Antimony-trioxide- and arsenic-trioxide-induced apoptosis in myelogenic and lymphatic cell lines, recruitment of caspases, and loss of mitochondrial membrane potential are enhanced by modulators of the cellular glutathione redox system

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ORIGINAL ARTICLE

Antimony-trioxide- and arsenic-trioxide-induced apoptosis in myelogenic and lymphatic cell lines, recruitment of caspases, and loss of mitochondrial membrane potential are enhanced by modulators of the cellular glutathione redox system

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Abstract During the last years remission rates of more than 72% for arsenic(III)-oxide (As_2O_3) treatment in relapsed or refractory acute promyelocytic leukemia have been published. As_2O_3 is under clinical investigation for therapy of leukemia and solid tumors. Due to the chemical affinity of arsenic and antimony, we analyzed the potency of antimony (III)-oxide (Sb_2O_3) to exert As_2O_3-like effects. Based on the same molar concentrations, lower efficacy in apoptosis induction and caspase-independent decrease of mitochondrial membrane potential was observed for Sb_2O_3. No difference in sensitivity to As_2O_3 or Sb_2O_3 was detected in CEM cells when compared to their multiple drug resistant derivatives. Apoptosis was induced by combining subapoptotic concentrations of Sb_2O_3 or As_2O_3 with subapoptotic concentrations of DL-buthionine-[*S*,*R*]-sulfoximine (BSO). Other modulators of the cellular redox system showed this effect to a lower extent and enhancement was not consistent for the different cell lines tested. Caspase inhibitors protected cell lines from Sb_2O_3- and As_2O_3-induced apoptosis. When BSO was added, the inhibitors lost their protective ability. The ability of modulators of the cellular redox system in clinically applicable concentrations to enhance the apoptotic effects of the two oxides in a synergistic way may be helpful to reduce their toxicity by optimizing their dose.

Keywords Apoptosis · Arsenic · Antimony · Buthionine sulfoximine · Glutathione · Redox system

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Introduction

During the last 10 years, several groups demonstrated remission of more than 72% for arsenic(III)-oxide (As_2O_3) treatment in relapsed or refractory acute promyelocytic leukemia (APL), as presented in various reviews [1–5]. As_2O_3 was established as a potent alternative in therapy of all-*trans* retinoic acid (ATRA)-resistant APL and shows, like Sb_2O_3, similar cellular effects to ATRA in APL: localization of the t(15;17) specific PML-RARα-fusion protein in

nuclear bodies and degradation of the fusion protein [6–9] and induction of differentiation and apoptosis [10–14]. Lately, we showed that induction of apoptosis by As_2O_3 in 22 myeloid and non-myeloid malignant cell lines of different sensitivity for cytostatic drugs, is based on breakdown of the mitochondrial membrane potential (Ψ_m) and therefore the redox system of the cell is one of the primary targets of As_2O_3. Activation of caspases is a downstream effect occurring after the breakdown of Ψ_m [15–17] and release of radical oxygen species (ROS) [18–21].

Both antimony and arsenic are members of the Vth group of the table of elements and share common chemical characteristics. Based on the affinity of As_2O_3 and Sb_2O_3 we analyzed whether Sb_2O_3 shows similar efficacy and identical mechanisms of action by investigation of the effects of Sb_2O_3 on representative myeloid cell lines of different sensitivity groups for As_2O_3 as classified in our previous report [22]. Given the increasing clinical use of As_2O_3, we were, in particular, interested in combinations that might be transferable to clinical application. Inhibitors of the cellular glutathione system were used to modulate the efficacy of As_2O_3 and Sb_2O_3 to induce apoptosis. The modulators analyzed in this report include the γ-glutamylcystein synthetase inhibitor DL-buthionine-[*S*,*R*]-sulfoximine (BSO), the glutathione peroxidase (GPx) inhibitor mercaptosuccinic acid (MS), as well as sodium ascorbate (NaAsc), sodium salicylate (NaSal), and 3-amino-1,2,4-azole (AT). Ascorbate was shown to decrease in glutathione-deficient rats and ascorbate spares glutathione and protects [23], NaSal potentates the toxicity of BSO in the E47 cells [24] and AT inhibits catalase in the presence of H_2O_2 and inhibits cyclizations leading to β-carotene and Φ-carotene accumulates. As far as data were achievable and tolerable concentrations of the different substances in vivo were available (BSO, NaAsc, NaSal), we have chosen concentrations within this range. For As_2O_3 and Sb_2O_3 we have chosen incubation times and concentrations as we previously published for As_2O_3 [15, 17, 22]. The As_2O_3 concentrations are in the range of peak values of clinically achievable As_2O_3 plasma levels as shown by pharmacokinetic analysis [10].

Materials and methods

Cell culture

Cell lines CCRF-CEM, HL-60, K-562, and LOUCY were purchased from The German Collection of Microorganisms

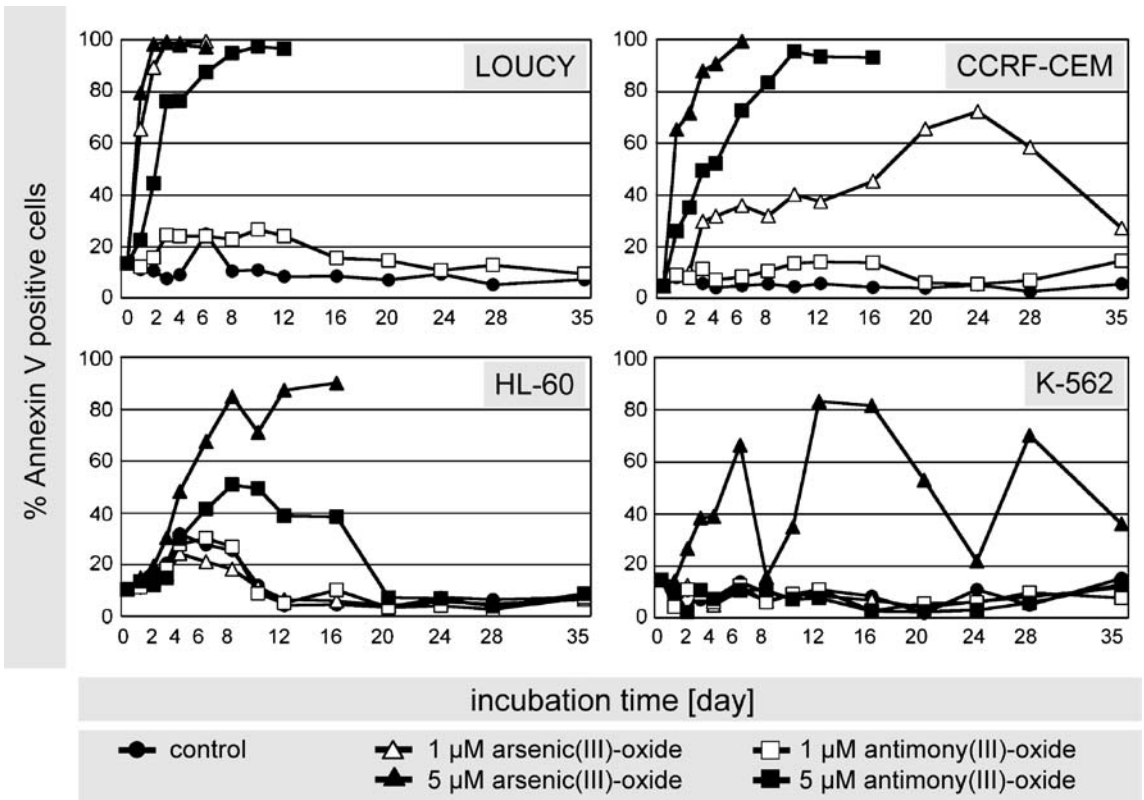

Fig. 1 Sb_2O_3- and As_2O_3-induced apoptosis in myeloid and lymphatic cell lines. Cell lines LOUCY, CCRF-CEM, HL-60, and K-562 were treated with PBS (*black circle*), 1 µM (*white square*) or 5 µM (*black square*) Sb_2O_2 or 1 µM (*white triangle*) or 5 µM (*black triangle*) As_2O_3 for 35 days. The percentage of 7-AAD-positive cells was determined before incubation and after 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, and 35 days by flow cytometry

and Cell Cultures (DSMZ, Braunschweig, Germany), CEM/C1, CEM/C2, HL-60/MX1, and HL-60/MX2 from the American Type Culture Collection (Manassas, USA). The doxorubicin resistant cell lines K-562(0.02) and K-562 (0.1) were described previously [22].

Induction of apoptosis with antimony(III)-trioxide (Sb_2O_3), arsenic(III)-trioxide (As_2O_3) and cytostatic drugs

Apoptosis was induced with freshly prepared aqueous stock solutions of 1 mM Sb_2O_3 (Fluka, Buchs, Switzerland) or 1 mM As_2O_3 (SIGMA, Deisenhofen, Germany) in PBS without Ca^{2+}/Mg^{2+} (Invitrogen/GIBCO Life Technologies, Karlsruhe, Germany). Due to its low solubility in H_2O, Sb_2O_3 had to be dissolved in HCl_{conc} before dilution in PBS. The pH of Sb_2O_3-solutions was determined for each assay and a separate control with the corresponding amount of PBS without Ca^{2+}/Mg^{2+} and the HCl_{conc}-adjusted adequate pH was used for each experimental approach. Stock solutions of 10 mM of (*S*)-(+)-camptothecin (SIGMA, Deisenhofen, Germany) in dimethyl sulfoxide (Sigma, Deisenhofen, Germany), 10 mM mitoxantrone hydrochloride (SIGMA, Deisenhofen, Germany) and 1 mg/mL doxorubicin hydrochloride (SIGMA, Deisenhofen, Germany) in 70 % ethanol (Carl ROTH GmbH, Karlsruhe, Germany) were used for further dilutions. Cells were seeded according to their optimal growth condition indicated by the provider.

Annexin V-FITC- and 7-AAD-staining

As described previously [22], the percentage of apoptotic cells was determined by two-color flow cytometry using an Annexin V-FITC (BD Pharmingen, Heidelberg, Germany) and 7-amino-actinomycin D (7-AAD) system (Sigma, Deisenhofen, Germany). Whenever possible, fluorescence data of 50.000 cells was acquired using fluorescence channels FL-1 and FL-3 of a FACScan (Becton Dickinson, Heidelberg, Germany). All experiments presented were performed three times, with the exception of those presented in Figs. 1, 5, and 6. In general, flow cytometry analysis was performed until the percentage of apoptotic cells was higher than 90% and acquisition of data was preformed for two further time points.

MitoTrackerRed CMXRos staining

MitoTrackerRed CMXRos (Molecular Probes, Leiden, The Netherlands) is a fluorescent dye that is enriched in mitochondria in a membrane potential dependent manner and reacts with mitochondrial proteins [25, 26]. Therefore, MitoTrackerRed CMXRos, can be used to detect cells with a loss of Ψ_m [27, 28]. The CMX group binds to SH groups of mitochondrial proteins and is retained in living cells, while unbound dye can be washed out of the cells. Staining was performed as described in a previous report in 1 mL cell suspension (ca. 5×10^5 to 1×10^6 cells) in the presence of 200 nM MitoTrackerRed CMXRos. After 45 min, cells were washed twice in PBS without Ca^{2+}/Mg^{2+} and the change of fluorescence intensity was measured by flow cytometry [15]. In order to compare the percentage of

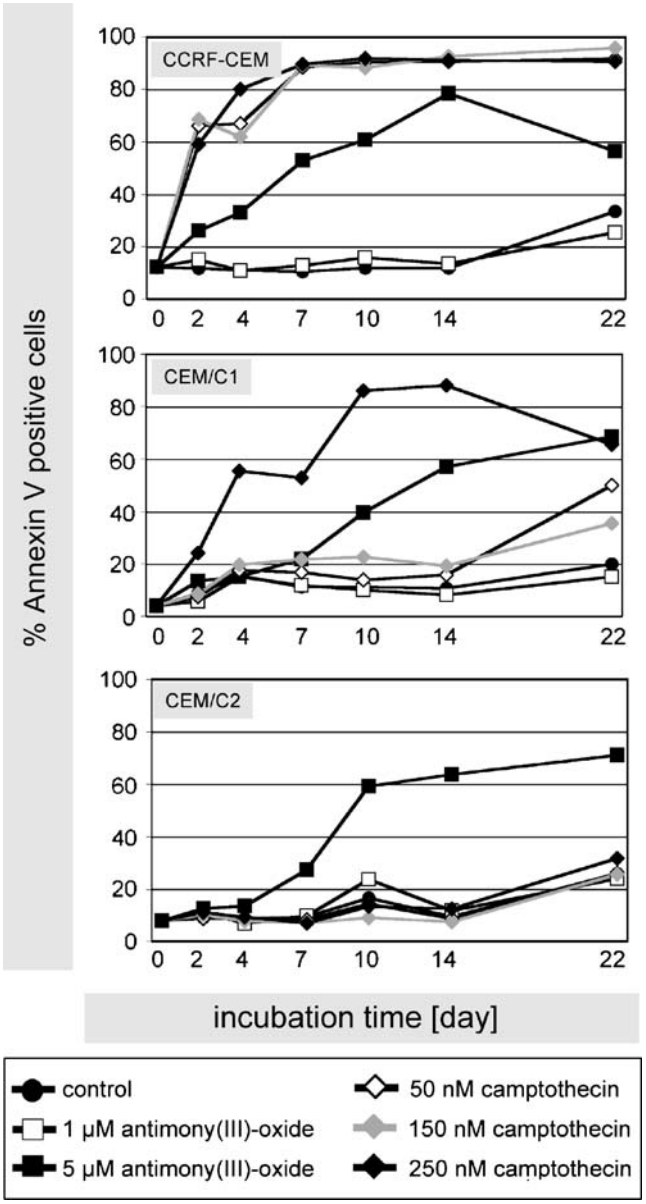

Fig. 2 Sb_2O_3-induced apoptosis occurs in camptothecin-resistant CCRF-CEM derivatives CEM/C1 and CEM/C2. Incubation of the cell lines CCRF-CEM, CEM/C1 and CEM/C2 was performed with PBS (*black circle*), 1 µM (*white square*) and 5 µM (*white square*) Sb_2O_2 as well as with 50 nM (*gray rhomb*), 150 nM (*white rhomb*) and 500 nM (*black rhomb*) camptothecin for 22 days. The percentage of 7-AAD-positive cells was determined before incubation and after 2, 4, 7, 10, 14, and 22 days

apoptotic cells (as measured by 7-AAD and Annexin V *positivity* in flow cytometry) and the percentage of cells with reduced Ψ_m, the following presentation was chosen for MitoTrackerRed CMXRos staining: the fluorescence intensity of untreated control cells was set to approximately 10^2 as described by the manufacturer. The percentage of cells with reduced staining pattern for MitoTrackerRed CMXRos (compared to the untreated controls) were referred as MitoTrackerRed CMXRos-*negative*. The percentage of the population with reduced fluorescence intensity (*negative* population) is shown in the diagrams, as these cells did not accumulate the dye in their mitochondria due to their *reduced* Ψ_m.

Incubation with caspase inhibitors

Cells were washed in PBS without Ca^{2+}/Mg^{2+} and resuspended at a density of $2–4\times10^5$ cells/mL in serum free QBSF51® medium (Sigma, Deisenhofen, Germany). Caspase inhibitors Boc-D(OMe)-Fmk, *Z*-VAD-FMK and Z-D(OMe)-E(OMe)-VD(OMe)-Fmk (all from ALEXIS Biochemicals, Grünberg, Germany) were dissolved in DMSO and used in a final concentration of 50 µM. CCRF-CEM and HL-60 cells were incubated (37°C, 6% CO_2, 98% humidity) in the presence of the inhibitors for 1 h prior to cultivation with Sb_2O_3 or As_2O_3.

Incubation with modulators of the cellular GSH-redox system

Fresh stock solutions of 100 mM DL-buthionine-[*S*,*R*]-sulfoximine (Sigma, Deisenhofen, Germany), 500 mM sodium salicylate (Sigma, Deisenhofen, Germany), 500 mM sodium ascorbate (Sigma, Deisenhofen, Germany), 100 mM mercaptosuccinic acid (Sigma, Deisenhofen, Germany) and 20 mM 3-amino-1,2,4-azole (Sigma, Deisenhofen, Germany) were prepared in PBS without Ca^{2+}/Mg^{2+} for each assay. All modulators of GSH were added together with PBS or the inducers of apoptosis (Sb_2O_3 and As_2O_3), when indicated.

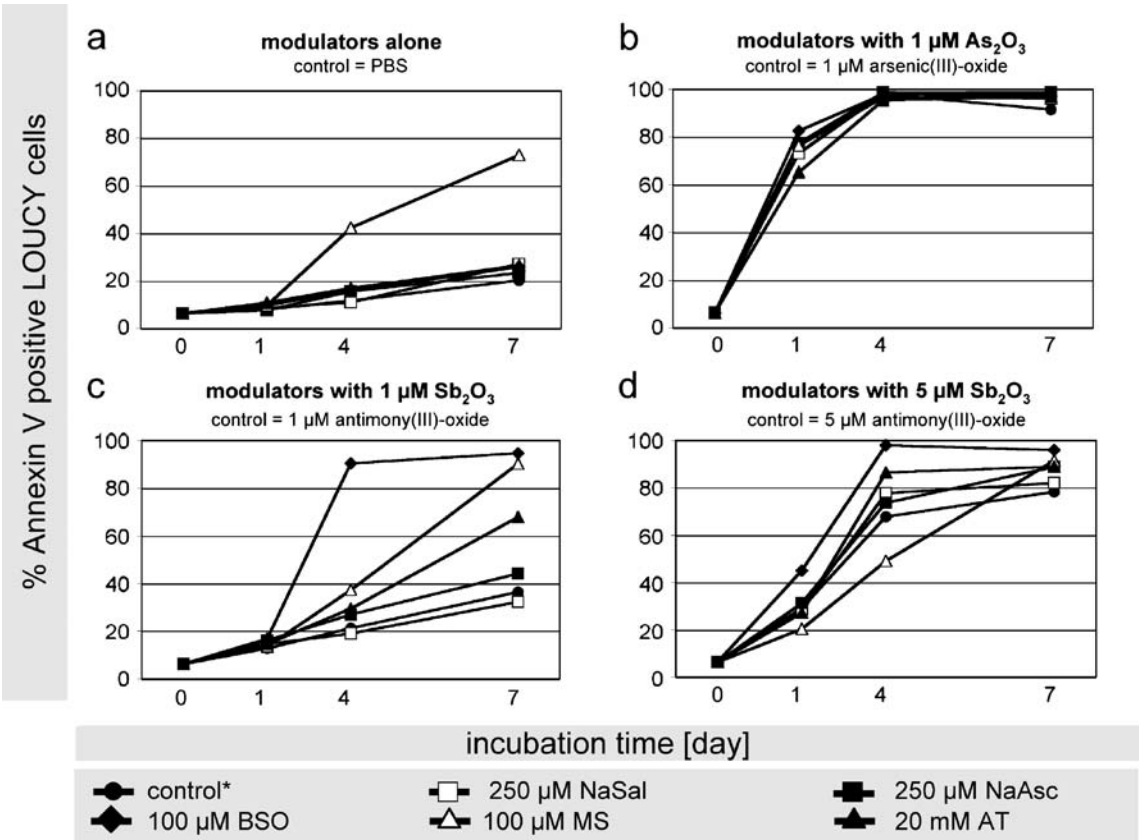

Fig. 3 Modulation of Sb_2O_3-induced apoptosis by NaSal, NaAsc, BSO, MS and AT in cell line LOUCY. Cell line LOUCY was incubated either with **a** PBS, **b** 1 µM As_2O_3, **c** 1 µM Sb_2O_3, or **d** 5 µM Sb_2O_3 alone (*black circle*) or in combination with 250 µM NaSal (*white square*), 250 µM NaAsc (*black square*), 100 µM BSO (*black rhomb*), 100 µM MS (*white triangle*) or 20 mM AT (*black triangle*) for 7 days. The percentage of cells positive for 7-AAD was determined before incubation and after 1, 2, 4, and 7 days. *The control shown in **a**, **b**, **c**, and **d** represents the corresponding assays with PBS or the inducers of apoptosis alone (Sb_2O_3 or As_2O_3) but without the modulators of Ψ_m

Results

Sb_2O_3 and As_2O_3 induce apoptosis in lymphohematopoietic cell lines

As we demonstrated in a recent report [22], concentrations of As_2O_3 that are achievable in the plasma of As_2O_3-treated APL-patients induced apoptosis in a variety of different lymphohematopoietic cell lines. We introduced a classification system according to the sensitivity of cell lines towards As_2O_3-induced apoptosis. To investigate the apoptosis-inducing potential of Sb_2O_3, we have chosen representative cell lines from the different sensitivity groups and incubated the cell lines LOUCY, CCRF-CEM, HL-60 and K-562 with either PBS, 1 μM As_2O_3, 5 μM As_2O_3, 1 μM Sb_2O_3 or 5 μM Sb_2O_3. Apoptosis was measured by staining with 7-AAD (Fig. 1) and Annexin V-FITC (data not shown). Based on experience with As_2O_3 that induced apoptosis in vivo and in some cell lines only after prolonged incubation [10, 22, 29, 30], we determined the percentage of apoptotic cells before incubation and after 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, and 35 days by flow cytometry.

Low concentration (1 μM) As_2O_3 induced apoptosis in LOUCY and CCRF-CEM-cells, whereas 1 μM Sb_2O_3 was not effective in any of the analyzed cell lines. When incubated with 5 μM As_2O_3, apoptosis occurred in all four cell lines. Only the MDR-1 positive cell lines K-562 was resistant to the high concentration of Sb_2O_3 (5 μM). We observed a high correlation between both methods used for the detection of apoptotic cells, Annexin V-FITC- and 7-AAD-staining: correlation coefficients were 0.999 for LOUCY, 0.994 for CCRF-CEM, 0.934 for HL-60, 0.815 for K-562, and 0.940 over all, respectively.

Sb_2O_3 and As_2O_3 induce apoptosis in cytostatic-resistant cell lines of the lymphohematopoietic system

Sensitivity to induction of apoptosis by As_2O_3 did not differ between CCRF-CEM and the less cytostatic-sensitive deriv-

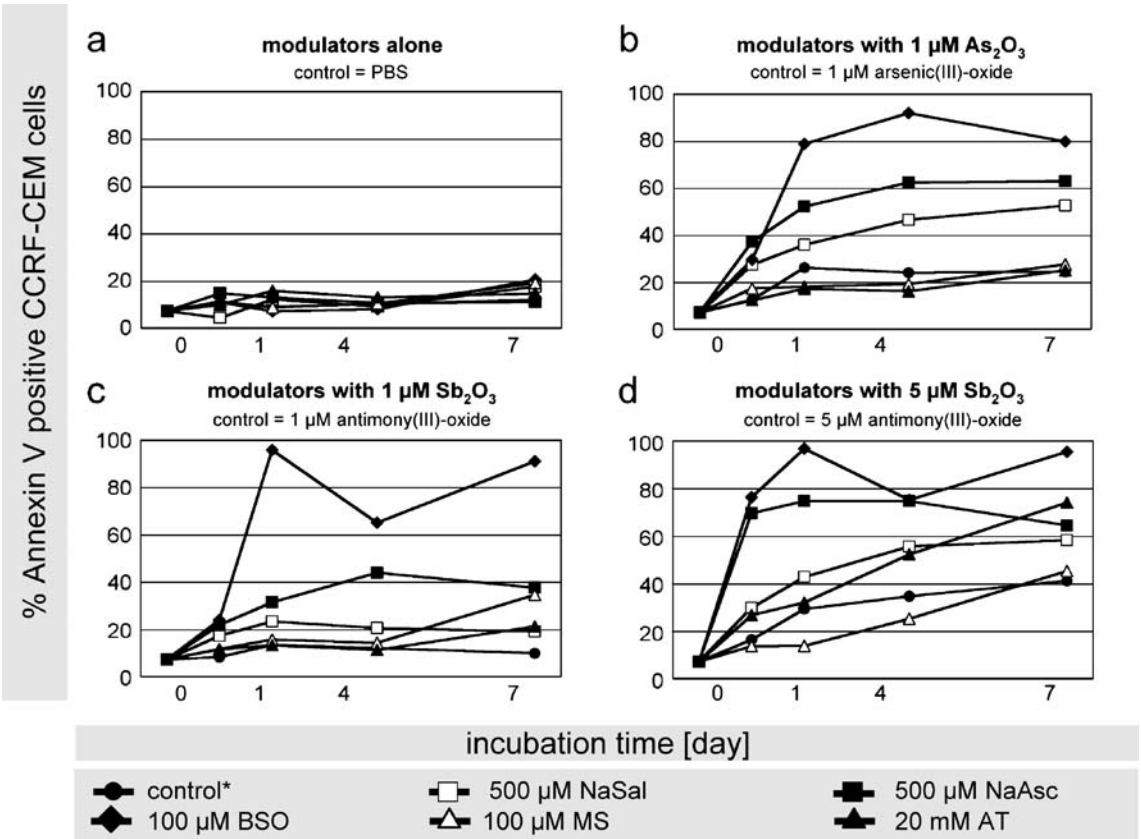

Fig. 4 Enhancement of Sb_2O_3-induced apoptosis by NaSal, NaAsc, BSO, MS, and AT in cell line CCRF-CEM. Cell line CCRF-CEM was incubated either with **a** PBS, **b** 1 μM As_2O_3, **c** 1 μM Sb_2O_3, or **d** 5 μM Sb_2O_3 alone (*black circle*) or in combination with 500 μM NaSal (*white square*), 500 μM NaAsc (*black square*), 100 μM BSO (*black rhomb*), 100 μM MS (*white triangle*) or 20 mM AT (*black triangle*) for 7 days. The percentage of cells positive for 7-AAD was determined before incubation and after 1, 2, 4, and 7 days. *The control shown in (a), (b), (c), and (d) represents the corresponding assays with PBS or the inducers of apoptosis alone (Sb_2O_3 or As_2O_3) but without the modulators of Ψ_m

atives CEM/C1 and CEM/C2 [22]. This prompted the question whether also Sb_2O_3 can induce apoptosis in these two cell lines with multiple drug resistance [31, 32]. Figure 2 summarizes data on Sb_2O_3- and camptothecin-treatment of CCRF-CEM, CEM/C1, and CEM/C2 cells which were incubated with Sb_2O_3 (1 μM or 5 μM) or camptothecin (50–500 nM; positive control for apoptosis induction) for up to 22 days. Based on equimolar concentrations, As_2O_3 was generally more potent as compared to Sb_2O_3.

CCRF-CEM cells were insensitive to 1 μM Sb_2O_3 but sensitive to 5 μM Sb_2O_3 and all concentrations of camptothecin. CEM/C1 cells showed less sensitivity to 50 and 150 nM camptothecin, however, were still sensitive to 5 μM Sb_2O_3. Cell line CEM/C2 that was resistant to camptothecin up to 500 nM still retained sensitivity to 5 μM Sb_2O_3. In contrast to As_2O_3 [22], no prolonged incubation time was necessary to induce apoptosis with increasing resistance to camptothecin for Sb_2O_3. Again, there was a high correlation between both methods used for the detection of apoptotic cells, Annexin V-FITC- and 7-AAD-staining: correlation coefficients were 0.973 for CCRF-CEM, 0.974 for CEM/C1, 0.961 for CEM/C2, and 0.978 over all, respectively.

The HL-60 mitoxantrone resistant derivatives HL-60/MX1 and HL-60/MX2 and the K-562 doxorubicin resistant derivatives K-562(0.02) and K-562(0.1) were insensitive to 1 μM Sb_2O_3 and 5 μM Sb_2O_3 (data not shown). As previously shown, these derivatives show reduced sensitivity to 1 μM As_2O_3 and 5 μM As_2O_3 as compared to their corresponding parental cell lines [22].

Increased sensitivity towards Sb_2O_3- and As_2O_3- induced apoptosis by modulators of the cellular GSH-system

We addressed the potential role of the glutathione redox system for Sb_2O_3- and As_2O_3-mediated apoptosis: cell lines LOUCY (Fig. 3), CCRF-CEM (Fig. 4), HL-60 (Fig. 5) and K-562 (Fig. 6) were treated with different substances known to influence Ψ_m or to modulate or inhibit the synthesis of glutathione.

Therefore, we incubated the indicated cell lines for 7 days (LOUCY) or 14 days (CCRF-CEM, HL-60, K-562) with

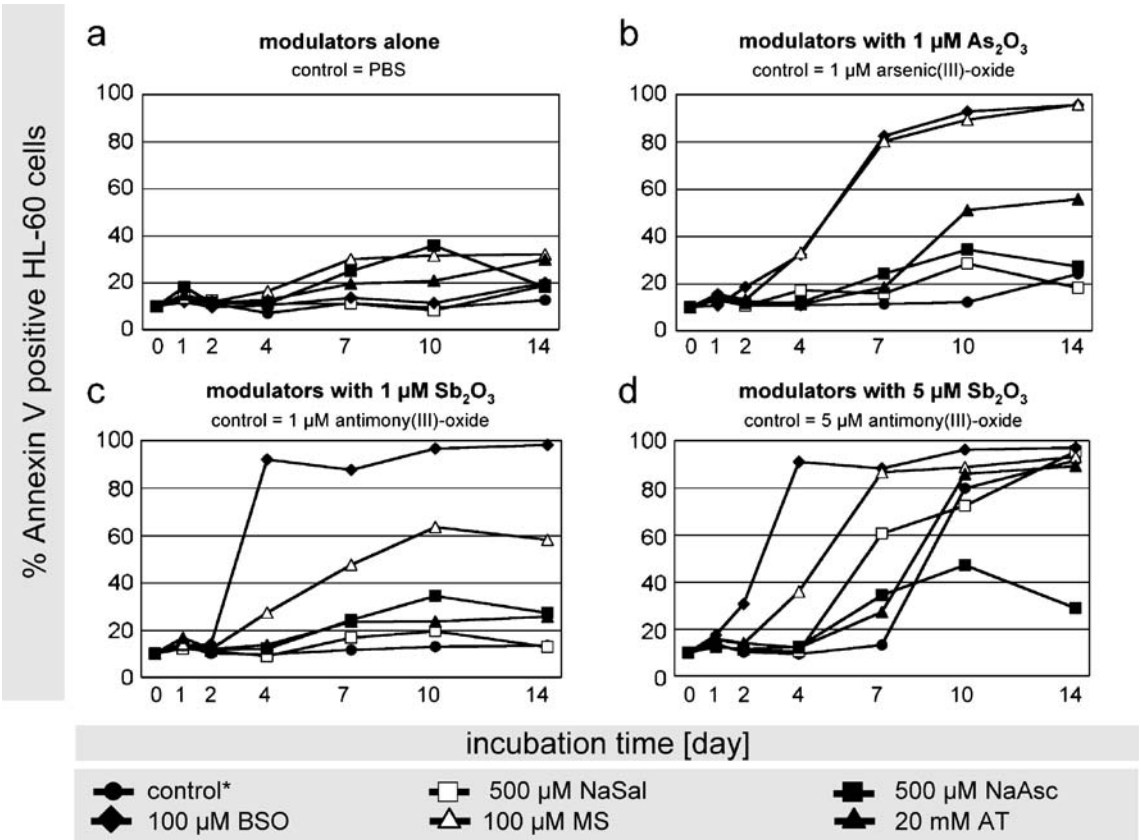

Fig. 5 Enhancement of Sb_2O_3-induced apoptosis by NaSal, NaAsc, BSO, MS, and AT in cell line HL-60. Cell line HL-60 was incubated either with **a** PBS, **b** 1 μM As_2O_3, **c** 1 μM Sb_2O_3 or **d** 5 μM Sb_2O_3 alone (*black circle*) or in combination with 1 mM NaSal (*white square*), 1 mM NaAsc (*black square*), 100 μM BSO (*black rhomb*), 100 μM MS (*white triangle*) or 20 mM AT (*black triangle*) for 14 days. The percentage of cells positive for 7-AAD was determined before incubation and after 1, 2, 4, 7, 10, and 14 days. *The control shown in **a**, **b**, **c**, and **d** represents the corresponding assays with PBS or the inducers of apoptosis alone (Sb_2O_3 or As_2O_3) but without the modulators of Ψ_m

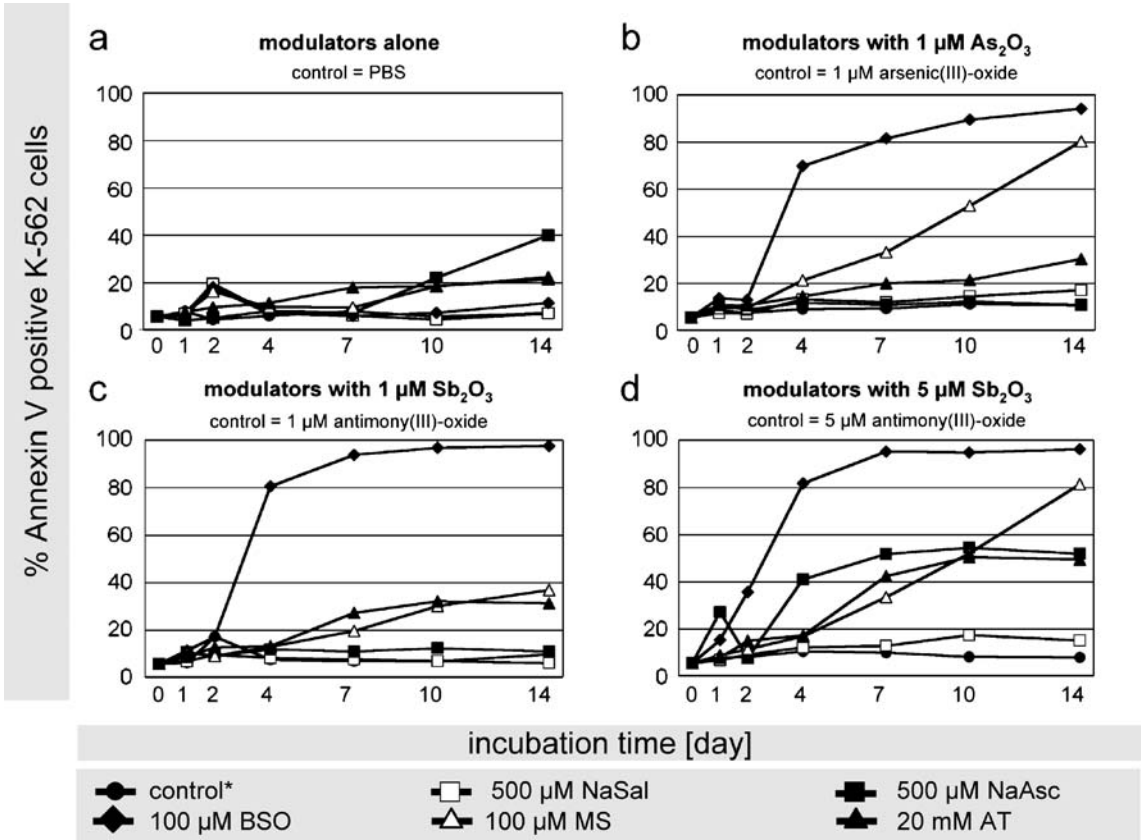

Fig. 6 Enhancement of Sb_2O_3-induced apoptosis by NaSal, NaAsc, BSO, MS and AT in cell line K-562. Cell line K-562 was incubated either with **a** PBS, **b** 1 µM As_2O_3, **c** 1 µM Sb_2O_3 or **d** 5 µM Sb_2O_3 alone (*black circle*) or in combination with 500 µM NaSal (*white square*), 500 µM NaAsc (*black square*), 100 µM BSO (*black rhomb*), 100 µM MS (*white triangle*) or 20 mM AT (*black triangle*) for 14 days. The percentage of cells positive for 7-AAD was determined before incubation and after 1, 2, 4, 7, 10, and 14 days. *The control shown in **a**, **b**, **c**, and **d** represents the corresponding assays with PBS or the inducers of apoptosis alone (Sb_2O_3 or As_2O_3) but without the modulators of Ψ_m

either PBS (Figs. 3a, 4a, 5a, 6a), 1 µM As_2O_3 (Figs. 3b, 4b, 5b, 6b), 1 µM Sb_2O_3 (Figs. 3c, 4c, 5c, 6c) or 5 µM Sb_2O_3 (Figs. 3d, 4d, 5d, 6d) alone or together with sodium salicylate (NaSal, 500 µM for LOUCY, CCRF-CEM and K-562, 1 mM for HL-60), sodium ascorbate (NaAsc, 500 µM for LOUCY, CCRF-CEM and K-562, 1 mM for HL-60), DL-buthionine-[*S*, *R*]-sulfoximine (BSO, 100 µM for all cell lines), mercaptosuccinic acid (MS, 100 µM for all cell lines) or 3-amino-1,2,4-azole (AT, 20 mM for all cell lines). The percentages of 7-AAD- and Annexin V-FITC (data not shown)-positive cells were determined before incubation and after 1, 2, 4, 7, 10, and 14 days. None of the modulators or inhibitors alone triggered apoptosis, with the exception of MS for cell lines LOUCY and HL-60 and NaAsc for cell line K-562, where an increase of the apoptotic population could be observed.

In general, strong enhancement of the two oxides Sb_20_3 (1 µM and 5 µM) and As_2O_3 (1 µM) by the inhibitor of the g-glutamylcystein synthetase BSO could be seen. Even when Sb_2O_3 or As_2O_3 were used in concentrations that were not or only weak effective by themselves (1 µM Sb_2O_3 for LOUCY, CCRF-CEM and HL-60; 5 µM Sb_2O_3 for K-562; 1 µM As_2O_3 for CCRF-CEM and HL-60, 5 µM As_2O_3 for K-562), an increase up to 100% of apoptotic cells was observed when 100 µM BSO were added to the assay. This phenomenon was consistently seen with all analyzed cell lines and was in particular remarkable with the cell line K-562 that was insensitive for Sb_2O_3-concentrations even higher than 5 µM (up to 10 µM, data not shown). Other modulators were less effective and did not demonstrate consistent synergistic results in all cell lines: NaAsc (250 or 500 µM) also induced slight increase in CCRF-CEM and K-562. In CCRF-CEM a synergistic effect was seen with 500 µM NaAsc and 1 µM Sb_2O_3 which both were insensitive as single agents. Prolonged incubation with MS (100 µM) did enhance As_2O_3- and Sb_2O_3-induced apoptosis in HL-60 and K-562, but not in CCRF-CEM. Both AT and NaSal did not show consistent effects with the inductors of apoptosis in the different cell lines.

A high correlation of Annexin V-FITC and 7-AAD-staining could be demonstrated for all assays: correlation coefficients were 0.993 for LOUCY, 0.956 for CCRF-CEM, 0.913 for HL-60, 0.885 for K-562, respectively.

Effects of caspase inhibitors on Sb_2O_3- and As_2O_3-induced apoptosis and breakdown of Ψ_m in the T-cell leukemia cell line CCRF-CEM

In order to study the role of caspases and Ψ_m on Sb_2O_3- and As_2O_3-induced effects, we pre-treated the T-cell line CCRF-CEM with various caspase inhibitors (Fig. 7).

Pre-incubation was performed with three different caspase inhibitors for 1 h prior to (apoptosis) induction with Sb_2O_3 (5 and 10 μM) or As_2O_3 (5 μM): Boc-D(OMe)-Fmk and Z-VAD-Fmk are potent pan-caspase inhibitors, whereas Z-D (OMe)-E(OMe)-VD(OMe)-Fmk preferentially inhibits Caspase-3 but also to lower extend Caspase-6, Caspase-7, Caspase-8, and Caspase-10. The percentage of Annexin V-*positive* and MitoTrackerRed CMXRos-*negative* cells was determined after 24 h of apoptosis induction. The inhibitors alone showed no increase in the percentage of apoptotic cells (as measured by Annexin V-FITC-staining; Fig. 7) or any change in Ψ_m (as measured by MitoTrackerRed CMXRos staining; Fig. 7). We did not observe effects of caspase inhibitors on the percentage of MitoTrackerRed CMXRos-negative cells, whereas the percentage of cells stained positive for Annexin V-FITC declined. The inhibitory effect of the caspase inhibitors on Annexin V-FITC-binding was most prominent after pre-incubation for 1 h but not detectible with concomitant addition together with Sb_2O_3 or As_2O_3 (data not shown).

Modulation of the intracellular GSH-level by BSO affects the anti-apoptotic potential of caspase inhibitor Z-VAD-Fmk in cell line HL-60

Pre-incubation of HL-60 cells was performed with the pan-caspase inhibitor Z-VAD-Fmk for 1 h prior to treatment with Sb_2O_3 (10 and 20 μM) or As_2O_3 (5 and 10 μM) alone or in combination with 30 μM or 100 μM BSO. In order to induce apoptosis within 48 h in HL-60 cells, higher concentrations of As_2O_3 and Sb_2O_3 were chosen. The percentage of Annexin V-*positive* and MitoTrackerRed CMXRos-*negative* cells was determined after 48 h of apoptosis induction (Fig. 8). The inhibitor Z-VAD-Fmk alone showed no increase in the percentage of apoptotic cells or in Ψ_m, whereas reduction of the decrease in Ψ_m of BSO-treated non-apoptotic HL-60 could be detected. When Z-VAD-Fmk-pre-incubated cells were treated with 10 μM As_2O_3, a reduction of the percentage of MitoTrackerRed CMXRos-negative cells could be observed, compared to cells treated with As_2O_3 alone. This effect was not detectable for Sb_2O_3, as Sb_2O_3-induced apoptosis occurred in a low percentage (<20%) of cells under these conditions. When HL-60 cells were pre-treated with the caspase inhibitor, the percentage of apoptotic cells declined for 10 μM As_2O_3 or 20 μM Sb_2O_3. However, when either 30 μM or 100 μM BSO was added, Z-VAD-Fmk lost is potency to reduce both apoptosis and decrease of Ψ_m. Moreover, even in cells pre-incubated with

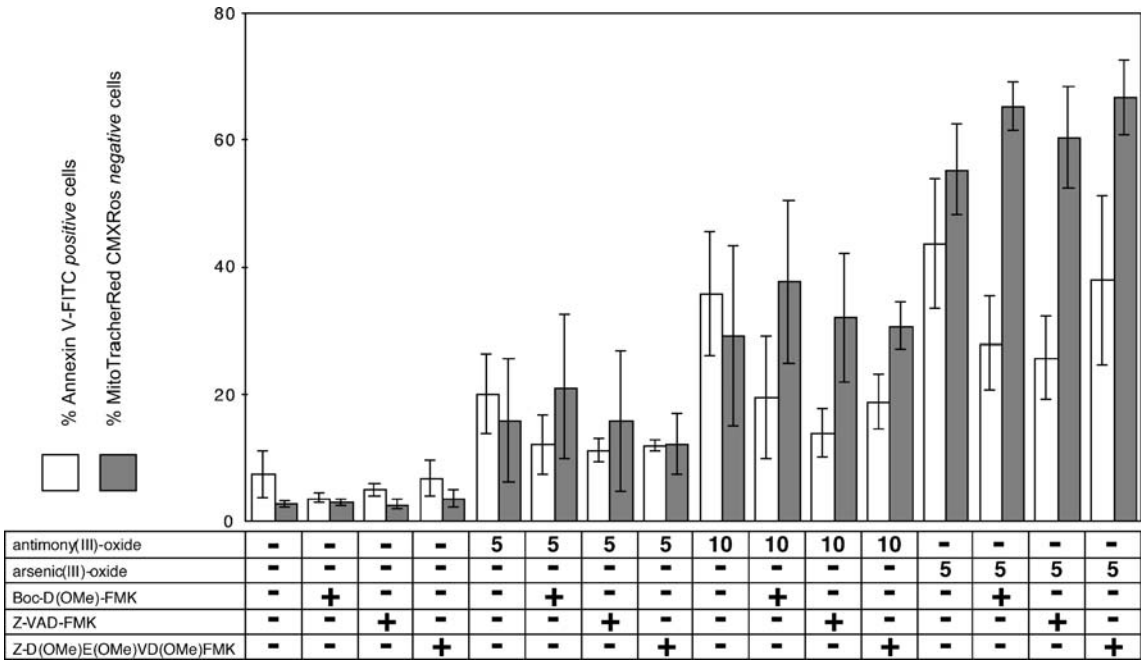

Fig. 7 Sb_2O_3- and As_2O_3-induced effects on Ψ_m cannot be blocked by caspase inhibitors in CCRF-CEM cells despite the percentage of apoptotic cells measured by Annexin V-FITC binding is reduced. After 1 h pre-incubation with the pan-caspase inhibitor Boc-D(OMe)-FMK or the multiple-caspase inhibitors Z-VAD-Fmk or Z-D(OMe)-E(OMe)-VD(OMe)-FMK, CCRF-CEM cells were co-incubated with 5 μM or 10 μM Sb_2O_3 or 5 μM As_2O_3 for 24 h. The percentage of cells *positive* for Annexin V-FITC (*white bars*) and *negative* for MitoTrackerRed CMXRos (*gray bars*) was measured by flow cytometry

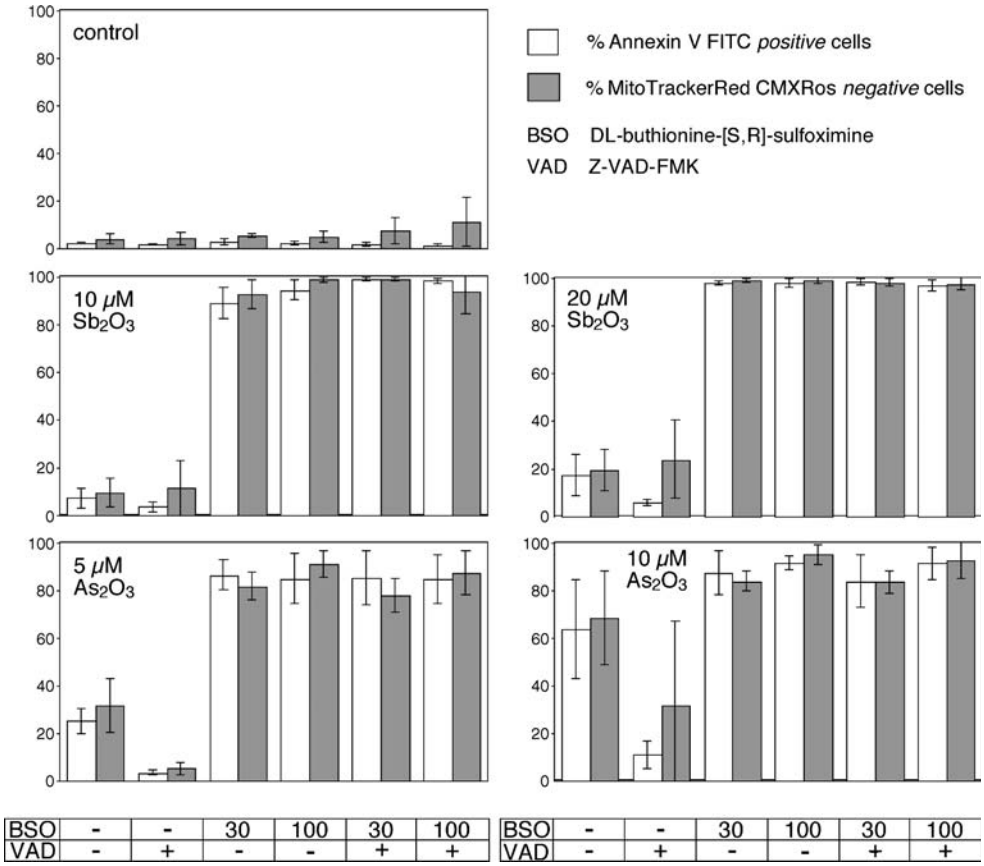

Fig. 8 Inhibition of Sb_2O_3- and As_2O_3-induced loss of Ψ_m and reduction of the percentage of apoptotic cells (measured by Annexin V-FITC binding) by caspase inhibitors is dependent from GSH-modulation in HL-60 cells. After 1 h pre-incubation with the pan-caspase inhibitor Z-VAD-Fmk, HL-60 cells were co-incubated with 30 μM or 100 μM BSO and PBS, 10 μM Sb_2O_3, 20 μM Sb_2O_3, 5 μM As_2O_3 or 10 μM As_2O_3 for 48 h. The percentage of cells *positive* for Annexin V-FITC (*white bars*) and *negative* for MitoTrackerRed CMXRos (*gray bars*) was determined by flow cytometry

the pan-caspase inhibitor Z-VAD-Fmk, both 30 μM and 100 μM BSO exerted the synergistic effect already described in Fig. 5 for lower As_2O_3 and Sb_2O_3 concentrations but longer incubation times.

Discussion

We were interested in magnifying the efficacy of As_2O_3 using substances that might be transferable to clinical application because the clinical applications of As_2O_3 gain in importance not only for therapy of relapsed and refractory APL [29, 30, 33–47], but also for treatment of multiple myeloma [48–50], myelodysplastic syndromes [51–53], and renal cancer [54]. Recently, two reports described apoptosis induction, caspase activation and ROS production by very high concentrations (up to 40 μM) of potassium antimonyl tartrate in lymphoid tumor cell lines [55] and growth inhibition and induction of apoptosis and reactive oxygen species [56]. Due to the chemical affinity of Sb_2O_3 and As_2O_3, we expected the same mechanism of apoptosis induction for the two substances. We observed Sb_2O_3-induced apoptosis in different myeloid and lymphatic cell lines (CCRF-CEM, HL-60, K-562), among them cell lines with reduced sensitivity to cytostatic agents (LOUCY, CEM/C1, CEM/C2). We were able to block Sb_2O_3-induced apoptosis by caspase inhibitors and, as presented previously for As_2O_3 [15], caspase-independent decrease of Ψ_m. We therefore postulate a common mechanism for Sb_2O_3 and As_2O_3 mediated apoptosis. However, based on the same molar concentrations, lower efficacy in apoptosis induction was observed for Sb_2O_3 than for As_2O_3.

Our special focus was set on substances that influence the glutathione redox state of the cell. Decrease of Ψ_m was shown to play a crucial role in As_2O_3 therapy-relevant effects [15, 17]. These effects are most probably based on inhibition of enzymes of the intracellular glutathione redox system, such as glutathione peroxidase GPx [57, 58], glutathione reductase GR and glutathione-*S*-transferase [58], resulting in an increase of ROS. In addition, a direct targeting of the mitochondrial permeability transition pore [59] was discussed as a possible mechanism. In this work, we were able to show enhancement of As_2O_3 and Sb_2O_3 effects by BSO, NaSal, NaAsc, MS and AT to different extents. As far as data on achievable and tolerable concentrations in vivo were available (BSO, NaSal, NaAsc), we have chosen concentrations within this range. Mainly BSO, an inhibitor of the rate limiting enzyme in glutathione (GSH)-synthesis γ-glutamylcystein synthetase [57, 60] proved to be a potent enhancer of As_2O_3 and Sb_2O_3. Our results obtained with As_2O_3 are in accordance with those from Dai et al. [61] and Zhu et al. [14], who

proved that BSO enhances apoptosis induction and decrease of Ψ_m in NB-4 and malignant lymphocytic cell lines and primary cultures of lymphocytic leukemia and lymphoma cells. Sub-apoptotic concentrations of As_2O_3 and Sb_2O_3 can be intensified by sub-apoptotic concentrations of BSO even in cell lines with reduced sensitivity for conventional cytostatic drugs. Repression of As_2O_3- and Sb_2O_3-induced apoptosis by the caspase inhibitor Z-VAD-Fmk can be overcome by co-treatment of BSO. Therefore, this substance may be useful in treatment of malignancies in case of reduced sensitivity for apoptosis induction by agents affecting Ψ_m or caspase activation.

Other known modulators of the glutathione redox system showed these effects to a lower extent and enhancement was not as consistent as with BSO, regarding the different cell lines treated. Nevertheless, the efficacy of enhancing As_2O_3- and Sb_2O_3-induced decrease of Ψ_m not only by BSO but also to lower extend by MS and ascorbic acid could be shown in this report. Ascorbic acid [61] and MS [62] also were shown to enhance the effects of As_2O_3 on mitochondria. In contradiction to our results, ascorbic acid was shown to protect from As_2O_3-induced toxicity [63]. However, evidence exists that ascorbic acid, widely praised as an antioxidant [64], can act as an oxidizing agent in the presence of ROS-inducing substances [65, 66]. Recently, promising results of a phase I/II combined trial of As_2O_3 and ascorbic acid were presented [47, 67–69].

Overall, we were able to decipher the mechanism of Sb_2O_3-induced apoptosis and show that the basic mechanisms involved are the same as described for As_2O_3. We also have blocked three different enzymes involved in oxidative stress detoxification (catalase, γ-glutamylcystein synthetase, glutathione peroxidase) and shown that only BSO, the inhibitor of the γ-glutamylcystein synthetase, was highly synergistic with sub-apoptotic concentrations of As_2O_3 or Sb_2O_3. The ability of specific drugs in clinically applicable concentrations to enhance the apoptotic effects of the two oxides in a synergistic way by may be helpful in reduction of their toxicity by the optimization of their doses. Moreover, further experiments may show how far our results on synergistic actions may be transferable for other combinations of drugs affecting the glutathione redox system and conventional cytostatic agents.

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