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Molecular Targets of Arsenic Trioxide in Malignant Cells

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ABSTRACT

Arsenic trioxide (As2O3; ATO) has considerable efficacy in the treatment of relapsed acute promyelocytic leukemia (APL), inducing partial differentiation and promoting apoptosis of malignant promyelocytes. Although initial studies focused on the role of the characteristic APL fusion protein, PML-RARα, in mediating response to ATO, subsequent investigations have revealed that ATO acts on numerous intracellular targets. ATO broadly affects signal transduction pathways and causes a wide range of alterations leading to apoptosis. Key mediators of sensitivity to ATO-induced apoptosis include intracellular glutathione and hydrogen peroxide (H2O2). The loss of inner mitochondrial membrane potential is also an important step in ATO-mediated cell killing. Cellular and physiologic pathways affected by ATO provide some clues as to the mechanisms for the biologic effects of ATO. Recent research has shown that hematologic cancers other than APL and solid tumors derived from several tissue types may be responsive to monotherapy or combination therapy with ATO. A better understanding of the mechanisms of action of ATO may help guide the use of ATO for the treatment of a wide variety of malignancies and allow its potential in cancer therapy to be fully realized. The Oncologist 2002;7(suppl 1):14-19

INTRODUCTION

Arsenicals have a long history of use in the treatment of leukemia, and recently, arsenic trioxide (As2O3; ATO) was approved by the Food and Drug Administration for use in the treatment of relapsed/refractory acute promyelocytic leukemia (APL). ATO induces a high rate of complete remissions (87%) in this disease. Treatment of APL patients with ATO is associated with the disappearance of the PML-RARα fusion transcript, the gene product of the chromosomal translocation t(15;17) characteristic of APL [1, 2]. As a result of this effect of ATO, 78% of APL patients achieve molecular remission after ATO therapy [1, 2].

Exactly how ATO mediates its clinical efficacy is not fully understood. Two main mechanisms of action of ATO
Degradation of the fusion protein, PML-RARα, is most likely the mechanism by which ATO induces cell differentiation in APL cells. Degradation of PML-RARα allows malignant promyelocytes to overcome their maturation block [3]. In clinical trials, cell samples taken from APL patients treated with ATO suggest that partial differentiation of the maturation-arrested leukemia cells contributes to the therapeutic effect [1]. ATO degrades the fusion protein and induces differentiation in APL cells whether or not they are resistant to retinoic acid. However, there is conflicting evidence whether ATO can synergize with retinoic acid in vitro or in vivo [4-6]. The partial differentiation effects of ATO appear to be unique to APL because of the direct effect of ATO on PML-RARα degradation.

ATO-induced apoptosis, in contrast, occurs via a variety of mechanisms, which appear to be independent of the presence of PML-RARα. The apoptotic effects of ATO occur, in part, through direct effects on mitochondria. ATO-induced apoptosis is associated with a loss of inner mitochondrial transmembrane potential and release of cytochrome c into the cytosol [7-9]. Experiments with purified mitochondria show that administration of ATO promotes opening of the permeability transition pore, releasing intermembrane proteins, which ultimately cause caspase activation [10]. Caspase induction and activation were also observed in APL cells from patients treated with ATO [1].

If PML-RARα expression were necessary for the induction of apoptosis by ATO, then ATO might be ineffective in other malignant cells, because the fusion protein is unique to APL. This does not appear to be the case. Studies conducted with a variety of malignant cell lines have shown that ATO-induced apoptosis is not limited to APL cells. ATO has been shown to stimulate programmed cell death in neuroblastoma, multiple myeloma, and other hematopoietic cell lines [11-13]. These data suggest that ATO may be effective in the treatment of a variety of hematologic malignancies.

Further evidence that PML-RARα is not necessary for ATO activity stems from studies in a subclone of NB4 cells resistant to all-trans-retinoic acid, in which PML-RARα is undetectable. ATO-induced growth inhibition and apoptosis in this subclone are comparable to the same effects in NB4 cells [5]. Additionally, NB4 subclones that are resistant to ATO-induced apoptosis remain sensitive to ATO-induced PML-RARα degradation and ATO-induced changes in PML subcellular localization [14].

Other studies, using a variety of leukemia cell lines that do not express PML-RARα, have also found that both ATO and melarsoprol (an organic arsenical compound) inhibit cell growth, downregulate the Bcl-2 protein, and induce apoptosis, with no evidence that these effects are mediated by native PML [15]. Cultures of murine embryonic fibroblasts and bone marrow progenitor cells, in which the PML gene was inactivated or not expressed, showed comparable responses to ATO, specifically, growth inhibition, apoptosis induction, and impairment of bone marrow proliferation [15]. Taken together, these results indicate that the cytotoxic activities of ATO are mediated by mechanisms independent of the PML-RARα fusion protein. These findings have prompted intense investigation of additional intracellular pathways by which ATO acts to inhibit growth and induce apoptosis.

Figure 1. Dual response of acute promyelocytic leukemia (APL) cells to arsenic trioxide (ATO). ATO at low concentrations induces differentiation of APL cells through degradation of PML-RARα, while ATO at high concentrations induces apoptosis through both PML-RARα-dependent and independent mechanisms. Abbreviations: RARE = retinoic acid response element; RXR = retinoid X receptor; RARα = retinoic acid receptor alpha; As2O3 = arsenic trioxide; PML/RARα = the characteristic APL fusion protein.
This article reviews the apoptotic mechanisms of ATO, the mediators that are known to increase cell susceptibility to ATO, and other important pathways contributing to ATO-induced apoptosis in malignant cells.

**APL Sensitivity to ATO-Induced Apoptosis and Growth Inhibition: The Role of Glutathione**

Although high concentrations of ATO may induce apoptosis in a number of different types of malignant cells, the fact that apoptosis and growth inhibition occur at lower ATO concentrations in APL cells (e.g., NB4 cells) indicates the increased sensitivity of these cells to the apoptotic effects of ATO. Certain mediators of ATO sensitivity have now been identified, raising the possibility that other malignant cells might be sensitized to arsenic by manipulation of the biochemical environment. By this means, the therapeutic potential of ATO could be extended to malignant diseases beyond APL.

The intracellular glutathione (GSH) redox system represents the best characterized mechanism of ATO sensitivity. Compared with other leukemia cells that are less sensitive to ATO, NB4 cells contain lower levels of glutathione peroxidase and catalase (which results in reduced redox buffering) and relatively higher levels of intracellular hydrogen peroxide ($\text{H}_2\text{O}_2$) [8, 16]. NB4 cells become resistant to ATO after administration of selenite, which increases glutathione peroxidase activity and decreases cellular $\text{H}_2\text{O}_2$ levels [6, 16]. If NB4 cells are treated with the catalase inhibitor aminotriazol, which increases intracellular $\text{H}_2\text{O}_2$ concentrations, their sensitivity to ATO is restored [16]. Malignant cells that are the most sensitive to ATO-induced apoptosis appear to be those with the lowest levels of intracellular GSH [17].

Experimental manipulations to alter GSH levels can change the apoptotic response to ATO. As shown in Figure 2, buthionine sulfoxide (BSO) and, in some cases, ascorbic acid can lower GSH in other malignant cells to levels that are equal to or lower than levels in NB4 cells. Such a decreased intracellular GSH has been shown to enhance sensitivity to ATO, whereas administration of free radical scavengers (i.e., N-acetylcysteine) increases GSH and abrogates the cytotoxic effects of ATO [17].

Thus, enzymes involved in regulating intracellular GSH and $\text{H}_2\text{O}_2$ levels play an important role in modulating sensitivity to the cytotoxic effects of ATO. Strategies to reduce intracellular GSH levels have been utilized in an attempt to enhance susceptibility to ATO-induced apoptosis. In ATO-resistant NB4 subclones, levels of GSH are twice as high as in normal NB4 cells that are sensitive to ATO. Administration of BSO, which diminishes GSH levels, promptly restores the sensitivity of these cells to ATO [18].

Ascorbic acid can act as an oxidizing agent in cancer cells and can also decrease GSH levels. Administration of ascorbic acid in conjunction with ATO has been reported to induce apoptosis in previously resistant malignant cells [17]. A potential synergy between ATO and ascorbic acid in the treatment of multiple myeloma is currently being investigated. Preliminary data suggest that this combination (ATO 0.15 mg/kg/day plus ascorbic acid 1,000 mg/day) is safe in heavily pretreated patients with relapsed or refractory multiple myeloma. Thus far, partial responses (≥25% decrease in disease markers) have been observed in two patients and stable disease (0%-25% decrease in disease markers) in four patients after these patients received a median of two cycles of treatment [19].

**Additional Intracellular Targets of ATO**

As arsenic has well-known carcinogenic properties as well as cancer-treating activities, dissection of the cell signaling pathways affected by arsenic has been an area of active investigation. Numerous intracellular targets that are affected by ATO have been elucidated. For instance, ATO shows a high binding affinity to proteins containing the vicinal sulphydryl (SH) groups, which are found in many enzymes. These enzymes may be inhibited by ATO binding. Some of the redox-sensitive enzymes that ATO may inhibit or activate include protein tyrosine phosphatases (PTPases) and c-Jun N-terminal kinase (JNK), as well as others involved in cellular signal transduction pathways (Table 1) [20-22]. In addition, ATO activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and other enzymes that are involved in the production of superoxides and other reactive oxygen species ultimately generating $\text{H}_2\text{O}_2$. Thus, regulatory redox buffering and relatively higher levels of intracellular $\text{H}_2\text{O}_2$ may induce apoptosis in previously resistant malignant cells.

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**Figure 2. Glutathione (GSH) levels in leukemic cell lines.** NB4 cells have the lowest GSH levels when compared with other leukemic cell lines. GSH levels can be decreased in all cell lines with the addition of BSO or AA. Abbreviations: BSO = buthionine sulfoxide; $\text{As}_2\text{O}_3$ = arsenic trioxide; AA = ascorbic acid. Adapted with permission from Dai et al. [17].
tional targets for arsenic. Arsenic stimulates JNK activity by for the treatment of cancers with p53 inactivation [23]. mediating pathways. For this reason, arsenic may be useful apoptosis independently of p53, acting instead via JNK-monomly mutated tumor-suppressor gene. Arsenic promotes with chemotherapy resistance, and p53 is the most com-
p53-mediated mechanisms. Inactivation of p53 is associated chemotherapeutic agents induce tumor cell apoptosis via induction of apoptosis by arsenic, as expression of a dominant negative mutant JNK nearly completely abrogated arsenic-induced apoptosis [23].

Interestingly, the pathway by which arsenic promotes apoptosis appears to be completely independent of p53. There was no difference in apoptosis induction following arsenic exposure of embryo fibroblasts with either active or inactivated p53 [23]. Furthermore, arsenic does not induce p53-dependent transcriptional activation [23]. This finding may have considerable clinical significance, as most cancer chemotherapeutic agents induce tumor cell apoptosis via p53-mediated mechanisms. Inactivation of p53 is associated with chemotherapy resistance, and p53 is the most commonly mutated tumor-suppressor gene. Arsenic promotes apoptosis independently of p53, acting instead via JNK-mediated pathways. For this reason, arsenic may be useful for the treatment of cancers with p53 inactivation [23].

PTPases all contain an SH group, making them potential targets for arsenic. Arsenic stimulates JNK activity by inhibiting a constitutive dual-specificity JNK phosphatase [20]. In nonstimulated cells, this phosphatase acts to maintain JNK activity at low basal levels. Its inhibition by arsenic leads to induction of JNK activity, which can alter gene transcription patterns, resulting in apoptosis [20].

### Gene Expression

Induction of JNK activity by arsenic occurs together with stimulation of activator protein 1 (AP-1) transcriptional activity and increased expression of the immediate early genes c-fos and c-jun. Both c-fos and c-jun are proto-oncogenes, the aberrant expression of which may contribute to tumor promotion. Upon activation of AP-1, numerous signal transduction pathways are engaged, changing the transcription of a variety of gene products [20]. For example, in a human bladder epithelial cell line, arsenic increased cell proliferation and AP-1 DNA binding [25].

Arsenic exposure not only increases expression of c-fos and c-jun, but it also alters the expression of several genes that modulate cell growth and cell cycle arrest, including early growth response (EGR-1), GADD153, and GADD45. The EGR-1 gene encodes for zinc-finger DNA binding transcription factors and influences growth stimulation in response to epidermal growth factor, nerve growth factor, or serum. In contrast, GADD153 and GADD45 are genes that are involved with the cellular stress response and growth arrest and may modulate the pathways leading to cell death [25].

Arsenic exposure also influences transcription of a wide range of genes that affect mitogen response, cell cycle progression, and programmed cell death. Future investigations may determine whether these different effects vary according to cell type, level or form of arsenic, or other factors.

### Induction of Reactive Oxygen Species

Oxidative damage has been postulated to be a key mechanism by which arsenic initiates the apoptotic process. The degree to which glutathione peroxidase and catalase levels influence susceptibility to arsenic-induced apoptosis has already been described. Arsenic-induced apoptosis is associated with the generation of reactive oxygen species (ROS) with subsequent accumulation of H2O2 in several experimental models [7, 16, 26]. Arsenic-induced apoptosis is inhibited when cells are treated with various antioxidants, free radical scavengers, or inhibitors of ROS-producing enzymes [7]. ATO-resistant NB4 cells have increased intracellular-reduced GSH compared with ATO-sensitive NB4 cells, allowing for effective elimination of free radicals [14, 18]. As a result, despite ATO-induced formation of ROS, these reactive oxygen molecules do not accumulate sufficiently to initiate apoptosis in ATO-resistant cells. Because

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**Table 1. Intracellular arsenic targets**

- Interaction with thiol groups, including those in catalytic sites of many enzymes
- Generation of reactive oxygen species and oxidative stress
- Inhibition or activation of redox-sensitive enzymes
  - Activation of JNK
  - Inhibition of dual-specificity phosphatase
  - Activation of AP-1 activity

Abbreviations: AP-1 = activator protein 1; JNK = c-Jun N-terminal kinase.
there is evidence that ATO induces JNK activity in NB4 cells in a dose-dependent manner, it will be interesting to evaluate JNK activation after depletion of GSH in NB4 cells and in ATO-resistant cells. Such an experiment might determine whether generation of ROS and activation of JNK is an important mechanism of the proapoptotic action of ATO.

**Summary**

Recently, patients with APL have experienced significant clinical gains after treatment with ATO. In both APL cells from patients and NB4 cells, ATO induces partial differentiation, inhibits growth, and promotes apoptosis. Although initial investigations focused on the role of PML-RARα in mediating the effects of ATO, it has become clear that ATO interferes with a variety of cellular processes by targeting numerous different intracellular molecules and thereby disrupting key signal transduction mechanisms and producing programmed cell death. These findings underscore the importance of understanding how differences in cell types or cellular environments might affect the action of ATO.

A determination of the factors that mediate sensitivity to ATO will allow use of this agent in such a way as to optimize therapeutic outcomes and minimize toxicity in the treatment of various malignancies. Intracellular GSH and H2O2 levels have been shown to be important mediators of ATO sensitivity, and strategies to alter the redox environment may allow normally ATO-resistant cells to become susceptible to ATO-induced apoptosis. One such strategy is already being studied in patients with multiple myeloma. Depending on the results of this and other trials, the therapeutic benefits of ATO, first observed in the treatment of APL, may be extended to other malignant cell types.

The mechanisms by which ATO promotes apoptosis are only beginning to be understood but appear to be distinct from those of traditional cytotoxic agents. To date, there is little evidence that the development of resistance to chemotherapy affects the response to arsenic. Thus, ATO represents a novel agent worth investigating, particularly in combination with other agents, for its effects in a variety of neoplastic diseases.

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**References**

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