuated upon treatment with Z-VAD-FMK (data not shown), confirming involvement of caspase-3 in THDA-mediated cell death.

Discussion

Many anti-cancer agents and DNA-damaging agents arrest the cell cycle at the G1, S, or G2/M phase and then induce apoptotic cell death [4, 5, 21]. Cell cycle check-points may function to ensure that cells have time for DNA repair, whereas apoptotic cell death may function to eliminate irreparable or unrepaired damaged cells. THDA had time- and dose-dependently antiproliferative effect on K562 cells, but the mechanism is poorly understood. The purpose of the present study was to elucidate molecular mechanism of action by which THDA inhibited proliferation of human leukemia K562 cells. As shown in Fig. 3, THDA induced a time- and dose-dependent accumulation of cells in the G2/M phase of the cell cycle. G2/M phase accumulation has been observed in cells exposed to DNA damaging agents such as γ-irradiation [22], microtubule-stabilizing agents [12], and topoisomerase inhibitors [23]. To the best of our knowledge, this is the first report describing the mechanism of G2/M phase arrest of THDA on K562 cells.

Unlike the p16 family, the p21 family of CDKIs has a broad range of specificity in the cell cycle proteins and is able to inhibit all the G1 cyclin-CDK complexes as well as cyclin B1-Cdk1 complexes [3, 24].

In this study, the G2/M phase arrest in K562 cells was associated with a marked up-regulation of p21 and p27 proteins, suggesting that the induction of p21 and p27 is an important event by THDA-induced antiproliferative effect. Additionally, our data suggest that THDA caused CDKIs upregulation involves p53-independentt pathway, as K562 cells lack functional p53.

A number of Cdks have been isolated and shown to regulate the cell cycle event in mammalian cells [24, 25]. Among Cdks that regulate cell cycle progression, Cdk1and Cdk2 kinases are activated primarily in association with cyclin A and B1 in the G2/M phase progression. In this study, we found that Cdk1, Cdk2 and cyclin A proteins were decreased in a time-dependent manner following the treatment with THDA, in contrast, the protein level of cyclin B1 was not changed (Fig. 4). Although cyclin A and cyclin B1 are known to be involved in G2/M cell cycle progression, the cyclinB1/Cdk1 complex is the primary regulator of transition from G2 to M

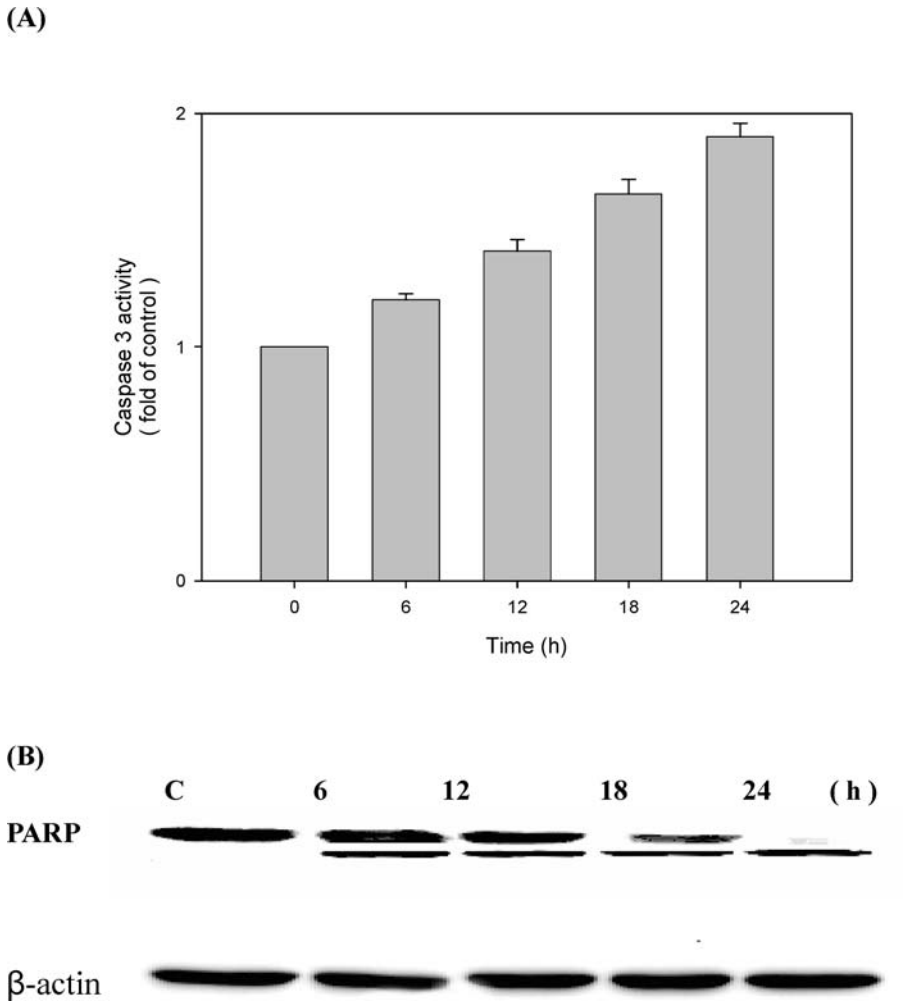

Fig. 5. Effects of THDA on caspase-3 activation and PARP cleavage. (A) Activation of caspase-3 by THDA. Cells were treated with 50 μM of THDA for 6, 12, 18, and 24 h. Cell lysates were prepared and enzymatic activity of caspase-3 protease was determined by incubation of 50 μg/ml total protein with colorigenic substrates for 2 h at 37 °C. The release of chromophore pNA was monitored spectrophotometrically (405 nm). (B) PARP cleavage. K562 cells were treated with 50 μM of THDA for 6, 12, 18, and 24 h. After treatment, the cytosolic fractions were resolved by SDS-PAGE, transferred onto cellulose membranes, and then probed with specific antibody. The amount of β-actin was measured as an internal control. Each blot is representative of three similar experiments.

phase [8]. Thus, our data suggest that cell cycle arrest is mediated by limitation of the supply of Cdk1 to Cdk1/cyclin B complex formation, which is an essential step in regulating passage into mitosis. Meanwhile, the Cdk 1/cyclin B1 kinase complex is maintained in an inactive state by reversible phosphorylations on tyrosine 15 and threonine 14 of Cdk1 [24]. At the onset of mitosis, both of these residues are dephosphorylated by the Cdc25 family of phosphatases, such as Cdc25B and Cdc25C, and this reaction is believed to be the rate-limiting step for entry into mitosis [24, 25]. However, the results of our study demonstrate that the expression level of Cdc25C protein remained unaltered, suggesting that this protein do not link to G2/M arrest. Recently, mitogen-activated protein kinase (MAPK) family of serine/threonine kinases has emerged as an important component of cellular signal transduction [26]. MAPK family members have been implicated in events such as apoptosis, cell cycle and differentiation. Three MAPK families have been described; the extracellular signal-regulated kinases (ERK), the c-jun N-terminal kinase/stress-activated protein kinases (JNK /SAPK) and the p38 kinases. In the future, we plan to elucidate interrelationship between these signal pathways.

In addition, our data showed that THDA markedly induced apoptosis in K562 cells. Several chemotherapeutic and chemopreventive agents have been shown to cause apoptotic cell death through mediation of caspases. Caspase-3 is an executioner caspase, which upon activation can systematically dismantle cells by cleaving key proteins such as PARP. Cleavage of pro-caspase-3 and PARP following treatment with THDA was observed in K562 cells (present study). In addition, THDA-induced cleavage of caspase-3 as well as PARP was significantly attenuated in the presence

of Z-VAD-FMK, and the growth inhibitory effect of THDA against K562 cells was also significantly attenuated upon treatment with Z-VAD-FMK (data not shown), suggesting a caspase-dependent mechanism in cell death by this agent.

In conclusion, THDA, a novel enediyne derivative, arrested the cell cycle at the G2/M phase and induced apoptosis of K562 cells, which is mediated by down-regulation of Cdk1, Cdk 2 and cyclin A in association with induction of p21 and p27, and a concomitant activation of caspase-3 in K562 cells. Finally, these results suggest that THDA may be useful as one of the investigational compounds in the treatment of leukemia.

Acknowledgments

This work was supported by grant NSC 93-2113-M-037-003 from the National Science Council, ROC.

References

1. Torres K, Horwitz SB: Mechanisms of taxol-induced cell death are concentration dependent. Cancer Res 58: 3620–3626, 1998
2. Gamet-Payrastre L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, Gasc N, Tulliez J, Terce F: Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res 60: 1426–1433, 2000
3. Murray AW: Revisiting the cell cycle: cyclins revisited. Cell 116: 221–234, 2004
4. Orren DK, Petersen LN, Bohr VA: Persistent DNA damage inhibits S-phase and G2 progression, and results in apoptosis. Mol Biol Cell 8: 1129–1142, 1997
5. Fujimoto K, Hosotani R, Doi R, Wada M, Lee JU, Koshiba T, Miyamoto Y, Tsuji S, Nakajima S, Imamura M: Induction of cell-cycle arrest and apoptosis by a novel retinobenzoic-acid derivative, TAC-101, in human pancreatic-cancer cell. Int J Cancer 81: 637–644, 1999
6. O'Connor PM, Ferris DK, Pagano M, Draetta G, Pines J, Hunter T, Longo D L, Kohn K W: G2 delay induced by nitrogen mustard in human cells affects cyclin A/cdk2, and cyclin B1/cdc-2 kinase complexes differently. J Biol Chem 268: 8298–8308, 1993
7. Lewin B: Driving the cell cycle: M phase kinases, its partners, and substrates. Cell 61: 743–752, 1990
8. Doree M, Galas S: The cyclin-dependent protein kinases and the control of cell division. FASEB J 8: 1114–1121, 1994
9. Sherr CJ: Cancer cell cycles. Science 274: 1672–1677, 1996
10. Coqueret O: New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? Trends Cell Biol 13: 65–70, 2003
11. King RW, Jackson PK, Kirschner MW: Mitosis in transition. Cell 79: 563–571, 1994
12. Ling YH, Consoli U, Tornos C, Andreeff M, Perez-Soler R: Accumulation of cyclin B1, activation of cyclin B1-dependent kinase and induction of programmed cell death in human epidermoid carcinoma KB cells treated with taxol. Int J Cancer 75: 925–932, 1998
13. Sleiman RJ, Catchpoole DR, Stewart BW: Drug-induced death of leukaemic cells after G2/M arrest: higher order DNA fragmentation as an indicator of mechanism. Br J Cancer 77: 40–50, 1998
14. Jones GB, Wright JM, Hynd G, Wyatt JK, Yancisin MJ, Brown MA: Org Lett 2: 1863–1866, 2000
15. Jones GB, Hynd G, Wright JM, Purohit A, Plourde GW, Huber RS, Mathews JE, Li A, Kilgore, MW, Bubley GJ, Yancisin M J, Brown MA: Target-directed enediynes: designed estramycins. J Org Chem 66: 3688–3695, 2001
16. Alabugin IV, Manoharan M, Kovalenko SV: Tuning rate of the bergman cyclization of benzannelated enediynes with ortho substituents. Org Lett 4: 1119–1122, 2002
17. Lin CF, Hsieh PC, Lu WD, Chiu HF, Wu MJ: A series of enediynes as novel inhibitors of topoisomerase 1. Bioorg Med Chem 9: 1707–1711, 2001
18. Lin CF, Lu WD, Hsieh PC, Kuo YH, Chiu HF, Wang CJ, Wu MJ: Cytotoxicities and topoisomerase 1 inhibitory activities of 2-[2-(2-alkynylphenyl)ethynyl]benzonitriles, 1-aryldec-3-ene-1,5-diynes, and related bis(enediynyl)arene compounds. Helv Chim Acta 8: 2564–2575, 2002
19. Lin CF, Lo YH, Hsieh MC, Chen YH, Wang JJ, Wu MJ: Cytotoxicities, cell cycle and caspase evaluations of 1,6-diaryl-3(Z)-hexen-1,5-diynes, 2-(6-aryl-3(Z)-hexen-1,5-diynyl)anilines and their derivatives. Bioorg Med Chem 13: 3565–3575, 2005
20. Su SH, Su SJ, Lin SR, Chang KL: Cardiotoxin III selectively enhances activation-induced apoptosis of human $CD8^+$ T lymphocytes. Toxicol Appl Pharmacol 193: 97–105, 2003
21. Kessel D, Luo Y: Cells in cryptophycin-induced cell-cycle arrest are susceptible to apoptosis. Cancer Lett 151: 25–29, 2000
22. Hyun JW, Cheon GJ, Kim HS, Lee YS, Choi EY, Yoon BH, Kim JS, Chung MH: Radiation sensitivity depends on OGG1activity status in human leukemia cell lines. Free Radic Biol Med 32: 212–220, 2002
23. Lock RB, Ross WE: Inhibition of p34cdc2 kinase activity by etoposide or irradiaton as a mechanism of G2 arrest in Chinese hamster ovary cells. Cancer Res 50: 3761–3766, 1990
24. Molinari M: Cell cycle checkpoints and their inactivation in human cancer. Cell Prolif 33: 261–274, 2000
25. Hartwell LH, Weinert TA: Checkpoints: controls that ensure the order of cell cycle events. Science 246: 629–634, 1989
26. Wada T, Penninger JM: Mitogen-activated protein kinases in apoptosis regulation. Oncogene 23: 2838–2849, 2004