[Frontiers in Bioscience 13, 561-568, January 1, 2008]

Shikonin analogue (SA) 93/637 induces apoptosis by activation of caspase-3 in U937 cells

Rajesh L. Thangapazham[1,2], Anoop K. Singh[1], Pankaj Seth[1], Namita Misra[3] , Shafali[3], Vijayavitthal T. Mathad[3], Kanwal Raj[3] Radha K. Maheshwari[1]

[1] Center for Combat Casualty and Life Sustainment Research, Department of Pathology, Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD 20814, USA, [2]Birla Institute of Technology and Science, Pilani 333031, India, [3] Central Drug Research Institute, Lucknow 226001, India

TABLE OF CONTENTS

1. ABSTRACT

β,β-dimethyl acryl shikonin is an extract from the root of plant *Arnebia nobilis* which has been shown to possess anti-cancer activity. However, its toxicity limited further development of shikonin as a therapeutic agent. Subsequently, several analogues of β,β-dimethyl acryl shikonin were synthesized. One of these analogues, shikonin 93/637 was found to be significantly less toxic compared to shikonin. This study is aimed to determine the cell cycle associated differences in the susceptibility of U937 cells to apoptosis induced by shikonin analogue 93/637 (SA). Lower concentrations of SA (~100nM) showed no significant changes in cell growth. However, higher concentrations (~500nM) resulted in growth inhibition of U937 cells after 48 h of treatment with SA as measured by MTT assay. Flow cytometric analysis showed that SA treatment resulted in blocking of cell cycle progression in G1 phase. Decreased expression of Cyclin D, CDK 4 and PCNA was observed with SA treatment corroborating the G1 block. DNA gel electrophoresis showed an oligonucleotide ladder pattern, a distinct characteristic of DNA fragmentation associated with programmed cell death. Ribonuclease protection assay revealed inhibition of bcl_2 expression at transcriptional level. SA treatment also resulted in induction of caspase-3 activity. The results suggest the involvement of bcl_2 and Caspase-3 in SA induced apoptosis of human U937 cells.

2. INTRODUCTION

Apoptosis is associated with a number of distinct morphological changes including cell shrinkage, chromatin degradation, cytoplasmic and nuclear condensation and blebbing (1-3). Several studies have demonstrated that susceptibility to apoptosis is related to cell cycle in a variety of cell lines (4-7). Earlier studies have shown that treatment with retinoic acid causes accumulation of neuroblastoma cells in G1 phase and also induces apoptosis, whereas cisplatin treatment induced G2/M accumulation and subsequently led to apoptosis (8). Apoptosis induced by DNA topoisomerase I inhibitor campothecin was shown to be limited to S-phase in HL-60 cells (7). Apoptosis is also known to be a major physiological mechanism of terminally differentiated hematopoietic cells. U937 cells are susceptible to apoptosis during treatment with number of different agents, such as tumor necrosis factor (TNF) (9), calcium ionophore (10), ceremide (11) and several microbial toxins (12).

Terminally differentiated hematopoietic cells such as monocytes and granulocytes are eliminated from the body after spending relatively short periods in a functional state (13). Elimination of these cells is believed to be important for the homeostasis of the self-defense mechanism. Survival of neoplastic cells is related to loss or

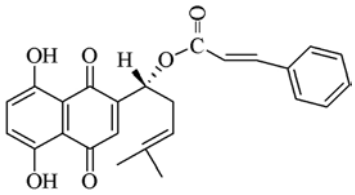

Figure 1. Structure of Cinnamoyl shikonin ([5,8-dihydroxy-2-1-(1'-Cinnamoyloxy-4'-methylpent-3-enyl)-1,4-naphthaquinone]).

suppression of normal apoptotic pathway (14), and many efforts are now directed towards ways of modulating or restoring an apoptotic pathway in malignant cells (15).

Previous studies from several independent laboratories have shown the usefulness of Shikonin-1 and its derivative in cancer, wound healing, anti-inflammatory and antimicrobial activities in variety of *in vitro* and *in vivo* models (16-20). It was reported that shikonin inhibited topoisomerase II by inducing topoisomerase II mediated cleavage of DNA (21). However, some toxicity of these derivatives restricted its development as an anticancer drug for clinical use. We have recently synthesized Shikonin ester labeled as shikonin analogue 93/637. In the present study, we have investigated the effect of SA 93/637 in human promonocytic cell line (U937). The results showed that SA suppressed cell growth of U937 cells in a dose dependent manner. Morphological, DNA and cell cycle analysis demonstrated that SA93/637 exerts its cytotoxic effect by either inducing apoptosis at higher concentrations (>500nM) or by inducing arrest at G0/G1 phase in cell cycle at lower concentrations (<100nM). These studies have greater clinical significance since the ability of tumor cells to respond to damage and eventually activate the apoptotic program might determine the success of chemotherapy.

3. MATERIALS AND METHODS

3.1. Materials

The structure of SA is shown as Figure 1. [5, 8-dihydroxy-2-1-(1'-Cinnamoyloxy-4'-methylpent-3-enyl)-1, 4-naphthaquinone] or Cinnamoyl shikonin is synthesized by hydrolyzing β,β dimethyl acryl shikonin isolated from the roots of *Arnebia noblis* (Boraginaceae) with sodium hydro-oxide to get Shikonin, which was then esterified with cinnamic anhydride or Cinnamoyl chloride or Cinnamic acid & D.C.C. A HPLC purified Shikonin analogue (5, 8-dihydroxy-2-(1'-cinnamoyloxy-4'-methylpent-3'-enyl)-1,4-napthaquinone; SA (93/637) was prepared at the Central Drug Research Institute, Lucknow, India. A stock of 10 mM was prepared in DMSO and stored at -20^{0}C. The effective concentration of DMSO was less than 0.05% in all the experiments.

3.2. Cell Culture

U937 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100units/ml penicillin, 100mg/ml streptomycin and 250 mg/ml fungizone (Gibco BRL, Gaithersburg,MD). The cells were passaged every third day and were diluted 1:2 one day before each experiment.

3.3. MTT cell viability assay

Cells were plated into 96 well tissue culture plates in a range of 3000 cells/well in a final volume of 100µl and were treated with varying doses of SA for 24, 48 and 72h. The chromogenic methyl thiazol tetrazolium bromide (MTT) dye, an indicator of metabolically active mass, was added to the cells and incubated for 4 h at 37^{0}C. Cells were lysed and the reduced intracellular formazan product was dissolved in a solution with 10% SDS and 0.01N HCl. The absorbance was recorded at 570 nm with a reference filter of 650 nm and percentage inhibition was plotted against untreated cells.

3.4. Flow Cytometry and DNA Histogram

Aliquots of 5x10^5 U937 cells were centrifuged at 1200 rpm for 10 min, and cell pellets were fixed with 70% ethanol overnight. The cells were then washed twice with PBS and resuspended in 1 ml of solution containing 3.4 mM sodium citrate, 50 µg/ml propidium iodide (Boehringer Mannheim, Indianapolis, IN) and 100 µg/ml RNAase A (Boehringer Mannheim, Indianapolis, IN) and stored in the dark for 30 min. Cells were analyzed on a FACScan flow cytometer. Samples were analyzed using an EPICS ELITE ESP (Beckman Coulter, Miami, FL) flow cytometer. Orange emission from propidium iodide was collected through a 575 nM band-pass filter using linear amplification. FL2 peak and FL2 integrated signals were collected to permit doublet discrimination. A minimum of 15,000 single events was collected for each sample. Cell cycle analysis of DNA histograms was performed with ModFit LT software (Verity Software House, Topsham, ME).

3.5. DNA fragmentation by electrophoresis

DNA from treated and untreated cells were isolated after lysis of approximately 2X10^7 cells in TE buffer containing TRIS 10mM, EDTA 10mM, SDS 0.5% and proteinase K 200µg/ml at 50^{0}C for 2h. The DNA was purified by precipitation of proteins in 1M NaCl. Samples were centrifuged at 2500g for 30min at 4^{0}C to pellet the proteins. The supernatant were then treated with DNAse free RNAse 25µg/ml) for 30min at 37^{o}C. The viscous solution was then transferred to an ice bath for 30min, and then centrifuged at 2500g for 30min at 4^{0}C to pellet the purified DNA. The purified strands of the DNA was quantitated and equal amount of DNA was electrophoresed in a 1.2% agarose gel. The separated bands were stained with ethidium bromide and visualized under UV light.

3.6. Ribonuclease Protection Assay

RNA was isolated from U937 cells using Trizol (Life Technologies Inc.) and was quantitated and equalized. 20µg of equalized RNA samples were then hybridized at 56^{0}C for 12-14 h with a ^{32}P alpha-UTP labeled probe that was prepared using the human apoptosis template (Pharmingen, San Diego, CA). Hybridized samples were later subjected to RNAse digestion for 45 min at 30^{0}C to remove the unhybridized RNAs. The ribonuclease protected bands were then resolved on denaturing urea-

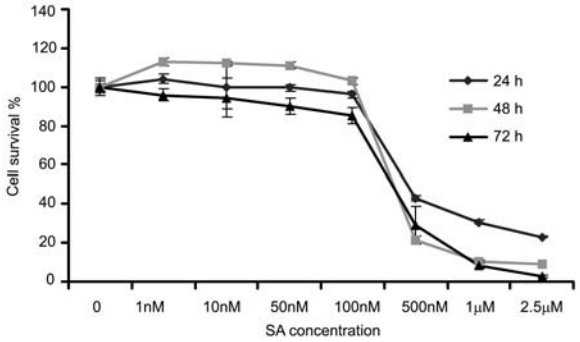

Figure 2. Effect of SA on U937 cells growth. Cells were plated into 96 well tissue culture plates in a range of 3-4x10^5 cells/ml in a final volume of 100µl and were treated with varying doses of SA for 6, 24 and 48 h. The chromogenic methyl thiazol tetrazolium bromide (MTT) dye, an indicator of metabolically active mass, was added to the cells and incubated for 3 h at 37^0C. Cells were lysed and the reduced intracellular formazan product was dissolved in a solution with 10% SDS and 0.01N HCl. The absorbance was recorded at 570 nm and percentage inhibition was calculated against untreated cells.

PAGE gels, followed by autoradiography. Multi-probe Ribonuclease protection assay system from Pharmingen, San Diego, CA was used. RPA is a highly sensitive and specific method for the detection and quantitation of mRNA species. RNA samples were prepared from 24 h SA treated U937 cells. Templates for apoptotic and anti apoptotic genes like $bclX_L$, bfl-1, bik, bak, bax, bcl_2 and Mcl-1 were used. L32 and GAPDH templates were used for the analysis of two house keeping gene transcripts.

3.7. Caspase-3 Cellular activity assay

Caspase-3 cellular activity in shikonin 93/637 treated cells was determined by using Caspase-3 cellular activity assay kit (Calbiochem, San Diego, CA-92039) following the instructions of the manufacturer. Briefly, U937 cell were grown and treated with SA (500nM) for 3h. 1x10^7 cells/ml per treatment were harvested by centrifugation and washed twice with phosphate buffered saline (PBS). Cells were lysed with the lysis buffer and then centrifuged to get clear supernatant. Caspase-3 activity was determined using the protocol described by the manufacturer (Calbiochem, San Diego, CA-92039). The absorbance was read at 405 nm in microtiter plate-reader and data was recorded at a regular interval for 240 min.

3.8. Western blotting

Cells were harvested, pelleted and were homogenized with ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X 100, 1% sodium deoxycholate, 0.1% SDS to which 1mM DTT, 1 mM PMSF, 1 µg/ml pepstatin A, 10 µg/ml Leupeptin, 10 µg/ml aprotinin, 25 mM NaF, and 100 µM Na_3VO_4 were added fresh). The homogenate was passed 10 times through a 25-guage needle and centrifuged at 14000 g for 20 min at 4°C. The supernatant protein extract was transferred to fresh tubes, aliquoted and stored at –80°C. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL). 50 µg of protein extracts in 13 µl of RIPA were thawed on ice and mixed with 2 µl of 10X sample reducing agent (Invitrogen, Carlsbad, CA) and 5 µl of 4X sample buffer (Invitrogen, Carlsbad, CA). Samples were heated at 100°C for 5 min. They were centrifuged and loaded on 4-12% Bis-Tris gels for fractionation at 120 V. Predetermined molecular weight standards (Invitrogen) were used as markers. Proteins on the gel were blotted onto nitrocellulose membranes at 225 mA for 120 min at 4°C. After transfer, the membranes were incubated with blocking buffer (5% skim milk in wash buffer [1× TBS and 0.1% Tween 20]) for 1 h at room temperature. Protein expression for cell cycle proteins was detected by incubating with appropriate primary antibodies (BD Biosciences, San Jose, CA) in blocking buffer. The membranes were washed three times with wash buffer and were then incubated with the appropriate secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:2000 blocking buffer. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were scanned and optical densities of the band were quantitated.

4. RESULTS

4.1. SA inhibits the growth and alters the morphology of U937 cells

Cell growth was assayed in culture exposed to varying doses of SA in 10% FCS-supplemented RPMI. Under these conditions, cell viability remained high in cultures treated with SA up to 200nM. However, there was a time and dose dependent inhibition of cell growth in cultures as measured by number of viable cells 24h after addition of SA (Figure 2). Gradual decrease in cell proliferation was observed between 100nM to 500nM doses of SA. Concentration lower than 50nM had no significant effect in cell proliferation and concentration exceeding 2.5 µM were toxic to the cells.

4.2. SA blocks the progression of U937 cells through G0/G1

The effect of SA on Go/G1-phase progression was evident as 12 h treatment with 10nM SA showed approximately ~64% cells in early G1-phase compared to 42% cells in untreated control. Most of the SA treated cells were found at the boundary of G1/S. Thus the cells damaged by SA co-related with a decrease in the relative fraction of the cell population in S and G2-phase (Table 1). However, higher treatment of SA93/637 resulted in more number of cells in S and G2 phase with greater number of cells undergoing apoptotic cell death (data not shown).

4.3. SA induces change in the expression of cell cycle regulatory proteins

The effect of SA on the expression of cell cycle regulatory proteins involved in G1 to S transition of cell cycle like cyclin D and CDK 4 was assessed by western blot. A dose dependent reduction in the expression of cyclin D and CDK 4 was observed with SA treatments. The data

Table 1. FACScan analysis of nuclear DNA stained with propidium iodide (PI)

Treatment / Phase	G1	S	G2
Control	42.6	45.9	11.5
SA 1 nM	62.5	35	2.4
SA 5 nM	63.7	35	0.8
SA 10 nM	63.9	35.5	0.7

The distribution of cells in different phases of cell cycle was analyzed using P.I staining. The data is summarized in a stacking bar form for simplicity. The X-axis indicates treatments whereas y-axis represents the percentage of cells in different phases of cell cycle. (A. Control; B. SA ; 500nM, C.1μM., D. 2.5μM)

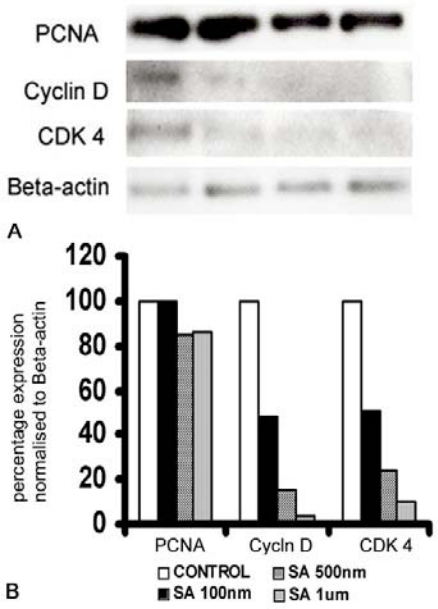

Figure 3. Effect of SA on Cyclin D, CDK 4 and PCNA. (A) Representative picture of Westernblot (B) Quantificaton of expression of cell cycle proteins.

shows a marginal decrease in the expression of PCNA, a proliferation biomarker which is considered to be a reliable index of the proliferation rate (Figure 3). These results indicate that SA treatment inhibits cell proliferation in U937 cells.

4.4. DNA Fragmentation of U937 cells upon SA treatment

Exposure of exponentially growing U937 cells to SA, led to apoptosis of large sub-population of cells. The effect was found to be rapid and within 6 h of the treatment as demonstrated by microscopic analyses and DNA ladder. We observed apoptotic cells, characterized by shrinkage of cells chromatin condensation and nuclear fragmentation. Fluorescence microscopic examination of P.I stained cells confirmed the presence of a nuclear fragmentation pattern typical of apoptosis as compared to normal nuclei (Data not shown). Moreover, DNA extracted from cultures treated with SA for 6 h showed DNA degradation into oligonucleosomes (Figure 4). Thus, growth inhibitory activity of SA at concentrations greater than 500 nM resulted from a sum of cytostatic and apoptotic effects (Figure 4; lane 3 and 4). There was no effect of vehicle control on DNA fragmentation (Figure 4; lane 2).

4.5. Inhibition of bcl_2 by SA at transcriptional level

Several reports have indicated that the ratio of bcl-2 to Bax determines the cell survival or death following an apoptosis stimulus. Our data clearly indicate that SA (500nM - 1μM) treatment for 12 h inhibited the expression of bcl_2 and bik. However, there were no significant alterations in bcl $_X$, bfl, bak, bax and, Mcl-1 (Figure 5; lane 1-4). Cells treated with SA for 24 h resulted in inhibition of all the genes at 1 μM dose as compared to untreated control. This could be due to cytotoxic effect of the drug at this higher dose for such a long time (Figure 5; lane 5-8). Kinetic data of cells treated with SA did not show significant alteration in gene expression after 3 h SA treatment. However, there was a slight inhibition in bcl_2 expression after 6 h treatment of SA at transcriptional level (data not shown). These studies suggest that inhibition of bcl_2 at higher doses of SA may be partly responsible for the apoptotic death.

4.6. Induction of Caspase-3 by SA treatment

To investigate whether caspase is activated during the apoptosis induced by the SA, we treated U937 cells with 500 nM of SA for 3 h. The results showed a marked increase in caspase-3 activity (Figure 6). This increase in Caspase-3 activity correlated well with that of SA induced DNA fragmentation. Caspase inhibitor almost completely inhibited the caspase activity of SA, suggesting that a caspase-3 like protease might be activated during SA-induced apoptosis in U937 cells.

5. DISCUSSION

Apoptosis shares a number of morphological features with mitosis, including cell rounding, surface bleebing, lamin disassembly and chromatin condensation (22,2). However, it is believed that cell differentiation is tightly regulated in a cell cycle dependent manner. Differentiation of hemopoietic cells are associated with the loss of cell cycling capacity and, as a consequence, terminally differentiated cells are arrested in G0/G1 phase. Thus, it is reasonable to speculate that susceptibility of these cells to apoptosis is closely related to a G0/G1 arrest. The results indicate that cells in G1 phase were susceptible to apoptosis induced by SA. The treatment duration of SA were so short (6 h) compared with the cell cycling time of U937 cells, therefore, even if cells were arrested in G1 phase while the progression through the remainder of the cycle was unperturbed, this time interval was inadequate for such a marked changes in cell cycle as observed with SA (<100 nM) to occur. At lower concentrations, SA retarded the progression of these cells, which was significantly halted at the sub-apoptotic doses. However, higher concentration of the SA (>500 nM) treated U937 cells actually moved faster through S and G2 phase and a block in G1 phase.

The present data demonstrating an effect of SA in promonocytic U937 cells of malignant lymphoma origin

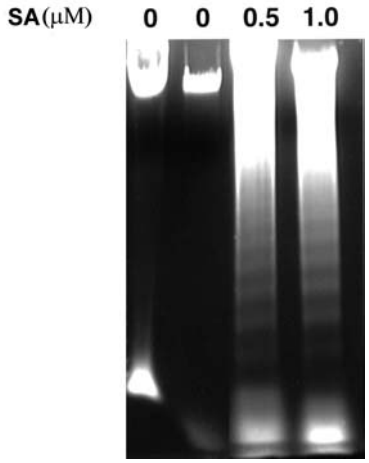

Figure 4. Electrophoresis of DNA in agarose gel: Agarose gel electrophoresis of DNA from cells cultured with medium alone (lane 1) or vehicle control (lane 2) or 500nM (lane 3) or 1 μM (lane 4) SA for 6 h. 15 μg of DNA were electrophoresed in 1.2 % agarose gel, stained with ethidium bromide, and photographed under UV illumination. DNA fragmentation with ladder pattern characteristic of apoptosis is determined in lane 3 and 4.

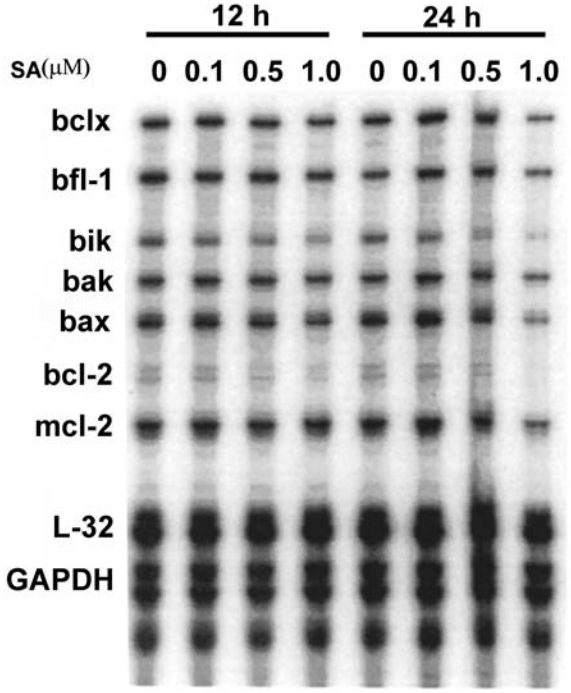

Figure 5. Multi-probe Ribonuclease protection assay for the expression of apoptotic genes: Lane 1-4: Cells treated with SA for 12 h (1; control, 2; SA 100 nM, 3; SA 500 nM, 4; SA 1 μm) or lane 5-8 treated for 24 h (5; control, 6; SA 100 nM, 7; SA 500 nM, 8; SA 1 μm). RNA samples were prepared from 12 and 24 h SA treated U937 cells. Templates for apoptotic and anti apoptotic genes like $bclX_L$, bfl-1, bik, bak, bax, bcl_2 and Mcl-1 were used. L32 and GAPDH templates were used for the analysis of two house keeping gene transcripts.

was of particular interest since these cells fail to express the tumor suppressor gene p53 (23). The absence of p53 function has been previously correlated with decrease in vulnerability to agents of apoptosis (14). An increase in p53 gene product can be associated with DNA repair and protection of cells against radiation damage (24). Since cell cycle checkpoint in G1 and G2 phase protect DNA damaged cells by delaying the entry into S-phase and mitosis, respectively. In our study, we have observed total abrogation of G2 checkpoint in presence of SA. The molecular basis of enhanced G2 checkpoint abrogation with defective p53 function remains to be investigated. Similar studies have earlier shown the involvement of the cdc2 kinase (25,26). The inability of malignant cells to express p53 also may be a factor related to development of multidrug resistance (15).

Studies from several independent laboratories have indicated the anti-tumor agent like camptothecin at concentrations lower than those which trigger immediate apoptosis perturb the cell cycle progression through S or G2 phase. It has been reported that usually the cell cycle phase which is perturbed by low concentration of the drug is the same at which higher concentration induces apoptosis (27). This hypothesis suggests that at lower concentration SA slows the progression of U937 cells through the G1 phase and attempts to repair the damage. Conversely, apoptosis is triggered at higher concentration when the damage is extensive and probably irreparable. There are several widely used drugs such as doxirubicin which have broad range of concentration to suppress cell cycle progression (5).

In the present study, we have observed apoptosis at higher concentrations with in 6 h. This may be due to the primary damage induced by SA, which appears to be associated with triggering apoptosis. The mechanism of delayed apoptosis, observed at lower doses after longer time intervals that are different and involve secondary lesions may be related to unbalanced growth, inefficiency of DNA repair, and other factors. Earlier b-hydroxyisovaleryl shikonin has been demonstrated to inhibit the growth of various cancer cell lines at low concentrations and induced apoptosis after 3h treatment with an activation of caspase-3 activity (28).

Studies have characterized genes such as bcl_2 in B- and T- cells which may contribute to the regulation of apoptosis (29,30). Whereas others have demonstrated that drug induced internucleosomal DNA fragmentation in human myeloid leukemia cells are associated with expression of early response genes (31). In the present report, we have demonstrated that bcl_2 is down regulated at higher doses of SA after 12 h treatment at transcriptional levels, which may be partly responsible for the death of U937 cells. There was no significant change in expression of bax, bcl_x, Mcl-1 and bak. Over expression of bcl_2 in lymphoid cells and in transgenic mice has been shown to prevent apoptosis induced by DNA damaging agents (32). It is also believed that although differentiation and apoptosis occur concomitantly, they are regulated independently in differentiating myeloid cells (33). Other

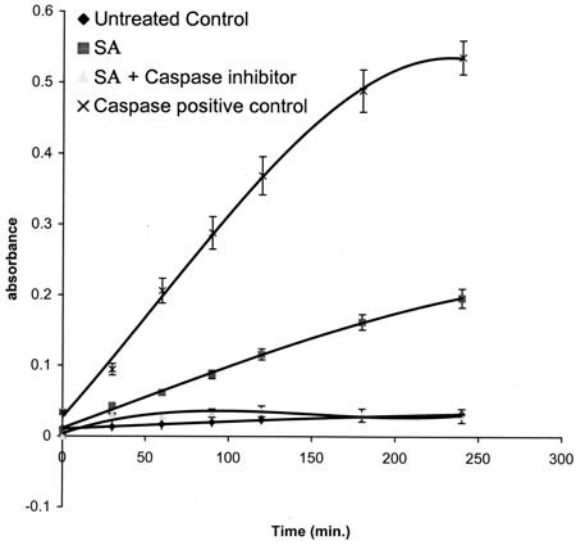

Figure 6. Induction of Caspase-3 activity in SA treated U937 cells: U937 cells were treated with 500 nM of SA for 3 h to undergo apoptosis. Cell extracts were prepared as described in materials and method section. Control cell extract was prepared from untreated cells. The positive control sample contained 30 U of caspase-3. Caspase-3 substrate, colorimetric (DEVD-pNA) cleavage rate were calculated from the slope and were divided with per mg of protein present in the sample.

studies have shown the importance of proteases in mediating apoptotic death induced by various agent (34,35). It is unclear how cell death is mediated after activation of caspases (36), however most of the known intracellular substrates of caspases are involved in cellular repair mechanism, regulation of cell cycle and maintenance of cell structure (37). Whether cleavage in one of substrates is an irreversible cause of cell death is not known. In our study, SA treatment resulted in activation of caspase-3 and could be partly responsible for the SA induced cell death.

Present study suggests that SA93/637 acts by a specific molecular mechanism of inducing cell death. Further, studies including the precise molecular mechanism of death initiation during cell cycle in a p53 negative, malignant cell line would improve understanding of the biological action on malignant cell. This may lead to the development of more effective shikonin-1 analogues against cancer.

6. ACKNOWLEDGMENTS

This work was supported by USUHS intra mural grant and grant G174HD from Naval Medical Research and Development Command and US-INDIA Foreign Currency Fund from US Department of State. The authors are grateful to Mr. Anuj Sharma for his help and valuable suggestions. The opinions or assertions contained herein are the private views of the authors and should not be construed as official or necessarily reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense.

7. REFERENCES

1. Martelli, A. M., I. Faenza, A. M. Billi, L. Manzoli, C. Evangelisti, F. Fala & L. Cocco: Intranuclear 3'-phosphoinositide metabolism and Akt signaling: new mechanisms for tumorigenesis and protection against apoptosis? *Cell Signal*, 18, 1101-7(2006)

2. van Engeland, M., H. J. Kuijpers, F. C. Ramaekers, C. P. Reutelingsperger & B. Schutte: Plasma membrane alterations and cytoskeletal changes in apoptosis. *Exp Cell Res*, 235, 421-30(1997)

3. Compton, M. M.: A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer Metastasis Rev*, 11, 105-19(1992)

4. Kamat, A. M., G. Sethi & B. B. Aggarwal: Curcumin potentiates the apoptotic effects of chemotherapeutic agents and cytokines through down-regulation of nuclear factor-{kappa}B and nuclear factor-{kappa}B-regulated gene products in IFN-{alpha}-sensitive and IFN-{alpha}-resistant human bladder cancer cells. *Mol Cancer Ther*, 6, 1022-30(2007)

5. Colombel, M., C. A. Olsson, P. Y. Ng & R. Buttyan: Hormone-regulated apoptosis results from reentry of differentiated prostate cells onto a defective cell cycle. *Cancer Res*, 52, 4313-9(1992)

6. Davidoff, A. N. & B. V. Mendelow: Unexpected cytokinetic effects induced by puromycin include a G2-arrest, a metaphase-mitotic-arrest, and apoptosis. *Leuk Res*, 16, 1077-85(1992)

7. Gong, J., X. Li & Z. Darzynkiewicz: Different patterns of apoptosis of HL-60 cells induced by cycloheximide and camptothecin. *J Cell Physiol*, 157, 263-70(1993)

8. Piacentini, M., L. Fesus & G. Melino: Multiple cell cycle access to the apoptotic death programme in human neuroblastoma cells. *FEBS Lett*, 320, 150-4(1993)

9. Afonso, V., G. Santos, P. Collin, A. M. Khatib, D. R. Mitrovic, N. Lomri, D. C. Leitman & A. Lomri: Tumor necrosis factor-alpha down-regulates human Cu/Zn superoxide dismutase 1 promoter via JNK/AP-1 signaling pathway. *Free Radic Biol Med*, 41, 709-21(2006)

10. Matsubara, K., M. Kubota, K. Kuwakado, H. Hirota, Y. Wakazono, A. Okuda, R. Bessho, Y. W. Lin, S. Adachi & Y. Akiyama: Variable susceptibility to apoptosis induced by calcium ionophore in hybridomas between HL-60 promyelocytic and CEM T-lymphoblastic leukemia cell lines: relationship to constitutive Mg(2+)-dependent endonuclease. *Exp Cell Res*, 213, 412-7(1994)

11. Woo, K. J., Y. H. Yoo, J. W. Park & T. K. Kwon: Bcl-2 attenuates anticancer agents-induced apoptosis by sustained activation of Akt/protein kinase B in U937 cells. *Apoptosis*, 10, 1333-43(2005)

12. Kochi, S. K. & R. J. Collier: DNA fragmentation and cytolysis in U937 cells treated with diphtheria toxin or other inhibitors of protein synthesis. *Exp Cell Res*, 208, 296-302(1993)

13. Mangan, D. F., B. Robertson & S. M. Wahl: IL-4 enhances programmed cell death (apoptosis) in stimulated human monocytes. *J Immunol*, 148, 1812-6(1992)

14. Symonds, H., L. Krall, L. Remington, M. Saenz-Robles, S. Lowe, T. Jacks & T. Van Dyke: p53-dependent apoptosis suppresses tumor growth and progression *in vivo*. *Cell*, 78, 703-11(1994)

15. Fisher, D. E.: Apoptosis in cancer therapy: crossing the threshold. *Cell*, 78, 539-42(1994)

16. Yang, F., Y. Chen, W. Duan, C. Zhang, H. Zhu & J. Ding: SH-7, a new synthesized shikonin derivative, exerting its potent antitumor activities as a topoisomerase inhibitor. *Int J Cancer*, 119, 1184-93(2006)

17. Mani, H., G. S. Sidhu, A. K. Singh, J. Gaddipati, K. K. Banaudha, K. Raj & R. K. Maheshwari: Enhancement of wound healing by shikonin analogue 93/637 in normal and impaired healing. *Skin Pharmacol Physiol*, 17, 49-56(2004)

18. Shen, C. C., W. J. Syu, S. Y. Li, C. H. Lin, G. H. Lee & C. M. Sun: Antimicrobial activities of naphthazarins from Arnebia euchroma. *J Nat Prod*, 65, 1857-62(2002)

19. Chen, X., J. Oppenheim & O. M. Howard: Shikonin, a component of antiinflammatory Chinese herbal medicine, selectively blocks chemokine binding to CC chemokine receptor-1. *Int Immunopharmacol*, 1, 229-36(2001)

20. Gaddipati, J. P., H. Mani, Shefali, K. Raj, V. T. Mathad, A. P. Bhaduri & R. K. Maheshwari: Inhibition of growth and regulation of IGFs and VEGF in human prostate cancer cell lines by shikonin analogue 93/637 (SA). *Anticancer Res*, 20, 2547-52(2000)

21. Fujii, N., Y. Yamashita, Y. Arima, M. Nagashima & H. Nakano: Induction of topoisomerase II-mediated DNA cleavage by the plant naphthoquinones plumbagin and shikonin. *Antimicrob Agents Chemother*, 36, 2589-94(1992)

22. Lazebnik, Y. A., S. Cole, C. A. Cooke, W. G. Nelson & W. C. Earnshaw: Nuclear events of apoptosis *in vitro* in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis. *J Cell Biol*, 123, 7-22(1993)

23. Calabresse, C., L. Venturini, G. Ronco, P. Villa, L. Degos, D. Belpomme & C. Chomienne: Selective induction of apoptosis in myeloid leukemic cell lines by monoacetone glucose-3 butyrate. *Biochem Biophys Res Commun*, 201, 266-83(1994)

24. Kastan, M. B., Q. Zhan, W. S. el-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein & A. J. Fornace, Jr.: A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, 71, 587-97(1992)

25. O'Connor, P. M., D. K. Ferris, I. Hoffmann, J. Jackman, G. Draetta & K. W. Kohn: Role of the cdc25C phosphatase in G2 arrest induced by nitrogen mustard. *Proc Natl Acad Sci U S A*, 91, 9480-4(1994)

26. O'Connor, P. M., D. K. Ferris, M. Pagano, G. Draetta, J. Pines, T. Hunter, D. L. Longo & K. W. Kohn: G2 delay induced by nitrogen mustard in human cells affects cyclin A/cdk2 and cyclin B1/cdc2-kinase complexes differently. *J Biol Chem*, 268, 8298-308(1993)

27. Bhuyan, B. K., T. J. Fraser, L. G. Gray, S. L. Kuentzel & G. L. Neil: Cell-kill kinetics of several S-phase-specific drugs. *Cancer Res*, 33, 888-94(1973)

28. Hashimoto, S., M. Xu, Y. Masuda, T. Aiuchi, S. Nakajo, J. Cao, M. Miyakoshi, Y. Ida & K. Nakaya: beta-hydroxyisovalerylshikonin inhibits the cell growth of various cancer cell lines and induces apoptosis in leukemia HL-60 cells through a mechanism different from those of Fas and etoposide. *J Biochem (Tokyo)*, 125, 17-23(1999)

29. Trescol-Biemont, M. C., C. Verschelde, A. Cottalorda & N. Bonnefoy-Berard: Regulation of A1/Bfl-1 expression in peripheral splenic B cells. *Biochimie*, 86, 287-94(2004)

30. McDonnell, T. J., G. Nunez, F. M. Platt, D. Hockenberry, L. London, J. P. McKearn & S. J. Korsmeyer: Deregulated Bcl-2-immunoglobulin transgene expands a resting but responsive immunoglobulin M and D-expressing B-cell population. *Mol Cell Biol*, 10, 1901-7(1990)

31. Gunji, H., S. Kharbanda & D. Kufe: Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-beta-D-arabinofuranosylcytosine. *Cancer Res*, 51, 741-3(1991)

32. Strasser, A., A. W. Harris & S. Cory: bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell*, 67, 889-99(1991)

33. Naumovski, L. & M. L. Cleary: Bcl2 inhibits apoptosis associated with terminal differentiation of HL-60 myeloid leukemia cells. *Blood*, 83, 2261-7(1994)

34. Mohan, J., A. A. Gandhi, B. C. Bhavya, R. Rashmi, D. Karunagaran, R. Indu & T. R. Santhoshkumar: Caspase-2 triggers Bax-Bak-dependent and -independent cell death in colon cancer cells treated with resveratrol. *J Biol Chem*, 281, 17599-611(2006)

35. Martin, S. J. & D. R. Green: Protease activation during apoptosis: death by a thousand cuts? *Cell*, 82, 349-52(1995)

36. Ashkenas, J. & Z. Werb: Proteolysis and the biochemistry of life-or-death decisions. *J Exp Med*, 183, 1947-51(1996)

37. Nicholson, D. W. & N. A. Thornberry: Caspases: killer proteases. *Trends Biochem Sci*, 22, 299-306(1997)

Key Words Apoptosis, Cell cycle, Shikonin analogue, Caspase-3

Send Correspondence to: Dr Radha K. Maheshwari, Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA, Tel: 301-295-3394, Fax: 301-295-1640, E-mail: rmaheshwari@usuhs.mil

http://www.bioscience.org/current/vol13.htm