**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 (Ph+). 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 (Ph+1). 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 CML (Ref. 1 and references therein). Until the 1950s, arsenic has been used in combination with radiotherapy to treat CML (2). More recently, it has been reported that As2O3 is capable of inducing complete remissions in patients with t(15;17) APL (4–6). The response to As2O3 in APL patients is genetically determined by expression of the PML/RARA fusion protein specific for t(15;17). Furthermore, transfection of PML/RARα into naturally As2O3-resistant U937 cells renders these cells sensitive to As2O3-induced apoptosis (7).

Therefore, we investigated whether there is also a functional relationship between the expression of the translocation product BCR-ABL and As2O3-induced apoptosis in Ph+ leukemia. Here, we show that As2O3 induced apoptosis in Ph+, but not in Ph− lymphoblastic cell lines. BCR-ABL mediated As2O3-induced apoptosis, analogous to PML/RARα. This activity was independent of the aberrant kinase activity of BCR-ABL. As2O3 was effective on Ph+ peripheral blasts of patients with CML in blast crisis but did not influence colony formation activity of primary CD34+ hematopoietic precursors.

The presented data establish 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 Suh 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(BCR-ABL) and p210(BCR-ABL) encoding cDNA was cloned into the ZnSO4 (Zn2+) -inducible pGMTSVneo expression vector (9). The BCR-ABL-positive U937 Mtp185 and Mtp210 bulk populations were obtained by electroporation of these p185 (BCR-ABL) - and p210 (BCR-ABL)-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-X (a-bcl-1), and anti-PARP (a-PARP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA; anti-bcl-2 (a-bcl-2) from DAKO (Hamburg, Germany); and anti-phosphotyrosine (a-PY) from Upstate Biotechnology, Inc. (Lake Placid, NY). All were applied according to widely used protocols.

**Apoptosis Assay.** Zn2+-treated U937 cells were washed twice with PBS to eliminate Zn2+, thus avoiding interference with apoptosis. All cell types were diluted to 1 × 105 cells/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 Ficol-Hypaque density-gradient centrifugation, followed by separation with the VarioMACS system, with the appropriate columns using the Direct CD34+ 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 0.9% semisolid methyl-cellulose Methocult complete medium (StemCell Technologies Inc., Vancouver, Canada) and incubated at 37°C in a humidified atmosphere of 5%
As$_2$O$_3$ Induces Apoptosis in BCR-ABL-positive Lymphoblastic Cell Lines. To determine whether Ph+ cell lines recapitulate the response of CMLs to As$_2$O$_3$, 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 As$_2$O$_3$. 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 As$_2$O$_3$ 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 As$_2$O$_3$ (BV173, 74%; SD-1, 49%; Sup-B15, 93%; Tom-1, 79%). None of the Ph− cell lines exhibited a significant response to the As$_2$O$_3$ 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 As$_2$O$_3$ in prolonged culture (data not shown).

As$_2$O$_3$ exerts its effect on Ph+ cells, regardless of the type of product known activity of As$_2$O$_3$ on Ph+ cells: MT B45, control cells transfected with the “empty” expression vector; P/R9, PML/RARA-expressing cells: BCR-ABL p185 and p210, p185$^{BCR-ABL}$, and p210$^{BCR-ABL}$-expressing cells. The U937 cells are treated with 1 μM As$_2$O$_3$ (As $-/-$) in the absence or presence of Zn$^{2+}$-induced protein expression (Zn $-/-$).

Fig. 1C demonstrates a typical 7-AAD FACs profile of BV173 cells exposed to increasing concentrations of As$_2$O$_3$. Already, 0.1 μM As$_2$O$_3$ induced a significant rate of apoptosis with respect to untreated cells. Furthermore, activity of As$_2$O$_3$ was dose dependent, as demonstrated by an increased percentage of apoptotic cells with rising dosages of As$_2$O$_3$.

Taken together, these data indicate a specific activity of As$_2$O$_3$ in BCR-ABL-expressing lymphoblastic cell lines comparable with the known activity of As$_2$O$_3$ on PML/RARA-positive cells. Moreover, As$_2$O$_3$ 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 As$_2$O$_3$ to the same extent as the CML specific p210$^{BCR-ABL}$.

As$_2$O$_3$-induced Apoptosis in Ph+ Cells Is Genetically Determined by the Presence of t(9;22). To examine whether As$_2$O$_3$-induced apoptosis in Ph+ cell lines is specifically mediated by BCR-ABL, and to exclude the possibility that the effect of As$_2$O$_3$ 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 Zn$^{2+}$-inducible metallothionein (MT-1) promoter (9).

U937 cells are myeloid precursors blocked at the promonocytic stage, which do not undergo As$_2$O$_3$-induced apoptosis (4, 7). In our experiments, we analyzed the effect of As$_2$O$_3$ 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 MTP210). On Zn$^{2+}$ 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 As$_2$O$_3$-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 Zn$^{2+}$ treatment was by itself not able to induce apoptosis in any cell lines (Fig. 2). On exposure to 1 μM As$_2$O$_3$ and in the absence of Zn$^{2+}$, MT B45 control cells showed a nearly identical apoptosis rate than with Zn$^{2+}$ treatment alone (20% and 19%, respectively). Even without Zn$^{2+}$-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 Zn\(^{2+}\) to induce protein expression prior to As\(_2\)O\(_3\) exposure, apoptosis increased dramatically in the MTP185 and MTPp210 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 bulk populations; data not shown), sensitivity of the p185\((\text{BCR-ABL})\) and p210\((\text{BCR-ABL})\)-expressing cells to As\(_2\)O\(_3\) reached nearly the same extent than the P/R9 clone (96%).

Taken together, these data indicate that the response to As\(_2\)O\(_3\) in Ph+ leukemia is genetically determined by the presence of the t(9;22) translocation products p185\((\text{BCR-ABL})\) or p210\((\text{BCR-ABL})\).

Response to As\(_2\)O\(_3\) 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 As\(_2\)O\(_3\)-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 As\(_2\)O\(_3\) after a 6-h exposure to 0.5 \(\mu\)M STI 571, 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 As\(_2\)O\(_3\). Fig. 3A shows one of three representative experiments that gave similar results.

To answer the question whether As\(_2\)O\(_3\) 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 As\(_2\)O\(_3\) treatment with an antiphospho-tyrosine monoclonal antibody (kindly provided by E. Buchdunger, Novartis, Basel, Switzerland). As shown previously, bcl-X\(_L\) expression plays an important role in protection from various apoptotic stimuli in BCR-ABL-transfected HL-60 cells (13). Two bcl-X gene products are known: bcl-X\(_L\), an inhibitor of apoptosis, and bcl-X\(_S\), a promoter of apoptosis (reviewed in Ref. 14). To answer the question whether variations of bcl-X isoform expression explains the mechanism of As\(_2\)O\(_3\)-induced apoptosis in BV173, we probed the above-mentioned immuno-blot with an antibody recognizing bcl-X\(_S\) as well as bcl-X\(_L\) (Santa Cruz Biotechnology). Only bcl-X\(_L\) was detected, and no difference in its expression level between untreated and As\(_2\)O\(_3\)-treated BV173 cells was seen (Fig. 3B).

Another key player discussed in the context of As\(_2\)O\(_3\)-induced apoptosis is caspase-3 (7, 15). Caspases constitute a family of cysteine proteases with aspartic acid substrate specificity, thought to be crucial for apoptosis in multicellular organisms (reviewed in Ref. 14). To address whether As\(_2\)O\(_3\)-induced apoptosis is mediated by caspase-3 activity, we probed the same samples with an antibody specific for PARP (Santa Cruz Biotechnology), a known substrate for several caspases, including caspase-3. In presence of activated caspase-3, PARP is cleaved and the 113 kDa species is replaced by a 81 kDa species, which is also recognized by the \(\alpha\)-PARP antibody. In our experiments, no cleavage of endogenous PARP was observed after 8, 24, 48, or 72 h of As\(_2\)O\(_3\) exposure (Fig. 3B), indicating that caspase-3 is not involved in As\(_2\)O\(_3\)-induced apoptosis of BCR-ABL-positive BV173 cells.

Taken together, these data give further proof that As\(_2\)O\(_3\) induces apoptosis independent of bcl-2 expression level and caspase-3-like activity.

As\(_2\)O\(_3\) Has No Effect on the CFUs of CD34+ Primary Hematopoietic Precursors. Clinical studies indicate that APL patients treated with As\(_2\)O\(_3\) alone do not experience aplasia of the bone marrow, commonly seen in cytotoxic chemotherapy regimen (6). To assess a possible effect of As\(_2\)O\(_3\) on normal hematopoietic progenitor cells, we exposed CD34+ cells isolated from two patients with Ph+ ALL in CR to 2 \(\mu\)M As\(_2\)O\(_3\) 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 As\(_2\)O\(_3\). 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/RARα-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. Furthermore, there was no notable difference regarding response to As$_2$O$_3$ between the ALL-derived (p185$^\text{BCR-ABL}$)-positive SupB15, TOM-1, SD-1, and the CML-derived (p210$^\text{BCR-ABL}$)-positive BV173 cell lines or between p185$^\text{BCR-ABL}$- and p210$^\text{BCR-ABL}$-transfected U937 cells. Thus, we conclude that p185$^\text{BCR-ABL}$, as well as p210$^\text{BCR-ABL}$, is able to mediate response to As$_2$O$_3$.

Moreover, sensitivity of BCR-ABL-transfected U937 cells 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$^\text{BCR-ABL}$ and p210$^\text{BCR-ABL}$. This effect of BCR-ABL is analogous to PML/RARα, which determines As$_2$O$_3$ sensitivity of APL blasts (6, 7). Other translocation products, such as HRX-AF4, the product of t(4;11), present in the SEM cells, did not mediate As$_2$O$_3$-induced apoptosis. Initial support for the hypothesis of a genetic determination of the As$_2$O$_3$ response in Ph+ leukemia was given by the fact that, to the best of our knowledge, only CML patients were reported to respond to treatment with arsenic derivatives (1, 2).

Nevertheless, the mechanism by which BCR-ABL mediates As$_2$O$_3$-induced apoptosis remains unclear. As$_2$O$_3$ does not interfere with the constitutive kinase activity of BCR-ABL, and response to As$_2$O$_3$ is not influenced by the abrogation of BCR-ABL kinase activity. Our data indicate that As$_2$O$_3$-induced apoptosis does not interfere with signaling pathways used by BCR-ABL to transform cells. This is, in particular, supported by the fact that the overall tyrosin-phosphorylation pattern of BV173 and SD-1 cells was unaltered in response to As$_2$O$_3$ (data not shown).

All originally BCR-ABL-positive cell lines, as well as BCR-ABL-transfected U937 cells, have shown clear evidence of apoptosis after 48–72 h on As$_2$O$_3$-treatment. As assessed by immunoblotting, bcl-2 expression was unaffected by As$_2$O$_3$-treatment up to 72 h. These data confirm recent data on PML/RARα-transfected U937 and PML/RARα-positive NB4 cells, which underwent apoptosis without down-regulation of bcl-2 (7, 16). In our study, we extended the investigation to bcl-X, another regulator of apoptosis that seems to be influenced by BCR-ABL (13). BV173 expressed high levels of bcl-XL, but As$_2$O$_3$-treatment neither led to down-regulation of bcl-XL nor to up-regulation of bcl-XS, the proapoptotic form of bcl-X. PARP cleavage is an important indicator of caspase activation during apoptosis. Activated caspase-3 is one of the PARP-cleaving caspases (17). We excluded an involvement of PARP-cleaving caspase activity in As$_2$O$_3$-treated BV173 cells. This confirms recent data that showed that PML/RARα is degraded by PARP-cleaving activity on retinoic acid treatment, but not by As$_2$O$_3$ (7, 18). Thus, we conclude that in BCR-ABL-transformed lymphoblasts As$_2$O$_3$-induced apoptosis is not mediated by any of the formerly discussed key players, such as bcl-2, bcl-X or caspase-3-like activity.

Because we show that the described effects of As$_2$O$_3$ are specific for Ph+ CML blasts, as well as for p185$^\text{BCR-ABL}$-expressing ALL-derived- and p210$^\text{BCR-ABL}$-expressing CML-derived cell lines, our data establish As$_2$O$_3$ as a potential agent for the treatment of patients with Ph+ leukemia.

**References**

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