# Modulation of reactive oxygen species by antioxidants in chronic myeloid leukemia cells enhances imatinib sensitivity through survivin downregulation

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**Survivin, a member of the inhibitor of apoptosis protein family and a target for new drugs, is modulated by reactive oxygen species in several types of neoplasms including leukemias. The aim of this study is to find mechanisms to enhance sensitivity to imatinib in imatinib-responsive cells. In this study, we demonstrated through fluorescein isothiocyanate-labeled annexin V for apoptotic cells detection and western blotting that by inhibiting catalase activity, imatinib apoptosis induction was significantly enhanced ($P<0.05$) through diminishing survivin expression in K562 cells. These findings might be of clinical relevance and might help improve the chemotherapeutic use of imatinib mesylate.** ***Anti-Cancer Drugs*** **19:975–981 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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## Introduction

Chronic myelogenous leukemia (CML) is a clonal hematologic malignancy characterized by the Philadelphia (Ph-1) chromosomal translocation t(9; 22)(q34; q11), which fuses the BCR gene on chromosome 22 to the ABL gene on chromosome 9 [1]. The *BCR-ABL* fusion gene, which has higher and aberrant tyrosine kinase activity than the normal c-abl-coded counterpart, is known to be essential for hematopoietic cell transformation and upregulates survivin, a member of the inhibitor of apoptosis protein family [2].

The clinical course of CML is triphasic and involves progression from a chronic phase (CML-CP), of variable duration, to an accelerated phase with a duration of 1–1.5 years followed by a fatal blast crisis phase (CML-BP) and associated with high levels of BCR-ABL tyrosine kinase activity [1].

Current therapies for CML include allogeneic bone marrow transplantation and tyrosine kinase inhibitors. Bone marrow transplantation is associated with substantial morbidity and mortality and is limited to patients for whom a suitable donor is available [1]. Since the discovery of the BCR-ABL-mediated pathogenesis of CML, efforts in developing inhibitory agents that target BCR-ABL kinase activity are progressively on course [3,4].

Imatinib (imatinib mesylate: Novartis Pharmaceuticals, Basel, Switzerland) is now a well-known selective inhibitor of the BCR-ABL tyrosine kinase activity in CML and acute lymphoblastic leukemia with Ph-1 chromosome [5–7]. However, 60% of the patients with CML-BP eventually relapse, indicating that there are leukemic cells that overcome the proapoptotic effect of imatinib and survive in the presence of pharmacological concentrations of the drug. Resistance to imatinib is a major problem in CML patients in BP and has been mainly associated with BCR/ABL tyrosine kinase domain point mutation reducing the affinity of the enzyme to imatinib [1,7,8].

Although BCR/ABL tyrosine kinase is known to play a central role in the pathogenesis of CML and, therefore, is the main target for therapeutic strategy, the specific mechanisms through which it mediates the disease are still unclear. It was, however, demonstrated that BCR/ABL tyrosine kinase activity induces high levels of intracellular reactive oxygen species (ROS) [9,10], which are involved in the genomic instability with malignant progression of Ph-1positive leukemias and imatinib resistance [10]. In contrast, it was shown that survivin is downregulated by ROS in different tumor cells [11–13] but is highly expressed in imatinib-responsive CML-BP cells [2]. Studies in different BCR/ABL-expressing cell lines showed that ROS production in these cells is

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inhibited by imatinib [11,12]. Additionally, disruption of survivin sensitizes BCR/ABL cells to imatinib-induced apoptosis [2].

It is well known that the dual (proapoptotic and antiapoptotic) responses of tumor cells to ROS arise from its concentration-dependent ability to induce proliferation and apoptosis pathways [14–17]. Therefore, a control of the cellular redox environment is essential for the survival of many malignant cell types. Taking these data into account, the aim of this study was to evaluate the role of the intracellular ROS amount modulated by inhibitors of antioxidants glutathione (GSH) and catalase in inhibiting survivin expression and enhancing apoptosis in K562, an imatinib-responsive CML cell line.

## Methods

### Cell culture

Human CML-BP BCR-ABL-positive K562, and the human promyelocytic leukemia cells BCR-ABL negative HL-60 (originally obtained from the American Type Culture Collection-ATCC, Manassas, Virginia, USA), were grown in a suspension culture with RPMI 1640 medium supplemented with 25 mmol/l HEPES buffer (Sigma, St Louis, USA) and 10% heat-inactivated fetal calf serum (FCS, Gibco BRL, Maryland, USA) at 37°C under humidified atmosphere of 95% air and 5% $CO_2$.

### Protein determination

Protein content was determined according to Lowry *et al.* [18], using bovine serum albumin fraction V (GE Healthcare, Uppsala, Sweden) as standard.

### Inhibition of catalase activity and glutathione synthesis in K562 cells

3-Amino-1,2,4-triazole (AT, Sigma) is an irreversible inhibitor of catalase activity by reaction with a catalase–$H_2O_2$ intermediate complex. After AT treatment, cells' catalase activity generally dropped to more than 95%. K562 cells, at a concentration of $5 \times 10^5$ cells/ml, were placed in a culture medium supplemented with 100 mmol/l AT for 2 h at 37°C [19] and catalase activity was checked after incubation. L-buthionine (*S,R*)-sulfoximine (BSO, Sigma) is a specific inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis [20]. After creating dose–response curves to establish the optimal concentration of BSO that inhibited GSH synthesis at the same time with low cytotoxic effects, K562 cells, at a concentration of $5 \times 10^5$ cells/ml, were placed in a culture medium supplemented with 25.0 μmol/l BSO for 24 h at 37°C. Treated cells were washed with PBS and then used in the assays.

### Analysis of intracellular reactive oxygen species levels

Production of ROS was measured with 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Oregon, USA), which is a cell-permeable dye and is oxidized inside the cells to fluorescent dichlorofluorescein (DCF) in the presence of $H_2O_2$ (Perhidrol, Merck, Darmstadt, Germany) [21]. Briefly, cells ($10^6$/ml) that were previously incubated or not with AT or BSO and treated with or without imatinib mesylate (provided by Novartis Brazil) at different concentrations (0.1, 0.25, and 0.5 μmol/l) were collected, washed and resuspended in 1 ml of RPMI with 10% FCS containing 300 μmol/l DCFH-DA. To monitor the effect of imatinib on intracellular ROS levels in K562 BCR-ABL positive cells, HL-60 cells were taken as a ROS/BCR-ABL negative control. Samples were incubated for 30 min in the dark at 37°C, washed to remove unreacted dye, read on the FL-1 channel of a Becton Dickinson FACScan (San Jose, California, USA), and analyzed using CellQuest software. In all experiments, the mean fluorescence intensity from 10 000 cells was analyzed and calculated. All data were expressed as mean ± SEM of DCF fluorescence of these cells and calculated as a percentage of increase in fluorescence of control cells (without treatment with AT, BSO, or imatinib).

### Cell viability

Cell viability was determined by measuring the mitochondrial conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, GE Healthcare) to a colored product [22]. K562 cells were seeded at a density of $2 \times 10^4$ cells into each well of a 96-well culture plate with 200 μl of the culture medium with 10% of FCS. Cells were treated with imatinib (0.5–10.0 μmol/l) for different incubation times (24, 48, and 72 h) or with a well-known ROS drug inductor, Trisenox (arsenic trioxide, $As_2O_3$, Cell Therapeutics, Seattle, Washington, USA) starting with the clinical concentration of 2.0 μmol/l, and then 5.0 and 10.0 μmol/l for 24 h of incubation. Four hours before the end of each time of incubation, the MTT reagent (0.5 mg/ml) was added to each well. The formazan crystals were solubilized with dimethylsulfoxide and measured at 570 nm [22].

### Apoptosis assay

Apoptotic cells were detected using fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) assay (Apoptosis Detection Kit–Genzyme Diagnostics, Genzyme Corporation, Cambridge, Massachusetts, USA) as described earlier [23]. In general, $10^6$ K562 cells in each condition previously treated or not with BSO or AT, were washed twice with PBS after 24 h of incubation with imatinib (0.5, 1.0, and 5.0 μmol/l). After incubation, the cells were labeled by annexin V and PI for apoptosis and detection of necrosis, in binding buffer according to the manufacturer's instructions. This assay distinguishes viable cells (annexin $V^-/PI^-$) from cells in early apoptosis (annexin $V^+/PI^-$), cells in late apoptosis or secondary necrosis (annexin $V^+/PI^+$), or cells undergoing necrosis (annexin $V^-/PI^+$). Fluorescence signals of FITC and PI were detected by FL1 at 530 nm and FL2 at 585 nm,

respectively, on a FACScan flow cytometer (Becton Dickinson). Data were analyzed using the CELLQuest (Becton Dickinson) software. For each analysis, 10 000 events were recorded.

### Western blot analysis

Cell lysates from K562 cells whether incubated earlier or not with AT or BSO, and treated or not with imatinib (1 and 5 μmol/l) or with Trisenox (2 μmol/l) for 24 h, were subjected to 15% SDS–polyacrylamide gel electrophoresis [23]. The gel was electrotransferred onto a nitrocellulose membrane (Hybond, GE Healthcare) in a buffer containing 25 mmol/l Tris, 192 mmol/l glycine, and 20% methanol at 2 mA/cm$^2$ for 2 h at 25°C. The membrane was blocked with Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 and 5% nonfat dry milk for 2 h. After this, the membrane was washed three times (10 min for each washing) with Tris-buffered saline containing 0.1% Tween 20 and then incubated overnight with an antisurvivin antibody (1 : 1000) (R&D Systems Inc., Minneapolis, Minnesota, USA) at 4°C. After washing (3 × 10 min), the membrane was incubated with antirabbit IgG antibody conjugated with horseradish peroxidase (1 : 1000), and the antibody complexes were visualized by the ECL detection system as according to the manufacturer's instructions (GE Healthcare) [23]. The membrane was reprobed with a monoclonal antibody against β-actin (1 : 2000) (Sigma) as a loading control. Immunoreactive bands were quantified using the Sigma Gel Program.

### Data analysis

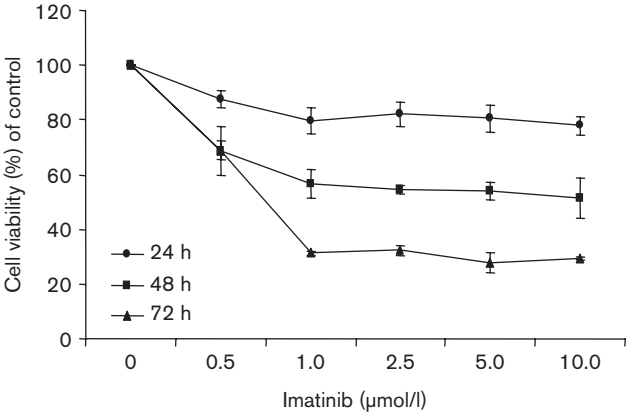
The values in this study were expressed as mean ± SE using at least three independent experiments. All data were analyzed using one-way analysis of variance (ANOVA). A $P$ value of less than 0.05 was considered as statistically significant.

## Results

### Dose–response and time–response curves of imatinib in K562 cells

To determine whether the time and dose of imatinib in which K562 cells were less responsive, imatinib dose–response curves for K562 cells were determined in three different times of incubation (24, 48, and 72 h). K562 cells were significantly ($P < 0.05$) less responsive when incubated for 24 h with imatinib, compared with 48 and 72 h, in all the concentrations tested (range: 0.5–10.0 μmol/l). Significant differences were observed in K562 cells viability percentage only between 0.5 and 10.0 μmol/l ($P < 0.05$), but not in 1.0–10.0 μmol/l ($P > 0.05$) of imatinib (Fig. 1).

**Fig. 1**




Dose and time response curves of K562–imatinib responsive. Imatinib dose–response curves for K562 cells were determined in three different times of incubations (24, 48, and 72 h). The cell viability was tested by MTT assay and expressed by percentage. K562 cells were significantly ($P < 0.05$) more resistant when incubated for 24 h with imatinib compared with 48 and 72 h. Significant differences are observed ($P < 0.05$) in K562 cells viability percentage only between 0.5 and 10.0 μmol/l, but not ($P > 0.05$) from 1.0 to 10.0 μmol/l of imatinib in 24, 48, and 72 h of incubation time. Values are mean ± SE of three independent experiments performed in triplicate.

### Effect of imatinib on intracellular reactive oxygen species production

As BCR-ABL tyrosine kinase induces production of ROS, we compared intracellular ROS amount in K562 and HL-60. Imatinib reduced the relative levels of intracellular ROS only in BCR-ABL-positive K562 cells. The intracellular ROS in K562 is five times higher than in BCR-ABL negative HL-60 cells (Fig. 2a).

Suppression of catalase activity by AT enhanced ROS in 26% and the inhibition of GSH synthesis by BSO increased the ROS amount in 13% in comparison with the control (Fig. 2b). Intracellular ROS levels, however, were significantly ($P < 0.05$) increased when K562 cells were previously treated with both inhibitors (Fig. 2b). Nevertheless, in all three conditions of K562 treated earlier (with AT, BSO, or both AT and BSO) and when incubated with imatinib for 1 h, ROS levels dropped to 50% in 0.25 and 0.5 μmol/l of imatinib (Fig. 2b).

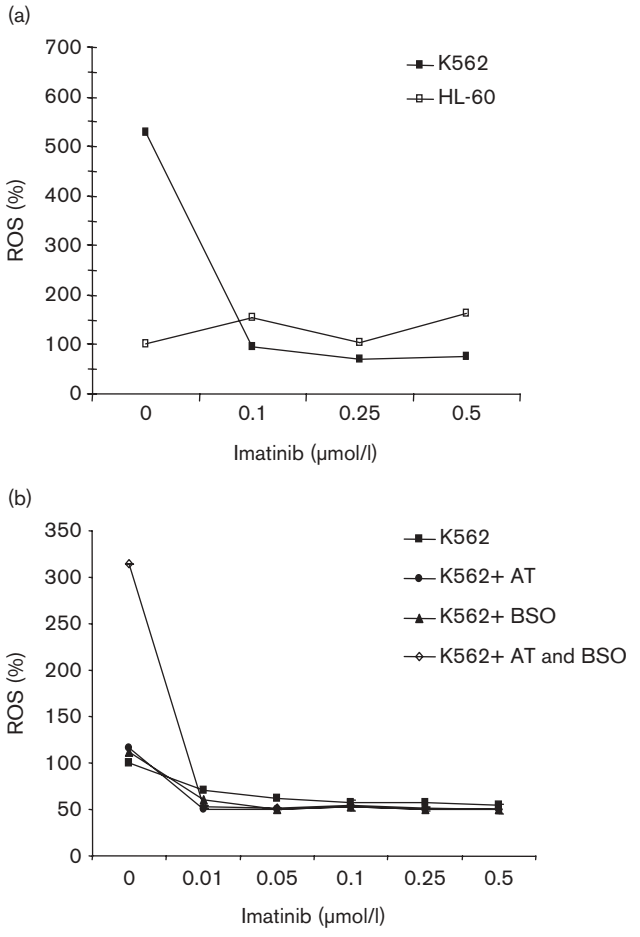
### Effect of imatinib on K562 cells apoptosis induction index in K562 cells

The efficacy of imatinib on apoptosis induction was evaluated, first in K562 cells after 24 h of incubation with imatinib at the concentrations of 0.5, 1.0, and 5.0 μmol/l (Fig. 3). Early apoptosis index induced by imatinib, was significantly higher ($P < 0.05$) than controls in all the concentrations tested (Fig. 3).

### Role of reactive oxygen species in imatinib apoptosis induction and survivin downregulation in K562 treated or not earlier with 3-amino-1,2,4-triazole or L-buthionine (*S,R*)-sulfoximine

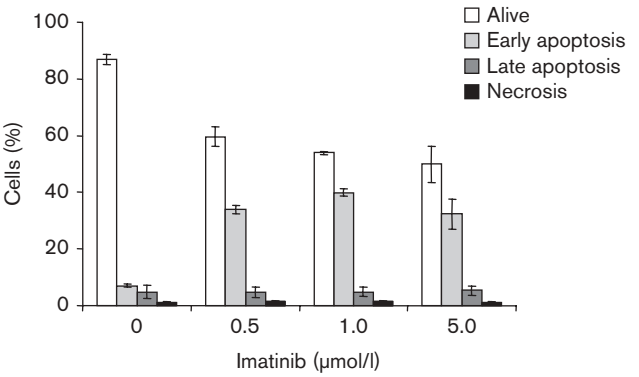
To address the question of whether the resistance to imatinib could also be counteracted by GSH, another ROS scavenger, K562 cells, treated earlier with BSO were also analyzed (Fig. 4a). Untreated K562 cells and those

**Fig. 2**




Intracellular reactive oxygen species (ROS) production. The intracellular ROS production was modulated by increasing concentration of imatinib (0.01–0.5 μmol/l) in positive BCR-ABL K562 and negative BCR-ABL HL-60 cells (a). The intracellular ROS production was modulated by increasing concentration of imatinib (0.01–0.5 μmol/l) in K562 cells previously treated or not with catalase activity inhibitor aminotriazol (AT), with glutathione synthesis inhibitor buthionine sulphoximine (BSO) or with both inhibitors (b). Intracellular ROS detection was performed with 2′,7′-dichlorofluorescein diacetate and the fluorescence measured by FACScan. Data are representative of three independent experiments.

**Fig. 3**




Effect of imatinib on K562 cells early apoptosis index after 24 h of incubation. Imatinib early apoptosis induction started with 0.5 μmol/l and was significantly ($P<0.05$) higher than control. No significant ($P>0.05$) differences were observed in late apoptosis and necrosis index in imatinib K562 cells treated and control. Values are mean ± SE of three independent experiments performed in triplicate.

treated earlier with AT or BSO were incubated with imatinib (0.5, 1.0, and 5.0 μmol/l) and hydrogen peroxide ($H_2O_2$) 1.0 μmol/l, a well-known apoptosis inductor inhibited by catalase and GSH (Fig. 4a). The effects of imatinib apoptosis were significantly ($P < 0.05$) enhanced only in K562 treated earlier with AT (Fig. 4a). In addition, survivin expression in cells treated earlier with AT and incubated with imatinib 1 and 5 μmol/l diminished in 30 and 70%, respectively (Fig. 4b and c), compared with K562 not treated with AT. Enhancement of K562 imatinib sensibility was not observed by GSH inhibition (Fig. 4a) even in experiments conducted for 48 and 72 h (data not shown). Survivin expression in cells treated with BSO remained the same as control (data not shown).

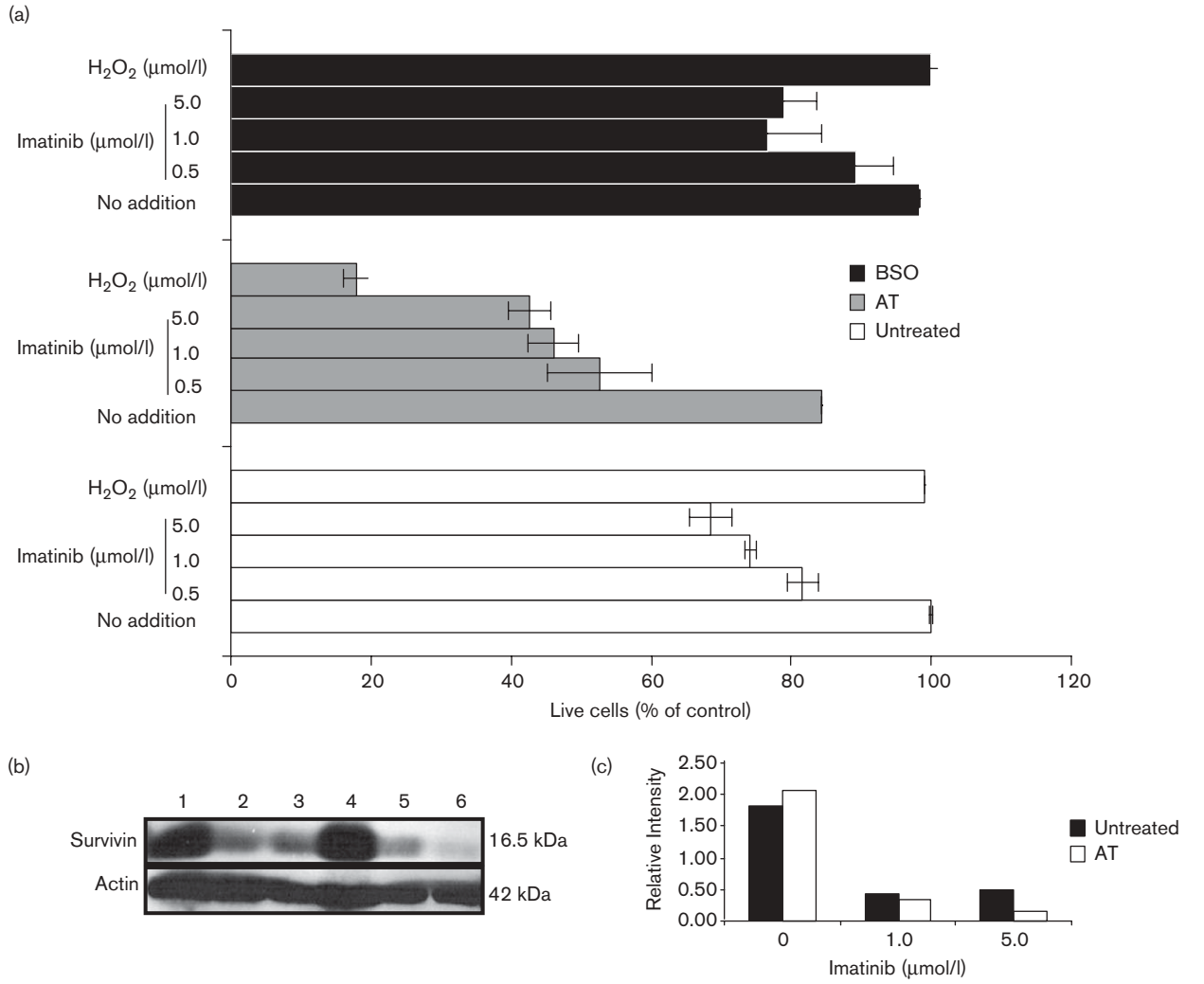
### Role of reactive oxygen species in Trisenox cell viability induction and survivin downregulation in K562 treated earlier or not with 3-amino-1,2,4-triazole or L-buthionine (*S,R*)-sulfoximine

Trisenox apoptosis activity is enhanced by GSH modulation in CML cells [24]. As $H_2O_2$ induces apoptosis through survivin inhibition in many tumor cells [11–13] and $H_2O_2$ is mainly the ROS-induced cell death of Trisenox, we decided to investigate whether Trisenox is able to induce survivin inhibition in K562 cells. First, a dose–response curve with K562 cells treated earlier with AT, BSO, and AT combined with BSO was created (Fig. 5a). Then, survivin expression was evaluated in K562 cells treated earlier or not with AT or BSO and incubated with Trisenox (2 μmol/l). Survivin expression levels remained the same in all conditions tested in comparison with the control (Fig. 5b).

## Discussion

Although a new range of anticancer agents that target BCR/ABL tyrosine kinase molecules has been developed [3,4,6], imatinib is considered a highly efficacious first-line treatment for patients newly diagnosed with CML [24]. However, distinct patterns of mutations in Abl kinase proteins that alter imatinib binding or favor kinase conformations inaccessible to imatinib, are a major problem in CML-BP patients and a common finding associated with clinical resistance [1,7,8]. Survivin overexpression has been observed in accelerated/blastic phase of CML patients [25]. In addition, survivin disruption sensitizes imatinib-responsive cells [2]. Moreover, it was described that GSH hinders the arsenic trioxide and sulindac apoptosis induction through ROS-dependent survivin downregulation in human lung cancer cells [11].

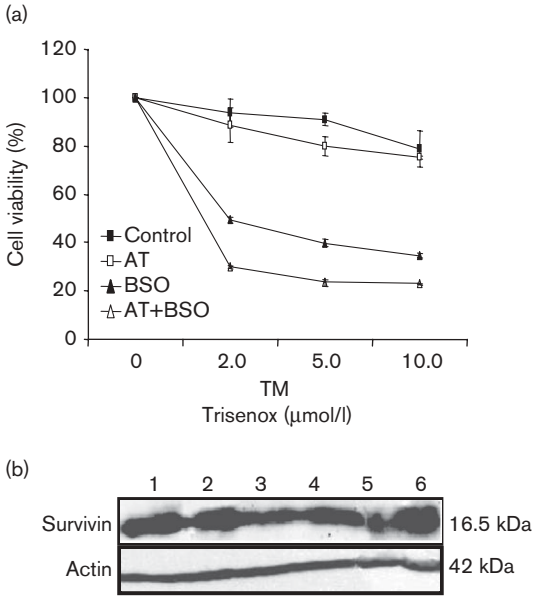
**Fig. 4**




Effect of catalase activity inhibition on the restoration of K562 imatinib (IM) sensitivity and survivin downregulation. K562 cells were previously treated with aminotriazole (AT) for 2 h for catalase activity inhibition and buthionine sulfoximine (BSO) for 24 h for glutathione synthesis inhibition as described in Materials and methods. No treated K562 cells and those previously treated with AT and BSO were incubated with IM mesylate (0.5, 1.0, and 5.0 μmol/l) and $H_2O_2$ 1.0 μmol/l for 24 h. The apoptosis was analyzed by annexin V-fluorescein isothiocyanate conjugated and propidium iodide and then by flow cytometer. Only cells previously treated with AT had their apoptosis significantly enhanced when compared with no treated cells ($P<0.05$). BSO had no effect on sensitivity to IM in K562 cells ($P>0.05$). Values are mean ± SE of three independent experiments (a). Downregulation of survivin by the IM/aminotriazole (AT) combination. Western blot for survivin in K562 cells (1) untreated, (2) incubated with IM 1 μmol/l and (3) IM 5 μmol/l, (4) previously treated with AT for catalase activity inhibition, (5) treated with AT + IM 1 μmol/l or (6) AT + IM 5 μmol/l for 24 h (b). Densitometric analysis and relative intensity of the modulation of survivin gene product. (c) Data are from one of two independent experiments with similar results.

In this study, we demonstrated the correlation among intracellular ROS levels, apoptosis index and survivin expression in K562 cells in the presence of increasing concentrations of imatinib. Survivin expression was ROS dose-dependent in K562 cells. Survivin expression levels were significantly reduced in cells incubated with imatinib for 24 h and treated earlier with AT (30 and 70%, respectively, for 1 and 5 μmol/l of imatinib) compared with AT untreated cells as observed through western blot analysis (Fig. 4b and c). The mean plasma concentration in CML patients 24 h after 400 mg of oral drug administration is 1 μmol/l [2,3,8–10]. Here we showed that the suppression of catalase activity in K562 cells enhanced apoptosis induction in 25% in the final concentration of 0.5 μmol/l of imatinib, which is lower than the mean plasma concentration. These effects were not observed when GSH synthesis was inhibited suggesting that survivin inhibition and apoptosis induction might not be through $H_2O_2$, a common substrate for both antioxidant catalase and GSH [16]. As expected, Trisenox, a well-known $H_2O_2$ apoptosis inductor [11,26], inhibited the growth of cells treated earlier with

**Fig. 5**




Trisenox, a known $H_2O_2$-apoptotic inductor, dose–response curves for K562 cells were determined in three different concentrations (2.0, 5.0, and 10.0 μmol/l). K562 cells not treated (control) and previously treated with aminotriazole (AT), buthionine sulfoximine (BSO), and a combined treatment with AT and BSO (AT + BSO) were incubated for 24 h. The cell viability was tested by MTT assay and expressed by percentage. No significant differences were observed between control and K562 cells previously treated with AT in the Trisenox clinical concentration (2.0 μmol/l). Significant differences were observed among cells previously treated with BSO and AT with BSO and control in all concentrations tested ($P < 0.05$). Values are mean ± SE of three independent experiments performed in triplicate. (a) Western blot for survivin expression in K562 cells (1) untreated, (2) incubated with Trisenox (2.0 μmol/l), (3) previously treated with AT for catalase activity inhibition, (4) treated with AT + Trisenox (2.0 μmol/l), or (5) previously treated with BSO for glutathione synthesis inhibition, and (6) incubated with BSO + Trisenox (2.0 μmol/l) for 24 h. Data are from one of two independent experiments with similar results (b).

BSO and enhancement of this inhibition was observed when both antioxidants were suppressed. Trisenox apoptosis induction, however, was not through survivin inhibition. It was described that catalase can also consume nitric oxide (NO) [27], a reactive nitrogen oxide species molecule known to induce apoptosis in many tumor cells [28] including leukemia [29]. As nitric oxide induces apoptosis through survivin downregulation [30,31], these findings might explain the current results. Altogether, the results reported here show that catalase could be a potential molecular target for increasing imatinib sensitivity in CML-BP cells.

Further studies with drugs that are clinically available and that inhibit catalase activity should be explored. It was described that an inotropic agent for treatment of congestive heart failure was able to inhibit catalase activity in myeloid cells [32]. Combinations of drugs with imatinib represent an emerging therapeutic concept for preventing resistance formation in CML [32]. Therefore, testing drugs that are commercially available with potentially less cytotoxicity, which modulates catalase activity might be an important path to increase imatinib sensitivity in imatinib-responsive cells and to hinder resistance formation, especially in CML-BP cells.

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