**BCR-ABL Mediates Arsenic Trioxide-induced Apoptosis Independently of Its Aberrant Kinase Activity**

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**Abstract**

In the prechemotherapy era arsenic derivatives were used for treatment of chronic myelogenous leukemia, a myeloproliferative disorder characterized by the t(9;22) translocation, the Philadelphia chromosome (Ph1). In acute promyelocytic leukemia response to arsenic trioxide (As2O3) has been shown to be genetically determined by the acute promyelocytic leukemia-specific t(15;17) translocation product PML/RARα. Hence, we reasoned that As2O3 might have a selective inhibitory effect on proliferation of BCR-ABL-expressing cells.

Here, we report that: (a) As2O3 induced apoptosis in Ph+ but not in Ph− lymphoblasts; (b) enforced expression of BCR-ABL in U937 cells dramatically increased the sensitivity to As2O3; (c) the effect of As2O3 was independent of BCR-ABL kinase activity; and (d) As2O3 reduced proliferation of chronic myelogenous leukemia blasts but not of peripheral CD34+ progenitors. In summary, these data establish As2O3 as a tumor cell-specific agent, making its clinical application in Ph+ leukemia feasible.

**Introduction**

Arsenic derivatives represent one of the oldest treatments for leukemia. In the last century, Fowler’s solution (potassium arsenite) was administered to patients suffering from leukemia. In the 1930s, treatment with Fowler’s solution achieved remarkable clinical response in CML1 (Ref. 1 and references therein). Until the 1950s, arsenic has been used in combination with radiotherapy to treat CML (2). More complete remissions in patients with t(15;17) APL (4–6). The rationale for this includes the basis for the application of As2O3 as a tumor cell-specific agent in the treatment of Ph+ CML, as well as Ph+ ALL.

**Materials and Methods**

**Cell Lines, Cell Culture, and Western Blotting.**

Nalm-6, MOLT-3, SEM, Jurkat, Daudi, BV173, SD-1, and U937 cells were maintained in RPMI 1640 supplemented with 10% FCS (Life Technologies, Inc., Karlsruhe, Germany). TOM-1 cells were maintained in Iscove Medium with 10% FCS and Sup B15 in RPMI 1640 with 15% FCS. The U937 MT B45 and PML/RARα-positive P/R9 cells were obtained as described previously (8, 9). The p185<sup>(BCR-ABL)</sup> and p210<sup>(BCR-ABL)</sup> encoding cDNA was cloned into the pGEMSVneo expression vector (9). The BCR-ABL-positive U937 MTP185 and MTPp210 bulk populations were obtained by electroporation of these p185<sup>(BCR-ABL)</sup> and p210<sup>(BCR-ABL)</sup>-carrying expression vectors and G418 selection. The expression of the exogenous protein by Zn2+ treatment was induced as described (9) and evaluated by Western blotting. Anti-ABL antibody (a-ABL), anti-bcl-2 (a-bcl-2), and anti-PARP (a-PARP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), 5-ethyl-2-(a-bcl-2) from DAKO (Hamburg, Germany), and anti-phosphotyrosine (a-FY) from Upstate Biotechnology, Inc. (Lake Placid, NY). All were applied according to the manufacturer’s instructions.

**Apoptosis Assay.**

Zn<sup>2+</sup>-treated U937 cells were washed twice with PBS to eliminate Zn<sup>2+</sup>, thus avoiding interference with apoptosis. All cell types were diluted to 1 × 10<sup>6</sup> cell/ml and exposed to a final concentration of 0.1–2 μM As2O3 (Sigma Chemical Co., St. Louis, MO). For analysis of the rate of apoptosis, the FACS-based 7-AAD method was used as described elsewhere (7).

**Patient Samples.**

Fresh CD34+ progenitor cells were purified from leukopheresis samples after 4–5 days of mobilization with granulocyte colony-stimulating factor (10 μg/kg body weight) of two patients with Ph+ ALLs in CR. The CML blasts derived from the peripheral blood of two patients with newly diagnosed myeloid or lymphatic blast crisis. CD34+ cells were isolated by Ficoll-Hypaque density-gradient centrifugation, followed by separation with the VarioMACS system, with the appropriate columns using the Direct CD34<sup>+</sup> Progenitor Isolation Kit according to manufacturer’s instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany).

**Methyl-Cellulose Assay.**

Fresh CD34+ cells and CML PMNCs were plated at 400 cells/ml on day 2 of treatment with As2O3 in 0.5% semisolid methyl-cellulose Methocult complete medium (StemCell Technologies Inc., Vancouver, Canada) and incubated at 37°C in a humidified atmosphere of 5%
As₂O₃ Induces Apoptosis in BCR-ABL-positive Lymphoblastic Cell Lines. To determine whether Ph+ cell lines recapitulate the response of CMLs to As₂O₃, analogous to the PML/RARA-expressing NB4 and U937 cells (4, 7), we exposed different ALL- and CML-derived lymphoblastic cell lines to 2 μM As₂O₃. SD-1, Tom-1, and Sup-B15 cells are p185(BCR-ABL)-positive ALL cell lines, and BV173 is a p210(BCR-ABL)-positive CML cell line (10). As control, we used Ph− lymphoblastic cell lines such as Nalm-6 (B-lineage ALL), MOLT-3 (T-lineage ALL), SEM [t(4;11)-positive B-lineage-ALL], Jurkat (leukemic T-cell lymphoma), and Daudi (Burkitt lymphoma).

Fig. 1A depicts the BCR-ABL protein expression levels of the above cited cell lines. The rate of apoptosis was determined by FACS analysis, measuring the percentage of 7-AAD-positive cells after 72–96 h of As₂O₃ exposure (11). In Fig. 1B, we show one representative experiment of three that gave similar results. In contrast to untreated cells (BV173, 16%; SD-1, 16%; Sup-B15, 26%; Tom-1, 35%) all Ph+ lymphoblastic cell lines showed a high rate of apoptosis on treatment with As₂O₃ (BV173, 74%; SD-1, 49%; Sup-B15, 93%; Tom-1, 79%). None of the Ph− cell lines exhibited a significant response to the As₂O₃ treatment (Fig. 1B). No average was calculated because the kinetics of apoptosis in the Ph+ cell lines differed between separate experiments, resulting in considerable variability, in particular, at the early time points. Moreover, in contrast to Ph− cells, none of the Ph+ cell lines recovered from treatment with As₂O₃ in prolonged culture (data not shown).

Fig. 1C demonstrates a typical 7-AAD FACS profile of BV173 cells exposed to increasing concentrations of As₂O₃. Already, 0.1 μM As₂O₃ induced a significant rate of apoptosis with respect to untreated cells. Furthermore, activity of As₂O₃ was dose dependent, as demonstrated by an increased percentage of apoptotic cells with rising dosages of As₂O₃.

Taken together, these data indicate a specific activity of As₂O₃ in BCR-ABL-expressing lymphoblastic cell lines comparable with the known activity of As₂O₃ on PML/RARA-positive cells. Moreover, As₂O₃ exerts its effect on Ph+ cells, regardless of the type of product of t(9;22) present [i.e., the ALL-specific p185(BCR-ABL) is able to mediate sensitivity to As₂O₃ to the same extent as the CML specific p210(BCR-ABL)].

As₂O₃-induced Apoptosis in Ph+ Cells Is Genetically Determined by the Presence of t(9;22). To examine whether As₂O₃-induced apoptosis in Ph+ cell lines is specifically mediated by BCR-ABL, and to exclude the possibility that the effect of As₂O₃ is due to a yet unknown common feature of the different Ph+ cell lines, we transfected U937 cells with different expression vectors. The expression vectors contained cDNA encoding either p185(BCR-ABL) or p210(BCR-ABL) under the control of the Zn²⁺-inducible metallothionein (MT-1) promoter (9).

U937 cells are myeloid precursors blocked at the promonocytic stage, which do not undergo As₂O₃-induced apoptosis (4, 7). In our experiments, we analyzed the effect of As₂O₃ on p185(BCR-ABL)- and p210(BCR-ABL)-expressing U937 cells. To avoid the bias of clonal variability, we used highly expressing bulk populations selected after transfection only for G418 resistance without further subcloning (MTP185 and MTPp210). On Zn²⁺ induction the transfected cells expressed the BCR-ABL fusion proteins to a similar level as BV173, SD-1, Sup-B15, and Tom-1 cells (Fig. 1A). As negative control for As₂O₃-induced apoptosis we used the MT B45 cells, transfected with the “empty” expression vector, and as positive control we used the PML/RARA-expressing P/R9 cells, as described previously (7). Twelve h of Zn²⁺ treatment was by itself not able to induce apoptosis in any cell lines (Fig. 2). On exposure to 1 μM As₂O₃ and in the absence of Zn²⁺, MT B45 control cells showed a nearly identical apoptosis rate than with Zn²⁺ treatment alone (20% and 19%, respectively). Even without Zn²⁺-induced protein expression there was a pronounced increase in apoptosis in BCR-ABL p210 and P/R9 cells (29% and 40%, respectively) when compared with MT B45 control.
cells (19%) and BCR-ABL p185 cells (12%; Fig. 2). This effect was most likely due to “leakage” protein expression from the transgenes, as demonstrated by Western blot (Fig. 1A). When cells were treated for 12 h with Zn²⁺ to induce protein expression prior to As₂O₃ exposure, apoptosis increased dramatically in the MTP185 and MTP210 populations (77% and 56%, respectively). Taking into account that the BCR-ABL-positive U937 cells are bulk populations, where only 50–70% of cells express the transgenes (determined by immunofluorescence studies and further subcloning of the populations; data not shown), sensitivity of the p185(BCR-ABL)⁻ and p210(BCR-ABL)⁺-expressing cells to As₂O₃ reached nearly the same extent than the P/R9 clone (96%).

Taken together, these data indicate that the response to As₂O₃ in Ph+ leukemia is genetically determined by the presence of the t(9;22) translocation products p185(BCR-ABL) or p210(BCR-ABL).

Response to As₂O₃ Is Independent of the Constitutive ABL Kinase Activity of BCR-ABL. BCR-ABL transforms cells via its aberrant constitutive kinase activity (3). To investigate a possible role of BCR-ABL kinase activity in As₂O₃-induced apoptosis, we exposed BV173 and SD-1 cells to the specific ABL-kinase inhibitor STI 571 (kindly provided by E. Buchdunger, Novartis, Basel, Switzerland). Cells were treated with As₂O₃ after a 6-h exposure to 0.5 μM STI571, to guarantee that ABL kinase activity was switched off. This is the lowest possible concentration of STI571, ensuring inhibition of BCR-ABL kinase activity. In BV173 cells as well as in SD-1 cells STI571 exhibited its known proapoptotic effects on BCR-ABL-transformed cells, but had no considerable influence on response to As₂O₃. Fig. 3A shows one of three representative experiments that gave similar results.

To answer the question whether As₂O₃ induces apoptosis by interfering with the BCR-ABL kinase activity, we probed immunoblots of cellular lysates of BV173 and SD-1 cells after 8, 24, 48, and 72 h of As₂O₃ treatment with an antiphospho-tyrosine monoclonal antibody. In our experiments, no cleavage of endogenous p113 kDa species is replaced by a 81 kDa species, which is also recognized by the anti-PARP antibody. In our experiments, no cleavage of endogenous PARP was observed after 8, 24, 48, or 72 h of As₂O₃ exposure (Fig. 3B), indicating that caspase-3 is not involved in As₂O₃-induced apoptosis of BCR-ABL-positive BV173 cells.

Taken together, these data give further proof that As₂O₃ induces apoptosis independent of bcl-2 expression level and caspase-3-like activity.

As₂O₃ Has No Effect on the CFUs of CD34⁺ Primary Hematopoietic Precursors. Clinical studies indicate that APL patients treated with As₂O₃ alone do not experience aplasia of the bone marrow, commonly seen in cytotoxic chemotherapy regimen (6). To assess a possible effect of As₂O₃ on normal hematopoietic progenitor cells, we exposed CD34⁺ cells isolated from two patients with Ph+ ALL in CR to 2 μM As₂O₃ and tested the CFU in a methyl-cellulose colony formation assay. The experiments were performed in triplicates. CD34⁺ cells were seeded in methyl-cellulose after 2 days of exposure to As₂O₃. There was no considerable difference regarding number, morphology, composition, or relationship between GM-CFU, BFU-E, and CFU mix between the treated and untreated population. A representative depiction of the growth pattern of the colonies of one of two patients’ samples is given in Fig. 4.
These data confirm the hypothesis that the toxic effect of As$_2$O$_3$ on normal hematopoietic precursors is minimal, as long as As$_2$O$_3$ is applied in clinically relevant concentrations.

As$_2$O$_3$ Significantly Reduces the CFU of Ph+ Blasts of CML Patients in Blast Crisis. In BV173 cells a 6-h exposure to As$_2$O$_3$ is sufficient to irreversibly induce apoptosis (data not shown). To examine the effect of As$_2$O$_3$ on primary blasts of CML patients, we treated PMNCs of five CML patients in myeloid or lymphatic blast crisis. On As$_2$O$_3$ exposure there was a very high variability in viability and total cell number between different experiments and patient samples, most likely due to the different sensitivity to culture conditions (data not shown). For that reason, PMNC samples of two patients, which showed no effect on As$_2$O$_3$ exposure regarding total cell number or viability, were seeded in a methyl-cellulose colony assay (see “Material and Methods”). At the 9th day, the number of CFUs of the As$_2$O$_3$-treated samples was significantly lower than in untreated control cells (Fig. 4). Interestingly, on As$_2$O$_3$-exposure most of the CFUs exhibited the characteristics of differentiated granulocytic colonies rather than BFU-E or CFUs (Fig. 4).

In summary, these data indicate that BCR-ABL increases sensitivity to As$_2$O$_3$-induced apoptosis in Ph+ CML blasts, but not in CD34$^+$ progenitors.

Discussion

Here, we show that As$_2$O$_3$, an agent known to induce apoptosis in PML/RAR$\alpha$-positive APL, also exhibits potent and specific activity against BCR-ABL-expressing cells. Without exception, all Ph+ lymphoblastic cell lines (SD-1, Tom-1, Sup-B15, and BV173) examined were highly sensitive to As$_2$O$_3$-induced apoptosis. In contrast, Ph- cell lines, including the t(4;11)-positive SEM cells, responded to As$_2$O$_3$-induced apoptosis also excludes the possibility that the effect of As$_2$O$_3$ is due to a common feature of bone marrow cells arrested at the B-cell precursor stage of differentiation. Instead, it demonstrates that As$_2$O$_3$-induced apoptosis is genetically determined by the presence of the (t(9;22))-specific chimeric gene products p185$^{(BCR-ABL)}$ and p210$^{(BCR-ABL)}$. This effect of BCR-ABL is analogous to PML/RAR$\alpha$, which determines As$_2$O$_3$ sensitivity of APL blasts (6, 7).

Discussion

As$_2$O$_3$ significantly reduces the CFU of Ph+ Blasts of CML Patients in Blast Crisis. In BV173 cells a 6-h exposure to As$_2$O$_3$ is sufficient to irreversibly induce apoptosis (data not shown). To examine the effect of As$_2$O$_3$ on primary blasts of CML patients, we treated PMNCs of five CML patients in myeloid or lymphatic blast crisis. On As$_2$O$_3$ exposure there was a very high variability in viability and total cell number between different experiments and patient samples, most likely due to the different sensitivity to culture conditions (data not shown). For that reason, PMNC samples of two patients, which showed no effect on As$_2$O$_3$ exposure regarding total cell number or viability, were seeded in a methyl-cellulose colony assay (see “Material and Methods”). At the 9th day, the number of CFUs of the As$_2$O$_3$-treated samples was significantly lower than in untreated control cells (Fig. 4). Interestingly, on As$_2$O$_3$-exposure most of the CFUs exhibited the characteristics of differentiated granulocytic colonies rather than BFU-E or CFUs (Fig. 4).

In summary, these data indicate that BCR-ABL increases sensitivity to As$_2$O$_3$-induced apoptosis in Ph+ CML blasts, but not in CD3+ progenitors.

References

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