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**Retinoid-Induced Apoptosis in HL-60 cells is Associated with Nucleolin Downregulation
and Destabilization of Bcl-2 mRNA[†]**

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Running Title: ATRA-induced bcl-2 mRNA destabilization

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Non-standard Abbreviations: ATRA, all-*trans* retinoic acid; PMSF, phenylmethylsulfonyl fluoride;

IDV, integrated density value; ARE, A+U rich element; NS, not statistically different compared to
the untreated control

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Abstract

All-*trans* retinoic acid (ATRA) induces differentiation of promyelocytic leukemia cells, but the mechanisms by which cellular differentiation leads to apoptosis are not well understood. Studies were done to address the question whether ATRA-induced apoptosis is a consequence of destabilization of bcl-2 mRNA and decreased cellular levels of the anti-apoptotic protein, bcl-2. ATRA induced differentiation of HL-60 cells along the granulocytic pathway within 48 hr. The half-lives of bcl-2 mRNA in HL-60 cells incubated with ATRA for 48 hr or 72 hr were reduced to 39 % and 7 % of the corresponding untreated control values, respectively. Cellular differentiation was accompanied by downregulation of the cytoplasmic levels of nucleolin, a bcl-2 mRNA stabilizing protein. Binding of a bcl-2 mRNA instability element (ARE-1) to nucleolin in S100 extracts from ATRA-treated cells was decreased to 15 % of control within 72 hr. The decay of 5' capped, polyadenylated bcl-2 mRNA transcripts containing ARE-1 was more rapid in S100 extracts from ATRA-treated cells compared to untreated cells. However, when recombinant nucleolin was added to extracts of ATRA-treated cells, the rate of bcl-2 mRNA decay was similar to the rate in extracts of untreated cells. These results provide evidence that ATRA-induced apoptosis is a consequence of cellular differentiation, which leads to nucleolin downregulation and bcl-2 mRNA instability.

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All-*trans* retinoic acid (ATRA) induces complete remission in the majority of patients having acute promyelocytic leukemia (James *et al.*, 1999). The remissions are a consequence of ATRA-induced terminal differentiation of the abnormal promyelocytes, which subsequently die by apoptosis (Lawson and Berliner, 1999; James *et al.*, 1999). Other myeloid cell lines also undergo neutrophil maturation following treatment with ATRA (Lawson and Berliner, 1999). In sensitive cell lines ATRA binds with high affinity to retinoic acid receptors, which heterodimerize and bind to specific retinoic acid response elements within the promoter regions of genes involved in myeloid differentiation and neutrophil function. The cell signaling pathways activated during ATRA-induced differentiation are beginning to be understood, but much less is known regarding the mechanisms by which retinoids trigger apoptosis in tumor cells. Bcl-2 protein (Ketley *et al.*, 1997; Nagy *et al.*, 1996; Ueno *et al.*, 1998) and mRNA levels (Delia *et al.*, 1992; Mengubas *et al.*, 1996; Agarwal and Mehta, 1997) decrease following ATRA-induced differentiation of HL-60 cells. It is of interest that constitutive overexpression of Bcl-2 protein in HL-60/Bcl-2 transfectants blocks ATRA-induced apoptosis but not differentiation (Naumovski and Cleary, 1994; Park *et al.*, 1994; Ueno *et al.*, 1998). Taken together, these results suggest that bcl-2 suppression is necessary for ATRA-induced apoptosis in HL-60 cells, while differentiation occurs independently of changes in Bcl-2 protein levels.

The biochemical mechanisms underlying the downregulation of bcl-2 mRNA and induction of apoptosis by ATRA have not been elucidated. Specifically, previous studies have not addressed the question whether the ATRA-induced down regulation of *bcl-2* mRNA is the result of decreased

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transcription or mRNA destabilization. The 3'-untranslated region (3'-UTR) of bcl-2 mRNA contains four AU-rich elements (AREs) that are associated with mRNA destabilization (Alberta *et al.*, 1994; Chen *et al.*, 1994). AREs generally contain multiple copies of the AUUUA pentamer within U-rich or A-U rich sequences and may also contain an UUAUUUA(U/A)(U/A) nonamer. They are characterized by the ability to promote rapid poly(A) shortening and subsequent decay of the transcript. The presence of these pentameric and nonameric motifs in a diverse group of at least 800 mRNAs suggests a common mechanism of closely regulating short-lived mRNAs (Bakheet *et al.*, 2001). Examination of various mRNAs indicates that the destabilizing effects of the ARE can be increased or decreased by interactions with U-rich sequences and with ARE-binding proteins (Chen and Shyu, 1995; Ross, 1995). Of particular significance, Schiavone *et al.* (2000) reported that a conserved ARE (ARE-1) in the 3'-UTR of bcl-2 mRNA is a functional destabilizing element, since cloning of the ARE into the 3'-UTR of β -globin mRNA increased the decay rate of β -globin mRNA *in vivo*. We have recently identified the RNA binding protein, nucleolin, as one of the bcl-2 ARE binding proteins in HL-60 leukemia cells that protects bcl-2 mRNA from degradation in an ARE-dependent manner (Sengupta *et al.*, 2004). Nucleolin also has been shown to bind to the 3'-UTR of amyloid precursor protein mRNA and human preprorenin mRNA, promoting stabilization of these messages (Malter, 2001; Westmark and Malter, 2001; Skalweit *et al.*, 2003). Additionally, nucleolin is required for the stabilization of IL-2 mRNA that occurs during T cell activation (Chen *et al.*, 2000).

Nucleolin expression is proliferation-dependent in human tumor cells, since the intracellular levels of this protein are directly related to the rates of cellular proliferation (Derenzini *et al.*, 1995).

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Accordingly, nucleolin mRNA (Murakami *et al.*, 1991) and protein (Tu *et al.*, 2003) levels decrease following induction of cellular differentiation, although the biological significance of these observations has not been determined. These observations raised the question whether ATRA-induced differentiation of HL-60 cells would lead to downregulation of nucleolin, decreased nucleolin binding to an ARE of bcl-2 mRNA, bcl-2 mRNA destabilization, and apoptosis induction. The studies described in this report were designed to address this sequence of events in an attempt to provide a biochemical mechanism for ATRA-induced apoptosis in HL-60 cells.

MATERIALS AND METHODS

Materials- Human HL-60 leukemia cells were obtained from Dr. Yi-Te Hsu of the Department of Biochemistry and Molecular Biology, Medical University of South Carolina. Recombinant nucleolin was generated using a bacterial expression vector (pET21a) containing c-DNA sequences that code for residues 284-707 of human nucleolin [1-283 Nuc-(His)₆] (Yang *et al.*, 2002). The histidine-tagged nucleolin fragment was expressed in *E. coli* and purified on a Ni⁺⁺-NTA column as previously described (Sengupta *et al.*, 2004). This nucleolin fragment includes the four consensus RNA binding domains (RBDs), the C-terminal nine RGG motifs, and retains the RNA binding and the native G-paired DNA-binding properties of full-length nucleolin (Hanakahi *et al.*, 1997, 1999). ATRA and actinomycin D were purchased from Sigma Chemical Co., St. Louis, MO, and stock solutions of each compound were prepared in ethanol. Antibodies for immunoblotting were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-human CD14 and CD11b monoclonal antibodies conjugated with FITC and PE, respectively, were purchased from Becton Dickinson, San Jose, CA.

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Immobilon-P PVDF transfer membranes were purchased from Fisher Scientific Company, Suwanee, GA. RNeasy kits and HotStarTaq DNA polymerase were obtained from Qiagen Inc., Valencia, CA. All other chemicals and supplies were obtained from Fisher Scientific Co. The ChemiImager digital imaging system was purchased from Alpha Innotech Corp., San Leandro, CA.

Cell Culture- HL-60 leukemia cells were grown at 37°C under 95% air-5% CO₂ in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (20,000 units/L) and streptomycin (20 mg/L). The tumor cells were counted using a Coulter model Z_f counter. All tissue culture reagents were obtained from Invitrogen, Carlsbad, CA.

Measurement of cellular differentiation and apoptosis- HL-60 cells were resuspended in fresh medium on day 0 at a density of 1 x 10⁵ cells/ml. The cells were then incubated with either 1 % ethanol (control), or 0.1, 1.0, or 10 μM ATRA for 24-96 hr. The cells were washed with phosphate-buffered saline (PBS) containing 0.1 % sodium azide and then resuspended in this solution. Cell differentiation was monitored by flow cytometric counting of cells that express the cell surface antigens CD11b or CD14 using either a PE-labeled anti-CD11b antibody or a FITC-labeled anti-CD14 antibody according to the manufacturer's instructions. The numbers of apoptotic and necrotic cells were determined by counting the FITC-labeled annexin V positive cells and the propidium iodide positive cells, respectively, in a flow cytometer. Viable cells are both annexinV and propidium iodide negative.

Western Blotting- Following ATRA treatment, HL-60 cells were washed twice with ice-cold PBS. To measure total cellular Bcl-2 protein the cells were sonicated in ice-cold PBS for 3 x 10 sec at

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intensity 4 in a VirSonic sonicator equipped with a microtip. Cytoplasmic S100 extracts were prepared by homogenization of the cells in ice-cold 10 mM Hepes/KOH buffer, pH 7.5 containing 200 mM manitol, 68 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM Dithiothreitol, and proteinase inhibitor cocktail (1 mM PMSF, 1 mM benzamidine, soybean trypsin inhibitor (10 µg/ml), leupeptin (50 µg/ml), pepstatin (1 µg/ml), and aprotinin (2 µg/ml). The homogenates were centrifuged at 800 x g for 10 min at 4⁰C, followed by successive centrifugation of the supernatants at 10,000 x g for 20 min at 4⁰C, and then at 100,000 x g for 50 min at 4⁰C to yield a S100 extract. Aliquots of the sonicates or S100 extracts were loaded onto a 8-16 % polyacrylamide SDS gel. Unless otherwise indicated each gel lane contained an equal amount of protein. Protein amounts were determined with the Bradford protein assay (Bradford, 1976). The proteins were transferred to an Immobilon-P PVDF transfer membrane using a semidry apparatus. When necessary the membranes were cut into segments to allow immunoblotting with different antibodies. Immunoblotting was carried out using 1:2000, 1:2000, and 1:5000 dilutions of anti-human bcl-2, nucleolin, and GAPDH monoclonal antibodies, respectively. Horseradish peroxidase-conjugated goat anti-mouse secondary antibody was diluted 1:5000. The antibodies were diluted in Tris-buffered saline containing 5 % powdered milk and 0.5 % Tween 20. Proteins were detected using the ECL-Plus reagent and Kodak X-OMAT film. The relative amounts of each protein were determined by counting the total number of pixels in each band (integrated density value, IDV) with a ChemiImager digital imaging system. The amount of a particular protein detected on the western blot was proportional to the total amount of protein loaded onto the electrophoresis gel.

Measurement of Bcl-2 mRNA Stability- HL-60 cells were resuspended in fresh medium on day

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0 at a density of 1×10^5 cells/ml and incubated with either 1 % ethanol (control) or 1.0 μM ATRA for 48-72 hr. The cells were then incubated with either 0.5 % ethanol or 1 $\mu\text{g/ml}$ actinomycin D in 0.5 % ethanol. Aliquots were removed from the cultures at time 0 and every 30 minutes over a 3 hr time course. Actinomycin D at this concentration did not induce any DNA fragmentation during this time period. At the various time points 2×10^7 cells were harvested by centrifugation, washed with PBS, and then the cell pellets were resuspended in Qiagen RLT buffer. The cells were sonicated 3 x10 sec at intensity 6 in a VirSonic sonicator equipped with a microtip. The sonicates were stored at -70°C until further processing. Total RNA was isolated from the sonicates using a Qiagen RNeasy kit, and the RNA concentrations were determined spectrophotometrically at 260 nm. Equal amounts of total RNA (2-5 μg) from each sample were reverse transcribed using MMLV reverse transcriptase and random hexamers. PCR amplification of the cDNAs was carried out with primer pairs for the *bcl-2* gene

(5'-GGAAGTGAACATTTTCGGTGAC-3' and 5'-GCCTCTCCTCACGTTCCC-3') and the β -*actin* gene (5'-GCGGGAAATCGTGCGTGACAT-3' and 5'-GATGGAGTTGAAGGTAGTTC-3').

The reaction mixture contained 200 μM dATP, dCTP, dGTP, and dTTP, primers at 1 μM each, 2.5 units of HotStarTaq DNA polymerase which lacks 3'-5' exonuclease activity, 2.5 mM MgCl_2 , and equal volumes of the cDNA products in a final volume of 50 μL . The HotStarTaq DNA polymerase was activated by a 15 min incubation at 95°C in the thermal cycler. This was followed by template denaturation for 1 min at 94°C , primer-template annealing for 1 min at 57°C , and then primer extension for 1 min at 72°C . After 26 cycles for *bcl-2* and 24 cycles for β -*actin*, the extension reaction was continued for an additional 7 min at 72°C . The PCR products were separated

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on a 1% agarose gel, stained with ethidium bromide, and product formation quantitated by determining the IDV of each band. Product formation was linear over the range of the amounts of cDNA and PCR cycles used.

Co-immunoprecipitation of Nucleolin and Bcl-2 ARE-1 RNA- HL-60 cells were incubated on day 0 with either 0 or 1 μ M ATRA. Following ATRA treatment for 72 hr, the cells were washed twice with PBS. The cell pellets were resuspended in lysis buffer containing 10 mM HEPES-KOH buffer, pH 8.0, 40 mM KCl, 3 mM MgCl₂, 10 % glycerol, 0.2 % Nonidet P40, 1 mM dithiothreitol, proteinase inhibitor cocktail and incubated on ice for 10 min. An S100 cytosolic fraction was prepared as described for Western blotting. Aliquots of the S100 fractions containing 200 μ g of protein were precleared with protein G agarose beads, and then incubated with 1 nM (70 fmoles) of ³²P-ARE-1 RNA (final specific radioactivity of 248 mCi/mmol) and 2 μ g of anti-nucleolin monoclonal antibody or mouse IgG (control antibody) for 3.5 hr min at 4 °C. The immunocomplexes were precipitated with protein G agarose beads, washed twice, and then analyzed by either liquid scintillation counting or Western blotting.

In Vitro mRNA Decay Assays- Groups of 5 x 10⁶ HL-60 cells were incubated with or without 1.0 μ M ATRA for 48-72 hr as described for the mRNA stability assays. The cells were washed twice with PBS at 4 °C. The cell pellets were then resuspended in lysis buffer and incubated on ice for 10 min. S100 extracts were prepared from the lysates as described above. RT-PCR fragments containing a portion of the bcl-2 coding region (nucleotides 600-750) or coding region plus ARE-1 (nucleotides 600-1057) were cloned into pCR4 (Bandyopadhyay *et al.*, 2003). Spe-I linearized pCR4-bcl-CR and pCR4-bcl-CR-ARE plasmids were used as templates for synthesis of the bcl-2

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coding region and bcl-2 coding region+ARE transcripts, respectively. 5'-capped ³²P-labeled transcripts were prepared using a mMessage mMachine T7 kit (Ambion) and following the manufacturer's instructions. Poly(A) tails of approximately 150 nucleotides were added to the 3'-ends of the transcripts using a poly(A) tailing kit (Ambion) and unincorporated nucleotides were removed by G-25 spin column chromatography. Decay reactions were carried out as previously described (Sengupta *et al.*, 2004). Approximately 150,000 cpm of capped and polyadenylated bcl-2 or bcl-2-ARE RNAs were used per decay reaction containing 9 µg of S100 extract protein in a final volume of 100 µl. To assay the affect of nucleolin on RNA decay, purified recombinant nucleolin [1-283 Nuc-(His)₆] was added to RNA decay reactions at a final concentration of 280 nM prior to addition of the S100 cell extracts. RNA was extracted with phenol-chloroform, ethanol precipitated and then electrophoresed on 6 % polyacrylamide gels containing 7 M urea. After electrophoresis, gels were fixed, dried and analyzed by phosphorimaging.

RESULTS

Effects of ATRA on Cell Growth, Differentiation and Apoptosis- HL-60 leukemia cells were incubated on day 0 with either 0, 0.1, 1.0, or 10 µM ATRA. Fig. 1 shows that the growth rate of cells incubated with 0.1 µM ATRA decreased about 50 % after the cells had undergone differentiation by 48 hr. Cell proliferation was almost completely inhibited following a 48 hr exposure to either 1 or 10 µM ATRA. Expression of the cell surface antigens, CD14 and the β₂-integrin CD11b, were monitored by flow cytometry to follow the differentiation of HL-60 cells into monocytes and granulocytes/monocytes, respectively (Perussia *et al.*, 1981; James *et al.*, 1997).

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Compared to untreated control cells, ATRA at concentrations from 0.1-10 μ M induced a maximal increase in CD11b expression of about 9-fold by 48 hr (Fig. 1b), but had no effect on CD14 when expression was monitored for 24- 96 hr after treatment (data not shown). This indicated that ATRA induced HL-60 differentiation along the granulocyte pathway.

By 72 hr about 15 % of the cells incubated with either 1 or 10 μ M ATRA had become annexin V positive (Fig. 2A) and showed a modest amount of high and low molecular weight DNA fragmentation (Fig. 2B, lanes 11 and 12), while cells treated with 0.1 μ M ATRA did not become apoptotic until about 96 hr. These results are consistent with previous reports that HL-60 cells incubated with ATRA undergo differentiation prior to entering apoptosis (James *et al.*, 1997; Lawson and Berliner, 1999; Ueno *et al.*, 1998).

ATRA Induces Destabilization of Bcl-2 mRNA- Following ATRA-induced differentiation, bcl-2 mRNA and protein levels decrease and the cells then enter apoptosis (Ketley *et al.*, 1997; Nagy *et al.*, 1996; Ueno *et al.*, 1998; Delia *et al.*, 1992; Mengubas *et al.*, 1996; Agarwal and Mehta, 1997). The reduced levels of bcl-2 mRNA that follow ATRA-induced cell differentiation could result from either decreased transcription or destabilization of bcl-2 mRNA. Since the *bcl-2* promoter does not have any retinoic acid response elements (Cheema *et al.*, 2003), it is unlikely that ATRA activation of retinoic acid receptors results in direct repression of transcription from the *bcl-2* gene. Thus, we examined the effect of ATRA on the cellular levels and stability of bcl-2 mRNA. Measurement of the levels of total bcl-2 mRNA by RT-PCR revealed that the bcl-2 mRNA / β -actin mRNA ratio did not change in untreated HL-60 cells between 0-72 hr (Fig. 3A). In contrast, the bcl-2 mRNA / β -actin mRNA ratio began to decrease relative to control by 24 hr after 1 μ M ATRA treatment and

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further declined to about 30 % of control by 72 hr. The effect of 1 μ M ATRA on bcl-2 mRNA stability was evaluated by incubating control and ATRA-treated cells with actinomycin D to prevent further RNA synthesis. Bcl-2 and β -actin mRNA levels were then measured by RT-PCR over a 3 hr time course. Actinomycin D did not induce any detectable DNA damage during this time period (data not shown). Fig. 3 B is a semi-log plot showing the decay of bcl-2 mRNA in either untreated cells or cells treated with 1 μ M ATRA for either 48 or 72 hr. Linear extrapolation of the data revealed that the half-life of bcl-2 mRNA was reduced from 7.9 hr ($r = 0.98$) in the control cells to 3.1 hr ($r = 0.98$) in cells incubated with 1 μ M ATRA for 48 hr. At 72 hr the half-lives of the control and ATRA-treated cells were 10.6 hr ($r = 0.77$) and 0.8 hr ($r = 0.99$), respectively. In contrast, incubation of HL-60 cells with 1 μ M ATRA for either 48 or 72 hr had no effect on the half-life of β -actin mRNA (data not shown). If destabilization has a critical role in the reduction of bcl-2 mRNA steady-state levels, then one would expect an equal or greater decrease in mRNA stability than in mRNA steady-state levels. A comparison of Figs. 3A and 3B suggests that the degree of bcl-2 mRNA destabilization observed was sufficient to account for the decrease in bcl-2 mRNA steady-state levels. At 48 hr the bcl-2 mRNA half-life in ATRA-treated cells was reduced to 39 % of the untreated control, while the bcl-2 mRNA steady-state level was 69 % of control. At 72 hr the bcl-2 mRNA half-life and steady-state level were 7 % and 41 % of control, respectively.

Bcl-2 mRNA destabilization induced by ATRA was followed by downregulation of Bcl-2 protein. The Bcl-2 /GAPDH protein ratio was not significantly different between control cells and cells incubated with 1 μ M ATRA for 48 hr. However, by 48 hr the bcl-2 mRNA half-life in ATRA-treated cells had decreased to 39 % of control (Fig. 3). Bcl-2 protein levels were significantly reduced

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to 46 % of control ($p < 0.02$) by 72 hr after 1 μ M ATRA treatment (Fig. 4). Thus, bcl-2 mRNA instability was induced by ATRA prior to the downregulation of Bcl-2 protein.

Mechanism of ATRA-Induced Bcl-2 mRNA Instability- We recently identified nucleolin as an ARE-binding protein involved in bcl-2 mRNA stabilization (Sengupta et al., 2004). Recombinant nucleolin binds specifically to a 139 base sequence in bcl-2-ARE-1 mRNA (Sengupta et al., 2004). Since nucleolin expression is highly proliferation-dependent in human tumor cells (Derenzini *et al.*, 1995), we addressed the question whether cell differentiation induced by ATRA is followed by downregulation of nucleolin expression. The decreased levels of this ARE-binding protein in ATRA-treated cells may in turn lead to bcl-2 mRNA instability. Because mRNA decay takes place in the cytoplasm, we monitored the effect of ATRA on cytoplasmic nucleolin levels as a function of time. Soluble cytoplasmic extracts (S100) were prepared from untreated and ATRA-treated HL-60 cells. Fig. 5 shows that in cells incubated with 1 μ M ATRA, the cytoplasmic nucleolin levels fell to 50 % of the control level between 24 and 48 hr. The ATRA-induced decrease in cytoplasmic nucleolin levels was coincident with the induction of bcl-2 mRNA instability (Fig. 3). Nucleolin downregulation was not seen prior to the induction of apoptosis with certain other anticancer drugs, indicating that nucleolin downregulation is not a general effect of anticancer drug treatment. An IC_{50} concentration of either cytosine arabinoside, merbarone, or VM-26 induced apoptosis in HL-60 cells within 6 hr, but had no effect on cytoplasmic nucleolin levels compared to the levels in untreated control cells (data not shown).

Further experiments were done to determine whether the downregulation of cytoplasmic nucleolin levels by ATRA results in decreased binding of nucleolin to exogenous bcl-2 ARE-1

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RNA. S100 fractions from untreated and ATRA treated cells were incubated with ^{32}P -ARE-1 and the nucleolin-ARE-1 complexes were co-immunoprecipitated with anti-nucleolin monoclonal antibody. The immunoprecipitates were analyzed by liquid scintillation counting and Western blotting. Of the four AREs in the 3'-UTR of bcl-2 mRNA, we chose to analyze transcripts of ARE-1 because this ARE has the highest concentration of AUUUA pentamers and has potent mRNA destabilizing activity in cell extracts (Sengupta *et al.*, 2004) and in intact cells (Schivone *et al.*, 2000; Bandyopadhyay *et al.*, 2003). Table 1 indicates that precipitation of ^{32}P -ARE-1 RNA was about 15-fold greater in untreated control extracts incubated with anti-nucleolin antibody (13.7 fmoles) compared to control extracts incubated with IgG (0.9 fmoles). In addition, the amount of ^{32}P -ARE-1 RNA precipitated in extracts of ATRA-treated cells was only 15 % of that recovered from control extracts (2.1 vs.13.7 fmoles). Western blot analysis revealed that the amount of nucleolin in the immunoprecipitates of ATRA-treated cells averaged 19 % (range of 11 % - 26 %) of the amount of nucleolin immunoprecipitated from untreated cells (data not shown). These results indicate that the decrease in cytoplasmic nucleolin levels induced by ATRA (Fig. 5) is accompanied by a similar decline in the binding of cytoplasmic nucleolin to exogenous bcl-2 ARE-1.

To directly examine the ability of nucleolin to stabilize bcl-2 mRNA, RNA decay assays were performed using the *in vitro* assay system developed by Ford and Wilusz (1999). Capped and polyadenylated mRNAs were used in these assays to mimic *in vivo* decay, which is thought to involve cap-stimulated deadenylation by poly (A)-specific ribonuclease (PARN) followed by rapid decay of the mRNA body (Ford and Wilusz, 1999). ^{32}P -labeled bcl-2 (coding region, bcl-

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CR) and bcl-2-ARE (coding region plus ARE-1, bcl-CR-ARE) transcripts were synthesized by *in vitro* transcription reactions in which the ⁷mGpppG cap was added co-transcriptionally. Poly(A) tails of ~150 nucleotides were subsequently added and the mRNAs were incubated with S100 extracts from control and ATRA-treated cells in the presence of poly(A) to activate deadenylation. At various times the reactions were stopped and RNA was isolated and its abundance determined by gel electrophoresis. Bcl-CR-ARE decayed faster than bcl-CR in extracts of 72 hr control cells (Fig. 6A). This is consistent with our previous observation that bcl-2 ARE-1 is an mRNA instability element (Sengupta *et al.*, 2004). Bcl-CR transcripts decayed at similar rates in extracts from cells incubated without ATRA or with 1 μM ATRA for 72 hr (Fig. 6A). In contrast, bcl-CR-ARE decayed faster in extracts from ATRA-treated cells compared to control cells. This indicates that ATRA stimulated ARE-dependent decay of the bcl-2 transcripts containing a portion of the bcl-2 coding region. Purified recombinant nucleolin [1-283 Nuc-(His)₆](280 nM) slowed the decay rate of bcl-CR-ARE when added to extracts of control and ATRA-treated cells, but had no effect on the decay rate of bcl-CR RNA (Fig. 6B). Similar assays with capped, polyadenylated CAT mRNA showed no effect of nucleolin on mRNA decay rates (Bandyopadhyay *et al.*, 2003). The results presented in Fig. 6 demonstrate that nucleolin increased the stability of bcl-2 mRNA in extracts of untreated HL-60 cells in an ARE-dependent manner. Nucleolin also blocked the decay of bcl-2-ARE mRNA in extracts from ATRA-treated cells. Taken together, our data support the hypothesis that downregulation of nucleolin levels by ATRA results in destabilization of bcl-2 mRNA.

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DISCUSSION

The studies described herein were designed to address the molecular mechanisms by which ATRA induces apoptosis in HL-60 leukemia cells. When taken together, our data suggest the following time-course of ATRA-induced events: ATRA → cell differentiation → cytoplasmic nucleolin downregulation → bcl-2 mRNA destabilization → Bcl-2 protein downregulation → apoptosis. According to this schema, ATRA-induced destabilization of bcl-2 mRNA leads to downregulation of Bcl-2 protein levels and subsequent induction of apoptosis. Bcl-2 mRNA destabilization does not induce cellular differentiation but rather is a consequence of differentiation. This is consistent with our observation that cellular differentiation preceded bcl-2 mRNA destabilization. In addition, others have shown that ATRA can still induce differentiation of HL-60 transfectants that overexpress Bcl-2 protein, but apoptosis is inhibited (Naumovski and Cleary, 1994; Park *et al.*, 1994; Ueno *et al.*, 1998). If a decrease in bcl-2 mRNA levels is necessary for the induction of cellular differentiation, then differentiation would have been blocked in the HL-60 transfectants incubated with ATRA. In contrast, ATRA-induced cell differentiation is proposed to affect bcl-2 mRNA stability indirectly by downregulating the cellular levels of nucleolin, a potent bcl-2 mRNA stabilization protein (Sengupta *et al.*, 2004). This is in agreement with the proliferation-dependence of nucleolin expression in tumor cells (Derenzini *et al.*, 1995), and the observations that nucleolin mRNA (Murakami *et al.*, 1991) and protein (Tu *et al.*, 2003) levels decrease in response to cellular differentiation.

Although it is possible that ATRA induced some repression of bcl-2 mRNA transcription, this does not appear to have a major role in determining the steady-state levels of bcl-2 mRNA in

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ATRA-treated HL-60 cells. Since the *bcl-2* promoter lacks retinoic acid response elements (Cheema *et al.*, 2003), it is unlikely that ATRA activation of retinoic acid receptors results in direct repression of *bcl-2* transcription. Hu *et al* (1995) reported that the rate of *bcl-2* mRNA transcription in ATRA-treated myeloid leukemia cells measured by nuclear run-on assays was decreased 2-fold, while the steady-state level of *bcl-2* mRNA seen on Northern blots was reduced 17-fold compared to untreated cells. Thus, ATRA decreased *bcl-2* mRNA levels primarily by a post-transcriptional mechanism. In the present study, the degree of *bcl-2* mRNA destabilization induced by ATRA appeared sufficient to completely account for the decrease in *bcl-2* mRNA steady-state levels.

Other anticancer agents are known to induce *bcl-2* mRNA instability. Liu *et al.* (1994) found that the rate of *bcl-2* mRNA synthesis in OV2008 cells was unaffected by taxol treatment; however, the half-life of *bcl-2* mRNA was about 3-fold lower in taxol-treated cells compared to untreated control cells (Liu and Priest, 1996). Taxol- and okadaic acid- induced apoptosis of HL-60 cells was preceded by *bcl-2* mRNA destabilization and down-regulation of *bcl-2* mRNA and protein levels, while *bcl-2* mRNA transcription rates were unaffected (Riordan *et al*, 1998; Bandyopadhyay *et al.*, 2003). In addition, incubation of HL-60 cells with taxol or okadaic acid resulted in proteolysis of nucleolin in a similar time frame as drug-induced *bcl-2* mRNA down-regulation. Proteolysis of nucleolin is enhanced following phosphorylation (Westmark and Malter, 2001), and nucleolin is threonine phosphorylated by p34^{cdc2} kinase during mitosis (Srivastava and Pollard, 1999; Ginisty *et al.*, 1999). These findings suggest that nucleolin functions as a *bcl-2* mRNA stabilizing factor, and that taxol and okadaic acid induce apoptosis in HL-60 cells through

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a process that involves down-regulation of nucleolin and destabilization of bcl-2 mRNA.

Analysis of the mRNA stability model proposed by Wilusz *et al.* (2001) has provided insights into possible mechanisms by which ATRA-induced downregulation of nucleolin can result in destabilization of bcl-2 mRNA. According to the Wilusz model, translation initiation factors eIF4E (4E) and eIF4G (4G) interact with poly(A) binding protein (PABP) and circularize the mRNA, which protects the 5' and 3' ends of the mRNA from decapping and deadenylase enzymes thereby enhancing mRNA stability. The presence of AREs in bcl-2 mRNA promotes rapid poly(A) shortening and renders the message intrinsically unstable. We have observed that the stabilization of bcl-2 mRNA by recombinant nucleolin *in vitro* was ARE-dependent (Figure 6). Thus, we propose that in HL-60 and other tumor cells where bcl-2 mRNA and protein levels are elevated, nucleolin and possibly other ARE-binding proteins stabilize the closed loop form of bcl-2 mRNA by enhancing the binding of PABP to the poly(A) tail of bcl-2 mRNA or to the translation initiation factors. Alternatively, nucleolin may protect the ARE from endonuclease attack. The resultant stabilization of the mRNA leads to increased translation and over-production of bcl-2 protein. ATRA-induced cell differentiation is followed by downregulation of nucleolin mRNA and protein. As a result of the decreased interaction of nucleolin with bcl-2 mRNA, the mRNA may be cleaved by an ARE-specific endonuclease or the affinity of PABP for the poly(A) tail of bcl-2 mRNA may be reduced. The latter effect would enable deadenylation by poly(A) ribonuclease (PARN), which would trigger rapid decay of the bcl-2 message.

Deciphering the role of nucleolin and other *trans*-acting factors in stabilizing bcl-2 mRNA may have broader implications to the mechanisms involved in the development of cancer. One

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idea that emerged from the present study is that overexpression of nucleolin in the cytoplasm is critical to the development of certain B cell lymphomas and leukemias. To explore this possibility, we are currently comparing the cytoplasmic nucleolin levels, bcl-2 mRNA stability, and binding of nucleolin to bcl-2 mRNA in normal B cells and in B cell chronic lymphocytic leukemia (CLL) cells from patients. Although CLL is an indolent disease during most of its clinical course, the leukemic cells accumulate during the indolent phase primarily by avoiding apoptosis (Klein *et al.*, 2000). CLL cells overexpress bcl-2 mRNA and protein, but lack the t(14;18) chromosomal translocation characteristic of B cell follicular lymphomas (Steube *et al.*, 1995; Robertson *et al.*, 1996). The results of our ongoing studies should reveal whether overexpression of bcl-2 protein in CLL cells is related to stabilization of bcl-2 mRNA by nucleolin.

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Figure Legends

Figure 1. Inhibition of cell growth and induction of differentiation by ATRA. HL-60 cells were incubated on day 0 with either 1 % ethanol (vehicle control), #; 0.1 μ M ATRA, ! ; 1 μ M ATRA, ▲; or 10 μ M ATRA, ◆. Cell growth (Fig. 1A) was measured by counting cells with a Coulter counter. Cell differentiation (Fig. 1 B) was monitored by flow cytometric counting of cells that express the cell surface antigen CD11b using a PE-labeled anti-CD11b antibody. Results shown in Fig.1A are the means of 8 experiments \pm 1 SE. Results in panel Fig. 1B are the means of 2 experiments.

Figure 2. Time Course of Apoptosis Induction by ATRA. HL-60 cells were incubated on day 0 with either 1 % ethanol, #; 0.1 μ M ATRA, ! ; 1.0 μ M ATRA, ▲; or 10 μ M ATRA, ◆. The numbers of apoptotic cells (Fig. 2A) were determined by counting the FITC-labeled annexin V positive cells in a flow cytometer. Results are the means of 2 experiments. DNA fragmentation induced by ATRA is shown in Fig. 2B. The cells were lysed with SDS and proteinase k in the wells of a 2 % agarose gel. The gel was stained with ethidium bromide and the bands visualized with a ChemiImager digital imaging system. Lanes 1, 5, 9, and 13, vehicle control at 24, 48, 72 and 96 hr, respectively; lanes 2, 6, 10, and 14, 0.1 μ M ATRA at 24, 48, 72 and 96 hr, respectively; lanes 3, 7, 11, and 15, 1.0 μ M ATRA at 24, 48, 72 and 96 hr, respectively; lanes 4, 8, 12, and 16, 1.0 μ M ATRA at 24, 48, 72 and 96 hr, respectively; extreme right lane, λ Hind III ladder.

Figure 3. ATRA-induced Destabilization of Bcl-2 mRNA. (A) Levels of total intracellular bcl-2 mRNA measured by RT-PCR in control cells (■) and cells treated with 1.0 μ M ATRA for 0-72 hr

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(▲). Results are the means of 3 determinations \pm 1 SD. (B) Semilog plot showing the decay of bcl-2 mRNA. Cells were treated with either 0 or 1.0 μ M ATRA for 48-72 hr and then incubated with 1 μ g/ml actinomycin D in 0.5 % ethanol. Aliquots were removed from the cultures at time 0 and every 30 minutes over a 3 hr time course. Results are the means of duplicate determinations \pm range and are expressed as percentages of the time 0 bcl-2 mRNA / β -actin mRNA ratio. Symbols: control day 2, ■; 1 μ M ATRA day 2, ▲; control day 3, □; 1 μ M ATRA day 3, Δ .

Figure 4. Effect of ATRA on Bcl-2 Protein Levels. HL-60 cells were incubated on day 0 with either 1 % ethanol, #; 0.1 μ M ATRA, !; 1.0 μ M ATRA, ▲; or 10 μ M ATRA, ◆. Following ATRA treatment for 0 - 4 days, the cells were sonicated and aliquots of the sonicates containing equal amounts of protein were loaded onto a 8-16 % polyacrylamide SDS gel. Immunoblotting was carried out using anti-human bcl-2 and GAPDH monoclonal antibodies. Results are expressed as the bcl-2 /GAPDH ratio and are the means of 3 experiments with duplicate determinations per experiment. * p = 0.05; ** p = 0.02; *** p = 0.01, paired t-test..

Figure 5. Effect of ATRA on Cytoplasmic Nucleolin Levels. HL-60 cells were incubated on day 0 with either 0 ■; or 1 μ M ATRA, ▲. Following ATRA treatment for 0-96 hr, an S100 cytoplasmic fraction was prepared and aliquots of the supernatants containing equal amounts of protein were loaded onto a 8-16% polyacrylamide SDS gel. Immunoblotting was carried out using anti-human nucleolin polyclonal and GAPDH monoclonal antibodies. Results are expressed as the cytoplasmic nucleolin / GAPDH ratio and are the means of 4 experiments. *p <0.02, one-tailed t-test.

Figure 6 Effect of Nucleolin on the Decay of Bcl-2-ARE mRNA in HL-60 Cell Extracts.

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5'-capped polyadenylated ^{32}P -RNAs were incubated with S100 extracts prepared from either untreated control HL-60 cells or from cells incubated with 1 μM ATRA for 72 hr. At the indicated times aliquots of the reaction mixtures were removed and analyzed by polyacrylamide gel electrophoresis and phosphoimaging. The effect of nucleolin on the decay of bcl-2 mRNA transcripts was assessed by the addition of [1-283 Nuc-(His)₆] to the S100 extracts before incubation with the mRNAs. The results are expressed as the percentage of full-length mRNA remaining as a function of time of incubation in the S100 extracts. Results are the means of two separate experiments with the maximum variability being less than 20 % of the mean. *A*, Decay of bcl-CR RNA in extracts of untreated control of ATRA-treated cells. *B*, Decay of bcl-CR-ARE RNA in extracts of control and ATRA-treated cells.

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Table 1. Effect of ATRA on the Binding of Nucleolin to Bcl-2 ARE-1 RNA^a

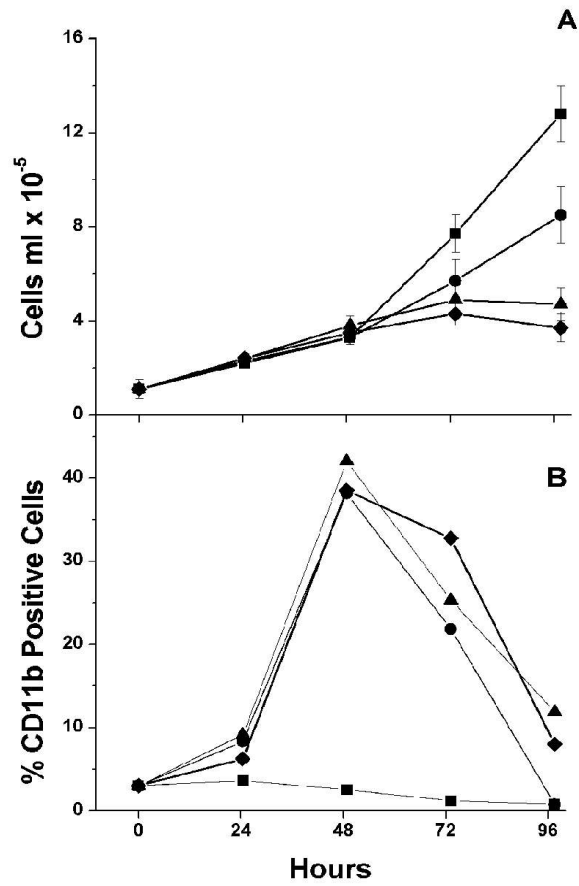
| Control / IgG | Control / Nucleolin Ab | ATRA / IgG | ATRA / Nucleolin Ab |
|----------------------|-------------------------------|-------------------|----------------------------|
| 0.9 ± 0.3 | 13.7 ± 4.0 ^b | 1.0 ± 0.4 | 2.1 ± 0.5 |

^aResults are expressed as the mean femtomoles ± SE of ³²P-ARE-1 RNA co-immunoprecipitated with either mouse IgG or anti-nucleolin monoclonal antibody. Combined results of 3 experiments with 3 observations per experiment.

^bp < 0.02 compared to ATRA / Nucleolin Ab group, one-tailed t-test.

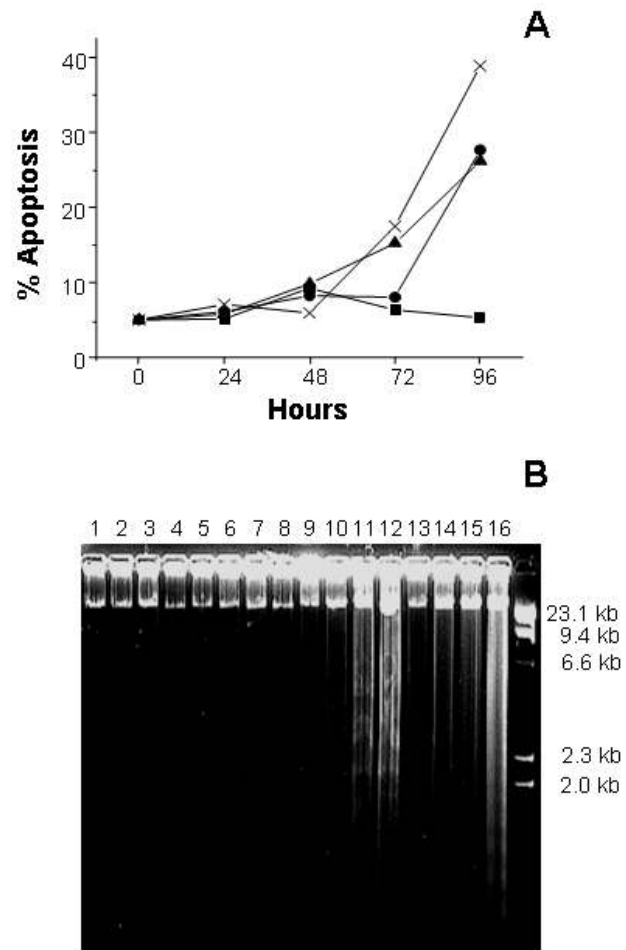
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Figure 1



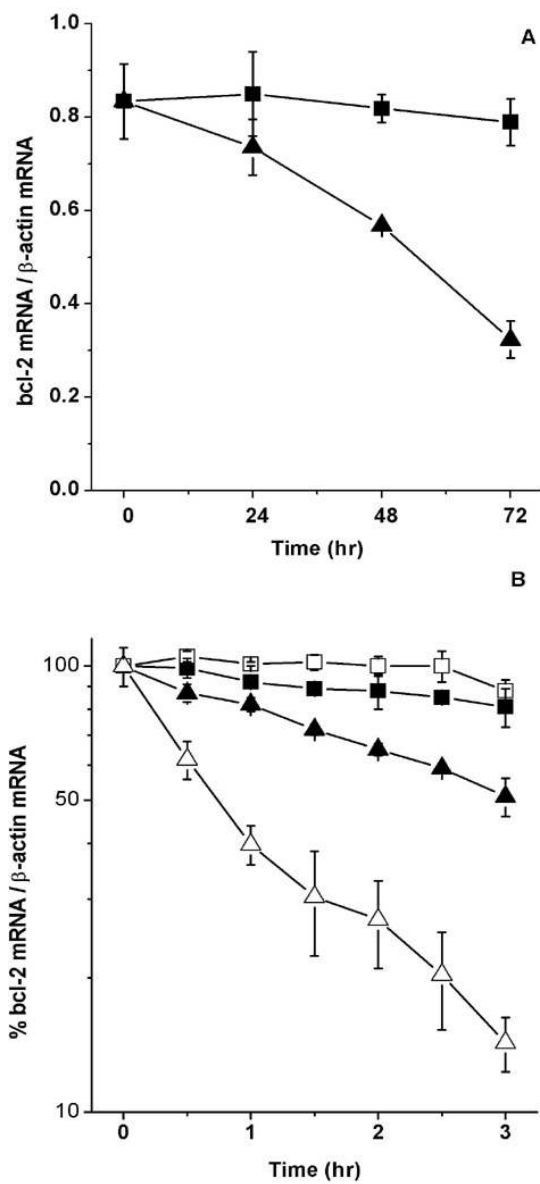
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Figure 2



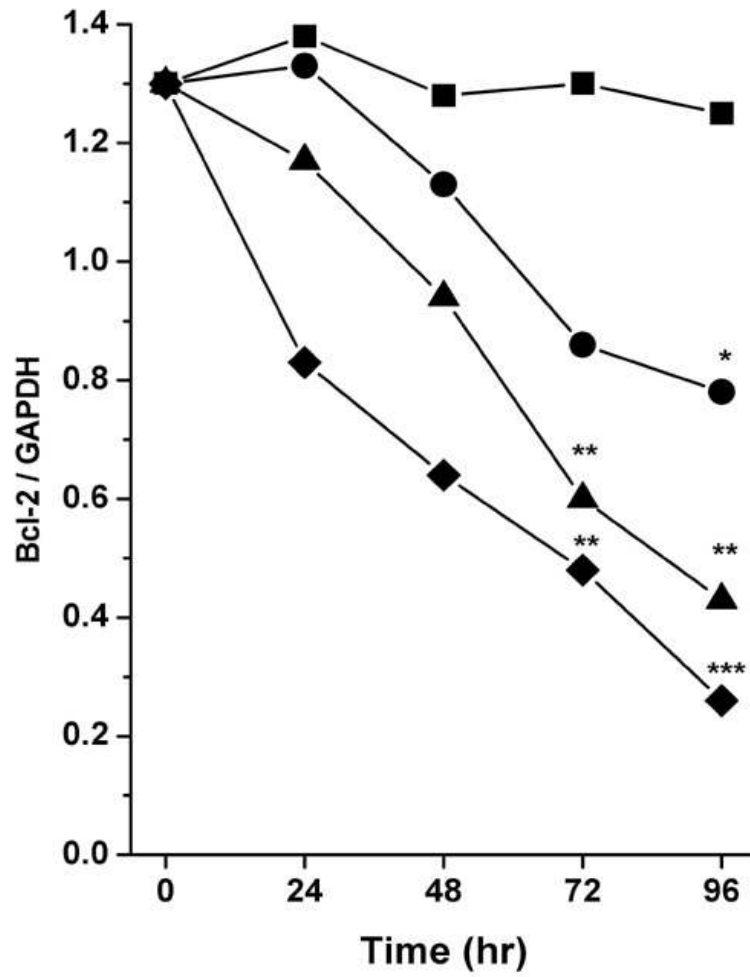
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Figure 3



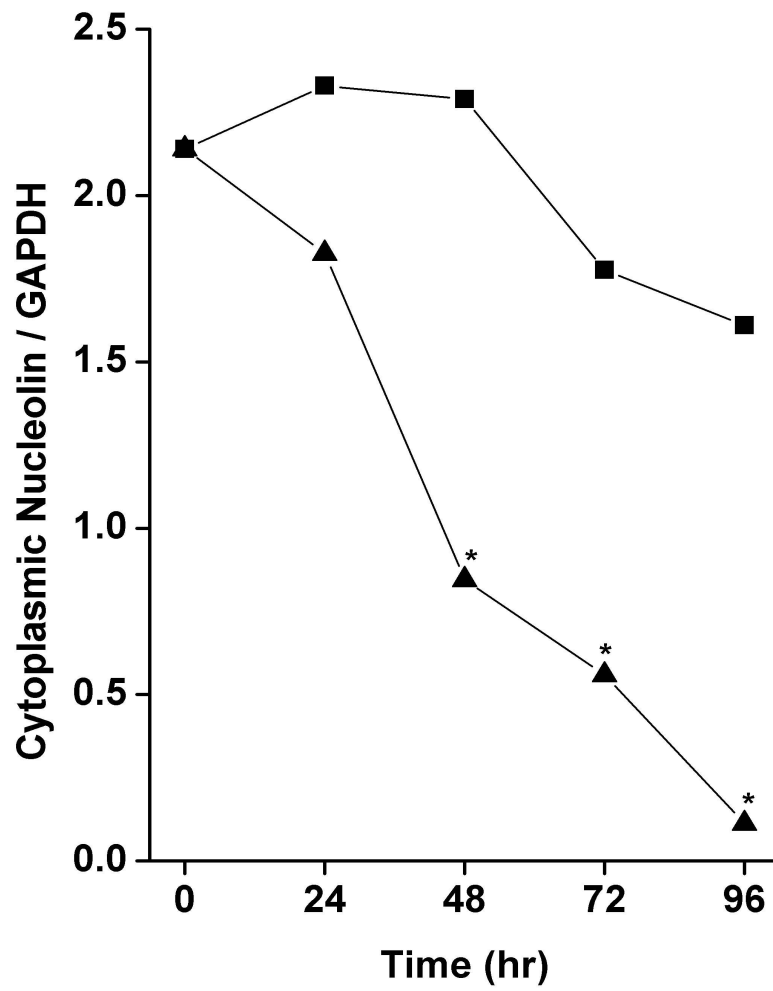
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Figure 4



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Figure 5



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Figure 6

