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ORIGINAL ARTICLE

Trolox enhances the anti-lymphoma effects of arsenic trioxide, while protecting against liver toxicity

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Arsenic trioxide (As_2O_3) is an effective therapy in acute promyelocytic leukemia (APL), but its use in other malignancies is limited by the higher concentrations required to induce apoptosis. We have reported that trolox, an analogue of α-tocopherol, increases As_2O_3-mediated apoptosis in a variety of APL, myeloma and breast cancer cell lines, while non-malignant cells may be protected. In the present study, we extended previous results to show that trolox increases As_2O_3-mediated apoptosis in the P388 lymphoma cell line *in vitro*, as evidenced by decrease of mitochondrial membrane potential and release of cytochrome c. We then sought to determine whether this combination can enhance antitumor effects while protecting normal cells *in vivo.* In BDF_1 mice, trolox treatment decreased As_2O_3-induced hepatomegaly, markers of oxidative stress and hepatocellular damage. In P388 tumor-bearing mice, As_2O_3 treatment prolonged survival, and the addition of trolox provided a further significant increase in lifespan. In addition, the combination of As_2O_3 and trolox inhibited metastatic spread, and protected the tumor-bearing mice from As_2O_3 liver toxicity. Our results suggest, for the first time, that trolox might prevent some of the clinical manifestations of As_2O_3-related toxicity while increasing its pro-apoptotic capacity and clinical efficacy in hematological malignancies.
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Introduction

Arsenic trioxide (As_2O_3), first used in traditional Chinese medicine, is highly effective in the treatment of patients with acute promyelocytic leukemia (APL).[1–3] Although the precise mechanism of action of As_2O_3 in APL is unclear, *in vitro* studies reported that As_2O_3 leads to cellular redox status perturbation, cellular signaling modulation, differentiation, growth inhibition and apoptosis.[4] Clinically achievable concentrations, that is between 1 and 2 μM of As_2O_3, induce apoptosis and inhibit growth of various malignant cells, including multiple myeloma and human T lymphotropic retrovirus type I-associated adult T-cell leukemia cells.[5–7] Recently, Rousselot *et al.*[8] documented *in vivo* activity of arsenic in the treatment of multiple myeloma using a SCID mouse xenotransplantation model. In another xenotransplantation model, As_2O_3 (3.75 mg/kg) induced a significant reduction of L540Cy Hodgkin tumors.[9] Subsequent *in vivo* studies characterized the effectiveness of arsenic in various hematological malignancies, and multiple phase I/II clinical trials are underway to evaluate its feasibility, safety and potential efficacy. Arsenic has also been tested in non-hematological cancer. Using an orthotopic prostate metastasis model, As_2O_3 alone provided a dose-dependent inhibition of both primary and metastatic lesions, although an increased survival rate was only obtained in the group treated with the combination of As_2O_3 and buthionine sulfoxamine (BSO), an inhibitor of γ-glutamyl cysteine synthase.[10]

In spite of these and other studies showing sensitivity to arsenic treatment *in vitro* and *in vivo*,[5–14] the degree of sensitivity has been consistently less than in APL cells, and clinical trials in different hematological malignancies and solid tumors have had mixed results.[15–18] These initial investigations suggest that arsenic trioxide, as a single agent, may have limited clinical activity outside APL. Therefore, combinations with other agents should be explored to increase antitumor efficacy and the therapeutic index of As_2O_3.

Several compounds have been reported to increase As_2O_3-mediated apoptosis *in vitro.*[5,19,20] BSO modulates the cellular glutathione system and can significantly potentiate the effects of As_2O_3, converting arsenic-resistant cell lines to a sensitive phenotype.[21,22] Although *in vivo* effects have been reported for this combination,[23] the observed additive toxicity may not be selective for cancer cells, and BSO itself has not been successfully developed for clinical use. Ascorbic acid (AA), a key antioxidant molecule, augments the toxicity of As_2O_3 *in vitro*,[24,25] but controversy exists regarding its mechanism of action,[26] and its potential for utility in the clinic is under study.

We have demonstrated recently that trolox (6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid) enhances the sensitivity of APL to As_2O_3 *in vitro.* We extended these results to NB4, an arsenic-resistant subclone of NB4, the IM9 multiple myeloma cell line and a variety of breast cancer cell lines.[27] In all these malignant cell lines, treatment with As_2O_3 and trolox increases intracellular oxidative stress, as evidenced by elevated heme oxygenase-1 (HO-1) protein levels, JNK activation and protein and lipid oxidation. Importantly, we found that trolox could protect non-malignant cells from As_2O_3-mediated cytotoxicity *in vitro*, suggesting that it may diminish or overcome the adverse effects associated with As_2O_3 monotherapy *in vivo*, potentially increasing the therapeutic index. As a presumed antioxidant, trolox has been used to mitigate the toxic effects of several compounds in animal models. Trolox reduced liver necrosis in a model of hepatic ischemia reperfusion in rats[28] and decreased streptozotocin-induced liver and kidney damage in mice.[29]

In this study, we addressed the effects of As_2O_3 and trolox on the viability of lymphoma P388 cells *in vitro* and in a mouse

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tumor model. We also investigated mechanisms underlying the pro-apoptotic properties of this combination, and its potential toxic effects in mice. We show that the combination of As_2O_3 and trolox decreases arsenic toxicity in non-tumor and tumor-bearing mice, while increasing the survival time and limiting the metastatic spread in mice bearing P388 lymphoma cells.

Materials and methods

Growth assays

P388 cells (provided by Dr Jing, Mount Sinai Medical Center, New York) were treated with various concentrations of As_2O_3 +/− 100 μM trolox for 6 days. Viable cells were counted by trypan blue exclusion on days 1, 3 and 6. Logarithmic growth phase was maintained at a density lower than 1×10^6 cells/ml through dilution as required, and media +/− treatment was replaced every third day.

Annexin V/propidium iodide staining

Cells were stained with annexin-V-FITC and propidium iodide (PI) in binding buffer according to manufacturer's (BD Pharmingen, San Diego, CA, USA) and analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA). Apoptotic cells (Annexin V positive/PI negative) were quantified using the CellQUEST software (Becton Dickinson).

Detection of the mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in $\Delta\Psi_m$ were determined with the J-aggregate-forming lipophilic cationic fluorochrome JC-1 (Molecular Probes, Eugene, OR, USA). Cells were incubated with 2.5 mg/ml JC-1 for 15 min. Cells were washed two times with PBS, resuspended in phosphate buffered solution (PBS), and analyzed on a FACScan. Data were analyzed and expressed as the ratio of mean fluorescence intensity between FL2 (polarized, dimeric) and FL1 (depolarized, monomeric) fluorescence.

Preparation of S-100 fractions and assessment of cytochrome c release

Cells were harvested, washed with ice-cold PBS and resuspended in five volumes of buffer (75 mM NaCl, 8 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM EDTA, 350 μg/ml digitonin, 1 mM dithiothreitol, 0.1 mM. phenylmethylsulfonyl fluoride, 10 μM aprotinin and 10 μM leupeptin). After 30 min on ice, the cells were centrifuged twice at 750 g, 10 min at 4°C. Cytosolic S-100 fractions (supernatants) were obtained by centrifugation at 100 000 g for 60 min at 4°C. Cytochrome *c* release into the S-100 fraction for each condition was assessed by western blot analysis.

Western blotting

Livers were disrupted by a Polytron homogenizer (Brinkmann, Westbury, NY, USA). Debris was removed by centrifugation at 700 *g* for 15 min, followed by centrifugation of the supernatant twice at 14 400 *g* for 15 min and finally at 100 000 × *g* for 1 h at 4°C. Proteins were separated and probed as described previously[27] with cytochrome *c* (1:500: BD Pharmigen), HO-1 (1:1000, Stressgen), and HSP70 (1:5000: Stressgen). Immunostaining for β-actin confirmed equal protein loading.

Cytochrome c oxidase (CcO) activity and cellular ATP levels

Mouse liver mitochondria were isolated using a mitochondria isolation kit (Sigma). CcO activity was calculated based on the rate of oxidation of ferrocytochrome *c* (decrease in absorbance at 550 nm). Ferrocytochrome *c* concentrations were determined using a kit (Sigma), with values expressed as μmol/min/mg mitochondrial protein. The intracellular ATP concentration was determined with a luminescent ATP detection kit (ATPLite; PerkinElmer Life Sciences) and was measured using a multiplate reader. ATP was calculated from a standard curve and was expressed as μM ATP/mg wet tissue.

In vivo *toxicity experiments*

All procedures conformed to the NIH guidelines for the care and use of laboratory animals, and were approved by the McGill University Animal Care Committee. BDF_1 mice (Charles River Laboratories, Wilmington, MA, USA) were randomly divided into eight groups of five mice. Each group received trolox (2.5, 10, 20 or 50 mg/kg), As_2O_3 (7.5 mg/kg) or the combinations of trolox and As_2O_3 interperitoneally. On alternate days for a total of 14 injections. Animals were weighed every other day. One day after the last dose of arsenic, blood was collected by cardiac puncture. Serum was separated and total protein levels, glucose content, activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (AKP) were assayed using commercially available kits. Mice were killed by cervical dislocation. Liver was extracted and washed in ice-cold isotonic saline solution and weighed. Liver samples were fixed in 10% phosphate-buffer formalin (pH 7.4), embedded in paraffin, sectioned, stained with hematoxylin–eosin, and examined under bright field microscope by a pathologist. Lymphocyte infiltration and number of binucleated cells were quantified in 10 random 0.159 mm^2 fields/sample.

In vivo *anti-tumor experiments*

P388 cells were injected i.p. in DBA/2 mice (Charles River Laboratories, Wilmington, MA, USA). After 15 days, cells were collected from the peritoneum, washed and resuspended in PBS. For experiments, 0.1 ml containing 2×10^6 cells obtained from the ascites was inoculated i.p. in BDF_1 mice. Mice were randomly divided into six groups each with eight mice. After 24 h, each group was given saline, As_2O_3 (7.5 or 10 mg/kg) and trolox (50 mg/kg) alone or in combination i.p. On alternate days for a total of 14 injections. The percentage increase in lifespan (ILS) over control was calculated as follows: ILS% = 5T/C%−100, where *T* is the test mean survival time, and C is the control mean survival time. Macroscopically visible lesions were counted in the liver, stomach, pancreas and intestine by a pathologist blinded to the treatment groups. Sections of liver were stained for hematoxylin and eosin to verify that the counted visible liver lesions were indeed liver metastases.

Statistical analysis

Significance was determined by analysis of variance followed by Newman–Keuls post-tests using Prism version 3.0 (GraphPad software, San Diego, CA, USA). The combination index (CI), an indication of the interaction between two drugs, was determined by the formula $a/A + b/B = 1$, where *a* is the IC_{50} of As_2O_3 + trolox at a concentration *b*; *A* is the IC_{50} of As_2O_3; and *B* is the IC_{50} of trolox. According to this formula, when $CI < 1$, the interaction is synergistic, when $CI = 1$, the interaction is additive, and when $CI > 1$, the interaction is antagonistic.[30]

Results

Trolox significantly enhances As_2O_3-induced apoptosis of murine lymphoma P388 cells

Based on our finding of synergy in NB4, AsR2, IM9 and a variety of breast cancer cells lines,[27] we first investigated whether trolox would increase the *in vitro* efficacy of As_2O_3 in a lymphoma cell line for which there is an established animal model. As Figure 1a shows, treatment of P388 cells for 6 days with 2 and 4 μM As_2O_3 reduced the viable cell number by 19.0 and 51.7% of control, respectively ($P<0.001$). Thus, P388 cells are less sensitive to As_2O_3 than some leukemic cell lines, in which 0.5 and 1 μM As_2O_3 are sufficient to induce a similar effect. Trolox (100 μM) alone had no effect on cell number at any time point. However, if the cells were treated with 2 or 4 μM As_2O_3 and 100 μM trolox in combination, 41.2 and 81.3% reductions in cell number were observed ($P<0.001$, when compared to As_2O_3 alone at either dose). In all cases, trypan blue-positive cells were less than 3%. A difference was also seen at 72 h, where addition of trolox enhanced the action of 4 μM As_2O_3 by decreasing cell number by 29% compared to As_2O_3 alone. The CI value was determined to be 0.619, documenting a synergistic interaction between As_2O_3 and trolox. A variety of complementary techniques were then performed to analyze whether the observed growth inhibitory effects were the result of the induction of apoptosis in P388 cells. Using annexin V/propidium iodide staining, we found that 24% of the cells treated with 2 μM As_2O_3 were apoptotic after 48 h (Figure 1b). This percentage was nearly doubled when trolox was added. Similarly, the addition of trolox to 4 μM As_2O_3 increased apoptosis from 33.2 to 58.5% ($P<0.001$). Consistently, control and cells treated with trolox exhibited JC-1 orange fluorescence due to the formation of JC-1 aggregates, indicating that the mitochondria were polarized (Figure 1c). Exposure to As_2O_3 induced a very rapid decline in $\Delta\Psi_m$, as revealed by complete loss of JC-1 orange fluorescence and a shift to JC-1 green fluorescence due to the formation of JC-1 monomers. Consistently, a dose-dependent decrease of $\Delta\Psi_m$ was observed in the cells treated with the combination of As_2O_3 and trolox. As a consequence of the decreased mitochondrial membrane potential, cytochrome c may be released from the mitochondria, providing another marker of apoptosis. As shown in Figure 1d, cytoplasmic cytochrome c content was increased when trolox and As_2O_3 were used in combination at both As_2O_3 doses. Thus, our data indicate that trolox increases As_2O_3-induced apoptosis in P388 cells.

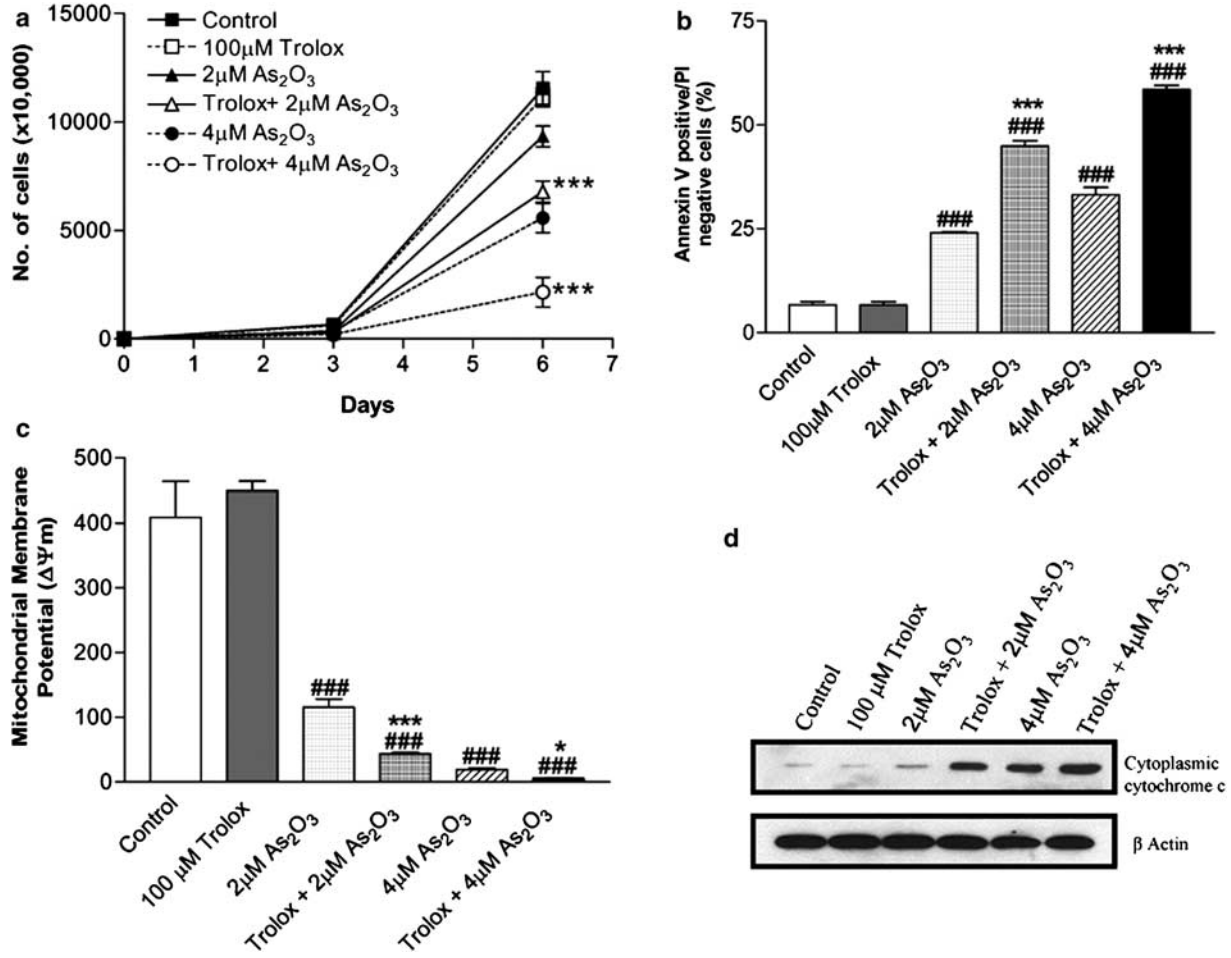

Figure 1 Trolox enhances As_2O_3-mediated growth inhibition and apoptosis in murine P388 lymphoma cells. (**a**) P388 cells were treated with media (■), trolox (□), 2 μM As_2O_3 (▲), 4 μM As_2O_3 (●) and the combination of trolox with 2 μM As_2O_3 (△) and 4 μM As_2O_3 (○). Viable cells were counted by trypan blue exclusion on days 1, 3 and 6. Bars denote standard deviations. Asterisks indicate significant differences ($P<0.001$) from As_2O_3-treated cells. (**b**) P388 cells were treated with As_2O_3 and trolox for 48 h. Apoptosis was detected by annexin V-FITC and PI staining. Apoptotic cells (Annexin V positive/PI negative) were quantified using the CellQUEST software. (**c**) Ratios of mean fluorescence intensity (JC-1 orange fluorescence and JC-1 green fluorescence) were calculated to determine changes in $\Delta\Psi_m$. (**d**) S-100 fractions were isolated and cytochrome *c* release into the S-100 fractions for each condition was assessed by western blot analysis. Asterisks indicate a significant difference (*$P<0.05$, ***$P<0.01$) from As_2O_3-treated cells. Number signs indicate a significant difference ($P<0.001$) from controls.

Trolox decreases As_2O_3-mediated toxicity in vivo *in BDF_1 mice*

In our previous work, we demonstrated that trolox decreases cytotoxicity of As_2O_3 in mouse embryonic fibroblasts.[27] In addition, we have determined that trolox protects the non-tumorigenic murine hepatocyte AML cells from As_2O_3 toxicity *in vitro* (data not shown), suggesting that synergistic toxicity of the combination could be specific to tumor cells. To test this hypothesis *in vivo*, we first conducted toxicological studies to define the maximum tolerable dose of trolox in BDF_1 mice.

Trolox treatment was well tolerated and not toxic at the doses studied (2.5, 10, 20, and 50 mg/kg), as indicated by assessment of body weight over the course of the study; the average body weight did not differ significantly from the control animals in any treatment group. The higher dose approaches its maximum solubility limit in 300 μl, the maximum volume that can be injected in mice intraperitoneally.

Liver damage has been reported to be a marker of arsenic toxicity in different experimental animals.[31,32] Therefore, we asked whether trolox could affect As_2O_3-associated hepatocellular damage *in vivo*. Mice were randomly divided into groups of five mice and treated with two doses of trolox and a dose of As_2O_3 (7.5 mg/kg) reported to be moderately toxic.[24] None of the animals exhibited discomfort or obvious distress throughout the duration of the experiment. No significant differences in weight were observed in any of the treated groups compared to control. As depicted in Figure 2a, moderate hepatomegaly was observed in the As_2O_3-treated group. The average liver weights in the control and trolox groups were quite similar, with an average of 1.07 ± 0.14 g, while in the As_2O_3-treated group, average liver weight was increased to 1.53 ± 0.35 g ($P<0.05$). However, in the groups treated with the combination of As_2O_3 and trolox, the hepatomegaly was abrogated, with an average liver weight of 1.10 ± 0.1 g ($P<0.05$ As_2O_3 vs. As_2O_3 + trolox).

When hepatocellular injury occurs, the associated plasma membrane leakage can be detected biochemically by assaying aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in serum. Figures 2b and c show that both

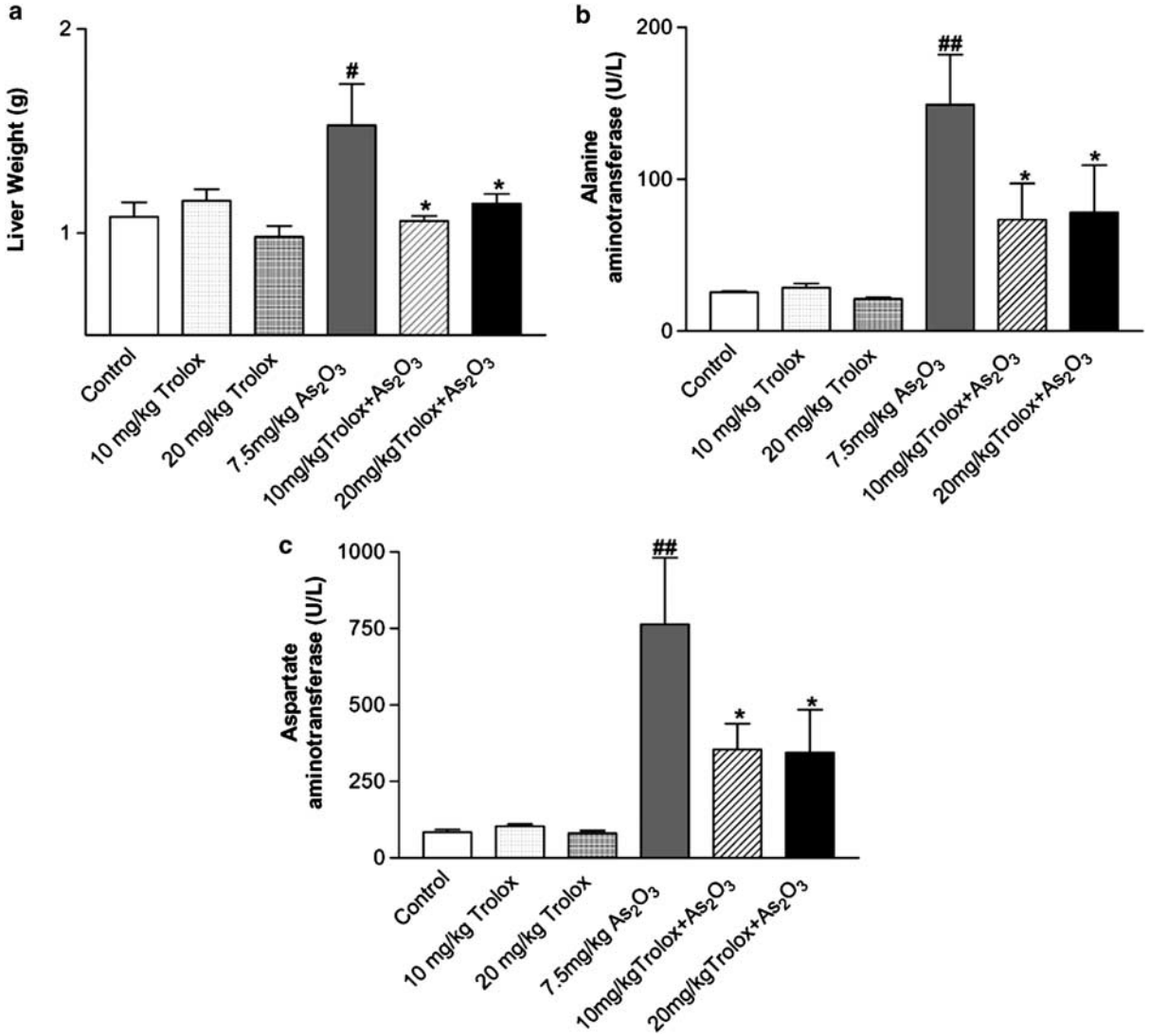

Figure 2 Trolox decreases As_2O_3-mediated liver toxicity *in vivo*. Animals were treated as indicated in Materials and methods section. One day after the last dose of arsenic, animals were killed and the livers were weighed (**a**). Blood was collected by cardiac puncture. Serum activities of alanine aminotransferase (**b**) and aspartate aminotransferase (**c**) were assayed using commercially available kits in all the animals. Asterisks indicate a significant difference ($P<0.05$) from As_2O_3-treated group. Number signs indicate a significant difference ($^{\#}P<0.05$, $^{\#\#}P<0.01$) from control group.

enzymatic activities were increased in the As_2O_3-treated group by 4.2- and 3.5-fold compared to that of the control group, respectively. However, in the animals treated with the combination of As_2O_3 and either 10 or 20 mg/kg trolox, a significantly decreased induction of AST and ALT activities was observed ($P<0.05$). The activity of alkaline phosphatase, an indicator of cholestasis, was not significantly affected by any of the treatments (data not shown), suggesting that As_2O_3 can induce a direct injury to the hepatocytes without blocking bile excretion. We did not observe any change in glucose or total protein levels in any of the groups (data not shown) or any fulminant hepatic failure, perhaps due to the short duration of the experiment.

Histopathological analysis of liver samples demonstrated a cell injury pattern in the As_2O_3-treated group (Figure 3a), characterized by hepatocellular degeneration, inflammatory infiltrates composed of fibrinous exudates and polymorphonuclear leukocyte aggregates, and areas with focal necrosis. A marked increase in binucleated cells was observed, suggesting regeneration of hepatocytes after acute toxicity. We tested whether the addition of trolox would modify this cellular pattern of toxicity. Lymphocyte foci and binucleated cells were counted in randomly selected fields by a pathologist blinded to the treatment groups. A significant decrease in lymphocyte infiltration and binucleated cells was observed after treatment with As_2O_3 and trolox compared to As_2O_3 alone (Figures 3b and c). These results indicate that trolox significantly protects hepatocytes from As_2O_3-mediated toxicity.

Trolox decreases As_2O_3-mediated oxidative stress and ameliorates the As_2O_3-mediated decrease in cellular metabolic rate in BDF_1 mice

Oxidative damage may be a key mechanism by which arsenic mediates its toxic effects. Because trolox decreases As_2O_3-induced liver toxicity, we hypothesized that the addition of trolox reduces hepatocellular oxidative stress induced by As_2O_3. HO-1, an oxidative stress-responsive protein,[33] was not detected in the liver of control animals or animals treated with trolox alone, but was markedly induced by As_2O_3. The addition of trolox significantly decreased As_2O_3-mediated HO-1 induction in all the animals (Figure 4a). The 70 kDa heat shock

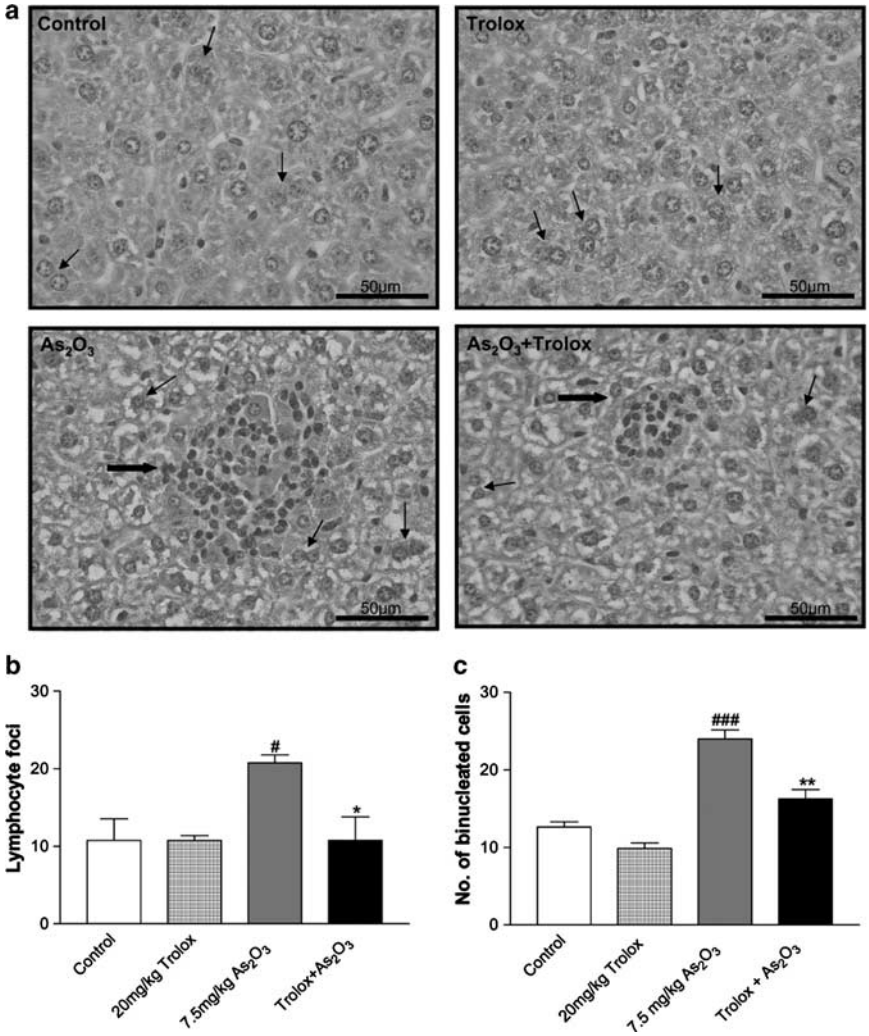

Figure 3 Trolox modulates As_2O_3 effects on liver morphology. (**a**) Photomicrographs (× 40) of the liver samples from animals treated with saline solution (Control), 50 mg/kg trolox (Trolox), 7.5 mg/kg As_2O_3 (As_2O_3) and the combination of As_2O_3 and trolox (As_2O_3 + Trolox). Inflammatory infiltrates composed of fibrinous exudates and polymorphonuclear leukocytes are depicted using big arrows. Representative examples of binucleated cells, an indication of hepatocellular regeneration following a toxic treatment, are shown using small arrows. Quantification of lymphocyte foci (**b**) and binucleated cells (**c**) are also shown. Asterisks indicate significant differences ($^{*}P<0.05$, $^{**}P<0.01$) from As_2O_3-treated group. Number signs indicate significant differences ($^{\#}P<0.05$, $^{\#\#\#}P<0.001$) from control group.

proteins (HSP-70 family) are important for protein folding and help to protect cells from stress. HSP-70 expression has been used as an indicator of As_2O_3 exposure in different experimental models.[34] As depicted in Figure 4b, HSP-70 protein levels were enhanced in the livers of As_2O_3-treated group, while they were decreased to near basal levels when the animals were treated with the combination of As_2O_3 and trolox. The HSP70 antibody recognizes the inducible form of HSP-70 (HSP-72) and the constitutive form HSP-73, explaining the basal levels in the control and trolox-treated groups.

Thus, having established that As_2O_3 induces liver oxidative stress, and that the addition of trolox significantly restores hepatocellular redox homeostasis, we further analyzed whether the hepatocellular metabolic rate was affected by this combination. Sulfhydryl groups in many enzyme systems react with arsenicals, which may result in a block of the Krebs cycle, interrupting oxidative phosphorylation, which in turn causes marked depletion of ATP stores.[7] The activity of cytochrome *c* oxidase in the liver is considered to be a good metabolic marker for functional activity of cells. Therefore, we asked whether

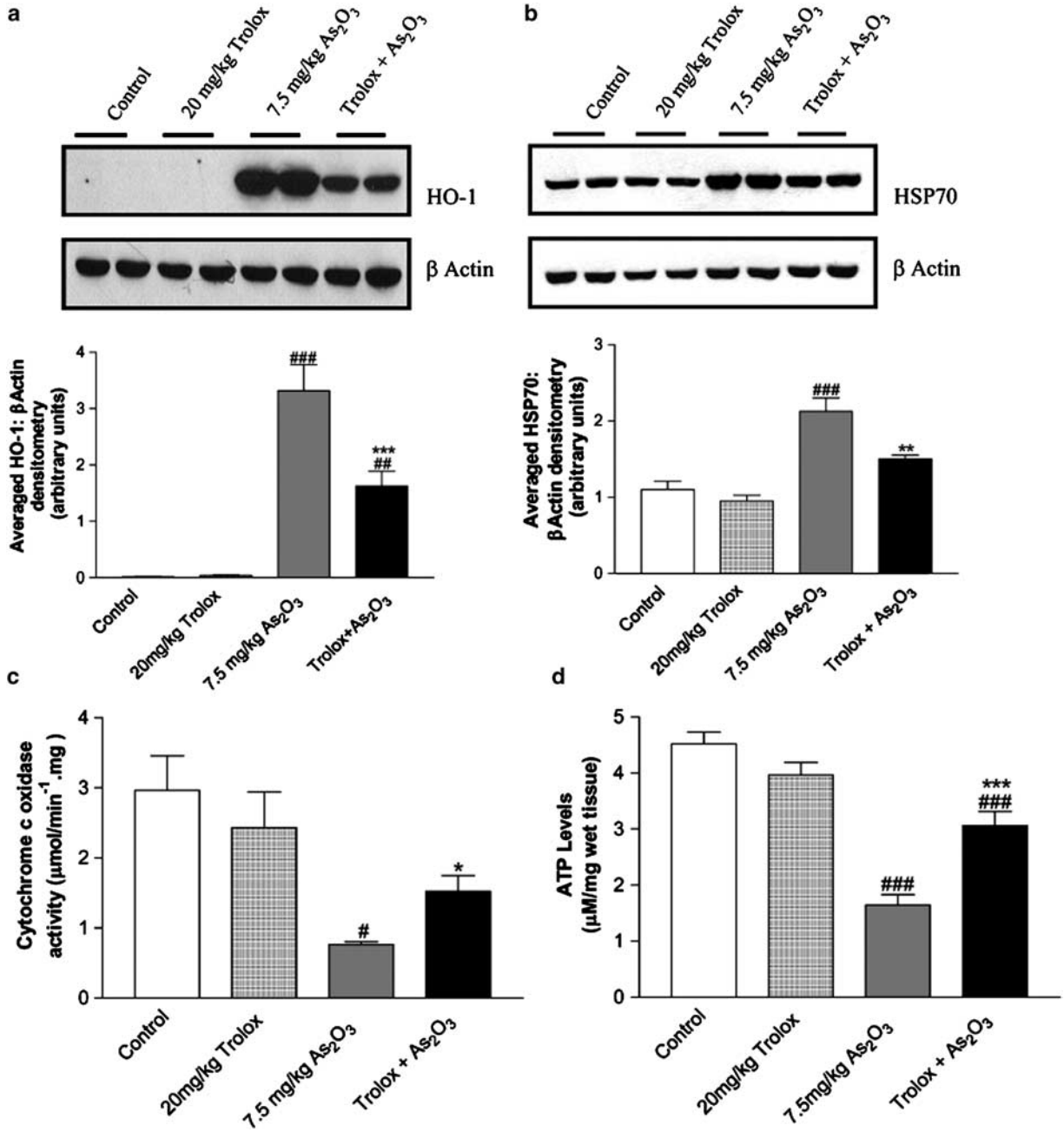

Figure 4 Trolox protects mice against As_2O_3-mediated oxidative stress and blocks As_2O_3-mediated decrease in hepatic metabolic rate. Animals were treated as indicated in Materials and methods section. Western blotting was performed to determine total cellular HO-1 (**a**) and HSP-70 (**b**) protein levels with β-actin as loading control in liver samples from all the animals. Densitometric analyses of blots from six animals were performed. (**c**) Mitochondria from liver were extracted and cytochrome c oxidase activity was assayed. (**d**) ATP concentrations in hepatocytes were measured with a luminescent ATP detection kit. Asterisks indicate significant differences ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$) from As_2O_3-treated group. Number signs indicate significant differences ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$) from control group.

As_2O_3 could reduce CcO activity and ATP stores and whether trolox might play a role in the restoration of the basal levels. Figure 4c shows that the hepatic CcO enzymatic activity of animals treated with As_2O_3 was decreased by 74.2%, while this activity was only decreased by 48.6% with the combination of As_2O_3 and trolox ($P<0.05$). As predicted, As_2O_3 treatment induced a 63.5% decrease in liver ATP levels, while the animals treated with As_2O_3 and trolox only showed a 32.2% reduction ($P<0.001$) (Figure 4d). These results again show protective effects of trolox on arsenic-mediated liver toxicity.

Trolox increases As_2O_3-mediated antitumor effects in BDF_1 mice bearing lymphoma P388 cells while protecting against liver toxicity

On the basis of the *in vitro* potency and favorable *in vivo* toxicity profiles, As_2O_3 and trolox were evaluated for *in vivo* antitumor efficacy in mice bearing P388 murine lymphoma tumors. The dose selection for As_2O_3 (7.5 and 10 mg/kg body weight) was based on the relatively low toxicity seen in our initial study of non-tumor-bearing mice. Trolox was given at 50 mg/kg, which was not toxic in our preliminary results but approached the maximum solubility. As shown in Figure 5a, As_2O_3 treatment prolonged survival, with median survival times of 20 and 18 days for 7.5 and 10 mg/kg As_2O_3, respectively, as compared to 14 days for controls ($P<0.001$). The median survival time for animals treated with the combination of As_2O_3 and trolox was further prolonged to 24.5 and 22 days compared to that of with As_2O_3 alone ($P<0.001$). Treatment with 7.5 mg/kg As_2O_3 provided a 46.4% ILS (Figure 5b). When this dose was combined with trolox, we observed a 73.5% ILS. Animals treated with 10 mg/kg As_2O_3, experienced an increased life span of only 28.6% ($P<0.001$). We observed moderate weight loss and lethargy in these mice (data not shown), suggesting that this dose is toxic. However, the addition of trolox doubled the increase in lifespan of 10 mg/kg As_2O_3 alone without evidence of increased toxicity.

The effects of the combination of As_2O_3 and trolox on tumor metastases were profound. At the time of killing or death due to tumor progression, metastases were present in all of the eight (100%) saline-treated control animals as seen macroscopically and in histological sections (Figure 5c and data not shown). Treatment with 7.5 and 10 mg/kg As_2O_3 reduced the number of animals with metastases to 62.5 and 37.5%, respectively. The

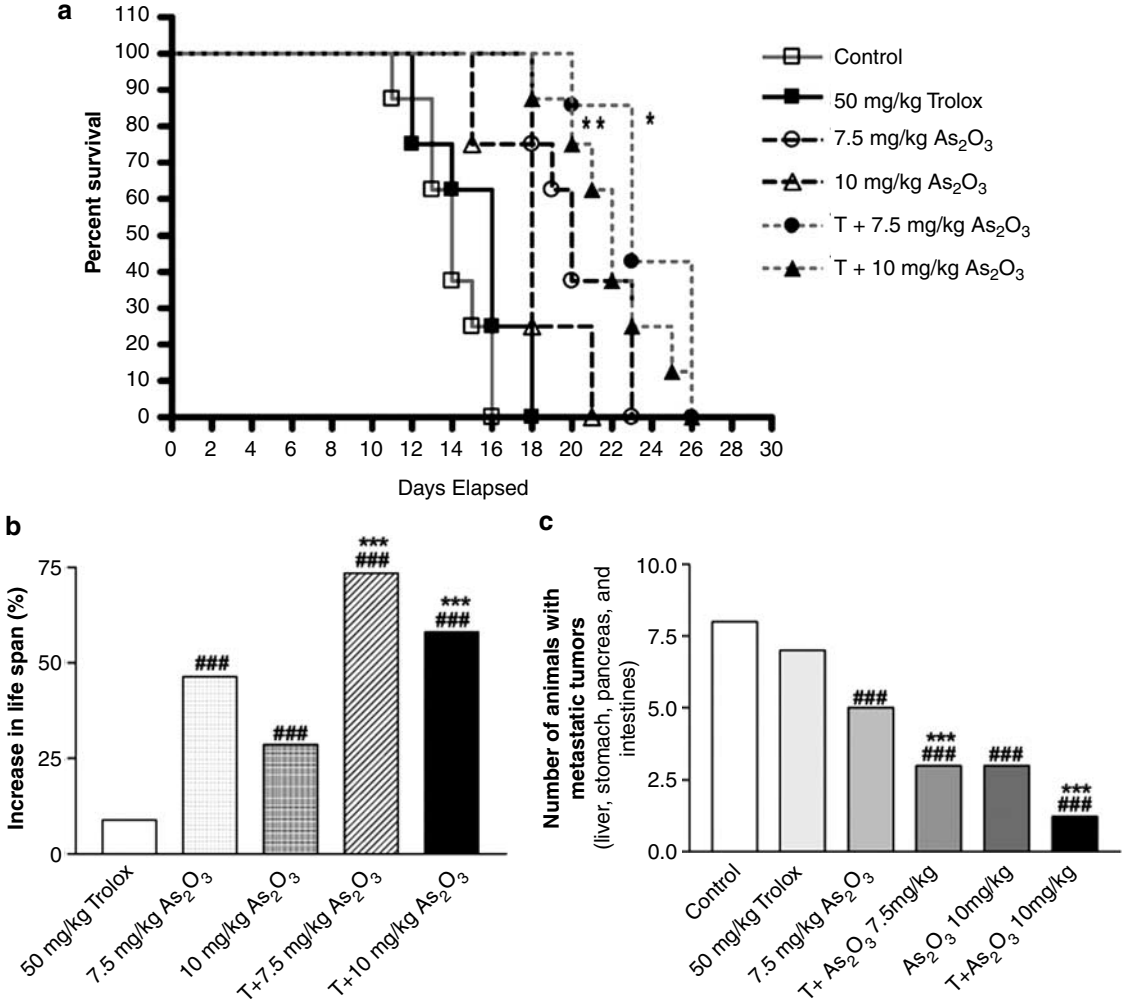

Figure 5 Trolox increases As_2O_3 antitumor effects in BDF_1 mice. Animals bearing P388 lymphoma cells were treated as indicated in Materials and methods section. Animal deaths were tabulated and Kaplan–Meier curves were generated to depict percent survival (**a**). Increase in lifespan of treated animals relative to controls was calculated (**b**). Macroscopically visible lesions were counted in liver, stomach, pancreas and intestine in all the animals (**c**). Asterisks indicate significant differences from As_2O_3-treated groups (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Number signs indicate significant differences ($P<0.001$) from controls.

incidence of metastases was significantly decreased to 37.5 and 12.5% when trolox was combined with 7.5 and 10 mg/kg As_2O_3 ($P<0.001$). We examined livers from tumor-bearing mice to analyze whether trolox could also modulate As_2O_3-mediated oxidative stress and its effects on the metabolic rate of these animals. We found that the non-treated, tumor-bearing animals had a higher baseline of liver oxidative stress than non-tumor-bearing mice, as demonstrated by an increase in HO-1 protein levels (first lane of Figure 6a, compared to Figure 4a). Interestingly, treatment with trolox alone decreased the hepatic oxidative stress. As_2O_3 treatment caused some further increase in HO-1 expression at both 7.5 and 10 mg/kg (Figure 6a and data not shown). However, addition of trolox significantly decreased HO-1 protein levels at both doses of As_2O_3 studied.

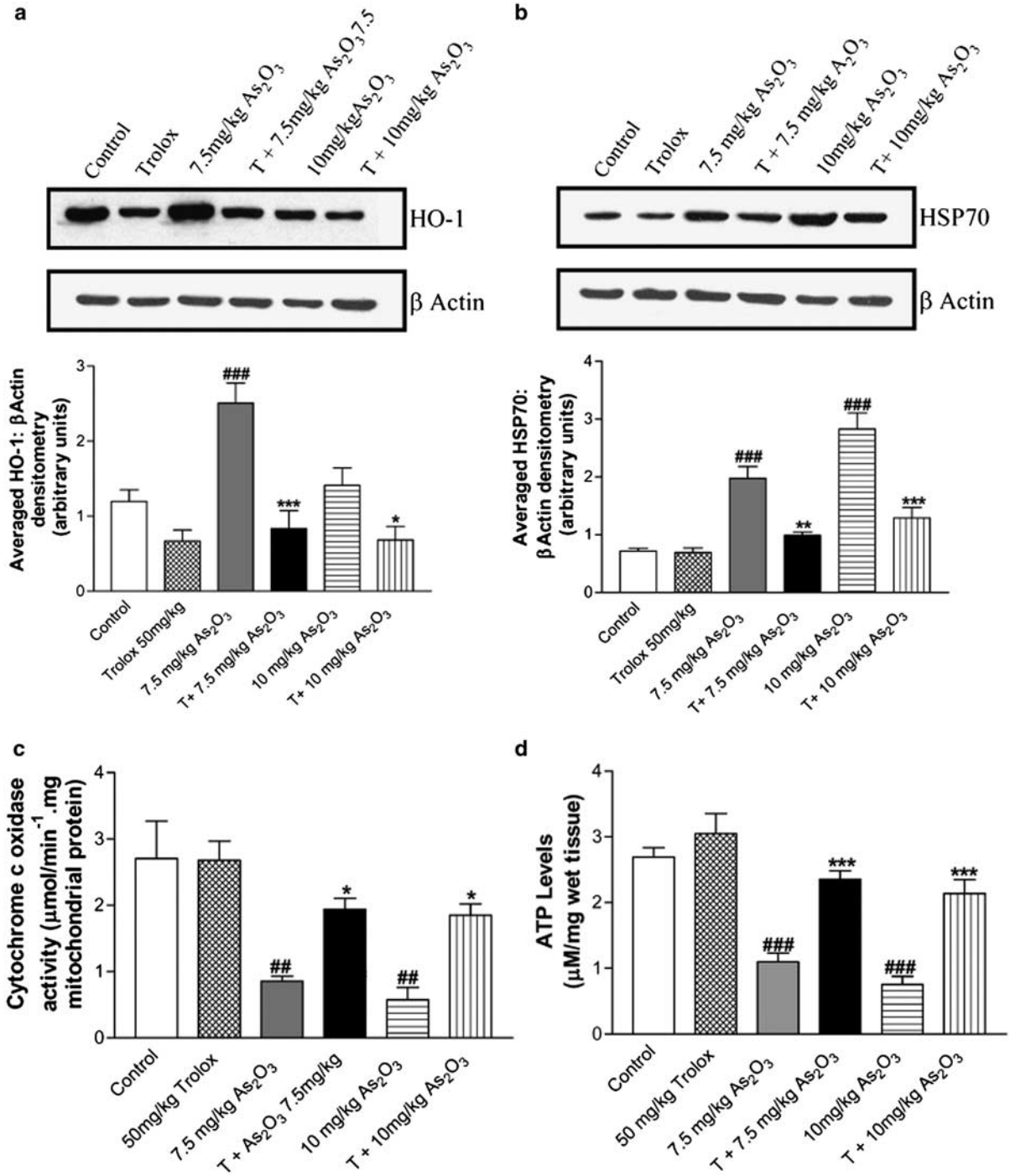

Figure 6 Trolox protects tumor-bearing mice against As_2O_3-mediated toxicity. Animals were treated as indicated in Materials and methods section. Western blotting was performed in liver samples from all the animals to determine HO-1 (**a**) and HSP-70 (**b**) protein levels with β-actin as loading control. Densitometric analyses of blots were performed using eight animals per group. (**c**) Mitochondria from liver were extracted and cytochrome *c* oxidase activity was assayed. (**d**) ATP concentrations in hepatocytes were measured with a luminescent ATP detection kit. Asterisks indicate significant differences (*$P<0.05$, ***$P<0.001$) from As_2O_3-treated group. Number signs indicate significant differences ($^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$) from control group.

Similarly, HSP70 expression was consistently increased by As_2O_3, and this effect was reduced by trolox (Figure 6b). We then explored the effects of the As_2O_3 and trolox combination on hepatic metabolic rate using assays of CcO and ATP as shown before. Consistently, CcO activity (Figure 6c) and ATP stores (Figure 6d) were markedly reduced in the animals treated with As_2O_3, but significantly restored in the animals treated with the combination of As_2O_3 and trolox. In addition, serum ALT levels were less elevated when As_2O_3 was given with trolox (data not shown). These results show that although tumor-bearing mice have baseline liver damage, consistent with our data for non-tumor-bearing mice, trolox protects the liver from arsenic-mediated toxicity.

Discussion

Although activity in many malignant cell lines requires concentrations of As_2O_3 that are not clinically achievable, our previous work identified trolox as a compound that might have a dual role depending on the cellular microenviroment. In malignant cells, trolox synergizes with As_2O_3 to increase its toxicity while in non-malignant cells, trolox decreases As_2O_3-mediated cellular damage. This is the first report showing that the combination of As_2O_3 and trolox *in vivo* targets malignant cells and limits cancer metastases, while decreasing damage to normal cells.

In this study, we used the mildly As_2O_3-resistant P388 lymphoma cell line to assess the potential synergy of As_2O_3 and trolox *in vitro* and *in vivo*. We found that the combination of As_2O_3 and trolox enhanced growth inhibition and apoptosis of P388 cells. These data support a synergistic effect of trolox in enhancing As_2O_3 toxicity consistent with previously published work in NB4, AsR2, IM9, MCF7, T47D and MDA-231 cells.[27] These *in vitro* effects provided the rationale for experiments using the P388 lymphoma cell line to determine how trolox would affect As_2O_3 activity and toxicity *in vivo*. We first performed *in vivo* studies with As_2O_3 and trolox alone or in combination to investigate whether trolox would affect arsenic toxicity in BDF_1 mice. We observed As_2O_3-induced liver toxicity, which has been previously reported to involve tissue necrosis and other histological and biochemical changes in several animal models.[35] In these non-tumor-bearing mice, we found that trolox protects normal hepatic cells from As_2O_3 toxicity. This is the first study to report *in vivo* protective effects of trolox on arsenic-induced hepatotoxicity, although several studies have demonstrated its efficacy in preventing toxicity of other metals.[36] In addition, studies have shown that a trolox derivative (U-83836E) appeared to be beneficial in reducing lipid peroxidation products and in partially preventing the decrease in glutathione and antioxidant enzymes induced by methanol in liver, serum[37] and brains[38] of rats. Lower doses of trolox than used in the present study clearly reduced methyl-mercury-induced toxicity in rats.[39] These studies suggest that trolox may decrease toxicity associated with a broad spectrum of compounds.

Based on our *in vitro* results and the favorable *in vivo* toxicity profile, we evaluated As_2O_3 and trolox for *in vivo* antitumor efficacy in the peritoneal P388 murine lymphoma model. This model was used to test the combination of ascorbic acid and arsenic, which also was reported to be effective *in vitro* in various cell lines. In addition, this model has been used to test the effects of different antineoplastic drugs.[40–42] Trolox doses ranging from 2 to 100 mg/kg have been used in rats and rabbits.[43–45] The intraperitoneal route was chosen in an attempt to avoid the potential confounding factors on As_2O_3 absorption through the gastrointestinal tract and to compare parenteral arsenic effects with previous reports in the literature.[46,47] We showed that the addition of trolox to As_2O_3 given to mice bearing P388 lymphoma cells significantly increased their survival time. It has been reported, using the same model, that ascorbic acid enhanced antitumor properties of As_2O_3.[24] Dai *et al*. observed an increase in survival time with the combination of AA and As_2O_3, although they used a lower dose of As_2O_3 (5 mg/kg), which did not increase the lifespan of the mice as a single agent. Using 7.5 mg/kg As_2O_3, we obtained a significant prolongation of survival that was markedly improved by the addition of trolox. Most notably, our studies show the potential for As_2O_3 and trolox given as a combination to limit metastases. It remains critical to reconcile these two opposite effects of the As_2O_3 and trolox combination, that is, a cooperative action of As_2O_3 and trolox against lymphoma growth and metastases with the concomitant protection by trolox of normal cells *in vivo*. Our data suggest that trolox may behave as a pro-oxidant in cancer cells exposed to As_2O_3, while having antioxidant properties in normal cells. Therapeutic enhancement by antioxidants is counterintuitive to the apparent role of ROS in apoptosis. However, the pro- or antioxidant effects of many redox-active compounds may vary substantially as a function of the cellular redox microenvironment and models employed. For example, it has been proposed that the selectivity of certain chemotherapeutic agents to cancer cells may be due, in part, to the relatively low concentrations of antioxidant enzymes documented in some malignant cells.[48,49] It is noteworthy that normal cells in general are more efficient in eliminating ROS than malignant cells. Furthermore, a specific ROS or its intracellular localization could be the critical determinant of cell death or survival. Finally, it has been demonstrated that vitamin E[50] may modulate tumor cell permeability by altering levels of lipid peroxidation in surface membranes, raising the possibility that trolox differentially augments As_2O_3 uptake by malignant cells.

Our observation of reduced toxicity of As_2O_3 *in vivo*, delayed death in mice bearing P388 tumor cells and reduction of metastatic spread in this model suggests that the combined use of As_2O_3 and trolox may increase the therapeutic index of arsenic and possibly decrease the development of secondary tumors in patients with advanced lymphoma and other malignancies.

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