Targeted Degradation of the AML1/MDS1/EVI1 Oncoprotein by Arsenic Trioxide

David Shackelford,1 Candia Kenific,1 Agnieszka Blusztajn,1 Samuel Waxman,2 and Ruibao Ren1

1Rosenstiel Basic Medical Sciences Research Center, Department of Biology, Brandeis University, Waltham, Massachusetts and 2Division of Hematology/Oncology, Department of Medicine, Mount Sinai School of Medicine, New York, New York

Abstract
Arsenic trioxide (ATO) has been found to be an effective treatment for acute promyelocytic leukemia patients and is being tested for treating other hematologic malignancies. We have previously shown that AML1/MDS1/EVI1 (AME), a fusion gene generated by a t(3;21)(q26;q22) translocation found in patients with chronic myelogenous leukemia during blast phase, myelodysplastic syndrome, or acute myelogenous leukemia (AML), impairs hematopoiesis and eventually induces an AML in mice. Both fusion partners of AML1, AML1/MDS1, and MDS1/EVI1, encode transcription factors and are also targets of a variety of genetic abnormalities in human hematologic malignancies. In addition, aberrant expression of ectopic viral integration site 1 (EVI1) has also been found in solid tumors, such as ovarian and colon cancers. In this study, we examined whether ATO could target AME and related oncoproteins. We found that ATO used at therapeutic levels degrades AME. The ATO treatment induces differentiation and apoptosis in AME leukemic cells in vitro as well as reduces tumor load and increases the survival of mice transplanted with these cells. We further found that ATO targets AME via both myelodysplastic syndrome 1 (MDS1) and EVI1 moieties and degrades EVI1 via the ubiquitin-proteasome pathway and MDS1 in a proteasome-independent manner. Our results suggest that ATO could be used as a part of targeted therapy for AME-, AML1/MDS1-, MDS1/EVI1-, and EVI1-positive human cancers. (Cancer Res 2006; 66(23): 11360-9)

Introduction
AML1/MDS1/EVI1 (AME) is a fusion gene generated by a t(3;21)(q26;q22) translocation found in patients with chronic myelogenous leukemia (CML) during blast phase, therapy-related myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML), and in rare cases of de novo AML (1). We previously reported that expression of the AME fusion protein in mouse bone marrow cells by retroviral transduction impaired hematopoiesis and eventually induced an AML in mice (2). Our results established a causal role of AME in myeloid malignancies.

Both fusion partners of AME encode transcription factors and are also targets of a variety of other genetic abnormalities found in human hematologic malignancies. AML1 (also termed as CBFA2 or RUNX1) is a DNA-binding subunit of core binding factor (CBF) and functions as a heterodimer with the non-DNA-binding subunit CBFβ (3). It plays an essential role in definitive hematopoiesis and blood vessel development (3) and is a target of multiple chromosomal abnormalities in human hematologic malignancies (4).

The other partner of AME, the ectopic viral integration site 1 (EVI1) gene, encodes a zinc finger-containing transcription factor and was first identified as a common site of retroviral integration in murine myeloid tumors (5, 6). MDS1/EVI1 is a longer alternatively spliced form of EVI1 (7, 8). EVI1 is an essential gene required for proper murine development and is expressed in a variety of tissues (9). Disruption of the murine EVI1 gene by targeted mutagenesis in embryonic stem cells resulted in death of mouse embryos at ~10.5 days after coitus (10). The Evi1−/− embryos at day 10.5 exhibit widespread hypocellularism, hemorrhaging, and disruption in the development of paraxial mesenchyme. In addition, defects in the heart, somites, and cranial ganglia were detected and the peripheral nervous system failed to develop (10). These data suggest that EVI1 has important roles in general cell proliferation, vascularization, and cell-specific developmental signaling. In the hematopoietic system, EVI1 is predominantly expressed in hematopoietic stem cells (HSC) during development and during adult life (11–13). HSCs from Evi1−/− mice were defective in their self-renewal proliferation and repopulation capacities (13). Consistent with the function of promoting cell proliferation, EVI1 was found to interfere with transforming growth factor-β (TGF-β) signaling and antagonizes the growth-inhibitory effects of TGF-β (1). It also enhances activator protein-1 activity by activating the c-Fos promoter. In HSCs, EVI1 up-regulates the expression of GATA2, an essential transcription factor for proliferation of adult HSCs (13).

Aberrant expression of EVI1 has been found in some CML in blast crisis, MDS, and AML that are associated with the t(3;3)(q21;q26) and inv(3)(q21q26) chromosome abnormalities (8). In addition, aberrant expression of EVI1 or MDS1/EVI1 has also been frequently found in AML, MDS, and other hematologic malignancies without detectable chromosomal rearrangement (1). Furthermore, EVI1 overexpression has been found in several solid tumors, such as ovarian and colon cancers (14, 15). Experiments in vitro showed that overexpression of the EVI1 protein blocks granulocytic differentiation of the myeloid precursor cell line 32Dcl3, inhibits erythropoietic responsiveness in erythroid progenitors, and up-regulates the megakaryocytic differentiation of murine embryonic stem cells (16–18). In vivo, enforced expression of EVI1 using the murine Sca-1 promoter in transgenic mice improved normal erythropoiesis and expression of EVI1 in bone marrow cells by retroviral transduction and transplantation induces either B-lymphoid malignancy with low efficiency or severe pancytopenia resembling MDS after a long latent period (19–21). These results show that aberrant expression of the EVI1 proto-oncogene plays a critical role in the pathogenesis of hematologic malignancies.

Therapies, such as imatinib, which target oncoproteins that play causal roles in tumorigenesis, are effective in treating cancers.

Requests for reprints: Ruibao Ren, Rosenstiel Basic Medical Sciences Research Center, MS029, Brandeis University, Waltham, MA 02454-9110. Phone: 781-736-2486; Fax: 781-736-2405; E-mail: ren@brandeis.edu.

Editors: Anna Marie Price and Jennifer Hocker

Cancer Res 2006; 66: (23). December 1, 2006 11360 www.aacrjournals.org

Downloaded from cancerres.aacrjournals.org on April 15, 2017. © 2006 American Association for Cancer Research.
Arsenic trioxide (ATO) has been found as an effective treatment for patients suffering from acute promyelocytic leukemia (APL) that harbors the t(15;17) translocation (22). The t(15;17) translocation generates the promyelocytic leukemia (PML)/retinoic acid receptor α (RARα) fusion protein, which ATO has been shown to specifically induce degradation of in human APL cells (22–24). Low doses of ATO induced partial differentiation, whereas higher doses trigger apoptosis in APL cells (22). Terminal differentiation can be induced in these cells when ATO was used in combination with cyclic AMP or granulocyte macrophage colony-stimulating factor (25, 26). It is thought that degradation of PML/RARα frees the cellular block in differentiation and allows the APL cells to respond to therapy.

The success of ATO in treating APL patients has prompted many to consider using the drug to treat hematologic malignancies harboring other translocations and/or mutations (27). In this study, we examined whether ATO could target AME and related oncoproteins for degradation.

**Materials and Methods**

**DNA constructs.** The human AME, MDS1/EVI1, EVI1, AML1/MDS1, and AMLΔ genes were each cloned into a bicistronic murine stem cell virus (MSCV) vector downstream of the encephalomyocarditis virus internal ribosomal entry site (IRES) and two myc tag sequences as described previously (2, 20). An enhanced green fluorescent protein (GFP) gene was cloned into the EcoRI site upstream of the IRES on the MSCV vector.

**Cell culture and virus preparation.** NIH3T3 mouse fibroblasts and Bosc-23 cells were grown as described previously (28, 29). Helper-free retroviruses were generated by transient transfection of retroviral vectors into Bosc-23 cells as described previously (29). NIH3T3 cells were infected with virus containing AME, EVI1, MDS1/EVI1, AML1-MDS1, or AMLΔ constructs. GFP-positive NIH3T3 cells were sorted and cultured in DMEM. Primary AML cells (BM6-4 cells) expressing AME were isolated from secondary recipient mice suffering from an AML-like disease (2) and cultured in DMEM and supplemented with 10% (v/v) fetal bovine serum (FBS), 20% (v/v) WEHI supernatant, and 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Grand Island, NY). Cells were grown and either left untreated or treated with ATO (Cell Therapeutics, Inc., Seattle, WA), lactacystin or MG132 (dissolved in DMSO; Calbiochem, San Diego, CA), and/or cycloheximide (Sigma, St Louis, MO).

**Immunoprecipitations and Western blot analysis.** Cells from the bone marrow of diseased mice were treated with solution ACK [0.15 mol/L NaCl, 1.0 mmol/L KHCO₃, 0.1 mmol/L NaEDTA (pH 7.5)] to lyse RBCs. Bone marrow, NIH3T3, and Bosc-23 cells were isolated to make whole-cell lysates. Cells were washed twice in cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer [1.0% NP-40, 0.25% deoxycholate, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA] containing protease inhibitors [pepatatin, leupeptin, aprotinin (Calbiochem), phenylmethylsulfonyl fluoride (Sigma), and Complete protease inhibitor cocktail (Roche, Mannheim, Germany)] to a concentration of 10 to 20 million cells/mL. Immunoprecipitations were carried out by preclearing whole-cell lysates with protein A agarose beads (Amersham, Piscataway, NJ). Lysates were then incubated at 4°C for 1 to 2 hours with either protein A beads or 9E10 bound protein A beads. Beads were washed twice with RIPA buffer and boiled with equal volume of 2× loading buffer. Equal volumes of samples were run on a 6% to 18% gradient polyacrylamide gel and transferred to nitrocellulose. The membrane was incubated with either a primary mouse anti-myc (9E10, 1:100), mouse anti-dynamin (1:1,000; Transduction Laboratories, Lexington, KY), or mouse anti-ubiquitin (1:500), anti-Blimp-1 (1:500), anti-C/EBPα (1:1,000), anti-PU.1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-p53 (1:500; BD PharMingen, San Diego, CA) antibodies followed by a secondary anti-mouse horseradish peroxidase antibody (1:2,000; Southern Biotechnology Associates, Birmingham, AL). Signals were detected by chemiluminescence. AME and dynamin expressions were quantified by densitometry using Alpha Innotech densitometry software (Alpha Innotech, San Leandro, CA).

**Flow cytometry.** Cells were collected, washed twice in PBS, and resuspended in fluorescence-activated cell sorting (FACS) staining buffer (PBS/5% FBS/0.1% sodium azide). Cells were stained with the following mouse antibodies from Becton Dickinson (San Jose, CA): phycoerythrin (PE)-conjugated F4/80 or e-C1 (2B8) and allophycocyanin (APC)-conjugated Mac-1 (MI/70). All cells were blocked with anti-mouse CD16-CD32 (Fc block), then washed twice in FACS staining buffer, and resuspended in 2 μg/mL propidium iodide in FACS staining buffer. Staining intensities of viable cells were measured by using a FACSCalibur (Becton Dickinson). Viable GFP (+) and GFP (−) cells were sorted by using FACSStar or FACSaria (Becton Dickinson). Flow cytometry data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

**Bone marrow transplantation and treatment with ATO.** Bone marrow cell isolation and transplantation were done as described (28, 29). Male BALB/c recipient mice were sublethally irradiated (450 rads) and received 1 × 10⁶ BM6-4 cells. At 5 or 21 days after bone marrow transplantation, mice were given 5 mg/kg ATO or vehicle (PBS) by daily ip injection. WBC counts were taken every week, and their peripheral blood was scanned by flow cytometry to detect GFP+ cells. Moribund mice were sacrificed and their spleens were weighed. Mice used in this project are housed in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited Foster Animal Research Facility at Brandeis University (Waltham, MA). All experiments involving mice are approved by Institutional Animal Care and Use Committee of Brandeis University.

**Apoptosis assay.** BM6-4 cells were seeded at a concentration of 2 × 10⁶/mL and treated with 0.5 to 2.0 μmol/L ATO (5 mmol/L stock) to induce apoptosis. Cells were collected after 1, 3, and 5 days, then treated as described by the Annexin V staining protocol (BD PharMingen). Briefly, cells were resuspended in Annexin V buffer to a concentration of 1 million/mL, and 100,000 cells were then stained with 5 μL of PE-conjugated Annexin V antibody (BD PharMingen) and 5 μL 7-aminoactinomycin D (7-AAD) and incubated at room temperature for 15 minutes. Annexin V buffer (400 μL) was then added to each sample with gentle mixing. Staining intensities of viable cells were measured by using a FACSCalibur (Becton Dickinson). Flow cytometry data were analyzed using FlowJo software.

**Cytospin.** Hematopoietic cells were spun down at 1,200 rpm for 6 minutes with a Cytospin3 (Shandon, Pittsburgh, PA) and then fixed and stained with the Hema3 solutions. Slides were mounted with coverslips and pictures were taken on an Olympus (Tokyo, Japan) 1870 microscope.

**Reverse transcription-PCR and real-time PCR.** Total cellular RNA from BM6-4 cells was isolated and DNase digested according to the manufacturer’s protocol using an RNasea RNA purification kit (Qiagen Sciences, Germantown, MD). RNA (2 μg) was reverse transcribed to cDNA according to the manufacturer’s protocol using the SuperScript III First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). Real-time PCR analysis was done on a Rotorgene 3000 PCR machine (Corbett Research, Sydney, Australia) with a primers pair for the AML1/MDS1 fusion portion of the AME gene and a primer pair for the β-actin gene. The AML1/MDS1 forward and reverse primer sequences (from 5′ to 3′) were GAGGGTCAAGCCGACACACAC and GCATCTGGCATTTTCTCCAAAG. The β-actin forward and reverse primer sequences were AGAGGGAATCTGGCGTGGA and CAATGGGT-GACCTGGGCGGT. Expression of the AME gene was normalized with the β-actin transcript copy number, and the ratio of AME transcript copy number to β-actin transcript copy number was referred to as the relative copy number. The efficiency of each primer pair was accounted for by forming standard curves from serial dilutions of appropriate genomic DNA preparations during each real-time run. All samples in a single PCR run were assayed in triplicate. All data represent the average of at least three independent experiments, with the error bars displaying the average deviation.

**Results**

ATO degrades the AME protein in a dosage-dependent manner. As mentioned earlier, we have found that AME induces an AML in a mouse bone marrow transduction and transplantation.
model (2). We further found that the primary leukemic cells derived from bone marrow of mice with AME-induced AML can grow in culture at the presence of interleukin-3. We used one of the AME primary leukemic cell lines, named BM6-4, to study the effects of ATO on the AME expression and on the biology of AME leukemic cells.

When BM6-4 cells were treated with clinically relevant concentrations of ATO (0.5-2.0 μmol/L) in culture, there was a marked reduction in the expression of AME (Fig. 1A and B). Within 24 hours, the AME protein levels decreased 5- and 3-fold in cells treated with 2 and 1 μmol/L ATO, respectively (Fig. 1A). At this time point, not much change in the AME protein expression was seen in cells treated with 0.5 μmol/L ATO. At 72 hours after treatment, the AME protein was not detectable in cells treated with 2 μmol/L ATO and decreased approximately 5- and 2.5-fold in cells treated with 1 and 0.5 μmol/L ATO, respectively (Fig. 1B). No significant decrease in total cell number was observed after days 1 and 3 of 0 to 1.0 μmol/L ATO treatments. Cells treated with 2.0 μmol/L ATO showed a decrease of 50% in total cell number by day 3. Cell lysates were normalized to a concentration of 1 × 10^6 live cells/mL. These results show that ATO induces down-regulation of the AME protein in a dosage-dependent manner. A similar result was seen in another AME leukemic cell line (data not shown). We further confirmed this result in the Bosc-23 cell line, a derivative of the HEK293 human embryonic kidney cell line, by transiently transfecting the MSCV-GFP-IRES-AME retroviral construct into the cells. The AME-expressing Bosc-23 cells treated with ATO for 24 hours also showed a marked decrease in the expression of AME, dynamin, and GFP (Fig. 1C). The expression of AME was quantified with Alpha Innotech software and represented as the 9E10/dyn ratio.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** ATO down-regulates AME. A and B, Western blot analysis of whole-cell lysates from BM6-4 cells treated with or without ATO. BM6-4 cells were either treated with 1× PBS (lane 1) or 0.5, 1.0, or 2.0 μmol/L ATO (lanes 2-4) for 24 (A) or 72 (B) hours, and their lysates were run on a 6% to 18% gradient gel. The expression of myc-tagged AME, dynamin (as a loading control), and GFP was probed with the anti-myc (9E10), anti-dynamin, and anti-GFP antibodies. AME and dynamin protein expression was quantified with Alpha Innotech software and represented as the 9E10/dyn ratio. C, Western blot analysis of whole-cell lysates from Bosc-23 cells transiently transfected with the MSCV-GFP-IRES-AME vector. Cells were treated with vehicle (lane 1) or 0.5, 1, or 2 μmol/L ATO (lanes 2-4) for 24 hours, lysed, and ran on a 6% to 18% gradient gel. The expression of AME, dynamin, and GFP was probed by antibodies described above. D, top, real-time PCR analysis of the relative copy number of AME normalized against β-actin in BM6-4 cells that were either untreated or treated with 2 μmol/L ATO for 24 hours; bottom, AME/actin protein expression in the same pool of BM6-4 cells untreated or treated with 2 μmol/L ATO for 24 hours as those in top. E, Western blot analysis of whole-cell lysates from BM6-4 cells untreated or treated with ATO (2 μmol/L) and/or cycloheximide (25 μg/mL) as indicated. The expression of myc-tagged AME, p53, and dynamin was probed with the anti-myc (9E10), anti-p53, and anti-dynamin antibodies.

Cancer Res 2006;66:(23). December 1, 2006 11362 www.aacrjournals.org
expression of AME in a dose-dependent fashion (Fig. 1C, comparing lanes 2-4 with lane 1).

In all the above experiments, no decrease of GFP, which was coexpressed with AME from the same transcript, was detected in cells treated with ATO (data not shown; Fig. 1). This result suggests that the down-regulation of AME by ATO is post-translational. To further test this possibility, we used real-time PCR on BM6-4 cells untreated or treated with 2 μmol/L ATO to measure expression of AME RNA. We measured the relative copy number of AME by normalizing the expression of AME RNA with expression of β-actin RNA. Figure 1D (top) shows that there was no significant difference in the relative copy number of AME between untreated (days 0 and 1) and ATO-treated cells (day 1). The relative copy number value of AME in cells treated with 2 μmol/L ATO was 0.92 compared with untreated cells, which had relative copy number values of 1.0 (day 0) and 1.16 (day 1). As a control, the AME protein expression was analyzed in the same pool of cells by Western blot and quantitated by densitometry to determine the relative AME to actin ratio as shown in Fig. 1D (bottom). There was a significant decrease (P < 0.0001) in AME protein expression in ATO-treated cells (day 1) compared with untreated cells (day 1) as expected. These results suggest that ATO does not down-regulate transcription of AME but rather targets the AME fusion protein.

To further examine whether down-regulation of AME by ATO is post-translational, we treated BM6-4 cells with or without cycloheximide and/or 2 μmol/L ATO. The tumor suppressor protein p53 was used as a control for this experiment. Untreated cells and those treated with 2 μmol/L ATO showed a reduction of p53 by 24 hours, which may be due to cells entering stationary phase in culture. Reduction of p53 levels was seen 6 hours after treatment (Fig. 1E, lane 4), indicating that cycloheximide was effective in suppressing new protein synthesis. Decrease of p53 levels was delayed (from 6 to 10 hours) in cells treated with both ATO and cycloheximide, suggesting that ATO may prolong the half-life of p53 in the cell. In contrast, cells treated with both ATO and cycloheximide showed loss of AME much faster than cells treated with ATO or cycloheximide alone (Fig. 1D). These results further show that ATO degrades AME via a post-translational mechanism.

ATO induces differentiation of AME leukemic cells. We next examined the biological effect of ATO treatment on AME leukemic cells. Expression of cell surface markers and cell morphology was analyzed by flow cytometry and cytospin, respectively, at 1, 3, and 5 days after ATO treatment.

Flow cytometry analysis shows that BM6-4 cells began expressing F4/80, a monocytic cell differentiation marker, and higher levels of Mac-1 when exposed to ATO (Fig. 2A). Most untreated cells retained Mac-1low and F4/80 when exposed to ATO (data not shown; Fig. 2A). This result suggests that the down-regulation of AME by ATO is post-translational. To further test this possibility, we used real-time PCR on BM6-4 cells untreated or treated with 2 μmol/L ATO to measure expression of AME RNA. We measured the relative copy number of AME by normalizing the expression of AME RNA with expression of β-actin RNA. Figure 1D (top) shows that there was no significant difference in the relative copy number of AME between untreated (days 0 and 1) and ATO-treated cells (day 1). The relative copy number value of AME in cells treated with 2 μmol/L ATO was 0.92 compared with untreated cells, which had relative copy number values of 1.0 (day 0) and 1.16 (day 1). As a control, the AME protein expression was analyzed in the same pool of cells by Western blot and quantitated by densitometry to determine the relative AME to actin ratio as shown in Fig. 1D (bottom). There was a significant decrease (P < 0.0001) in AME protein expression in ATO-treated cells (day 1) compared with untreated cells (day 1) as expected. These results suggest that ATO does not down-regulate transcription of AME but rather targets the AME fusion protein.

To further examine whether down-regulation of AME by ATO is post-translational, we treated BM6-4 cells with or without cycloheximide and/or 2 μmol/L ATO. The tumor suppressor protein p53 was used as a control for this experiment. Untreated cells and those treated with 2 μmol/L ATO showed a reduction of p53 by 24 hours, which may be due to cells entering stationary phase in culture. Reduction of p53 levels was seen 6 hours after treatment (Fig. 1E, lane 4), indicating that cycloheximide was effective in suppressing new protein synthesis. Decrease of p53 levels was delayed (from 6 to 10 hours) in cells treated with both ATO and cycloheximide, suggesting that ATO may prolong the half-life of p53 in the cell. In contrast, cells treated with both ATO and cycloheximide showed loss of AME much faster than cells treated with ATO or cycloheximide alone (Fig. 1D). These results further show that ATO degrades AME via a post-translational mechanism.

ATO induces differentiation of AME leukemic cells. We next examined the biological effect of ATO treatment on AME leukemic cells. Expression of cell surface markers and cell morphology was analyzed by flow cytometry and cytospin, respectively, at 1, 3, and 5 days after ATO treatment.

Flow cytometry analysis shows that BM6-4 cells began expressing F4/80, a monocytic cell differentiation marker, and higher levels of Mac-1 when exposed to ATO (Fig. 2A). Most untreated cells retained Mac-1low and F4/80 (Fig. 2A, a-c and d-f, red curve). Cells treated with 1.0 and 2.0 μmol/L ATO expressed mature monocytic cell markers as soon as 24 hours after ATO treatment (Fig. 2A, b, c, e, and f) but expressed monocytic cell markers by days 3 and 5 (Fig. 2A, b, c, e, and f). Flow cytometry data were not collected on cells treated with 2.0 μmol/L ATO at day 5 because majority of the cells were dead. In contrast to the increased expression of MAC-1, expression of c-Kit was reduced over time in a dosage-dependent manner (data not shown).

Consistent with the expression of monocytic differentiation markers, ATO-treated BM6-4 cells underwent drastic morphologic changes in 1 to 5 days after ATO treatment in a dosage-dependent manner (Fig. 2B, d-f, g-i, and j-l). Before the ATO treatment, BM6-4 cells retain immature myeloid cell morphology with a high nuclear/cytoplasmic ratio. Following the ATO treatment, cell morphology changed to resemble mature macrophages with increased cell size, plentiful cytoplasm, and one-sided small nucleus. In contrast, untreated cells underwent minimal spontaneous differentiation over time, yet most cells retained the morphology of immature myeloid cells (Fig. 2B, a-c). A similar result was seen in another AME leukemic cell line (data not shown).

Correlating with the monocytic cell differentiation, the expression of Blimp-1, a transcription factor that involved in the terminal differentiation of B and monocytic cells, was increased after 1-day treatment with 2.0 μmol/L ATO. Meanwhile, C/EBPα, a transcription factor required for granulocytic cell differentiation, was decreased 3 days after ATO treatment. The expression of PU.1, a transcription factor required for the development of multiple blood lineages, on the other hand, was not affected by the ATO treatment. All these data indicate that ATO induces BM6-4 cells to undergo monocytic cell differentiation in a dosage-dependent manner.

ATO induces apoptosis of AME leukemic cells. Numerous studies have shown that ATO induces apoptosis in a variety of cell lines, including APL cells and malignant lymphoma cells in a dosage-dependent fashion (30, 31). To examine whether ATO induces apoptosis in AME leukemic cells, we treated BM6-4 cells with 0.5 to 2.0 μmol/L ATO for 1, 3, and 5 days and then stained the treated cells with 7-AAD and Annexin V-PE. The Annexin V-PE and 7-AAD two-color flow cytometric analysis discriminated three populations: viable (bottom left), early apoptotic (bottom right), and both late apoptotic and necrotic cells (top right). As shown in Fig. 2D, a fraction of BM6-4 cells receiving no treatment (approximately 25% at day 3 and 40% at day 5) underwent spontaneous apoptosis. In comparison, larger amounts (82%) of BM6-4 cells treated with 2 μmol/L ATO underwent apoptosis by day 3 of ATO treatment and all cells were dead by day 5 of treatment. At day 5, approximately 60% and 91% of AME leukemic cells exposed to 0.5 and 1 μmol/L ATO, respectively, also had undergone apoptosis.

Interestingly, BM6-4 cells treated with 0.5 μmol/L ATO seemed to be fully differentiated by day 5 (Fig. 2A and B), yet ~40% of the cells were still viable and had not undergone apoptosis. Likewise, BM6-4 cells treated with 1 and 2 μmol/L ATO seemed to be fully differentiated by days 3 and 1, respectively (Fig. 2A and B), yet significant amounts of cells were still viable at the time. These results suggest that ATO induced two separate processes—an early differentiation phase and a late apoptosis phase.

ATO induces down-regulation of AME via the MDS1 and EVI1 moieties. Both fusion partners of AME, AML1 and MDS1/EVI1, are also targets of a variety of other genetic abnormalities found in human hematologic malignancies. We wanted to determine which moiety or moieties of AME (AML1, MDS1, or EVI1) were targeted by ATO. To do this, we stably expressed AME, MDS1/EVI1, EVI1, AML1/MDS1, and AML1A (Fig. 3A) in NIH3T3 cells and treated them with increasing amounts of ATO. ATO reduced the expression of AME 2- to 10-fold at a dosage of 5 to 10 μmol/L in NIH3T3 cells (Fig. 3B, top, lanes 4 and 5). The increase dosage of ATO needed to down-regulate AME in NIH3T3 cells may reflect a cell type–specific response to the drug. Similar studies on fibroblasts/NIH3T3 cells have used 6 to 100 μmol/L ATO to study its effects on signal transduction and apoptosis (32). Treatment with 10 μmol/L ATO also down-regulated the MDS1/EVI1, EVI1, and AML1/MDS1 proteins by 3- to 5-fold (Fig. 3B, top, lanes 4 and 5).

www.aacrjournals.org 11363 Cancer Res 2006; 66: (23). December 1, 2006
AML1/MDS1 seems to be expressed in two forms (Fig. 3B, middle bottom); the higher molecular weight form may be post-translationally modified. It is this higher molecular weight form of AML1/MDS1 that was down-regulated distinctly by the ATO treatment (Fig. 3B, middle bottom). The putative modification of AML1/MDS1 may be due to a specific conformation of the protein because other AML1- or MDS1-containing proteins do not show such a modified form.

In contrast, AML1Δ showed no decrease in expression at any of the ATO dosages (Fig. 3B, bottom, lanes 1-5). The lanes were loaded equally as shown by the expression of a housekeeping protein, dynamin (Fig. 3B, middle, lanes 1-5). In addition, GFP expression was unaffected by the ATO treatment (Fig. 3B, bottom, lanes 1-5). Consistent with the idea that ATO does not target AML1 sequences, AML1/ATO is not degraded by ATO treatment in Kasumi-1 cells. Together, these results show that ATO down-regulates AME through MDS1 and EVI1 moieties.

ATO targets EVI1 for degradation via the ubiquitin-proteasome pathway. We examined whether inhibition of proteasome could abrogate ATO-induced down-regulation of AME, MDS1/EVI1, EVI1, AML1/MDS1, or AML1Δ. The 20S proteasome inhibitor lactacystin was added to NIH3T3 cells expressing each of the above proteins at concentrations of 1.0 and 2.0 μmol/L with or without 10.0 μmol/L ATO (Fig. 4A). Higher dosage of lactacystin was not included in the experiment due to compounded toxicity in the presence of ATO. EVI1 expression was significantly rescued by the addition of lactacystin in a dosage-dependent manner (Fig. 4A, top, comparing lane 3 with lanes 4 and 6). However, no significant rescue was seen for AME, MDS1/EVI1, or AML1/MDS1 (Fig. 4A, middle top, middle, middle bottom, comparing lane 3 with lanes 4 and 6). These results suggest that ATO down-regulates EVI1 through the proteasome degradation pathway but down-regulates MDS1-containing proteins through a proteasome-independent pathway.

To confirm that EVI1 was targeted by the proteasome pathway, we examined whether the 26S proteasome inhibitor MG132 could also inhibit ATO-induced down-regulation of EVI1. Cells were treated with 100 or 500 nmol/L MG132 with or without 10.0 μmol/L ATO. Figure 4B shows that MG132 did rescue EVI1 expression in the

---

3 Jing and Waxman, unpublished data.
presence of 10.0 μmol/L ATO in a dosage-dependent manner (Fig. 4B, top, compare lane 2 with lanes 5 and 6). This result further indicates that ATO down-regulates EVII, at least in part, through the proteasome degradation pathway. The effect of proteasome inhibition was unable to be assessed in the BM6-4 cells because these cells are sensitive to low doses of proteasome inhibitors in the presence of ATO.

Most proteins degraded by the 20S/26S proteasome are modified by ubiquitination. We next investigated whether EVII and AME are ubiquitinated and whether ATO stimulates their ubiquitination. NIH3T3 cells expressing AME or EVII were treated with 5.0 μmol/L ATO, 1.0 μmol/L lactacystin, or in combination. Cells were lysed, and the proteins were immunoprecipitated and probed for ubiquitin or EVII. As shown in Fig. 5, a fraction of EVII in untreated cells is indeed ubiquitinated (Fig. 5A and B, lane 6) and addition of lactacystin alone increased the amounts of ubiquitinated EVII (Fig. 5A and B, lane 10). As expected, the ATO treatment led to EVII degradation (Fig. 5A, lanes 2 and 8) and addition of lactacystin in the presence of ATO partially rescued EVII from degradation (Fig. 5A, lanes 4 and 12). Interestingly, there is a 2.5-fold increase of the amount of ubiquitinated EVII in cells treated with both ATO and lactacystin compared with those not treated (Fig. 5B, comparing lane 6 with 12). In addition, cells treated with both ATO and lactacystin had nearly twice the ubiquitinated EVII than cells treated with just lactacystin alone (Fig. 5B, comparing lane 10 with 12). This result suggests that ATO down-regulates EVII through regulating its ubiquitination.

Immunoprecipitation of the full-length AME protein showed that the protein is also ubiquitinated in untreated cells (Fig. 5D, lane 5) and treatment with lactacystin led to a slight increase in the amount of ubiquitinated AME protein (Fig. 5D, lane 9). As expected, treatment with ATO alone led to the degradation of AME (Fig. 5C, lanes 2 and 7) and a decrease in ubiquitinated AME (Fig. 5D, lane 7). However, treatment with lactacystin did not rescue the AME protein from ATO-induced degradation (Fig. 5C, lane 4 and 11), although ubiquitinated AME was slightly increased (Fig. 5D, lane 11). This shows that, although the AME protein is ubiquitinated, its degradation could not be rescued by proteasome inhibitor. This result suggests that the proteasome-independent pathway targeting the MDS1 moiety of AME is codominant with the proteasome-dependent pathway targeting the EVII moiety.

ATO reduces AME expression and suppresses expansion of AME leukemia cells in mice. Having shown that ATO targets AME for degradation, we moved to examine the effect of ATO treatment on AME leukemic cells in vivo. We have previously shown that AME-induced AML can be transferred to secondary recipient mice (2). When BM6-4 cells were cultured and transplanted back into either lethally or sublethally irradiated recipient mice via tail vein injection, the animals developed an AML-like disease within 3 to 7 weeks after transplantation depending on the progression of the disease.

Figure 3. ATO targets both the MDS1 and EVII moieties. A, schematic representation of the AME, MDS1/EVI1, EVII, AML1/MDS1, and AML1Δ proteins. RHD, runt homology domain; Zn1, zinc finger domain 1; Zn2, zinc finger domain 2. B, Western blot analysis of whole-cell lysates from NIH3T3 cells stably expressing AME, MDS1/EVI1, EVII, AML1/MDS1, or AML1Δ. Samples were treated with 0 to 10 μmol/L ATO (lanes 1-5) for 24 hours, lysed, and run on a 6% to 18% gradient gel. The anti-myc (9E10) antibody was used to probe for myc-tagged AME, MDS1/EVI1, EVII, AML1/MDS1, or AML1Δ proteins. Expression of dynamin and GFP controls was detected by anti-dynamin and anti-GFP antibodies, respectively. AME, MDS1/EVI1, EVII, AML1/MDS1, and AML1Δ and dynamin protein expression was quantified with Alpha Innotech software and represented as the 9E10/dyn ratio. Both the high and lower molecular weight bands for AML1/MDS1 were measured by densitometry.
amounts of AME leukemic cells injected (data not shown). The immunophenotype of the AME leukemic cells in mice remains the same as in culture except for the amounts of c-Kit-positive cells, which were increased from 20% to 25% in culture to 50% to 70% in mice (data not shown).

We went on to test whether AME leukemic cells respond to the ATO treatment in vivo. Mice transplanted with BM6-4 were treated with vehicle or 5.0 mg/kg ATO for 28 days beginning at day 5 or day 21 after transplantation. The AME protein expression in the leukemic cells isolated from bone marrow of recipient mice that were treated with 5.0 mg/kg ATO or vehicle 4 weeks beginning at 21 days after transplantation was analyzed by immunoblotting (Fig. 6A). The mice receiving 5.0 mg/kg ATO (Fig. 6A, lanes 2-6) had an ~3-fold reduced AME expression compared with mice receiving vehicle (P < 0.01; Fig. 6A, lanes 7-10). It was noted that several of the mice that received ATO expressed higher molecular weight forms of AME (Fig. 6A, lanes 3 and 5), which may be due to ATO-induced ubiquitination of AME, as shown above. The lower efficiency of ATO in down-regulating AME in vivo compared with that in vitro (Fig. 1) might be due to insufficient delivery of ATO to the leukemic cells in mice.

Consistent with a reduction of the AME expression, the recipient mice that were treated with 5.0 mg/kg ATO for 4 weeks beginning at day 21 after transplantation had significantly reduced splenomegaly compared with those receiving vehicle (P < 0.002) as well as a 35% lower WBC count (P < 0.004; Fig. 6B and C). However, no significant survival benefit was seen for this treatment. One possibility is that the treatment was given too late. Indeed, treatment with 5.0 mg/kg ATO beginning at day 5 after transplantation significantly prolonged life of mice with AME-induced AML, with a mean survival of 50 days compared with 43 days for mice treated with vehicle (P < 0.0001; Fig. 6D). In addition, these mice also showed a delayed expansion of GFP + leukemia blasts in the peripheral blood compared with mice treated with vehicle (Fig. 6E). However, tumor cells did begin aggressively expanding in the animals once treatment was stopped (to avoid long-term toxicity of ATO) and the animals eventually succumbed to AML.

Discussion

In this study, we showed that ATO specifically targets the AME oncoprotein for degradation. The ATO treatment induces differentiation and apoptosis of AME leukemic cells in vitro and reduces tumor load as well as prolongs survival of mice transplanted with AME leukemic cells. ATO targets AME for degradation through the MDS1 and EVI1 moieties via both proteasome-dependent and proteasome-independent pathways.

Figure 4. Proteasome inhibition abrogates ATO-induced degradation of EVI1. A, NIH3T3 cells stably expressing AME, MDS1/EVI1, EVI1, AML1/MDS1, or AML1Δ were treated with 0 or 10 μmol/L ATO and/or 1 to 2 μmol/L lactacystin for 24 hours as indicated. Cells were lysed and run on a 8% to 18% gradient gel. Western blot analyses of myc-tagged AME, MDS1/EVI1, EVI1, AML1/MDS1, or AML1Δ proteins were done using the anti-myc (9E10) antibody. Expression of dynamin and GFP controls was detected by anti-dynamin and anti-GFP antibodies, respectively. AME, MDS1/EVI1, EVI1, AML1/MDS1, and AML1Δ and dynamin protein expression was quantified with Alpha Innotech software and represented as the 9E10/dyn ratio. B, Western blot analysis of whole-cell lysates from NIH3T3 cells that stably express the myc-tagged EVI1 protein. Cells were treated with or without ATO and/or MG132 for 24 hours, lysed, and run on a 6% to 18% gradient gel. Western blot analyses of myc-tagged EVI1, dynamin, and GFP protein expression were done as described above.
Arsenic is an ancient drug, used for many centuries to treat a broad range of ailments, including cancer, but was replaced as a cancer therapeutic in the early 20th century with the advent of modern chemotherapy. It was rediscovered in 1990s as a safe and effective treatment for patients suffering from APL (33, 34). The success in using ATO as a target therapy for APL underscores the importance of matching drugs to the appropriate patients. Our results suggest that ATO could be used as a part of targeted therapy for AME-, AML1/MDS1-, MDS1/EVI1-, and EVI1-positive human cancers.

The preliminary results of ongoing phase II studies conducted in patients with MDS suggest that ATO produces hematologic improvement, including durable transfection independence in up to 30% of patients (35, 36). Aberrant expression of EVI1 has been frequently found in patients with MDS (37, 38). AME, AML1/MDS1, and MDS1/EVI1 are also found to be associated with MDS cases (8). A recent study also showed that ATO and thalidomide combination produces multilineage hematologic responses in MDS patients, particularly in those with high pretherapy EVI1 expression (39). It is possible that EVI1-positive MDS is sensitive to ATO treatment. Our data provide a biochemical basis for this hypothesis and suggest that ATO may also be effective in treating AME-, AML1/MDS1-, and MDS1/EVI1-positive MDS. In addition to hematologic malignancies, ATO may target EVI1 in other cancers, such as ovarian and colon, which are frequently associated with high EVI1 expression (14, 15).

ATO effectively induces differentiation and apoptosis of AME leukemic cells in culture (Fig. 2). ATO has been shown to induce apoptosis in cells by a host of different mechanisms, such as the modulation of the glutathione redox system (30, 33, 40) as well as activation of caspases (41) and c-Jun NH2-terminal kinase (32). These mechanisms may contribute to the apoptosis of AME leukemic cells induced by ATO. In addition, AME may contribute to the block of myeloid cell differentiation and provide survival signals in leukemic cells. Down-regulation of AME may free the cellular block in differentiation and contribute to ATO-induced apoptosis.

In addition to the drastic in vitro response to ATO, AME leukemic cells were also sensitive to ATO in vivo. The ATO treatment suppressed expansion of AME leukemia cells and prolonged the life of the recipient mice. However, the therapeutic effect was not long lasting; once treatment stopped the leukemic cells quickly expanded and animals succumb to AML. The limited efficacy may attribute to several factors. One is that ATO half-life is relatively short (<6 hours) and may have not been delivered for maximal efficacy such that the local concentration of ATO was low. Secondly, the in vivo environment may render AME leukemic cells more resistant to ATO treatment. Lastly, the development of myeloblastic leukemia involves multiple genetic abnormalities. Targeting a single oncoprotein has not been effective, at least in cases of treating CML in advanced phases by imatinib and FLT3 mutation-positive AML, with FLT3 tyrosine kinase inhibitors (42, 43). It is possible that ATO may be more effective in combining with drugs that target additional oncogenic events.

It is interesting that ATO is capable of targeting both MDS1 and EVI1 sequences. This effect of ATO is quite specific; even Blimp-1, a transcription factor belonging to the same subclass of PR domain-containing zinc finger proteins as MDS1/EVI1, was not down-regulated by ATO (Fig. 2C). Our data show that ATO targets EVI1 for degradation, at least in part, through the ubiquitin-proteasome pathway. Arsenite has been shown to regulate ubiquitination both positively and negatively (44–49). One interesting finding is that arsenite induces Cdc25C degradation through the KEN-box and ubiquitin-proteasome pathway (50). The ubiquitination of Cdc25C may involve APC, a ubiquitin ligase complex that covalently attaches ubiquitin to several cell cycle regulatory proteins (50). A similar mechanism...
might be involved in the normal ubiquitin-proteasome–mediated turnover of EVI1.

In contrast to the degradation of EVI1, ATO targets MDS1-containing proteins for degradation largely through a proteasome-independent mechanism. This mechanism seems to be effective because ATO still down-regulates AME, MDS1/EVI1, and AML1/MDS1 in the presence of proteasome inhibitors, which abrogate ATO-induced degradation of EVI1. It would be interesting to elucidate the mechanism of stimulation of ubiquitination of EVI1 by ATO as well as the proteasome-independent mechanism by which ATO targets MDS1-containing proteins. Such research will help to identify new therapeutic targets for cancer therapies and to understand the normal regulation of these proteins.

**Acknowledgments**

Received 5/16/2006; revised 9/20/2006; accepted 9/26/2006.

Grant support: American Cancer Society research grant RSG-02-059-01-LIB (R. Ren) and the Samuel Waxman Cancer Research Foundation (R. Ren and S. Waxman).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank N. Farina and Dr. Y. Jing for technical assistance and Drs. George Acs and Arthur Zelent for critically commenting the article.
Degradation of AML1/MDS1/EVI1 by Arsenic Trioxide

References

15. Liu Y, Chen L, Ko TC, Fields AP, Thompson EA. Ev1 is a survival factor which conveys resistance to both TGFβ and taxol-mediated cell death via PTK/ATK. Oncogene 2006;25:3655–75.
Targeted Degradation of the AML1/MDS1/EVI1 Oncoprotein by Arsenic Trioxide

David Shackelford, Candia Kenific, Agnieszka Blusztajn, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/23/11360

Cited articles
This article cites 50 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/23/11360.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/66/23/11360.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.