Enhanced Bcr-Abl-specific antileukemic activity of arsenic trioxide through glutathione-depletion in imatinib-resistant cells

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ABSTRACT

The development of resistance to imatinib mesylate may partly depend on high Bcr-Abl-expression levels. Arsenic trioxide (ATO) has Bcr-Abl suppressing activity *in vitro*. Here we investigated means to improve ATO activity in CML by modulating cellular glutathione (GSH), a key regulator of ATO-activity in malignant disease. Our studies demonstrate that depletion of cellular glutathione using dl-buthionine-[S,R]-sulfoximine (BSO) enhances ATO activity against CML cells. GSH-depletion promotes enhanced Bcr-Abl specific activity of ATO through avid repression of Bcr-Abl protein levels and total cellular Bcr-Abl activity. These data provide a rationale for the clinical development of optimized ATO-based regimens through incorporation of GSH-modulators in CML treatment.

Key words: CML, Bcr-Abl, arsenic, glutathione, Imatinib, resistance.

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In chronic myeloid leukemia (CML), mechanisms of resistance to imatinib mesylate (Glivec), a selective Bcr-Abl kinase inhibitor, have been traced to Bcr-Abl reactivation, most frequently by mutations in the kinase domain of Bcr-Abl,[1] or to BCR-ABL amplification.[2] Bcr-Abl overexpression not only directly modulates sensitivity to imatinib,[3] it may also indirectly influence treatment response by influencing the rate of mutation development in the kinase domain.[4] We previously demonstrated the ability of arsenic trioxide (As_2O_3, ATO, Trisenox™, Cell Therapeutics, Seattle, USA) to make imatinib resistant cell lines and primary cells susceptible to imatinib-induced growth inhibition and apoptosis.[5] Furthermore, we were able to confirm previous reports[6] that ATO-induced downregulation of Bcr-Abl protein expression leads to reduced total cellular Bcr-Abl activity in imatinib-resistant cell lines that are characterized by Bcr-Abl dependent resistance.[5] Nimmanapalli *et al.* have traced the mechanism of ATO-dependent Bcr-Abl-suppression to specific inhibition of translation.[7] Given the inverse relationship between glutathione-levels and the antileukemic activity of ATO,[8,9] we asked whether ATO-activity could be increased by modulation of cellular glutathione (GSH). The effects of GSH-modulation on the antileukemic activity of ATO in CML were studied using dl-buthionine-[S,R]-sulfoximine (BSO), a specific and irreversible inhibitior of the rate limiting enzyme in GSH-biosynthesis γ-glutamylcysteine synthetase.[10]

Design and Methods

Cellular material and cell culture

Cell line experiments were performed using the established imatinib-sensitive AR230-s and KCL22-s, and their imatinib-resistant counterparts AR230-r, and KCL22-r.[11] In AR230-r, and KCL22-r cells, imatinib-resistance (defined as unaffected viability in the presence of 1 μM imatinib) was originally induced through continuous culture of cells in the presence of imatinib (1 μM). In AR230-r cells, imatinib resistance was traced to Bcr-Abl overexpression, whereas no specific resistance mechanism was detected in KCL22-r cells.[11] Also, known ATO-sensitive cell lines (K562, NB4) were used as controls.[5,8] All cell lines were cultured in RPMI 1640 medium supplemented with 2% L-glutamine (Gibco BRL), 1% penicillin-streptomycin and heat-inactivated fetal bovine serum (FBS, Gibco BRL, Wiesbaden, Germany). Primary cells were obtained from peripheral blood

Table 1. Antiproliferative activity of ATO in relation to cellular GSH.

Cell line	*IC50 ATO [μM]*	*GSH [nmol/mg]*
K562	0.9±0.5	13.2±2.7
AR230-s	2.6±0.5	34.8±10.6
KCL22-s	2.6±0.5	43.3±10.0
AR230-r	6.9±1.4	51.1±15.7
KCL22-r	2.8±0.5	36.4±10.9
NB4	0.4±0.03	14.7±3.9

Antiproliferative activity of ATO in various cell lines (left column) after 3 days of treatment was analyzed using the MTS proliferation assay. Drug concentrations (μM) required for 50% growth inhibition (IC50) are given (center column) and compared to GSH-levels (nmol/mg) of the respective cell lines (right column). GSHHigh cells show IC50 values for ATO >2 μM (AR230-s, AR230-r1, KCL22-s, KCL22-r1). Results are given as means of at least 3 experiments ± standard deviation.

specimens of four patients with blast crisis CML resistant to imatinib. Informed consent was obtained from all patients according to the declaration of Helsinki. Cell culture was performed in 5% CO2/95% O2 air, in a 37°C fully humidified incubator.

Drugs

As_2O_3 stock solution (Trisenox 1mg/mL, approximately 5 mM) was stored at room temperature. L-buthionine sulfoximine (Sigma, St. Louis, MO, USA) was freshly prepared for each experiment as a 10 mM solution in water and filtered sterilized. It was added to the cells at the clinically relevant concentration of 100 μM.[12] A 10 mmol/L stock solution of imatinib mesylate (Novartis, Basel, Switzerland) was prepared in sterile phosphate-buffered saline and stored at –20°C.

Proliferation assay (MTS assay)

Cellular growth was evaluated using the MTS Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer´s instructions.

Quantification of intracellular glutathione (GSH)

Intracellular GSH content was determined using the Glutathione Assay Kit (Calbiochem, San Diego, CA, USA) according to the manufacturer´s instructions. To adjust cellular GSH to total cellular protein, pelleted cells were lysed in 1N NaOH and protein content was analyzed using the Bradford method.

Western blot

Western blot analysis was performed as previously described,[13] using anti-abl (8E9, BD Pharmingen, Heidelberg, Germany) and anti-pTyr (4G10, Upstate Biotechnology, Lake Placid, NY) antibodies. Equal protein loading was ensured by blotting for actin with the Actin (Ab-1) kit (Oncogene, Boston, MA, USA).

Apoptosis assay

Detection of apoptotic cells was performed using Annexin V-FLUOS staining kit (Roche, Mannheim, Germany) according to the manufacturer´s instructions. Stained cells were analyzed by flow cytometry on a FACSscan run on the CellQuest software (Becton Dickinson, Oxford, UK).

Data analysis

Statistical analysis was performed to assess significant differences between treatment conditions using the t-test on the SAS software (SAS release 8.02).

Results and Discussion

GSH^{high} CML cells show innate resistance against ATO, which can be overcome by GSH-depletion

In pilot experiments, the antiproliferative activity of ATO was determined in a panel of CML cell lines sensitive or resistant to the Bcr-Abl-inhibitor imatinib and correlated to the cellular GSH levels (only selected data are shown, Table 1). Intracellular GSH measurements were performed after pre-treatment with BSO (100 μM) for 12 hours, and after continued treatment for 24 hours and 48 hours with BSO (100 μM) and/or ATO (1 μM), respectively. While the CML cell line K562 shows ATO-sensitivity similar to the highly sensitive promyelocytic NB4 cells, AR230-s/r, and KCL-s/r are intrinsically resistant against ATO with IC50-values beyond 2 μM. This concentration represents the upper margin of the clinically useful range.[14] The most sensitive NB4 cells express low GSH levels (14.7±3.9 nmoL/mg), while highly resistant AR230-r cells show a fourfold increased GSH-content. Depletion of cellular GSH by treatment with BSO (Figure 1A) shows no cytotoxicity in trypan blue viability assays (Figure 1B) using AR230-s/r, and KCL22-s/r cells. However, the combination of ATO with BSO is synergistic leading to a rapid loss of cell viability within 24 hrs (Figure 1B).

Therefore, similar to PML/RARα positive leukemia,[8,15] high intracellular GSH is a determinant of ATO-sensitivity in BCR-ABL expressing cells. Consistent with viability data, 24 hours co-treatment with 1 μM ATO and 100 μM BSO leads to a 3- (AR230-r, KCL22-s, KCL22-r) to 6-fold (AR230-s) increase in the fraction of apoptotic annexinV-positive (Ann^+)/ PI-negative (PI-) cells when compared to untreated controls (Figure 1C). By contrast, a moderate or no increase in apoptotic cells was observed in all 4 cell lines when treated with 1 μM ATO or 100 μM BSO alone.

Combined treatment with ATO and BSO leads to rapid downregulation of Bcr-Abl protein levels

At 1-2 μM, ATO has previously been shown to downregulate ectopically expressed or endogenous Bcr-Abl in cell lines and primary CML cells via translational mechanisms after 48 hours treatment.[7,13] Therefore, the impact of BSO co-treatment on the Bcr-Abl-specific activity of ATO in AR230-r and KCL22-r cells was investigated. Treatment with 1 μM ATO and 100 μM BSO for 24

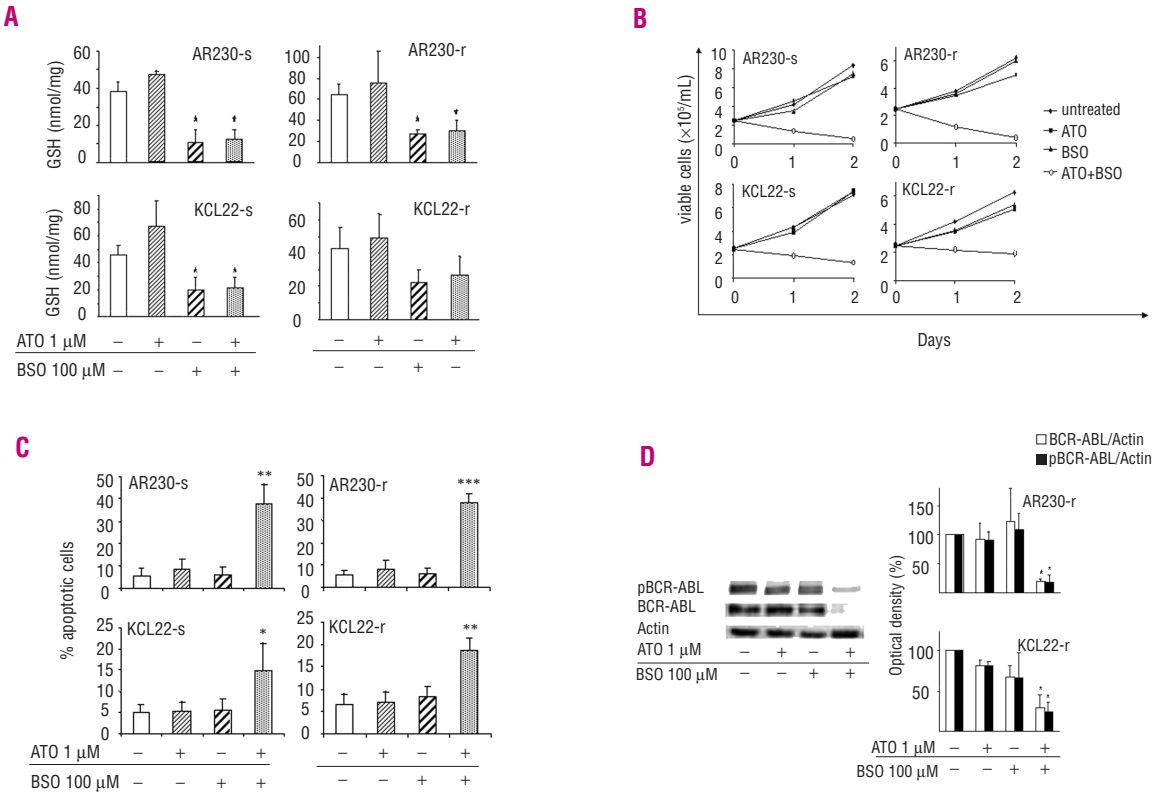

Figure 1. Depletion of cellular GSH in GSH^{High} cells by treatment with BSO translates in enhanced growth inhibition in proliferation assays. (A) Intracellular GSH (in nmoL/mg total cellular protein, y-axis) after 12 hours pre-treatment with 100 μM BSO and subsequent exposure to 1 μM ATO and/or continued treatment with 100 μM BSO for 24 hours. Values are given as means of at least 3 experiments ± standard deviation for AR230-s, AR230-r, KCL22-s and KCL22-r cells. Trypan blue viability count of the respective cell lines every 24 h for two consecutive days (x-axis) (B). Combination treatment (p) was compared to single agent treatment or no treatment at all. Data are graphed as viable cells ×10^5/mL (y-axis). Synergistic induction of apoptosis in GSH^{High} cell lines by combined treatment with ATO/BSO (C). Mean percentage of apoptotic cells (y-axis) after ATO-treatment with and without 100 μM BSO. Columns represent compiled data derived from three independent experiments ± standard deviation. *: $p < 0.05$; **: $p < 0.001$; ***: $p < 0.0001$. Synergistic Bcr-Abl protein suppression by combination treatment with ATO/BSO. (D) Representative immunoblot of at least 3 independent experiments for detection of tyrosine phosphorylated Bcr-Abl (pBcr-Abl), total cellular Bcr-Abl and Actin in imatinib-resistant AR230-r cells (left). Results of densitometric analysis of immunoblots derived from whole cell lysates of AR230-r and KCL22-r cells (right). Bcr-Abl (white) and tyrosine phosphorylated Bcr-Abl (black) were normalized to Actin expression levels as loading control. Data are calculated as percent change of optical density (y-axis) vs control (untreated). Statistical analysis reveals significant ($p < 0.05$) downregulation of total Bcr-Abl and pBcr-Abl as soon as 24 hours after exposure to combined treatment.

hours results in downregulation of Bcr-Abl protein and autophosphorylation levels when compared to untreated controls (Figure 1D). Enhanced Bcr-Abl suppression is associated with enhanced inhibition of proliferation (Figure 1B) and induction of cell death by apoptosis (Figure 1C). The nearly complete abrogation of Bcr-Abl protein expression observed in the Bcr-Abl overexpressing AR230-r cells, is particularly important for clinical resistance or refractoriness to imatinib. Bcr-Abl-amplification is a known mechanism of resistance in patients treated with imatinib.[2,16] In cell lines, the expression level of Bcr-Abl determines the sensitivity to imatinib[17] but also determines the rate of development of resistance eventually triggered by kinase domain mutations of Bcr-Abl.[4] Kinase domain mutations are the most frequently detected resistance mechanism in patients.[18] A reduced development of resistance can be expected when imatinib is combined with agents that have the potential to

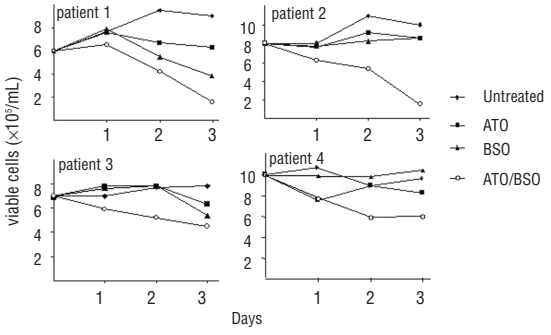

Figure 2. Enhanced antileukemic activity by combination treatment with ATO/BSO in primary blasts of imatinib-resistant CML patients. Trypan blue viability count of PB MNCs derived from four CML patients in blast crisis resistant to imatinib. Viability was analyzed after 1, 2 and 3 days (x-axis) of *in vitro* exposure to ATO and/or BSO in liquid media (RPMI1640 + 10% FBS). Data are graphed as viable cells ×10^5/mL (y-axis).

suppress Bcr-Abl protein expression. Bcr-Abl protein suppressors such as ATO/BSO may therefore serve as a starting point in the search for agents that can reduce the incidence of imatinib resistance by modulating Bcr-Abl expression. Finally, high expression levels of Bcr-Abl in CML stem cells[19] is thought to impart imatinib resistance in the leukemogenic stem cell compartment, which cannot be overcome even by high affinity second generation inhibitors such dasatinib.[19] Nimmanapalli *et al.*[7] have already demonstrated the Bcr-Abl-suppressing activity of ATO in CD34+ CML cells. However, the drug concentration of 2 μM used is at the upper end of the clinically useful range. Also, ATO alone is not able to induce apoptosis in nonproliferating CD34+ progenitors.[20] Therefore, combined treatment with BSO may strengthen ATO activity against the stem cell compartment while allowing less toxic drug concentrations of ATO. However, further experiments are needed to support this hypothesis.

BSO enhances the cytotoxic activity of ATO in primary CML cells

Based on the evidence that BSO enhances the cytotoxic activity of ATO in immortalized CML cell lines, MNC derived from 4 patients with imatinib resistant CML in blast crisis were analyzed in proliferation assays (Figure 2). One μM ATO applied as a single agent, does not decrease viability similar to the results seen with the AR230-s/r, and KCL22-s/r cell lines. In primary cells, BSO alone shows some antileukemic activity consistent with earlier reports.[21] However, combined treatment with 1 μM ATO and 100 μM BSO leads to maximum decrease in cell viability in all four patient samples compared to single agent treatment.

In conclusion, our data support the development of arsenicals in combination with GSH-depleters to combat abundant Bcr-Abl expression as a mechanism of imatinib resistance.

Authors' Contributions

HK: performed experiments, wrote manuscript draft; NH: performed primary cell experiments; BS: revised manuscript draft, performed Western studies; MS: treated patients, provided sample material, revised manuscript draft; CL: treated patients, provided sample material, revised manuscript draft; JM: created imatinib resistant cell lines, revised manuscript; RH: supervised study, revised manuscript; AH: supervised study, revised manuscript; PLR: designed study, wrote the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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