# Arsenic but not all-trans retinoic acid overcomes the aberrant stem cell capacity of PML/RARα-positive leukemic stem cells

Xiaomin Zheng, Anita Seshire, Brigitte Rüster, Gesine Bug, Tim Beissert, Elena Puccetti, Dieter Hoelzer, Reinhard Henschler, Martin Ruthardt

*From the Laboratory of Tumor Stem Cell Biology, Department of Hematology, J.W. Goethe University, Frankfurt, Germany (XZ, AS, GB, TB, EP, DH, MR); Department of Transfusion Medicine and Immunohematology, J.W. Goethe University, Frankfurt, Germany (BR, RH).*

*Funding: This study was funded by a grant from the Deutsche José Carreras Leukämie-Stiftung (R04/05) to MR and RH XZ and AS are both supported by a fellowship for young investigators from the Deutsche José Carreras Leukämie-Stiftung (DJCLS-F04/04 and DJCLS-F06/05, respectively).*

*Manuscript received July 14, 2006. Manuscript accepted February 6, 2007.*

*Correspondence: Martin Ruthardt, M.D., Med. Klinik II/Hämatologie , Klinikum der J.W. Goethe Universität Frankfurt, Theodor Stern Kai 7, 60590 Frankfurt, Germany. E-mail: ruthardt@em.uni-frankfurt.de*

## ABSTRACT

### Background and Objectives

Stem cells play an important role in the pathogenesis and maintenance of most malignant tumors. Acute myeloid leukemia (AML) is a stem cell disease. The inefficient targeting of the leukemic stem cells (LSC) is considered responsible for relapse after the induction of complete hematologic remission (CR) in AML. Acute promyelocytic leukemia (APL) is a subtype of AML characterized by the t(15;17) translocation and expression of the PML/RARα fusion protein. Treatment of APL with all-trans retinoic acid (ATRA) induces CR, but not molecular remission (CMR), because the fusion transcript remains detectable, followed by relapse within a few months. Arsenic induces high rates of CR and CMR followed by a long relapse-free survival (RFS). Here we compared the effects of ATRA and arsenic on PML/RARα-positive stem cell compartments.

### Design and Methods

As models for the PML/RARα-positive LSC we used: (i) $Sca1^+/lin^-$ murine HSC retrovirally transduced with PML/RARα; (ii) LSC from mice with PML/RARα-positive leukemia; (iii) the *side population* of the APL cell line NB4.

### Results

In contrast to ATRA, arsenic abolishes the aberrant stem cell capacity of PML/RARα-positive stem cells. Arsenic had no apparent influence on the proliferation of PML/RARα-positive stem cells, whereas ATRA greatly increased the proliferation of these cells. Furthermore ATRA induces proliferation of APL-derived stem cells, whereas arsenic inhibits their growth.

### Interpretations and Conclusions

Taken together our data suggest a relationship between the capacity of a compound to target the leukemia-initiating cell and its ability to induce long relapse-free survival. These data strongly support the importance of efficient LSC-targeting for the outcome of patients with leukemia.

Key words: acute promyelocytic leukemia, arsenic, all-trans retinoic acid, leukemic stem cell

Haematologica 2007; 92:323-331

Acute myeloid leukemia (AML) is characterized by an accumulation of abnormal hematopoietic progenitor cells in the bone marrow and the peripheral blood, which exhibit the morphology of a certain stage of myeloid differentiation. This defines the respective subtype according to the French-American British (FAB) classification of AML.[1] The AML phenotype seems to be maintained by an increased proliferation of the blast cells, which is considered to result from the combination of two components: (i) a differentiation block preventing progenitor cells reaching the post-proliferative stage and subsequently undergoing programmed cell death;[2] (ii) an increased capacity for self-renewal of the leukemic progenitors.[3,4] Acute promyelocytic leukemia (APL) is a well characterized subtype of AML, classified as FAB M3.[5] It can be distinguished from other AML subtypes based on distinct cytogenetic, biological and clinical features.[6] More than 95% of patients with APL harbor the t(15;17) translocation, which encodes the PML/RARα fusion protein.[5] In addition, APL is clinically characterized by (i) the achievement of complete remission (CR) in about 90% of patients upon treatment with all-trans retinoic acid (ATRA);[7] and (ii) induction of CR in 72–96% of patients upon exposure to low dose arsenic trioxide ($As_2O_3$).[8]

Treatment with ATRA as a single agent results in CR but not complete molecular remission (CMR), because the t(15;17)-associated PML/RARα fusion transcript remains detectable. In about 29% of patients CMR can be induced by ATRA if double the dosage is administered as a liposomal formulation.[9] The great majority of patients will experience a relapse within a few months.[6] Nevertheless, when used in combination with conventional chemotherapy, the efficacy of ATRA is increased dramatically, improving the long-term survival of APL patients by up to 75%.[7] In contrast to ATRA, $As_2O_3$ as a single agent is able to induce both CR and CMR followed by long-term relapse-free survival in about 50% of APL patients, even following relapse after treatment with chemotherapy regimens containing ATRA .[8] Both $As_2O_3$ and ATRA have tumor cell-specific activity in APL, with almost no toxic effect on normal hematopoiesis. In fact, treatment of APL patients with either $As_2O_3$ or ATRA is not accompanied by the bone marrow aplasia normally seen with conventional chemotherapy. The CR induced by ATRA in APL patients is related to the induction of terminal differentiation of the APL blasts.[6,10] $As_2O_3$ induces CR by stimulating both a high rate of apoptosis and a considerable rate of differentiation in APL cells.[8]

The tumor-specific activities of ATRA and $As_2O_3$ are strictly determined by the cytogenetic features of APL. The sensitivity of APL patients to treatment is genetically determined by the presence of the t(15;17) translocation.[11] Expression of functional PML/RARα is necessary for the response to these drugs[12] and sensitizes several cell types to the differentiation-inducing activity of ATRA or to the apoptosis induced by $As_2O_3$.[6,12] In contrast, another APL-specific fusion protein – the t(11;17)-associated PLZF/RARα – mediates neither ATRA-induced differentiation nor $As_2O_3$ -induced apoptosis, and t(11;17)-positive APL patients do not respond to treatment with either drug.[12-15] The questions of why ATRA-monotherapy, in contrast to $As_2O_3$, is not able to eradicate the leukemic population completely and how it increases the response to chemotherapy remain to be resolved. It has recently been shown that, in normal hematopoiesis, ATRA does not only induce differentiation but also has a strong effect on stem cells, which leads to an enrichment of selected immature progenitors with stem cell features.[16] In fact, exposure to ATRA leads to a significant increase of self-renewal in early murine $Sca1^+/lin^-$ hematopoietic stem cells (HSC).[16,17]

We, therefore, hypothesized that ATRA might have a dual effect on the APL leukemic cell population: induction of differentiation in the mature *blast* population resulting in a CR and, on the other hand, induction of proliferation and/or self-renewal of the leukemic stem cells (LSC), the persistence of which is documented by the failure to achieve a CMR and which thus become the source of relapse. This PML/RARα-positive LSC population represents only 1 in $10^4$ to $2.5x10^6$ cells.[18] Such a frequency of cells would not be detected by the current definition of CR, but would fall within the range of sensitivity of clinically relevant reverse transcriptase polymerase chain reaction (RT-PCR-based) systems for the detection of minimal residual disease (MRD).[19] In contrast, $As_2O_3$ is able to induce CMR and does not seem to exert any toxic effect on HSC. APL patients treated with $As_2O_3$ do not undergo bone marrow aplasia,[20] and normal $CD34^+$ human bone marrow cells have unaltered colony-forming potential in semi-solid medium, even upon exposure to 2 μM $As_2O_3$.[21] We, therefore, compared the effects of ATRA with those of $As_2O_3$ on cell populations with stem cell capacity expressing PML/RARα.

## Design and Methods

### Cell culture and chemicals

NB4 cells were maintained in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS) (Invitrogen) or in serum-free medium (X-VIVO10 - Cambrex, Verviers, Belgium). ATRA (Sigma, Steinheim, Germany) and $As_2O_3$ were used at final concentrations of 1 μM and 2 μM, respectively. These are the clinically relevant concentrations achieved in APL patients.

### Murine HSC: isolation, retroviral transduction, replating efficiency and differentiation

$Sca1^+/lin^-$ cells were isolated from female C57BL/6N mice aged 6 to 12 weeks (Charles River, Sulzfeld, Germany). Isolation of $Sca1^+/lin^-$ HSC, retroviral transduction, serial replatings for the assessment of replating

efficiency, and differentiation analysis were performed as described before.[4,17] The Sca1$^+$/lin$^-$ cell populations contain about 10% of early HSC. Therefore 5×10$^3$ cells were plated in the semi-solid medium for the determination of the colony-forming unit (CFU)-potential and replating efficiency. The SV129 mouse with PML/RARα-positive leukemia was obtained by the inoculation of Sca1$^+$/lin$^-$ cells retrovirally transduced with PML/RARα. Leukemic mononuclear bone marrow cells were enriched on a Ficoll density gradient.

### Day 12 CFU-spleen assay

Sca1$^+$/lin$^-$ cells isolated from C57BL/6N mice were retrovirally transduced with PML/RARα or PLZF/RARα and cultured as previously described[4] for 2 days with or without 1 μM ATRA or plated in semi-solid medium with or without 1 μM ATRA or 2 μM $As_2O_3$. The cells were harvested or washed out from the semi-solid medium with phosphate-buffered saline (PBS) and inoculated into lethally irradiated (11 Gy) female C57BL/6N recipient mice. The CFU-spleen (CFU-S) assay was performed as previously described.[17]

### Statistical analysis

Paired Student's *t*-tests were performed using GraphPad Prism 3.0 software (GraphPad Software, San Diego, California, USA).

### Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated by the Trizol reagent according to the manufacturer's instructions (Invitrogen). First strand DNA was obtained according to standard protocols. The PML/RARα fusion region was amplified by conventional RT-PCR (sensitivity of 5×10$^{-3}$-10$^{-4}$ using the following primers: PML-A1, CAGTGTACGCCTTCTCCATCA, and RARA-B, GCTTGTAGATGCGGGGTAGA). The annealing temperature was 55 °C.

### PKH26 cell membrane labeling

The PKH26 Red Fluorescent Cell Linker Kit (Sigma) was used to label cell membranes with PKH26. PML/RARα-positive or mock-infected control cells were stained with PKH26 according to the manufacturer's instructions and staining was detected by FACS.

### Side population of NB4 cells

Cells (1×10$^6$ cells/mL) were incubated in prewarmed DMEM with 2% FCS (Invitrogen) containing freshly added Hoechst 33342 (Molecular Probes, Strasbourg, France) at a final concentration of 5 μg/mL for 100 minutes at 37°C with intermittent mixing. In some experiments, cells were incubated with 50 μM verapamil (Sigma) for 20 min. before Hoechst 33342 staining. At the end of the incubation, cells were spun down in the cold and resuspended in ice-cold Hank's balanced salt solution (HBS) (Invitrogen) with 2% FCS and 10 mM HEPES buffer. Propidium iodide (Sigma) was added at a final concentration of 2 μg/mL for 5 minutes before FACS analysis, which allowed discrimination between dead and living cells. Samples were analyzed on a Becton Dickinson FACSAria. The Hoechst dye was excited with the 407 nm violet laser and its fluorescence measured with a 450/40 nm side population filter (Hoechst blue) and a 620 LP EFLP optical filter (Hoechst red). A 595 DCLP filter was used to separate the emission wavelengths.

## Results

### ATRA selects a subpopulation which maintains the aberrant replating efficiency of PML/RARα-positive Sca1$^+$/lin$^-$ cells (SL cells)

It has recently been shown that ATRA has an effect on stem cells.[16] ATRA treatment of early HSC selects immature progenitors with an increased self-renewal potential.[16,17] SL cells expressing PML/RARα are characterized by a dramatically increased replating efficiency, which correlates with an aberrant potential for self-renewal.[4] To answer the questions of whether and how ATRA influences the biology of PML/RARα-expressing primary HSC, we studied the effect of this drug on the aberrant replating efficiency of PML/RARα-expressing murine SL cells. As an ATRA-resistant control, we included cells expressing PLZF/RARα, the fusion protein expressed in patients with t(11;17)-positive APL. In contrast to PML/RARα, PLZF/RARα does not mediate ATRA induced differentiation in APL blasts[14] and, therefore, t(11;17)-positive APL patients do not undergo CR upon treatment with ATRA.[5] We retrovirally transduced murine SL cells with PML/RARα and PLZF/RARα, and performed serial replatings in semi-solid medium in the absence or presence of 1 μM ATRA. The proviral constructs used for the retroviral transduction are shown in Figure 1A. The expression of the transgenes was confirmed by the analysis of EGFP-positive cells (*data not shown*) and by western blotting (Figure 1B). Each plating round was grown for 10 days. As shown in Figure 1C, exposure to ATRA slightly increased the replating efficiency of mock-transduced control cells up to the third plating, in agreement with the known positive effects of ATRA on the self-renewal of HSC. For PML/RARα- and PLZF/RARα-positive SL cells, exposure to ATRA reduced the number of colony-forming units (CFU) in the first three plating rounds but increased the overall replating efficiency, as revealed by an increased number of serial replating rounds than for the controls. From the fourth to the fifth plating, the ATRA-treated cells gave rise to more CFU than did the untreated cells.

The maturation level of the CFU of the first plating was assessed by determining the expression of differentiation-specific (Gr-1 and Mac-1) and stem cell-specific (Sca-1, c-Kit) surface markers. Whereas ATRA induced a slight

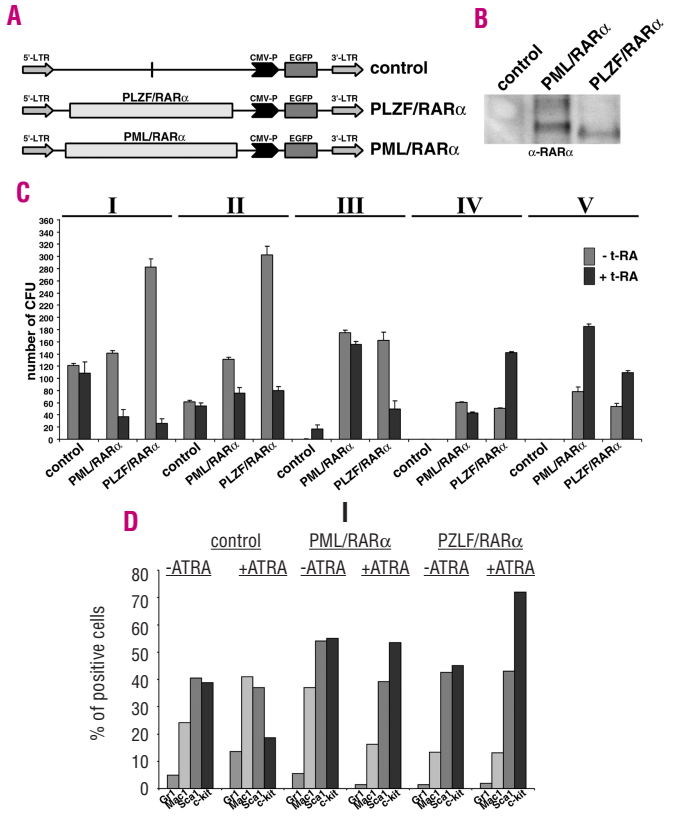


**Figure 1.** Effect of t-RA on the replating efficiency and differentiation of Sca$^+$/lin$^-$ HSC expressing the APL-associated fusion proteins PML/RARα and PLZF/RARα. **A.** The proviral constructs used for the transduction of SL cells. The transgenes are driven by the 5'-LTR (long-term repeat), whereas the GFP-reporter gene is under the control of the CMV promoter (CMV-P). **B.** Western blot analysis of transgene expression in the SL cells. The blot was incubated with an anti-RARα antibody as indicated. **C.** Replating efficiency of murine Sca$^+$/lin$^-$ HSC expressing PML/RARα and PLZF/RARα upon exposure to 1 μM ATRA. Numbers of platings (I, II.. ) and CFU numbers are provided. Bar graphs show means of triplicates with standard deviations (SD) of one representative experiment out of three performed. **D.** Differentiation of PML/RARα- and PLZF/RARα-positive Sca$^+$/lin$^-$ CFC from the first plating of the experiment shown in A upon exposure to 1 μM t-RA. Cells were cultured in semi-solid medium for 10 days. c-Kit and Sca-1 were used as stem cell markers and Gr-1 and Mac-1 as myeloid differentiation markers.

increase in Gr-1 and Mac-1, as well as a reduction in c-Kit expression, in the mock-infected control cells, both PML/RARα- and PLZF/RARα-positive HSC showed no increase in Gr-1 and Mac-1, or reduction in Sca-1 and c-Kit as a sign of differentiation (Figure 1D). The reduction of the number of CFU, combined with an increased number of serial replating rounds, is in accordance with the dual effect of ATRA: induction of differentiation in the more mature blast population and increased self-renewal at the stem cell level.

In summary, these data show that ATRA is unable to abolish aberrant replating efficiency or to induce differentiation of early HSC expressing APL-associated fusion proteins, regardless of whether PML/RARα, the mediator of ATRA signaling, or PLZF/RARα, the inhibitor of ATRA signaling, is expressed.

### *Exposure of PML/RARα-positive SL cells to ATRA does not abrogate their stem cell capacity*

A major feature of HSC is their capacity to engraft *in vivo*, as assessed by *in vivo* reconstitution assays such as the CFU-S assay.[22] The capacity to give CFU-S at day 12 characterizes multipotent early hematopoietic progenitors, whose stem cell capacity is confirmed by their transplantability into secondary recipients. To confirm the unaltered stem cell capacity of PML/RARα-positive SL cells upon exposure to ATRA, we investigated the ability of colony-forming cells (CFC) from plating rounds one and four to produce CFU-S on day 12 after inoculation into lethally irradiated recipient mice. Viability and equal cell numbers were verified prior to inoculation by dye exclusion (*data not shown*). As shown in Figure 2A, exposure to ATRA did not reduce the capacity of the PML/RARα-positive CFC to produce CFU-S after the first plating. In fact, the number of CFU-S visible on the spleen surface did not differ between ATRA-treated and untreated HSC (Figure 2A). To prove that the CFU-S contain cells with stem cell capacity we performed a second transplantation by harvesting the cells from the spleen colonies and inoculating them into secondary recipients. Regardless of whether they had been exposed to ATRA, these cells continued to produce CFU-S (Figure 2A).

To prove the significance of more advanced replating rounds for the detection of cells with stem cell capacity, we performed a CFU-S assay with the CFC from the fourth plating in the absence or presence of ATRA. As shown in Figure 2B, this did not produce visible CFU-S on the spleen surface, but histologically detectable cell formations were present, independently of the exposure to ATRA (Figure 2B). The same results were obtained with PLZF/RARα-positive CFC derived from the fourth plating (Figure 2B). The presence of the fusion transcripts was confirmed by RT-PCR in all experiments (*data not shown*). Taken together, these data show that SL cells expressing APL-associated fusion proteins maintain their stem cell capacity even in the presence of ATRA, as demonstrated by their unaltered capacity to engraft *in vivo*.

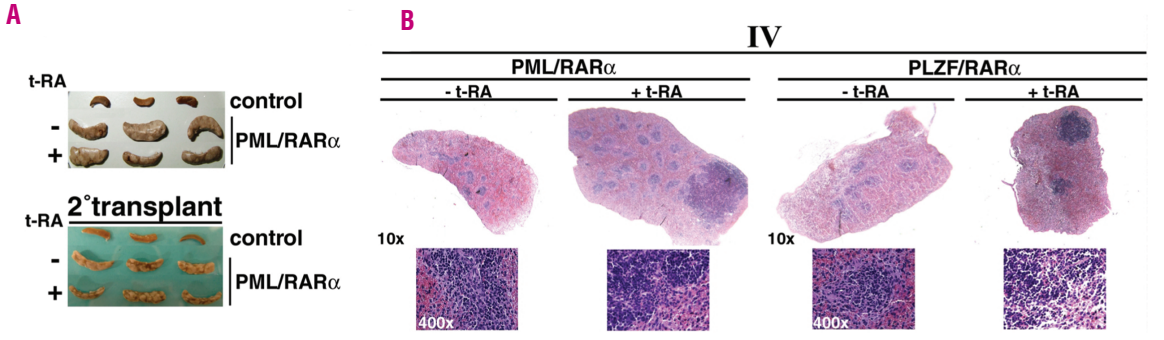


**Figure 2.** Effect of t-RA on the stem cell capacity of Sca$^+$/lin$^-$ HSC expressing the APL-associated fusion proteins PML/RARα and PLZF/RARα. **A.** CFU-S from cells harvested from the first plating in the absence or presence of t-RA ($^-/^+$). control: non-transplanted mice; 2$^{nd}$ transplant: CFU-S induced by secondary transplantation of mononuclear cells isolated from the original CFU-S assay. **B.** Hematoxylin-eosin -staining of CFU-S from PML/RARα- or PLZF/RARα-positive cells harvested from the 4$^{th}$ plating. Original magnification ×10 and ×400.

### *$As_2O_3$ but not ATRA abrogates the clonogenicity of mononuclear cells derived from PML/RARα-positive murine APL*

Like ATRA, $As_2O_3$ has a tumor cell-specific activity in APL.[20] However, it is able to induce CMR, which is associated with a high rate of long-term relapse-free survival.[8] Based on our findings that ATRA increased the self-renewal of PML/RARα-positive HSC we addressed the question of whether these differences in long-term outcome are related to a different effect on the stem cell capacity of the LSC in APL. Blasts from APL patients do not give origin to CFU in the commonly used semi-solid media. We, therefore, investigated the effects of ATRA and $As_2O_3$ on the CFU-potential of PML/RARα-positive mononuclear bone marrow cells (MNBMC) derived from an APL mouse. The tumor burden was about 90% of the cells, as revealed by FACS analysis (*data not shown*). Given the fact that the CFU-potential in MNBMC is at least ten times lower than that of Sca1$^+$/lin$^-$ cells, we plated $5\times10^4$/mL in a semi-solid medium in the absence or presence of 1 μM ATRA or 2μM $As_2O_3$. As shown in Figure 3, in contrast to ATRA, $As_2O_3$ abolished the CFU-potential of the PML/RARα-positive MNBMC. These findings indicate that $As_2O_3$ and ATRA exert different effects on the PML/RARα-positive LSC.

### *In contrast to ATRA, $As_2O_3$ abolishes the aberrant self renewal potential of PML/RARα-positive SL cells*

To confirm our hypothesis of different effects of $As_2O_3$ and ATRA on the PML/RARα-positive LSC we performed stem cell assays with PML/RARα-positive HSC in the presence or absence of $As_2O_3$ and ATRA. Hence we retrovirally transduced murine Sca-1$^+$/lin$^-$ HSC with PML/RARα and plated them in semi-solid medium to determine their replating efficiency upon exposure to 2 μM $As_2O_3$ or 1 μM ATRA. As shown in Figure 4A, $As_2O_3$ had no toxic effects on mock-transduced control cells. In contrast, it reduced the replating efficiency of PML/RARα-positive HSC almost to the level of control cells, whereas ATRA stimulated the replating efficiency of both PML/RARα-positive and mock-transfected cells (Figure 4A). ATRA not only increased the number but also the cellularity of the PML/RARα-positive CFU compared to untreated PML/RARα-positive CFU (Figure 4B). To prove that the influence of $As_2O_3$ on the replating efficiency of PML/RARα-positive HSC is related to a reduction in their stem cell capacity, we harvested the cells after the first plating and inoculated them into lethally irradiated recipient mice for a CFU-S assay. Viability and equal cell numbers were verified prior to inoculation by dye exclusion (*data not shown*). Exposure to $As_2O_3$ completely abrogated the ability of PML/RARα-positive HSC to produce CFU-S, indicating the complete abolition of their stem cell

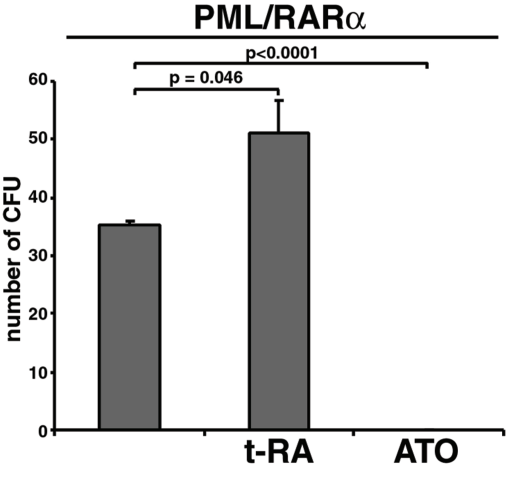


**Figure 3.** Effect of ATRA and $As_2O_3$ on LSC expressing PML/RARα upon treatment with ATRA or $As_2O_3$ CFU potential of MNBMC derived from a PML/RARα-positive leukemic mouse in a 129SV genetic background in the presence of 1 μM ATRA or 2 μM $As_2O_3$. Bar graphs show means of triplicates with SD. Statistical relevance was tested by the Student's t-test (values <0.05 are statistically significant).

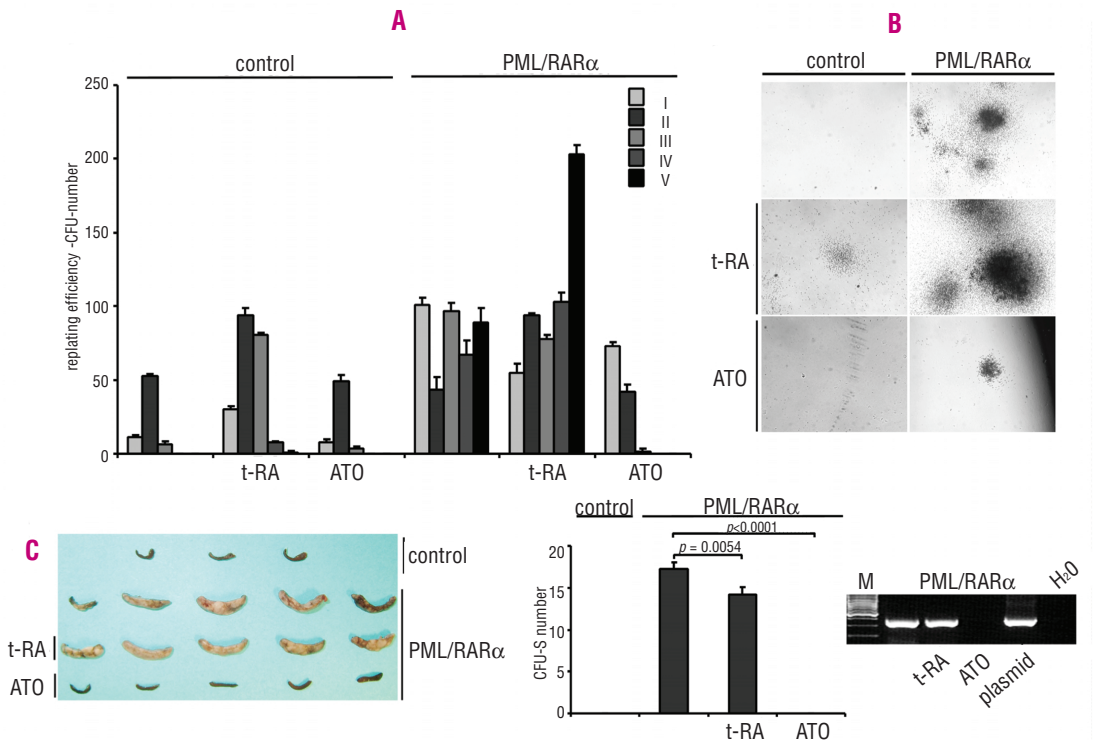


**Figure 4.** Comparison of the effects of t-RA and $As_2O_3$ on the stem cell capacity of HSC expressing PML/RARα **A.** Replating efficiency of murine $Sca^+/lin^-$ HSC expressing PML/RARα upon exposure to 1 μM t-RA or 2 μM $As_2O_3$ (ATO). Numbers of platings (I, II..) and CFU numbers are provided. Bar graphs show means of triplicates with SD of one representative experiment out of three performed. **B.** Morphology of the CFU in the fourth plating round in which controls were negative. Original magnification ×25. **C.** CFU-S from cells harvested from the first plating in the absence or presence of t-RA and $As_2O_3$ (ATO). Bar graphs show the average CFU-S number of six mice/group with SD. Statistical relevance was tested by Student's t-test (values <0.05 are statistically significant). The presence of the PML/RARα-fusion transcript was revealed by RT-PCR analysis of the spleen cells of one out of six mice for each group. pCDNA3-PML/RARα diluted to a concentration of 5 molecules/100 μL (plasmid), was used as positive control.

capacity (Figure 4B). No CFU-S were seen in the mice inoculated with these $As_2O_3$-treated HSC, whereas ATRA only slightly reduced the number of CFU-S as compared to untreated PML/RARα-positive HSC (Figure 4C). RT-PCR did not reveal any fusion transcript in the spleens of mice inoculated with $As_2O_3$-treated cells, further confirming that the stem cell capacity of PML/RARα-positive HSC was completely eradicated (Figure 4C). Taken together, these data show that, in contrast to ATRA, $As_2O_3$ is able to abrogate the stem cell capacity of PML/RARα-positive HSC.

### *In contrast to $As_2O_3$ ATRA accelerates proliferation of PMLRARα-positive SL-cells*

We next investigated the influence of $As_2O_3$ and ATRA on the proliferation of PML/RARα-positive HSC. We seeded murine $Sca1^+/lin^-$ HSC that had been retrovirally transduced with PML/RARα in liquid culture containing murine interleukin-3, murine interleukin-6 and murine stem cell factor in the presence of either $As_2O_3$ or ATRA. After 4 days, we plated $5\times10^3$ cells in semi-solid medium for replating efficiency assays to prove the integrity of the cells under these conditions. The rest of the cells were stained with PKH26 to label the membrane and returned to liquid culture at a density of $10^5$ cells/mL for an additional 11 days. Here we show that 4 days in liquid culture did not interfere with the CFU potential of the cells and that the exposure to $As_2O_3$ also under these conditions abolished the replating efficiency of the PML/RARα-positive HSC (Figure 5A). The PKH26 membrane labeling of the cells was measured by FACS on days 5 and 11 after staining. The different extinction kinetics of the PKH26 labeling showed that the expression of PML/RARα reduced the proliferation of the SL cells as compared to that of mock-infected controls. In contrast, exposure to ATRA strongly increased the proliferation of PML/RARα-positive SL cells as compared to untreateds control and PML/RARα-positive SL cells, whereas $As_2O_3$ had no apparent influence on the proliferation of these cells (Figure 5B). In summary these data show that, in contrast to $As_2O_3$, ATRA drastically increases the proliferation of PML/RARα-positive HSC.

### *Opposite effects of ATRA and $As_2O_3$ on the "side population" of the APL cell line NB4*

Blasts derived from APL patients do not engraft in immunodeficient mice.[18] The patient-derived NB4 APL cell line only engrafts in NOD/SCID mice with a low efficien-

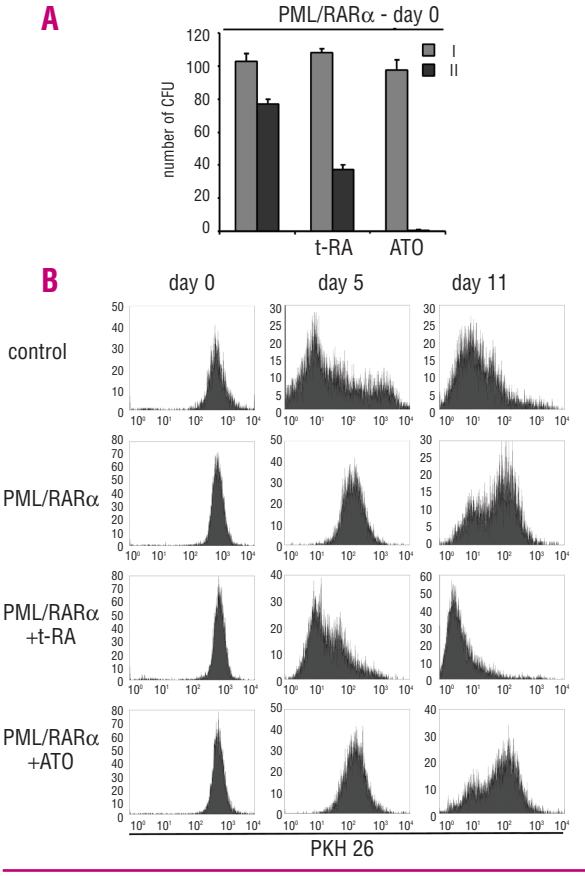


**Figure 5.** Comparison of the effects of ATRA and $As_2O_3$ on the proliferation of HSC expressing PML/RARα. A. CFU potential of the HSC expressing PML/RARα upon exposure to 1 μM t-RA or 2 μM $As_2O_3$ after 4 days in liquid culture, on the day of PKH26 staining (day 0). B. Replication rate of PML/RARα-positive HSC determined by PKH26 labeling in the absence or presence of t-RA or $As_2O_3$ (ATO). The progressive extinction of the PKH26 labeling by cell replication was measured by FACS on days 5 and 11.

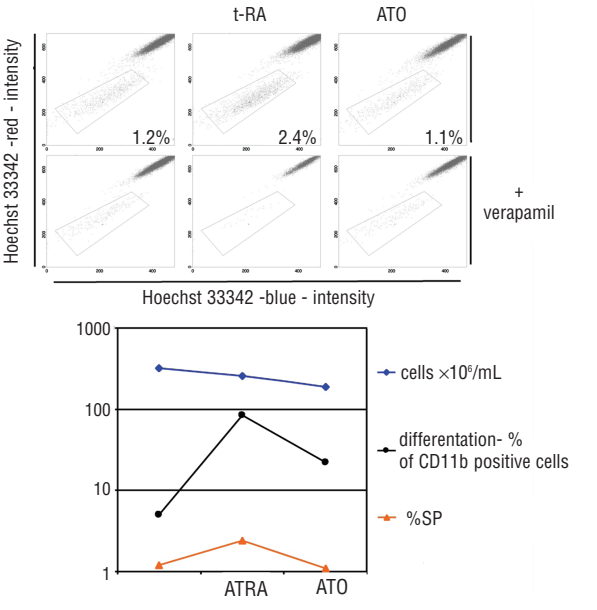


**Figure 6.** Influence of ATRA and $As_2O_3$ on the side population of NB4 cells. NB4 side population (SP) defined by exclusion of Hoechst 33342 in the absence or presence of verapamil. Total cell number, percentage of cells positive for CD11b, a marker of granulocytic differentiation and the percentage of cells representing the side population.

cy. In fact 6 weeks after the inoculation of $5\times10^6$ NB4 cells into NOD/SCID mice, it was possible with a very sensitive real-time PCR to detect the presence of NB4 cells, whose presence was not influenced by a prior treatment with ATRA (*data not shown*). In other cell lines the stem cell population is contained in the so-called *side population* determined by staining exclusion in the presence or absence of ABC transporter inhibitors such as verapamil.[23,24] We found that the NB4 cell line has a side population which represents not more than 1% of the whole population (*data not shown* and Figure 6A). To confirm our results obtained with PML/RARα-transduced murine HSC, we investigated the effect of ATRA and $As_2O_3$ on the side population of NB4 cells as a model of APL-derived LSC. Thus we exposed NB4 cells to 1 μM ATRA or 2 μM $As_2O_3$ for 48 h. At this time point, the cell growth arrest was complete and ATRA-induced differentiation was already advanced, as revealed by the presence of 91% CD11b-positive cells (Figure 6B). Notwithstanding the cell growth arrest in both ATRA- and $As_2O_3$-treated NB4 cells, ATRA nearly doubled the percentage of cells in the side population, whereas $As_2O_3$ did not influence the percentage of the side population (Figure 6A). In summary, these data show that ATRA induces proliferation of APL-derived stem cells, whereas $As_2O_3$ inhibits their growth.

## Discussion

AML is a disease of stem cell origin and the malignant LSC are biologically distinct from more differentiated blast cells.[3] The frequency of LSC, which must be considered the source of the disease as well as of clinical relapse, is very low.[18] Although these cells can, at present, only be defined functionally, there is growing evidence that the little progress made in therapy in the last decades is most likely due to the fact that the current therapy regimens do not target the source of AML – the LSC. With the exception of stem cell transplantation protocols, none of the current therapeutic approaches – whether conventional or experimental – has considered the specific effects on the LSC.

The AML M3 subtype, APL, is a model disease in which ATRA and $As_2O_3$, as single agents, are able to induce very similar CR rates but completely different rates of CMR and thus of long-term outcome.[8]

Our data presented here suggest that these differences might be due to different effects of ATRA and $As_2O_3$ on the APL LSC. Our data suggest that ATRA exerts a dual effect on PML/RARα-positive cells: amplification of the stem cell compartment by increasing proliferation and

self-renewal and induction of terminal differentiation of the more committed blast cells. The induction of terminal differentiation of the more mature leukemic blasts is demonstrated by the high differentiation rate of NB4 cells, as well as by the reduction in CFU numbers in the first plating rounds of PML/RARα-positive SL cells upon exposure to ATRA. The selection of a LSC population is indicated by the fact that upon exposure to ATRA, the stem cell capacity of PML/RARα-positive SL cells is unaltered and the NB4 side population increases in size. The enlarged side population is associated with unaltered engraftment of these cells in NOD-SCID mice, as compared to untreated NB4 cells (*data not shown*). Thus, ATRA selects small subpopulations which maintain fundamental stem cell features, such as the potential for self-renewal and engraftment *in vivo*, and which are presumably the LSC or the *leukemia-initiating cells* – the source of relapse, if not targeted by additional therapy. The effects of ATRA on the APL stem cell compartment were until now almost invisible, because of the dramatic effects of this drug on committed blasts. In fact, ATRA induces more than 95% differentiation or apoptosis, covering important effects on the resting 5% of cells which do not undergo these processes. The PML/RARα-expressing stem cell models presented here were designed to amplify these effects.

We and others recently reported similar effects to those of ATRA for the histone deacetylase inhibitor valproic acid.[17,25] Histone deacetylase inhibitors have attracted considerable attention because of their ability to overcome the differentiation block in leukemic blasts, an effect achieved either alone or in combination with differentiating agents, such as ATRA.[26] However, exposure to valproic acid is frequently accompanied by hypergranulocytosis in patients with AML.[27] In fact, although valproic acid, similarly to ATRA, induces differentiation or apoptosis in leukemic blasts, it stimulates both proliferation and self-renewal of HSC.[17] This effect is also seen in the stem cells of different AML subtypes (*Bug et al.* in press).

As a monotherapy, ATRA is able to induce long-term relapse-free survival only if administered at a high dosage and as a liposomal formulation in a low number of patients.[7,9] However, following the introduction of therapy regimens combining ATRA with an anthracycline, APL became a curable disease with an excellent prognosis.[7] Nothing is known about how ATRA increases the cytotoxic effect of the anthracyclines. Most authors attribute the good outcome of chemotherapy regimens containing ATRA to the combination of differentiation induced by ATRA and the cytotoxic effect of the anthracyclines.[7,28] Anthracyclines induce a CR rate of between 55 and 88%, followed by a high rate of relapse when not combined with ATRA.[28] Together with the efficacy of ATRA monotherapy discussed above, these data strongly imply that ATRA has an effect on the cytotoxicity of anthracyclines in APL. Our data show that the expression of PML/RARα alone decreases the replication rate of early HSC, whereas ATRA accelerates the replication rate of PML/RARα-positive HSC. The apoptosis induced by anthracyclines requires progression through cell cycle check points. Thus, one could hypothesize that ATRA increases the response of APL patients to chemotherapy, not only by inducing differentiation of the blast population, but mostly by accelerating cell cycle progression of the LSC compartment. The main effect of ATRA in APL could be to sensitize the LSC to the cytotoxic effects of anthracyclines by increasing their proliferation rate.

In contrast to ATRA, $As_2O_3$ targets PML/RARα-positive LSC. It interferes efficiently with unique stem cell functions, such as replication rate, replating efficiency and self-renewal. Hence, our findings strongly suggest that the high rate of CMR and long-term relapse-free survival induced by $As_2O_3$ in APL patients is due to the fact that it effectively targets the APL LSC. The data we present here support recent clinical studies showing that when $As_2O_3$ is used as a single agent for induction, consolidation, and maintenance in the treatment of newly diagnosed cases of APL, it is associated with durable remissions, which are comparable to those achieved with conventional ATRA plus chemotherapy regimens.[8,29-31] However, longer follow-up is required to exclude the possibility of late relapses.[29,30] Regarding the mechanisms of action of ATRA, the effects on the PML/RARα-positive HSC can be considered an amplification of the physiological effects of ATRA on normal HSC. These are characterized by an enhanced generation of CFU-S and both short- and long-term repopulating cells in cultures of SL cells, as well as by an enhanced mitogenic activity of human $CD34^+$ cells.[16,17] On the other hand, the effect of $As_2O_3$ seems to be similar to that seen in other PML/RARα-positive cell models, in which the biological effects are mediated directly by PML/RARα.[12,15]

In conclusion, our data show for the first time that there might be a direct relationship between the capacity of compounds to effectively target the LSC and their capacity to eradicate the leukemia and, thereby, to induce CMR and long-term relapse-free survival. Thus, in order to increase the curative potential of leukemia therapies, future studies need to include the effect of given compounds on the stem cell compartment to determine their ability to eradicate the LSC.

### Author Contributions

*XZ: performed experiments; AS: generated and provided reagents; BR: performed experiments; GB: assisted in designing experiments and writing the paper; TB: performed experiments; EP: assisted in designing experiments and writing the paper; DH: assisted in designing experiments and writing the paper; RH: designed experiments and assisted in writing the paper; MR: designed the study, designed experiments and wrote the paper.*

### Conflict of Interest

*The authors reported no potential conflicts of interest.*

## References


1. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, et al. Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. Ann Intern Med 1985; 103:620-5.
2. Tenen DG. Disruption of differentiation in human cancer: AML shows the way. Nat Rev Cancer 2003;3:89-101.
3. Jordan CT, Guzman ML. Mechanisms controlling pathogenesis and survival of leukemic stem cells. Oncogene 2004; 23:7178-87.
4. Zheng X, Beissert T, Kukoc-Zivojnov N, Puccetti E, Altschmied J, Strolz C, et al. {gamma}-catenin contributes to leukemogenesis induced by AML-associated translocation products by increasing the self-renewal of very primitive progenitor cells. Blood 2004;22:22.
5. Melnick A, Licht JD. Deconstructing a disease: RARa, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. Blood 1999;93:3167-215.
6. Grignani F, Fagioli M, Alcalay M, Longo L, Pandolfi PP, Donti E, et al. Acute promyelocytic leukemia: from genetics to treatment. Blood 1994;83:10-25.
7. Degos L, Wang ZY. All trans retinoic acid in acute promyelocytic leukemia. Oncogene 2001;20:7140-5.
8. Douer D, Hu W, Giralt S, Lill M, DiPersio J. Arsenic trioxide (trisenox) therapy for acute promyelocytic leukemia in the setting of hematopoietic stem cell transplantation. Oncologist 2003;8:132-40.
9. Estey E, Koller C, Tsimberidou AM, O'Brien S, Beran M, Cortes J, et al. Potential curability of newly diagnosed acute promyelocytic leukemia without use of chemotherapy: the example of liposomal all-trans retinoic acid. Blood 2005;105: 1366-7.
10. Chomienne C, Ballerini P, Balitrand N, Daniel MT, Fenaux P, Castaigne S, et al. All-trans retinoic acid in acute promyelocytic leukemias. II. In vitro studies: structure-function relationship. Blood 1990;76:1758-64.
11. Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M, Alcalay M, et al. The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 1993; 74:423-31.
12. Sternsdorf T, Puccetti E, Jensen K, Hoelzer D, Will H, Ottmann OG, et al. PIC-1/SUMO-1-modified PML-retinoic acid receptor a mediates arsenic trioxide-induced apoptosis in acute promyelocytic leukemia. Mol Cell Biol 1999;19:5170-8.
13. Licht JD, Chomienne C, Goy A, Chen A, Scott AA, Head DR, et al. Clinical and molecular characterization of a rare syndrome of acute promyelocytic leukemia associated with translocation (11;17). Blood 1995;85:1083-94.
14. Ruthardt M, Testa U, Nervi C, Ferrucci PF, Grignani F, Puccetti E, et al. Opposite effects of the acute promyelocytic leukemia PML-retinoic acid receptor α (RARα) and PLZF-RARα fusion proteins on retinoic acid signalling. Mol Cell Biol 1997;17:4859-69.
15. Koken MH, Daniel MT, Gianni M, Zelent A, Licht J, Buzyn A, et al. Retinoic acid, but not arsenic trioxide, degrades the PLZF/RARalpha fusion protein, without inducing terminal differentiation or apoptosis, in a RA-therapy resistant t(11;17)(q23;q21) APL patient. Oncogene 1999;18:1113-8.
16. Collins SJ. The role of retinoids and retinoic acid receptors in normal hematopoiesis. Leukemia 2002;16: 1896-905.
17. Bug G, Gul H, Schwarz K, Pfeifer H, Kampfmann M, Zheng X, et al. Valproic acid stimulates proliferation and self-renewal of hematopoietic stem cells. Cancer Res 2005; 65: 2537-41.
18. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, et al. A cell initiating human acute myeloid leukemia after transplantation into SCID mice. Nature 1994;367:645-8.
19. Lo Coco F, Diverio D, Falini B, Biondi A, Nervi C, Pelicci PG. Genetic diagnosis and molecular monitoring in the management of acute promyelocytic leukemia. Blood 1999;94:12-22.
20. Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY, et al. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood 1997;89:3354-60.
21. Puccetti E, Guller S, Orleth A, Bruggenolte N, Hoelzer D, Ottmann OG, et al. BCR-ABL mediates arsenic trioxide-induced apoptosis independently of its aberrant kinase activity. Cancer Res 2000;60:3409-13.
22. Coulombel L. Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. Oncogene 2004;23:7210-22.
23. Hirschmann-Jax C, Foster AE, Wulf GG, Nuchtern JG, Jax TW, Gobel U, et al. A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci USA 2004;101:14228-33.
24. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci USA 2004;101:781-6.
25. De Felice L, Tatarelli C, Mascolo MG, Gregorj C, Agostini F, Fiorini R, et al. Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. Cancer Res 2005;65:1505-13.
26. Ferrara FF, Fazi F, Bianchini A, Padula F, Gelmetti V, Minucci S, et al. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. Cancer Res 2001;61:2-7.
27. Bug G, Ritter M, Wassmann B, Schoch C, Heinzel T, Schwarz K, et al. Clinical trial of valproic acid and all-trans retinoic acid in patients with poor-risk acute myeloid leukemia. Cancer 2005;104:2717-25.
28. Tallman MS, Nabhan C, Feusner JH, Rowe JM. Acute promyelocytic leukemia: evolving therapeutic strategies. Blood 2002; 99:759-67.
29. Ghavamzadeh A, Alimoghaddam K, Ghaffari SH, Rostami S, Jahani M, Hosseini R, et al. Treatment of acute promyelocytic leukemia with arsenic trioxide without ATRA and/or chemotherapy. Ann Oncol 2006;17: 131-4.
30. Mathews V, George B, Lakshmi KM, Viswabandya A, Bajel A, Balasubramanian P, et al. Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: durable remissions with minimal toxicity. Blood 2006; 107:2627-32.
31. Miller WH Jr. Molecular targets of arsenic trioxide in malignant cells. Oncologist 2002;7 Suppl 1:14-9.