

# **The Effects of Arsenic Trioxide and Zoledronic Acid on Malignant Plasma Cells Derived from Bone Marrow Cells of Multiple Myeloma Patients**

*AH Emami<sup>1</sup>, M Yousefi<sup>2</sup>, A Mirshafiey<sup>2</sup>, M Momeny<sup>2</sup>, \*MR Khorramizadeh<sup>2,3</sup>*

<sup>1</sup>*Dept. of Internal Medicine, School of Medicine, Tehran University of Medical Sciences, Iran*

<sup>2</sup>*Dept. of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Iran*

<sup>3</sup>*Dept. of Medical Biotechnology, School of Advanced Medical Technologies, Tehran University of Medical Sciences, Iran*

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## **Abstract**

**Background:** Multiple myeloma (MM) is a disease of plasma cells that has fatal consequences. New agents associated with molecular targets have prompted clinical investigators to design new treatment strategies initially for advanced MM and later for newly diagnosed MM, with encouraging preliminary results. We devised a project to assess the mechanisms of action of two drugs, Arsenic trioxide (ATO) and Zoledronic acid (Zometa) on Bone marrow mononuclear cells (BMMCs) derived from patients. **Methods:** Bone marrow samples were collected from 10 patients after receipt of formal consent. BMMCs were collected from samples. In two parallel sets of experiments, BMMCs were treated with 0.5, 2, 6  $\mu$ M ATO and 0.1, 10, 100  $\mu$ M Zometa, for 72 h. The following analyses were then performed on treated cells as compared to untreated cells (assumed as control): cytotoxicity using Micro culture tetrazolium test (MTT assay); matrix metalloproteinase-2 zymography; comparative gene expression analysis of IL-6, vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1). **Results:** MTT assay showed significant proliferation inhibition in ATO high dose treatment (6  $\mu$ M). However, no significant inhibitory effect of Zometa was seen. Zymography analyses showed significant decrease in gelatinolytic activity in treated cells. Analyses of gene expression using Real-Time RT-PCR methodology showed significant decrease in IL-6, ICAM-1, and VEGF genes as normalized against Hypoxanthine phosphoribosyltransferase normalizer and as compared with untreated cells. **Conclusion:** Both ATO and Zometa could significantly decrease MM cells critical phenotype and genotype. This finding could support the hypothesis that ATO or Zometa could inhibit growth and metastasis of malignant cells.

**Keywords:** *Multiple myeloma, Arsenic trioxide, Zoledronic acid, Real time PCR, Gene expression.*

## **Introduction**

Multiple Myeloma (MM) is an incurable B-cell neoplasia characterized by proliferation of a clone of malignant plasmacells in the bone marrow (BM) and less frequently at extramedullary sites (1). MM is the second most common hematological cancer in the world which accounts for 1% of cancer diagnoses and 2% of cancer-related deaths (2, 3). In MM patients malignant transforming of plasmacells leads to immortalization, growth-factor independent survival and evasion from apoptosis in MM cells which secrete monoclonal immunoglobulin (Ig) in serum or/and urine (1, 4). The main clinical features of disease are primary amyloidosis due to monoclonal Ig deposit

in critical tissues (5), osteolytic lesions, anemia, renal failure, recurrent infections and to a less extent, bleeding, peripheral neuropathy and hyper viscosity syndrome (6).

Although the pathogenesis of the disease still remains somewhat unclear, however, it is well-established that cellular (stromal cells, osteoblasts, osteoclasts and endothelia) and protein components of BM microenvironment play a crucial role in MM development and pathogenesis (7). The interaction between MM cells with bone marrow stromal cells (BMSCs) and extra cellular matrix (ECM) is implicated in proliferation, survival and metastasis potential of MM cells. This interaction is occurred by adhesion molecules such as ICAM-1,

LFA-1, VCAM-1 and VLA-4 and induces the secretion of cytokines and growth factors via nuclear factor kappa B (NFκB) dependent pathway (8).

IL-6 is the most significant cytokine in MM biology (9, 10) which is secreted by MM cells and stromal cells through their interactions. The roles of IL-6 in MM pathogenesis can be summed up as follows: Proliferation of malignant plasmacells via the RAS-MAP kinase pathways (11), increasing cell survival via the JAK-STAT pathways (12), anti-apoptotic effects via the PI3-AKT pathways (13), enhancing production of vascular endothelial growth factor (VEGF) which is a pivotal factor in angiogenesis, growth and migration of MM cells (14, 15).

Furthermore, MM cells and BM microenvironment interactions induces multidrug resistance phenotype of malignant plasmacells (16) and secretion of matrix metalloproteinase-2 and 9 (MMP-2 and 9) which degrade extracellular matrix and results in cell migration and metastases (17, 18).

As mentioned above we can come to a conclusion that in MM patients BM microenvironment cross talk confers MM cells survival, growth, migration and drug resistance; hence therapeutic agents that inhibit this interaction between MM cells and BM cells and components will benefit most from the other drug related therapies.

In the present study, we investigated the effects of Arsenic Trioxide (AS<sub>2</sub>O<sub>3</sub>: ATO) and Zoledronic Acid (ZA; Zometa) on MM cells survival, MMP-2 activity and expression of some key genes in MM pathogenesis such as IL-6, VEGF and ICAM-1.

## Material and Methods

### *ATO and Zometa treatment*

Bone marrow samples were collected from 10 patients after receipt of formal consent and bone marrow mononuclear cells (BMMCs) was collected by histopaque 1.077 (Sigma). the BMMC cells obtained from MM patients were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 1 mM glutamine (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 1.5 g/l sodium bicarbonate (Sigma, St Louis, MO). The cell cul-

tures were maintained at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were treated with varying concentrations of ATO (0.5, 2 and 6 μM) and Zoledronate (0.1, 10 and 100 μM) for 72 h. The medium was replaced every 24 h during this treatment period. The final concentrations of DMSO did not exceed than 0.1% in all treatments.

### *Micro culture tetrazolium test (MTT assay)*

The inhibitory effect of both drugs on growth and proliferation of cells was assessed by the uptake of thiazolyl blue tetrazolium bromide (MTT, Sigma) by viable cells. Cells were plated onto 96-well plates (Orange Scientific, Brussels, Belgium) at a density of  $2 \times 10^4$  cell/100 μl/well. After incubation at 37 °C for 24 h, the medium was replaced with either control medium or medium containing either ATO or Zoledronate at desired concentrations every 24 h for 72 h. 50 μl of MTT solution (0.5 mg/ml) was added to each well and cells were further incubated at 37 °C for 1 h. Following solubilization of precipitated formazan with 100 μl DMSO, the optical densitometry was measured at a wavelength of 550 nm.

### *Gelatin Zymography*

This technique has been used for the detection of gelatinase (collagenase type IV or matrix metalloproteinase type 2, MMP-2), in conditioned-media according to Khorramizadeh et al. with some modifications (19). Briefly, aliquots of conditioned media were subjected to electrophoresis in polyacrylamide gel containing gelatin (2 mg/ml), under non-reducing conditions. The gels underwent electrophoresis for 3 h at a constant voltage of 80 volts. After electrophoresis, the gels were washed and gently shaken in three consecutive washings in 2.5% Triton X100 solution. The gel slabs were then incubated at 37 °C overnight in 0.1 M Tris HCl gelatinase activation buffer (pH 7.4) containing 10 mM CaCl<sub>2</sub> and subsequently stained with 0.5% Coomassie Blue (Sigma, Germany). After intensive destaining, proteolysis areas appeared as clear bands against a blue background. Using a UVI pro gel documentation system (GDS-8000 system), quantitative evaluation of both surface and intensity of lysis bands, on the basis of grey levels, were measured.

**Quantitative real-time RT-PCR**

RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to isolate total RNA from cultured cells and then 1 µg of extracted RNA was reverse transcribed into cDNA using QuantiTect reverse transcriptase (Qiagen). QuantiFast SYBR Green technology (Qiagen) was employed to perform quantitative PCR on an ABI PRISM 7500 Sequence Detection System (PE Applied Biosystems, Foster City, CA) applying the following thermal cycling conditions: an initial activation step for 5 min at 95 °C followed by 40 cycles including a dena-

turation step for 10 s at 95 °C and a combined annealing/extension step for 30 s at 60 °C.

The primers used are listed in Table1. Hypoxanthine phosphoribosyltransferase (HPRT) was used as a normalizer and the fold change in expression of each target mRNA relative to HPRT was calculated based on  $2^{-\Delta\Delta ct}$  comparative expression method.

**Statistical analyses**

The differences in cell proliferation and gelatinase activity were compared using the Student's *t* test. *P* < 0.05 were considered significant.

**Table 1:** Nucleotide sequences of the primers used for real-time RT-PCR

Target Genes	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
IL-6	NM_000600	ATGAACCTCCTTCTCCACAAGCGC	GAAGAG CCCTCA GGCTGGACTG
VEGF	NM_003376	ATCGAGTACATCTTCAAGCCAT	CTTTCTTTGGTCTGCATTCACA
ICAM-1	NM_000201	GAGCTG TTTGAGAACACC TC	TCACACTTCACTGTCACCTC
HPRT	NM_000194	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA

**Results**

ATO significantly decreased BMNCs proliferation as assessed by MTT assay:

MTT assay was performed to determine whether ATO and Zometa inhibit the proliferation and growth of BMNCs. Concentration-dependent experiments showed that ATO considerably inhibited the proliferation of BMNCs (*P* < 0.001). In comparison, no significant inhibitory effect of Zometa was seen (Fig. 1).

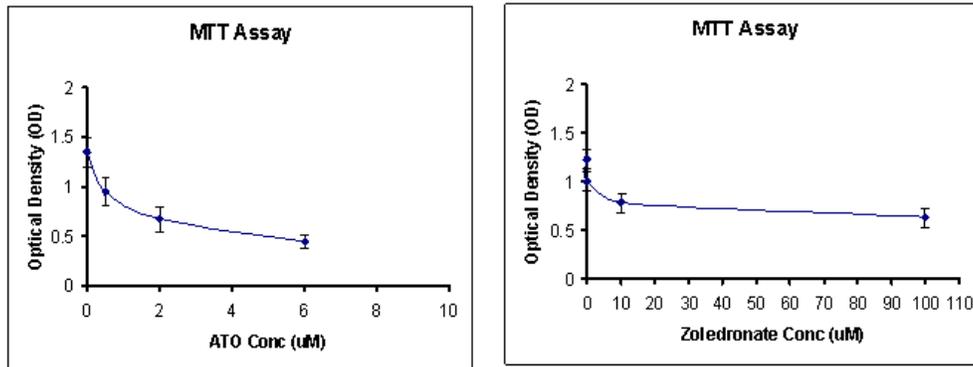
Effect of ATO and Zometa on the modulation of MMP-2 production:

We studied the different ATO and Zometa concentrations in reduction of MMP-2 gelatinolytic activity in BMNCs. ATO and Zometa decreased

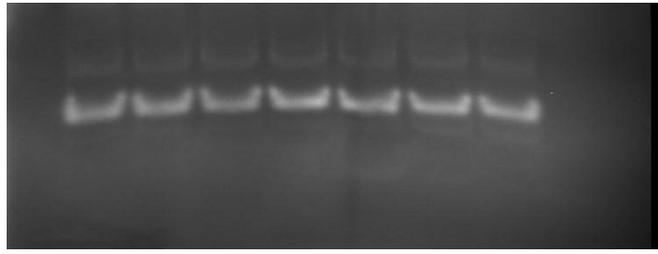
MMP-2 gelatinolytic activity in a dose dependent manner after 72 h of incubation in serum free media. This effect was significant in 6 µM dose of ATO and 100 µM dose of Zometa (*P* < 0.05 vs. control) (Fig. 2, 3).

ATO and Zometa down-regulated mRNA levels of IL-6, ICAM-1 and VEGF:

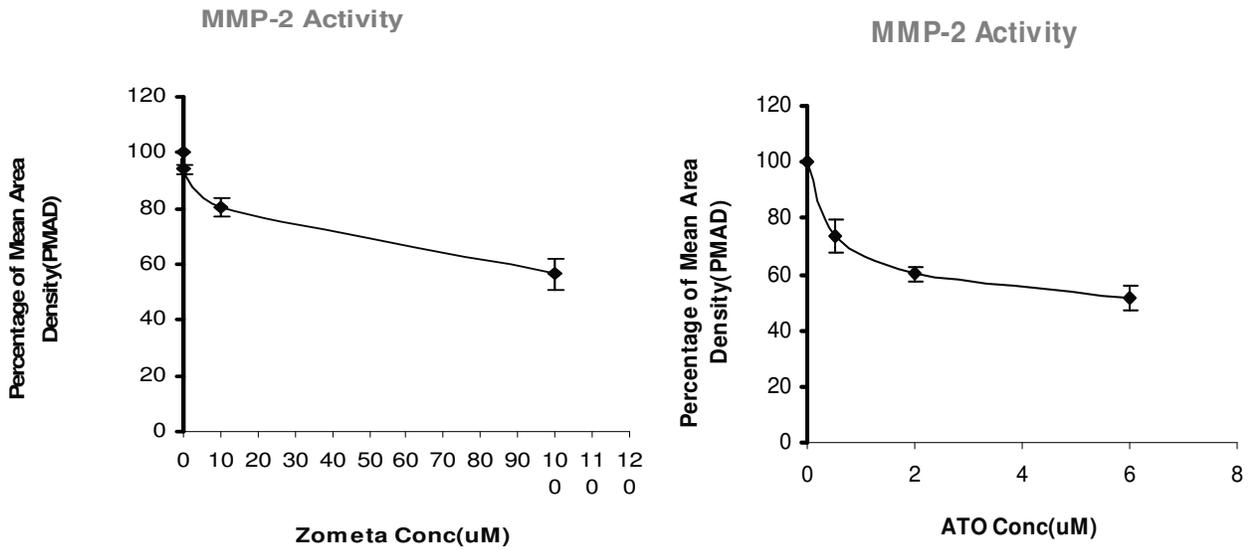
To explore whether ATO and Zometa is capable to transcriptionally down-regulates mRNA levels of IL-6, ICAM-1 and VEGF; we evaluated the effect of ATO and Zometa on mRNA levels of these genes. As depicted by Fig. 3, ATO and Zometa significantly reduced transcriptional levels IL-6, ICAM-1 and VEGF of in a dose-dependent manner (Fig.4).



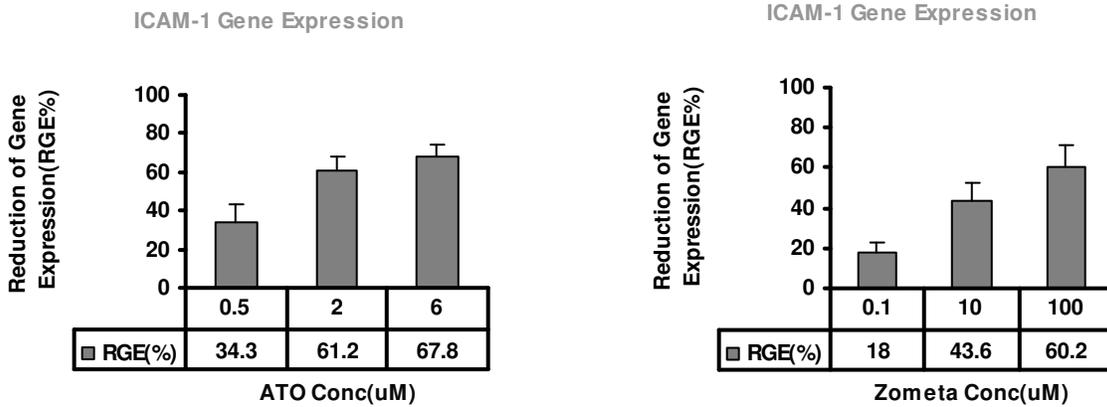
**Fig.1:** Effects of ATO and Zometa on proliferation of BMNCs



**Fig. 2:** Zymogram Gel from left to right: 1, 2, 3, (treatments with 0.1, 1, 100  $\mu$ M of Zometa) 4, 5, 6, (treatments with 0.5, 2, 6  $\mu$ M of ATO) 7: standard MMP-2(HT-29 cell line supernatant)



**Fig. 3:** Effect of ATO and Zometa on MMP-2 enzymatic activity



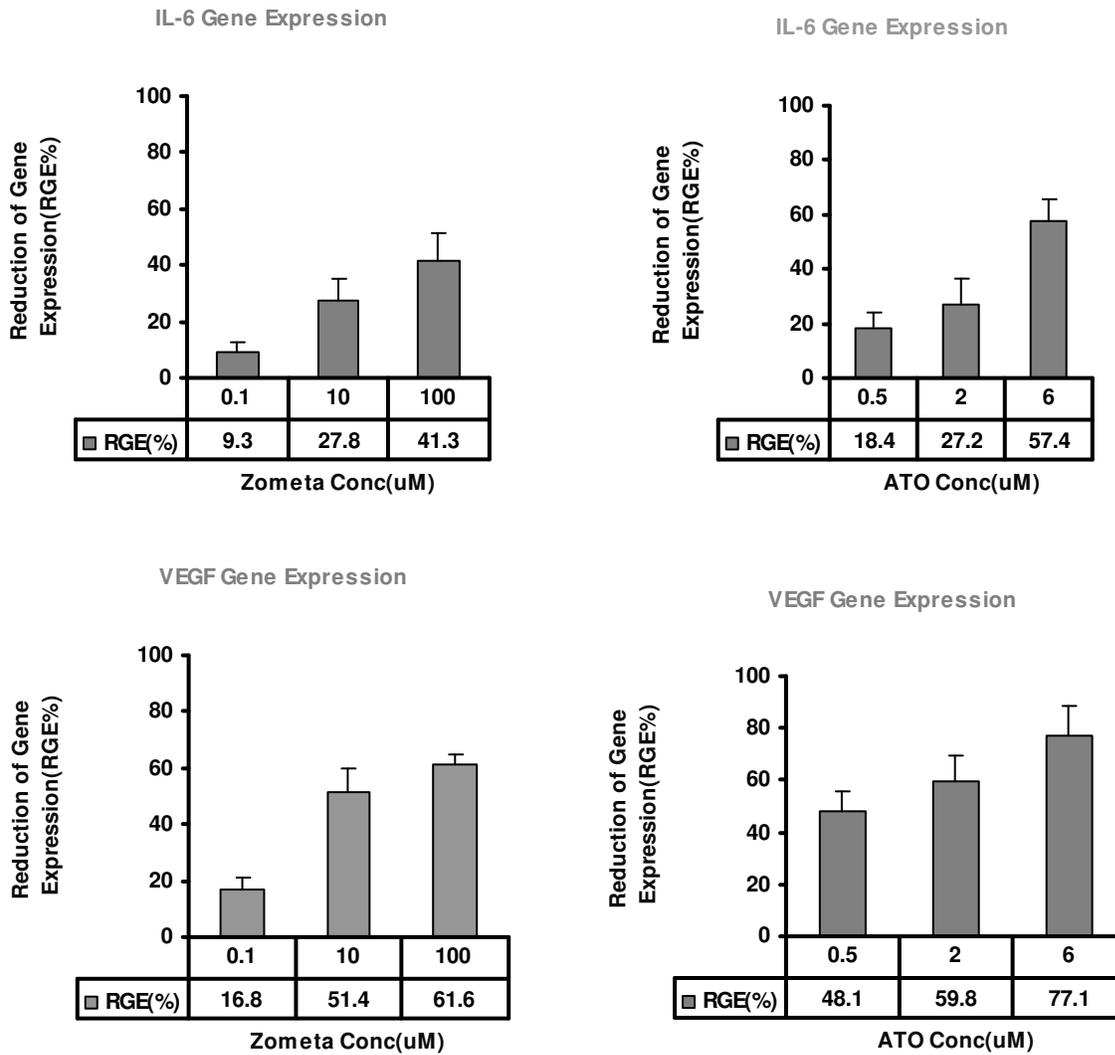


Fig. 4: Relative Expression of Target Genes, as Normalized against HPRT house-keeping Gene

## Discussion

In multiple myeloma disease, malignant plasma cells proliferate enormously in bone marrow milieu and invade to other ossicular locations. The metastases of malignant plasma cells could lead to such clinical complications as accidental bone breaking, renal failures, anemia, and immunodeficiency. The interactions between plasma cells and BM microenvironment could also induce overproduction of matrix metalloproteinases (MMPs), angiogenic factors (e.g. VEGF) that, in turn, might extravagate malignant plasma cell metastases. Multiple myeloma has been implicated as a prototypic

disease model for therapeutic research purposes to assess the interactions between tumor cells and their local microenvironment (20). The interaction of MM cells with BMSCs is considered as a critical component of the sophisticated biological network engulfed the malignant cells and their BM milieu. BMSCs are known as a heterogeneous compartment of mesenchymal cells similar to fibroblast morphological features that has been shown to maintain normal hematopoiesis (21, 22). In the course of MM disease, this hematopoietic support could culminate in unfavorable pathophysiological effects as abnormal sur-

vival and drug resistance of MM cells. To address this issue, some recent studies have applied targeted therapy of MM disease based upon tumor microenvironmental interactions. It has been shown that ATO inhibits growth of Human Multiple Myeloma Cells in the Bone Marrow Microenvironment. (23) As<sub>2</sub>O<sub>3</sub> has also been reported to induce apoptosis of MM cells and overcomes the protective effect of IL-6 in the BM milieu. In addition, As<sub>2</sub>O<sub>3</sub> reduces TNF- $\alpha$ -induced adhesion to BMSCs and the resultant induced secretion of cytokines (IL-6 and VEGF) that promote MM cell growth, survival, and migration. Importantly, As<sub>2</sub>O<sub>3</sub> inhibits the growth even of MM cells adherent to BMSCs at clinically achievable concentrations (24). Furthermore, in some reports, zometa has been shown to down-regulate the adhesion molecules of bone marrow stromal cells in multiple myeloma. It has also been reported to interfere with myeloma BMSCs by reducing proliferation, increasing apoptosis, and modifying the pattern of expression of adhesion molecules, especially those involved in plasma cell binding (25).

These studies provide the rationale for in vitro studies of As<sub>2</sub>O<sub>3</sub> and Zometa in MM. In the present study, we have shown that Arsenic trioxide and Zometa effectively inhibit the functional activities of malignant plasma cells at levels of cell proliferation, MMP enzymatic activity, and gene expression of IL-6, ICAM-1, and VEGF. The concomitant inhibition of key genes could advocate the idea that the two drug agents act on a common transcription factor like NF- $\kappa$ B that has been previously shown to induce MMPs, VEGF, ICAM-1, and IL-6 gene expression. The exact signaling effectors of ATO and Zometa, however, need to be elucidated. Collectively, our results show that both ATO and Zometa could modulate metastatic and angiogenic functions of malignant plasma cells. These effects could inhibit the migration and further complications of plasma cell tissue invasions. Most MM patients are elderly individuals that hardly tolerate and respond to such modality therapies as BM transplantations or high-dose chemotherapies. Thus, we believe

that drugs in these classes will need to be combined to achieve complete eradication of MM cells, and our present study has somewhat elucidated their mechanisms of action at cellular function, gene expression, and enzyme activity levels in order to provide the framework for rational combination of clinical trials to overcome drug resistance and improve patient outcome. The obtained effects of ATO and Zometa could be promising in sophisticated therapeutic process of multiple myeloma disease.

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The authors declare that they have no conflict of interests.

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