[CANCER RESEARCH 60, 3409–3413, July 1, 2000]

Advances in Brief

BCR-ABL Mediates Arsenic Trioxide-induced Apoptosis Independently of Its Aberrant Kinase Activity[1]

Elena Puccetti, Saskia Güller, Anette Orleth, Nicole Brüggenolte, Dieter Hoelzer, Oliver Gerhard Ottmann, and Martin Ruthardt[2]

Medizinische Klinik III/Abteilung Hämatologie, Johann Wolfgang Goethe-Universität, D-60590 Frankfurt, Germany [E. P., S. G., N. B., D. H., O. G. O., M. R.], and Istituto di Medicina Interna e Scienze Oncologiche, Policlinico Monteluce, Perugia University, 06100 Perugia, Italy [A. O.]

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 (As_2O_3) has been shown to be genetically determined by the acute promyelocytic leukemia-specific t(15;17) translocation product *PML/RARα*. Hence, we reasoned that As_2O_3 might have a selective inhibitory effect on proliferation of *BCR-ABL*-expressing cells.

Here, we report that: (*a*) As_2O_3 induced apoptosis in Ph+ but not in Ph− lymphoblasts; (*b*) enforced expression of *BCR-ABL* in U937 cells dramatically increased the sensitivity to As_2O_3; (*c*) the effect of As_2O_3 was independent of *BCR-ABL* kinase activity; and (*d*) As_2O_3 reduced proliferation of chronic myelogenous leukemia blasts but not of peripheral CD34+ progenitors. In summary, these data establish As_2O_3 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[3] (Ref. 1 and references therein). Until the 1950s, arsenic has been used in combination with radiotherapy to treat CML (2). More than 95% of CMLs are Ph+ and, thus, express the $p210^{(BCR\text{-}ABL)}$. The other possible t(9;22) translocation product, $p185^{(BCR\text{-}ABL)}$, is present predominantly in adult Ph+ ALLs (20–25% of adult ALLs; Ref. 3). In both cases, the t(9;22) translocation leads to mutated forms of the genes encoding the tyrosine kinases BCR and ABL, which become constitutively activated, thereby inducing aberrant proliferation and neoplastic transformation (3).

Recently, it has been reported that As_2O_3 is capable of inducing complete remissions in patients with t(15;17) APL (4–6). The response to As_2O_3 in APL patients is genetically determined by expression of the *PML/RARα* fusion protein specific for t(15;17). Furthermore, transfection of *PML/RARα* into naturally As_2O_3-resistant U937 cells renders these cells sensitive to As_2O_3-induced apoptosis (7).

Therefore, we investigated whether there is also a functional relationship between the expression of the translocation product *BCR-ABL* and As_2O_3-induced apoptosis in Ph+ leukemia.

Here, we show that As_2O_3 induced apoptosis in Ph+, but not in Ph− lymphoblastic cell lines. *BCR-ABL* mediated As_2O_3-induced apoptosis, analogous to *PML/RARα*. This activity was independent of the aberrant kinase activity of *BCR-ABL*. As_2O_3 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 As_2O_3 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 $p185^{(BCR\text{-}ABL)}$ and $p210^{(BCR\text{-}ABL)}$ encoding cDNA was cloned into the $ZnSO_4$ (Zn^{2+})-inducible pGMTSVneo expression vector (9). The *BCR-ABL*-positive U937 MTp185 and MTp210 bulk populations were obtained by electroporation of these $p185^{(BCR\text{-}ABL)}$- and $p210^{(BCR\text{-}ABL)}$-carrying expression vectors and G418 selection. The expression of the exogenous protein by Zn^{2+} treatment was induced as described (9) and evaluated by Western blotting. Anti-*ABL* antibody (*α-ABL*), anti-*bcl-X* (*α-bcl-X*), and anti-*PARP* (*α-PARP*) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-*bcl-2* (*α-bcl-2*) from DAKO (Hamburg, Germany); and anti-phosphotyrosine (*α*-PY) from Upstate Biotechnology, Inc. (Lake Placid, NY). All were applied according to widely used protocols.

Apoptosis Assay. Zn^{2+}-treated U937 cells were washed twice with PBS to eliminate Zn^{2+}, thus avoiding interference with apoptosis. All cell types were diluted to 1×10^5 cell/ml and exposed to a final concentration of 0.1–2 μM As_2O_3 (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 leukapheresis 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 $CD34^+$ Progenitor Isolation Kit according to manufacturer's instructions (Myltenyi 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 As_2O_3, 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%

Received 2/4/00; accepted 5/17/00.

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.

[1] Supported by grants from Deutsche Forschungsgemeinschaft and "Dr. Mildred Scheel–Stiftung" der Deutschen Krebshilfe e.V. E. P. is a recipient of a fellowship from the "Deutsche José Carreras Leukämiestiftung e.V." (DJCLS-99/NAT-1).

[2] To whom requests for reprints should be addressed, at Med. Klinik III/Abtl. Hämatologie, Klinikum der J. W. Goethe Universität Frankfurt, Theodor Stern Kai 7, 60590 Frankfurt, Germany. Phone: 49-69-6301-6129; Fax: 49-69-6301-6131; E-mail: ruthardt@em.uni-frankfurt.de.

[3] The abbreviations used are: CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; APL, acute promyelocytic leukemia; FACS, fluorescence-activated cell-sorting; PARP, poly-(ADP ribose) polymerase; 7-AAD, 7-amino-actinomycin D; CFU, colony-forming unit; As_2O_3, arsenic trioxide; Ph+, Philadelphia chromosome; CR, complete remission; PMNC, peripheral mononuclear cells; PML/RARα, promyelocytic leukemia gene/retinoic acid receptor α; GM-CFU, granulocyte-macrophage CFU; BFU-E, blast-forming unit(s) erythroid.

CO_2. Each assay was plated as triplicate. After 9–14 days of incubation colonies (>50 cells) were counted.

Results

As_2O_3 Induces Apoptosis in *BCR-ABL*-positive Lymphoblastic Cell Lines. To determine whether Ph+ cell lines recapitulate the response of CMLs to As_2O_3, analogous to the *PML/RARα*-expressing NB4 and U937 cells (4, 7), we exposed different ALL- and CML-derived lymphoblastic cell lines to 2 μM As_2O_3. SD-1, Tom-1, and Sup-B15 cells are $p185^{(BCR\text{-}ABL)}$-positive ALL cell lines, and BV173 is a $p210^{(BCR\text{-}ABL)}$-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. 1*A* 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_2O_3 exposure (11). In Fig. 1*B*, 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_2O_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_2O_3 treatment (Fig. 1*B*). 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_2O_3 in prolonged culture (data not shown).

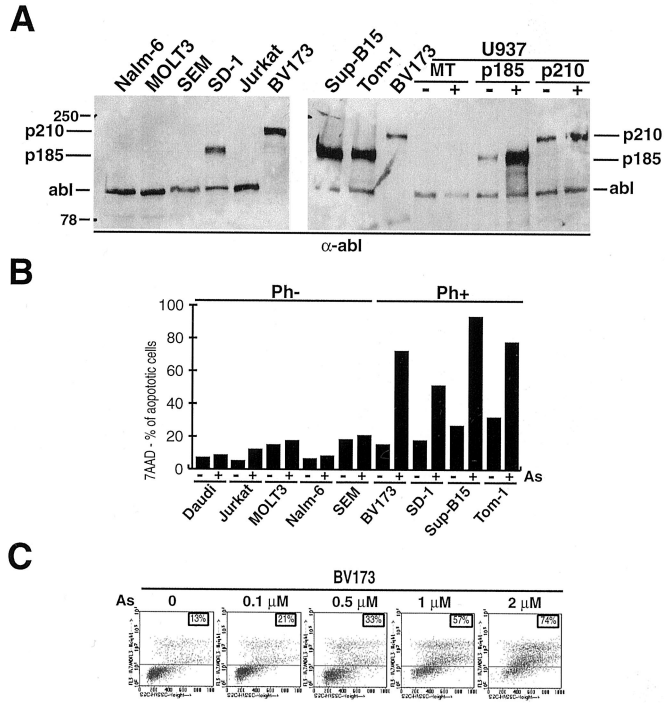

Fig. 1. *A*, Western blot analysis of *BCR-ABL* and *abl* expression levels of Ph+ and Ph− cell lines and Zn-induced $p185^{(BCR\text{-}ABL)}$ and $p210^{(BCR\text{-}ABL)}$ expression in U937 cells. U937 cells are stably transfected with a Zn-inducible MT-$p185^{(BCR\text{-}ABL)}$ and $p210^{(BCR\text{-}ABL)}$ expression vector in the presence (+) or absence (−) of Zn induction. Blots were stained with an anti-*abl* polyclonal antibody (Santa Cruz Biotechnology). Molecular weight markers are given. Each *lane* was loaded with lysates from 2×10^5 cells. The position of $p185^{(BCR\text{-}ABL)}$ and $p210^{(BCR\text{-}ABL)}$ protein is indicated. *B*, proapoptotic effect of As_2O_3 on Ph+ cell lines expressing $p185^{(BCR\text{-}ABL)}$ or $p210^{(BCR\text{-}ABL)}$, with respect to Ph− lymphoblastic cell lines–7-AAD analysis. All cells were treated with 2 μM As_2O_3 (As −/+). *C*, dose dependency of As_2O_3-induced apoptosis in BV173 cells–7-AAD analysis. Cells were exposed to increasing concentrations of As_2O_3.

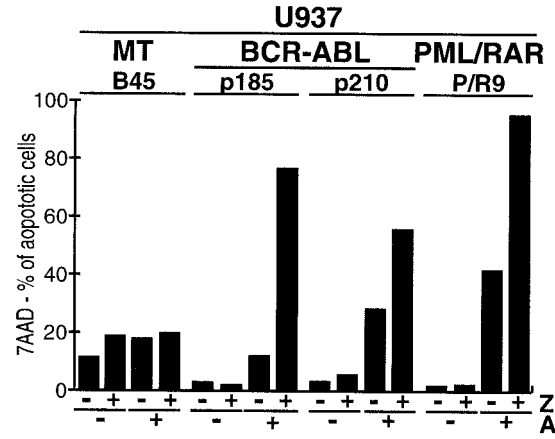

Fig. 2. Proapoptotic effect of As_2O_3 on $p185^{(BCR\text{-}ABL)}$- and $p210^{(BCR\text{-}ABL)}$-expressing U937 cells compared with PML/RARα-expressing U937 cells–7-AAD analysis. U937 cells: MT B45, control cells transfected with the "empty" expression vector; P/R9, PML/RARα-expressing cells; *BCR-ABL* p185 and p210, $p185^{(BCR\text{-}ABL)}$- and $p210^{(BCR\text{-}ABL)}$-expressing cells. The U937 cells are treated with 1 μM As_2O_3 (As −/+) in the absence or presence of Zn^{2+}-induced protein expression (Zn −/+).

Fig. 1*C* demonstrates a typical 7-AAD FACS profile of BV173 cells exposed to increasing concentrations of As_2O_3. Already, 0.1 μM As_2O_3 induced a significant rate of apoptosis with respect to untreated cells. Furthermore, activity of As_2O_3 was dose dependent, as demonstrated by an increased percentage of apoptotic cells with rising dosages of As_2O_3.

Taken together, these data indicate a specific activity of As_2O_3 in *BCR-ABL*-expressing lymphoblastic cell lines comparable with the known activity of As_2O_3 on *PML/RARα*-positive cells. Moreover, As_2O_3 exerts its effect on Ph+ cells, regardless of the type of product of t(9;22) present [*i.e.*, the ALL-specific $p185^{(BCR\text{-}ABL)}$ is able to mediate sensitivity to As_2O_3 to the same extent as the CML specific $p210^{(BCR\text{-}ABL)}$].

As_2O_3-induced Apoptosis in Ph+ Cells Is Genetically Determined by the Presence of t(9;22). To examine whether As_2O_3-induced apoptosis in Ph+ cell lines is specifically mediated by *BCR-ABL*, and to exclude the possibility that the effect of As_2O_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 $p185^{(BCR\text{-}ABL)}$ or $p210^{(BCR\text{-}ABL)}$ 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_2O_3-induced apoptosis (4, 7). In our experiments, we analyzed the effect of As_2O_3 on $p185^{(BCR\text{-}ABL)}$- and $p210^{(BCR\text{-}ABL)}$-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. 1*A*). As negative control for As_2O_3-induced apoptosis we used the MT B45 cells, transfected with the "empty" expression vector, and as positive control we used the *PML/RARα*-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_2O_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

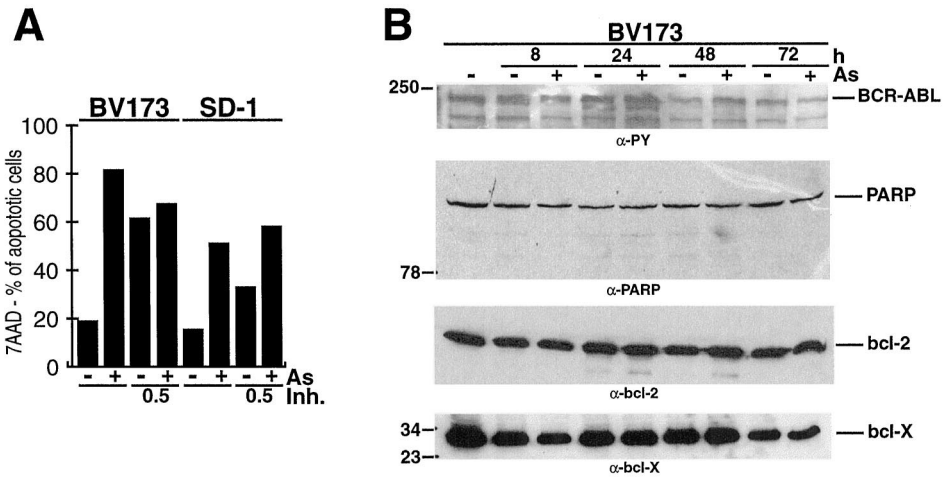

Fig. 3. *A*, role of the *ABL* kinase activity in As_2O_3-induced apoptosis in Ph+ cell lines. Treatment with 0.5 μM of the specific *ABL* kinase inhibitor STI 571 (*Inh*; Novartis) does not influence significantly response of BV173 ($p210^{(BCR\text{-}ABL)}$) and SD-1 ($p185^{(BCR\text{-}ABL)}$) cells to As_2O_3–7-AAD analysis. *B*, BV173 cells: effect of As_2O_3 on the kinase activity of *BCR-ABL*, *PARP*-cleaving caspase activity, and expression level of *bcl-2* and *bcl-X*. BV173 in the presence or absence of As_2O_3 treatment at 8, 24, 48, and 72 h (As −/+). Blots were stained with the indicated antibodies. Each *lane* was loaded with lysates from 2×10^5 cells and an equal amount of protein loaded on each *lane* was confirmed by Ponceau staining of the nitrocellulose membrane and Coomassie staining of the gel after protein transfer (data not shown).

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. 1*A*). When cells were treated for 12 h with Zn^{2+} to induce protein expression prior to As_2O_3 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 bulk populations; data not shown), sensitivity of the $p185^{(BCR\text{-}ABL)}$- and $p210^{(BCR\text{-}ABL)}$-expressing cells to As_2O_3 reached nearly the same extent than the P/R9 clone (96%).

Taken together, these data indicate that the response to As_2O_3 in Ph+ leukemia is genetically determined by the presence of the t(9;22) translocation products $p185^{(BCR\text{-}ABL)}$ or $p210^{(BCR\text{-}ABL)}$.

Response to As_2O_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_2O_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_2O_3 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 proapototic effects on *BCR-ABL*-transformed cells, but had no considerable influence on response to As_2O_3. Fig. 3*A* shows one of three representative experiments that gave similar results.

To answer the question whether As_2O_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_2O_3 treatment with an antiphospho-tyrosine monoclonal antibody (see "Material and Methods"). There was no decrease in the expression of phosphorylated *BCR-ABL* at any time point (Fig. 3*B*).

In summary, the response to As_2O_3 in Ph+ lymphoblasts seems to be independent of the constitutive *ABL* kinase activity of *BCR-ABL*.

In *BCR-ABL*-positive Cells As_2O_3 Activates Apoptosis without *Caspase-3* Activation or *bcl-2* Regulation. In APL, the role of *bcl-2*- and *caspase-3*-like activity in As_2O_3-induced apoptosis is controversially discussed. Reportedly, one of the mechanisms of decreased susceptibility to apoptosis in *BCR-ABL*-positive cells is due to upregulation of *bcl-2* (12). Therefore, we assessed *bcl-2* expression by immunoblotting in BV173 cells on As_2O_3 treatment. In comparison with untreated BV173 cells, no modification of *bcl-2* expression was noted at 8, 24, 48, or 72 h of As_2O_3 treatment (Fig. 3*B*).

As shown previously, *bcl-X* 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_2O_3-induced apoptosis in BV173, we probed the above-mentioned immunoblots with an antibody recognizing *bcl-*X_L as well as *bcl-*X_S (Santa Cruz Biotechnology). Only *bcl-*X_L was detected, and no difference in its expression level between untreated and As_2O_3-treated BV173 cells was seen (Fig. 3*B*).

Another key player discussed in the context of As_2O_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_2O_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 *α-PARP* antibody. In our experiments, no cleavage of endogenous *PARP* was observed after 8, 24, 48, or 72 h of As_2O_3 exposure (Fig. 3*B*), indicating that *caspase-3* is not involved in As_2O_3-induced apoptosis of *BCR-ABL*-positive BV173 cells.

Taken together, these data give further proof that As_2O_3 induces apoptosis independent of *bcl-2* expression level and *caspase-3*-like activity.

As_2O_3 Has No Effect on the CFUs of CD34+ Primary Hematopoietic Precursors. Clinical studies indicate that APL patients treated with As_2O_3 alone do not experience aplasia of the bone marrow, commonly seen in cytotoxic chemotherapy regimen (6). To assess a possible effect of As_2O_3 on normal hematopoietic progenitor cells, we exposed CD34+ cells isolated from two patients with Ph+ ALL in CR to 2 μM As_2O_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_2O_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.

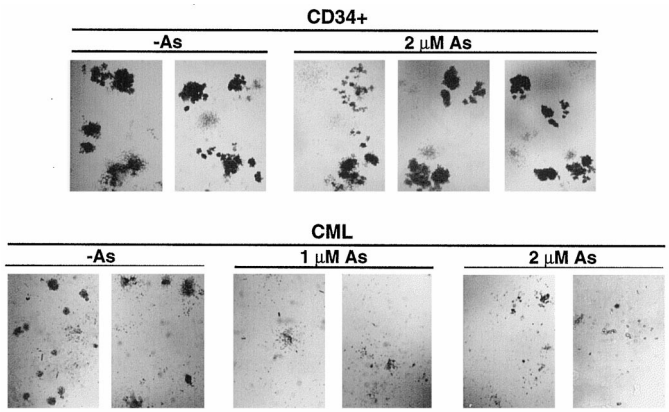

Fig. 4. Effect of As_2O_3 on normal CD 34+ progenitor cells and CML blasts. A methyl-cellulose colony formation assay on granulocyte colony-stimulating factor mobilized peripheral $CD34^+$ cells (*CD34+*) from a patient with Ph+ ALL in CR and on peripheral blast from a CML patient (*CML*) in blast crisis, both incubated for 2 days in liquid culture in the presence/absence of 2 μM and 1 and 2 μM As_2O_3, respectively.

These data confirm the hypothesis that the toxic effect of As_2O_3 on normal hematopoietic precursors is minimal, as long as As_2O_3 is applied in clinically relevant concentrations.

As_2O_3 Significantly Reduces the CFU of Ph+ Blasts of CML Patients in Blast Crisis. In BV173 cells a 6-h exposure to As_2O_3 is sufficient to irreversibly induce apoptosis (data not shown). To examine the effect of As_2O_3 on primary blasts of CML patients, we treated PMNCs of five CML patients in myeloid or lymphatic blast crisis. On As_2O_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_2O_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_2O_3-treated samples was significantly lower than in untreated control cells (Fig. 4). Interestingly, on As_2O_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_2O_3-induced apoptosis in Ph+ CML blasts, but not in CD34+ progenitors.

Discussion

Here, we show that As_2O_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_2O_3-induced apoptosis. In contrast, Ph− cell lines, including the t(4;11)-positive SEM cells, responded to As_2O_3-induced apoptosis. Furthermore, there was no notable difference regarding response to As_2O_3 between the ALL-derived ($p185^{(BCR\text{-}ABL)}$ positive) SupB15, TOM-1, SD-1, and the CML-derived ($p210^{(BCR\text{-}ABL)}$ positive) BV173 cell lines or between $p185^{(BCR\text{-}ABL)}$- and $p210^{(BCR\text{-}ABL)}$-transfected U937 cells. Thus, we conclude that $p185^{(BCR\text{-}ABL)}$, as well as $p210^{(BCR\text{-}ABL)}$, is able to mediate response to As_2O_3.

Moreover, sensitivity of *BCR-ABL*-transfected U937 cells to As_2O_3-induced apoptosis also excludes the possibility that the effect of As_2O_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_2O_3-induced apoptosis is genetically determined by the presence of the t(9;22)-specific chimeric gene products $p185^{(BCR\text{-}ABL)}$ and $p210^{(BCR\text{-}ABL)}$. This effect of *BCR-ABL* is analogous to PML/RARα, which determines As_2O_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_2O_3-induced apoptosis. Initial support for the hypothesis of a genetic determination of the As_2O_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_2O_3-induced apoptosis remains unclear. As_2O_3 does not interfere with the constitutive kinase activity of *BCR-ABL*, and response to As_2O_3 is not influenced by the abrogation of *BCR-ABL* kinase activity. Our data indicate that As_2O_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_2O_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_2O_3-treatment. As assessed by immunoblotting, *bcl-2* expression was unaffected by As_2O_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-*X_L, but As_2O_3-treatment neither led to down-regulation of *bcl-*X_L nor to up-regulation of *bcl-*X_S, 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_2O_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_2O_3 (7, 18). Thus, we conclude that in *BCR-ABL*-transformed lymphoblasts As_2O_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_2O_3 are specific for Ph+ CML blasts, as well as for $p185^{(BCR\text{-}ABL)}$-expressing ALL-derived- and $p210^{(BCR\text{-}ABL)}$-expressing CML-derived cell lines, our data establish As_2O_3 as a potential agent for the treatment of patients with Ph+ leukemia.

References

1. Li, Y. M., and Broome, J. D. Arsenic targets tubulins to induce apoptosis in myeloid leukemia cells. Cancer Res., *59:* 776–780, 1999.
2. Schulten, H. Lehrbuch der klinischen Hämatologie. Stuttgart, West Germany: G. Thieme Verlag, 1953.
3. Faderl, S., Talpaz, M., Estrov, Z., O'Brien, S., Kurzrock, R., and Kantarjian, H. M. The biology of chronic myeloid leukemia. N. Engl. J. Med., *341:* 164–172, 1999.
4. Chen, G. Q., Zhu, J., Shi, X. G., Ni, J. H., Zhong, H. J., Si, G. Y., Jin, X. L., Tang, W., Li, X. S., Xong, S. M., Shen, Z. X., Sun, G. L., Ma, J., Zhang, P., Zhang, T. D., Gazin, C., Naoe, T., Chen, S. J., Wang, Z. Y., and Chen, Z. *In vitro* studies on cellular and molecular mechanisms of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia: As2O3 induces NB4 cell apoptosis with down-regulation of Bcl-2 expression and modulation of PML-RAR α/PML proteins. Blood, *88:* 1052–1061, 1996.
5. Chen, G. Q., Shi, X. G., Tang, W., Xiong, S. M., Zhu, J., Cai, X., Han, Z. G., Ni, J. H., Shi, G. Y., Jia, P. M., Liu, M. M., He, K. L., Niu, C., Ma, J., Zhang, P., Zhang, T. D., Paul, P., Naoe, T., Kitamura, K., Miller, W., Waxman, S., Wang, Z. Y., de The, H., Chen, S. J., and Chen, Z. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): I. As2O3 exerts dose-dependent dual effects on APL cells. Blood, *89:* 3345–3353, 1997.
6. Shen, Z. X., Chen, G. Q., Ni, J. H., Li, X. S., Xiong, S. M., Qiu, Q. Y., Zhu, J., Tang, W., Sun, G. L., Yang, K. Q., Chen, Y., Zhou, L., Fang, Z. W., Wang, Y. T., Ma, J., Zhang, P., Zhang, T. D., Chen, S. J., Chen, Z., and Wang, Z. Y. Use of arsenic trioxide (As2O3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. Blood, *89:* 3354–3360, 1997.
7. Sternsdorf, T., Puccetti, E., Jensen, K., Hoelzer, D., Will, H., Ottmann, O. G., and Ruthardt, M. PIC-1/SUMO-1-modified PML-retinoic acid receptor α mediates ar-

senic trioxide-induced apoptosis in acute promyelocytic leukemia. Mol. Cell. Biol., *19:* 5170–5178, 1999.
8. Ruthardt, M., Testa, U., Nervi, C., Ferrucci, P. F., Grignani, F., Puccetti, E., Grignani, F., Peschle, C., and Pelicci, P. G. Opposite effects of the acute promyelocytic leukemia PML-retinoic acid receptor α (RAR α) and PLZF-RAR α fusion proteins on retinoic acid signalling. Mol. Cell. Biol., *17:* 4859–4869, 1997.
9. Grignani, F., Ferrucci, P. F., Testa, U., Talamo, G., Fagioli, M., Alcalay, M., Mencarelli, A., Grignani, F., Peschle, C., Nicoletti, I., and Pelicci, P. G. The acute promyelocytic leukemia-specific PML-RAR α fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell, *74:* 423–431, 1993.
10. Drexler, H. G., MacLeod, R. A., and Uphoff, C. C. Leukemia cell lines: *in vitro* models for the study of Philadelphia chromosome-positive leukemia (Editorial). Leuk. Res., *23:* 207–215, 1999.
11. Schmid, I., Uittenbogaart, C. H., Keld, B., and Giorgi, J. V. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. J. Immunol. Methods, *170:* 145–157, 1994.
12. Sanchez-Garcia, I., and Martin-Zanca, D. Regulation of Bcl-2 gene expression by BCR-ABL is mediated by Ras. J. Mol. Biol., *267:* 225–228, 1997.
13. Amarante-Mendes, G. P., McGahon, A. J., Nishioka, W. K., Afar, D. E., Witte, O. N., and Green, D. R. Bcl-2-independent Bcr-Abl-mediated resistance to apoptosis: protection is correlated with up-regulation of Bcl-xL. Oncogene, *16:* 1383–1390, 1998.
14. Yang, E., and Korsmeyer, S. J. Molecular thanatopsis: a discourse on the BCL2 family and cell death. Blood, *88:* 386–401, 1996.
15. Zhu, J., Koken, M. H., Quignon, F., Chelbi-Alix, M. K., Degos, L., Wang, Z. Y., Chen, Z., and de The, H. Arsenic-induced PML targeting onto nuclear bodies: implications for the treatment of acute promyelocytic leukemia. Proc. Natl. Acad. Sci. USA, *94:* 3978–3983, 1997.
16. Gianni, M., Koken, M. H., Chelbi-Alix, M. K., Benoit, G., Lanotte, M., Chen, Z., and de The, H. Combined arsenic and retinoic acid treatment enhances differentiation and apoptosis in arsenic-resistant NB4 cells. Blood, *91:* 4300–4310, 1998.
17. Sallmann, F. R., Bourassa, S., Saint-Cyr, J., and Poirier, G. G. Characterization of antibodies specific for the caspase cleavage site on poly(ADP-ribose) polymerase: specific detection of apoptotic fragments and mapping of the necrotic fragments of poly(ADP-ribose) polymerase. Biochem. Cell. Biol., *75:* 451–456, 1997.
18. Nervi, C., Ferrara, F. F., Fanelli, M., Rippo, M. R., Tomassini, B., Ferrucci, P. F., Ruthardt, M., Gelmetti, V., Gambacorti-Passerini, C., Diverio, D., Grignani, F., Pelicci, P. G., and Testi, R. Caspases mediate retinoic acid-induced degradation of the acute promyelocytic leukemia PML/RARα fusion protein. Blood, *92:* 2244–2251, 1998.