

Dose- and Time-Dependent Response of Human Leukemia (HL-60) Cells to Arsenic Trioxide Treatment

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Abstract: The treatment of acute promyelocytic leukemia (APL) has been based on the administration of all-trans retinoic acid plus anthracycline chemotherapy, which is very effective as first line therapy; however 25 to 30% of patients will relapse with their disease becoming refractory to conventional therapy. Recently, studies have shown arsenic trioxide to be effective in the treatment of acute promyelocytic leukemia. In this study, we used the human leukemia (HL-60) cell line as a model to evaluate the cytotoxicity of arsenic trioxide based on the MTT assay. Data obtained from this assay indicated that arsenic trioxide significantly reduced the viability of HL-60 cells, showing LD₅₀ values of $14.26 \pm 0.5 \mu\text{g/mL}$, $12.54 \pm 0.3 \mu\text{g/mL}$, and $6.4 \pm 0.6 \mu\text{g/mL}$ upon 6, 12, and 24 hours of exposure, respectively; indicating a dose- and time-dependent response relationship. Findings from the present study indicate that arsenic trioxide is highly cytotoxic to human leukemia (HL-60) cells, supporting its use as an effective therapeutic agent in the management of acute promyelocytic leukemia.

Keywords: Arsenic trioxide, HL-60 cells, exposure time, cytotoxicity, MTT assay

Introduction

Arsenic is a metalloid that has played a significant therapeutic role in various diseases for more than 2400 years [1, 2]. However, it has not been used clinically for many decades. Recently, arsenic trioxide was approved by U.S. Food and Drug Administration to be used clinically against acute promyelocytic leukemia (APL) [3, 4]. APL is a subtype of acute myelocytic leukemia with most cases carrying the characteristic chromosomal translocation t(15, 17) that results in the PML-RAR α fusion protein [5].

Although APL is highly responsive to arsenic trioxide, the mechanism by which arsenic trioxide is effective against APL remains unclear, despite studies suggesting that arsenic trioxide can promote degradation of the oncogenic PML-RAR α fusion protein [6, 7]. Paradoxically, arsenic is also an established human carcinogen [8, 9] that can induce reactive oxygen species (ROS), leading to DNA damage or cell death [10, 11]. Intoxication by this heavy metal can result from breathing sawdust, workplace air, ingesting contaminated water, food, or soil [12, 13]. Chronic exposure to arsenic is associated with anemia, peripheral neuropathy, liver and kidney damage, and irritation of the skin and mucous membranes [9]. Symptoms of chronic arsenic intoxication include the following: headache, fatigue,

confusion, polyneuritis with distal weakness, exfoliative dermatitis, leucopenia, hyperkeratosis, vomiting, and hyperpigmentation [12, 13].

Recent studies in our laboratory have demonstrated that arsenic trioxide is cytotoxic to human liver carcinoma (HepG₂) cells [14, 15]. The aim of the present study was to evaluate the cytotoxicity of arsenic trioxide to human leukemia (HL-60) cells, with a special emphasis on the assessment of time- and dose-response relationships.

Materials and Methods

Chemicals and Test Media

Arsenic trioxide (As₂O₃), CASRN 1327-53-3, MW 197.84, with an active ingredient of 100% (w/v) arsenic in 10% nitric acid was purchased from Fisher Scientific in Houston, Texas. Growth medium RPMI 1640 containing 1 mmol/L L-glutamine was purchased from Gibco BRL products (Grand Island, NY). Ninety six-well plates were purchased from Costar (Cambridge, MA). Fetal bovine serum (FBS), antibiotics (penicillin G and streptomycin), phosphate buffered saline (PBS), and MTT assay kit were obtained from Sigma Chemical Company (St. Louis, MO).

Tissue Culture

The HL-60 promyelocytic leukemia cell line was purchased from the American Type Culture Collection – ATCC (Manassas, VA). This cell line has been derived from peripheral blood cells of a 36-year old Caucasian female with acute promyelocytic leukemia (APL). The HL-60 cells grow as a suspension culture. The predominant cell population consists of neutrophilic promyelocytes [16, 17].

In the laboratory, cells were stored in the liquid nitrogen until use. They were next thawed by gentle agitation of their containers (vials) for 2 minutes in a water bath at 37°C. After thawing, the content of each vial of cell was transferred to a 25 cm² tissue culture flask, diluted with up to 10 mL of RPMI 1640 containing 1 mmol/L L-glutamine (GIBCO/BRL, Gaithersburg, MD) and supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin/streptomycin. The 25 cm² culture flasks (2 × 10⁶ viable cells) were observed under the microscope, followed by incubation in a humidified 5 % CO₂ at 37°C. Three times a week, they were diluted under same conditions to maintain a density of 5 × 10⁵/mL, and harvested in the exponential phase of growth. The cell viability was assessed by the trypan blue exclusion test (Life Technologies), and manually counted using a hemocytometer.

Cytotoxicity/MTT Assay

Principle of the Assay

This is a colorimetric assay that measured the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 4-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where is reduced to an insoluble, colored, formazan product. The cells are then solubilised with an organic solvent (DMSO or isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically [18, 19].

Test Protocol

Human leukemia HL-60 cells were maintained in RPMI 1640 containing 1 mmol/L L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) Penicillin/Streptomycin, and incubated at 37°C in humidified 5% CO₂ incubator. To 180 µL aliquots in six replicates of cell suspension (5 × 10⁵/mL) seeded to 96 well polystyrene tissue culture plates, 20 µL aliquots of arsenic trioxide solutions (0.04, 0.08, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10, and 20 µg/mL) were added to each well using distilled water as solvent. Cells incubated in culture medium alone served as a control for cell viability (untreated wells). All chemical exposures were carried in 96 well tissue culture plates for the purpose of chemical dilutions. Cells were placed in the humidified 5% CO₂ incubator at 37°C for 6, 12, and 24 hours respectively. After incubation, 20 µL aliquots of MTT solution (5 mg/mL in PBS) were added to each well and re-incubated for 4 hours at 37°C, followed by low centrifugation at 800 rpm for 5 minutes. Then, the 200 µL of supernatant culture medium were carefully aspirated

and 200 µL aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals, followed by incubation for 10 minutes to dissolve air bubbles. The culture plate was placed on a Biotex Model micro-plate reader and the absorbance was measured at 550 nm. The amount of color produced is directly proportional to the number of viable cell. All assays were performed in six replicates for each concentration and means ± SD values were used to estimate the cell viability. Cell viability rate was calculated as the percentage of MTT absorption as follows:

$$\% \text{ survival} = (\text{mean experimental absorbance} / \text{mean control absorbance}) \times 100.$$

Statistical Analysis

The cell viability was calculated using a computer software program developed by Xenometrix based of the optimal density readings at 550 nm [20]. Results were presented as means ± SDs. Statistical analysis was done using one way analysis of variance (ANOVA) for multiple samples and Student's t-test for comparing paired sample sets. *p*-values less than 0.05 were considered statistically significant. The percentages of cell viability were presented graphically in the form of histograms, using Microsoft Excel computer program.

Results

We used the MTT assay to examine the cytotoxic effect of arsenic trioxide (ATO) on HL-60 cells for 6, 12, and 24 hours, respectively (Figures 1, 2, and 3). Data generated from these studies clearly showed that ATO exposure significantly reduces the viability of HL-60 cells. After 6, 12, and 24 h of exposure, ATO exerted a significant cytotoxic effect on HL-60 cells, showing LD₅₀ values of 14.26 ± 0.5 µg/mL (Fig 1), 12.54 ± 0.3 µg/mL (Fig 2), and 6.4 ± 0.6 µg/mL ATO (Fig 3), respectively; indicating a dose- and time-dependent response relationship.

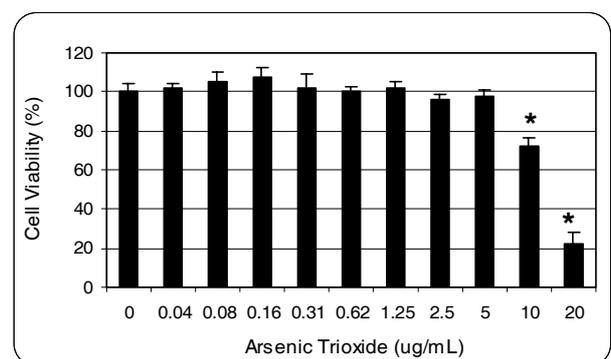


Figure 1: Toxicity of arsenic trioxide to human leukemia (HL-60) cells. HL-60 cells were cultured with different doses of arsenic trioxide for 6 hours as indicated in the Materials and Methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard deviation of 3 experiments with 6 replicates per dose. Cell Viability in 10 and 20 µg/mL are significantly different (*p* < 0.05) compared to the control according to ANOVA Dunnett's test.

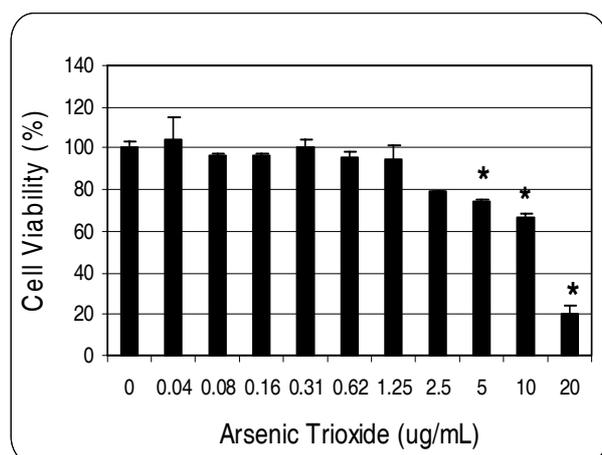


Figure 2: Toxicity of arsenic trioxide to human leukemia (HL-60) cells. HL-60 cells were treated with different doses of arsenic trioxide for 12 hours as indicated in the Materials and Methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard deviation of 3 experiments with 6 replicates per concentration. Cell Viability in 5, 10, and 20 $\mu\text{g/mL}$ are significantly different ($p < 0.05$) compared to the control according to ANOVA Dunnett's test.

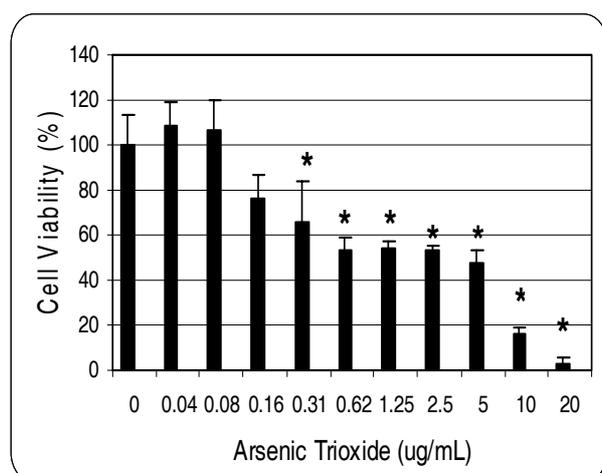


Figure 3: Toxicity of arsenic trioxide to human leukemia (HL-60) cells. HL-60 cells were cultured with different doses of arsenic trioxide for 24 hours as indicated in the Materials and Methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard deviation of 3 experiments with 6 replicates per dose. All values are significantly different ($p < 0.05$) compared to the control cells according to ANOVA Dunnett's test, except 0.04-0.08 $\mu\text{g/mL}$.

Figure 4 shows the time-response relationship with regard to the viability in different doses of ATO while figure 5 shows a similar relationship with regard to the lethal median dose (LD_{50}). Data presented in these figures indicate that the viability of HL-60 cells decreases with the increase in either arsenic trioxide dose and/or exposure time.

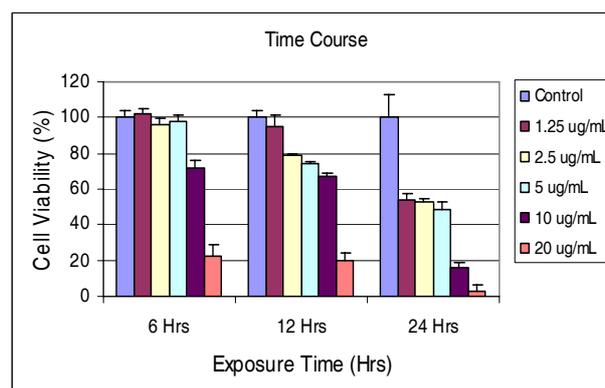


Figure 4: Time-response relationship with regard to the cytotoxicity of arsenic trioxide to human leukemia (HL-60) cells. HL-60 cells were cultured with different doses of arsenic trioxide for 6, 12, and 24 hours respectively as indicated in the Materials and Methods. Cell viability was determined based on the MTT assay. Each point represents a mean \pm SD of 3 experiments with 6 replicates per dose.

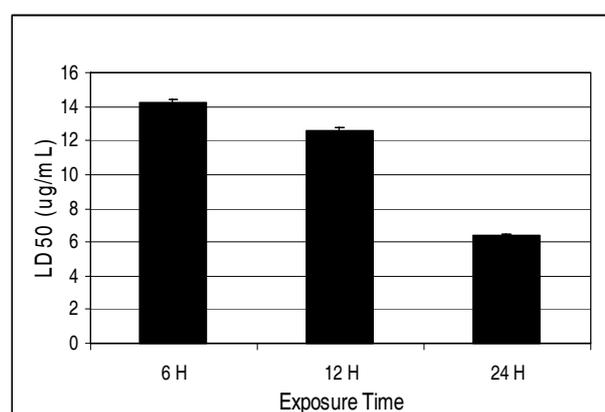


Figure 5: Time-response relationship with regard to the LD_{50} values of arsenic trioxide to human leukemia (HL-60) cells. $\text{LD}_{50} = 14.26 \pm 0.5 \mu\text{g/mL}$ for 6 h; $12.54 \pm 0.3 \mu\text{g/mL}$ for 12 h; and $6.35 \pm 0.6 \mu\text{g/mL}$ for 24 h of exposure.

Discussion

In the present study, we examined the cytotoxic effect of arsenic trioxide (ATO) on HL-60 cells. Data from this study clearly showed that ATO is highly cytotoxic to human leukemia (HL-60) cells, showing LD_{50} values of $14.26 \pm 0.5 \mu\text{g/mL}$, $12.54 \pm 0.3 \mu\text{g/mL}$, and $6.35 \pm 0.6 \mu\text{g/mL}$ for 6, 12, and 24 h of exposure, respectively. Recently, we reported that ATO is cytotoxic to human liver carcinoma (HepG₂) cells, showing a LD_{50} of $8.55 \pm 0.58 \mu\text{g/mL}$ after 48 hours of exposure [14, 15]. We found that low doses of ATO induce minimal toxicity in HL-60 cells upon 24 hours of exposure (Fig 3). Interestingly, such doses are similar to the therapeutically effective concentrations of ATO which have been shown to induce remission in APL patients with minimal toxicity [4]. Clinically, the standard dose for the treatment of patients with APL is

0.15 mg/kg per day which yields a maximum dose of 1-2 μM of ATO in the plasma [4]. High levels of ATO (5 $\mu\text{g}/\text{mL}$ and higher for 24 hrs) induce more than 50% of cell mortality [4]. In the present study, we have demonstrated that higher level of arsenic trioxide exposure inhibits cell proliferation and induces mortality of HL-60 cells in a dose- and time-dependent manner. Such effects have been observed with other test models [21-23], as well as in clinical studies [4, 24-27].

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