Arsenicals Inhibit Thioredoxin Reductase in Cultured Rat Hepatocytes

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · MARCH 2001

Impact Factor: 3.53 · DOI: 10.1021/tx0001878 · Source: PubMed

6 AUTHORS, INCLUDING:

Luz Maria Del Razo
Center for Research and Advanced Studies of…
117 PUBLICATIONS **4,238** CITATIONS
SEE PROFILE

David J Thomas
United States Environmental Protection Age…
139 PUBLICATIONS **7,010** CITATIONS
SEE PROFILE

Arsenicals Inhibit Thioredoxin Reductase in Cultured Rat Hepatocytes

Shan Lin,[†] Luz Maria Del Razo,[‡,§] Miroslav Styblo,[∥] Changqing Wang,[⊥] William R. Cullen,[⊥] and David J. Thomas*[,@]

Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, Center for Environmental Medicine and Lung Biology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada, and Pharmacokinetics Branch, Experimental Toxicology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

Received August 24, 2000

Thioredoxin reductase (TR), an NADPH-dependent flavoenzyme that catalyzes the reduction of many disulfide-containing substrates, plays an important role in the cellular response to oxidative stress. Trivalent arsenicals, especially methyl As that contains trivalent arsenic (MAs^{III}), are potent noncompetitive inhibitors of TR purified from mouse liver. Because MAs^{III} is produced in the biomethylation of As, it was postulated that the extent of inhibition of TR in cultured rat hepatocytes would correlate with the intracellular concentration of methyl As. Exposure of cultured hepatocytes to inorganic As^{III} (iAs^{III}), MAs^{III}, or aurothioglucose (ATG, a competitive inhibitor of TR activity) for 30 min caused a concentration-dependent reduction in TR activity. The estimated IC_{50} was $\gg$100 μM for iAs^{III}, ~10 μM for ATG, and ~3 μM for MAs^{III}. In hepatocytes exposed to 1 μM MAs^{III} for up to 24 h, the inhibition of TR activity was maximal (~40%) after exposure for 15 min. After exposure for 3 h [when most MAs^{III} has been converted to dimethyl As (DMAs)], TR activity in these cells had returned to control levels. Notably, exposure of the cell to 50 μM $DMAs^{III}$ did not affect TR activity. In hepatocytes exposed to 10 μM iAs^{III} for up to 24 h, the inhibition of TR activity was progressive; at 24 h, activity was reduced ~35%. Following exposure to iAs^{III} or MAs^{III}, the extent of inhibition of TR activity correlated strongly with the intracellular concentration of MAs. Taken together, these results suggest that arsenicals formed in the course of cellular metabolism of As are potent inhibitors of TR activity. In particular, MAs^{III}, an intermediate in the metabolic pathway, is an especially potent inhibitor of TR. Hence, the capacity of cells to produce or consume the intermediates in the pathway for As methylation may be an important determinant of susceptibility to the toxic effects of As.

Introduction

In many species, including humans, exposure to inorganic As (iAs)[1] results in the formation and excretion of methyl As (MAs) and dimethyl As (DMAs) as major metabolites. For example, following ingestion or inhalation of iAs, humans excrete iAs, MAs, and DMAs in urine (*1*−*3*). The metabolic pathway for the conversion of iAs into its methylated derivatives involves the oxidative methylation of As and the cycling of As between pentavalency and trivalency. The overall scheme has been summarized by Cullen and associates (*4*) as

$$As^{V}O_4^{3-} + 2e \rightarrow As^{III}O_3^{3-} + CH_3 \rightarrow CH_3As^{V}O_3^{2-} + 2e \rightarrow CH_3As^{III}O_3^{2-} + CH_3 \rightarrow (CH_3)_2As^{V}O_3^{-} + 2e \rightarrow (CH_3)_2As^{III}O^{-} + CH_3 \rightarrow (CH_3)_3As^{V}O + 2e \rightarrow (CH_3)_3As^{III}$$

The reduction of pentavalent (As^{V}) to trivalent As (As^{III}) in mammalian cells has been linked to the oxidation of glutathione (GSH) (*5*, *6*), or it can be catalyzed enzymatically (*7*, *8*). Arsenicals containing As^{III} (e.g., iAs^{III} and MAs^{III}) are preferred substrates for the enzymatically catalyzed methylation reaction that uses *S*-adenosylmethionine (AdoMet) as the methyl group donor (*9*, *10*). Hence, the formation of trivalent arsenicals is a critical step in the metabolism of As. The generation of trivalent

* To whom correspondence should be addressed: Pharmacokinetics Branch, ETD, NHEERL, MD-74, U.S. EPA, Research Triangle Park, NC 27711-2055. Telephone: (919) 541-4974. Fax: (919) 541-5394. E-mail: thomas.david@epa.gov.

[†] Curriculum in Toxicology, University of North Carolina at Chapel Hill.

[‡] School of Medicine, University of North Carolina at Chapel Hill.

[§] Present address: Section of Environmental Toxicology, Department of Pharmacology and Toxicology, CINVESTAV-IPN, P.O. Box 14-740, Mexico City, Mexico.

[∥] Department of Pediatrics, University of North Carolina at Chapel Hill.

[⊥] University of British Columbia.

[@] U.S. Environmental Protection Agency.

[1] Abbreviations: iAs (iAs^{III} or iAs^{V}), inorganic arsenic species; MAs (MAs^{V} or MAs^{III}), methylarsenic species; DMAs ($DMAs^{V}$ or $DMAs^{III}$), dimethylarsenic species; TR, thioredoxin reductase; Trx, thioredoxin; ATG, aurothioglucose; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.

10.1021/tx0001878 CCC: $20.00
Published on Web 02/01/2001

arsenicals in the cellular environment creates species that are more reactive than their pentavalent analogues. Recent studies in a variety of human and animal cell lines have demonstrated that MAs^{III} and $DMAs^{III}$ are more potent cytotoxins than either iAs^{III} or iAs^{V} (*11*, *12*). Arsenicals containing As^{III} inhibit NADPH-dependent flavoprotein oxidoreductases, including GSH reductase (NADPH:oxidized-glutathione oxidoreductase, EC 1.6.4.2) and thioredoxin reductase (TR, NADPH:oxidized-thioredoxin oxidoreductase, EC 1.6.4.5). Notably, MAs^{III} and $DMAs^{III}$ exceed iAs^{III}, iAs^{V}, MAs^{V}, and $DMAs^{V}$ in potency as inhibitors of both purified yeast GSH reductase and mouse liver TR (*13*, *14*). GSH reductase is competitively inhibited by $MAs^{III}I_2$ ($K_i \sim 74\ \mu M$) and the homologous arsinothiol $MAs^{III}(GS)_2$ ($K_i \sim 9\ \mu M$). TR is competitively inhibited by $MAs^{III}I_2$ ($K_i \sim 0.1\ \mu M$). The extreme sensitivity of TR to inhibition by MAs^{III} suggested that this enzyme might be a critical target for trivalent arsenicals in the cell. TR catalyzes the reduction of thioredoxin (Trx), a 12 kDa protein with redox-active Cys residues. Through its capacity to reduce thiols in proteins, Trx is an important component of the machinery that regulates the response of cells to oxidative stress (*15*, *16*). In addition, TR catalyzes the reduction of a number of other substrates, including dehydroascorbate, selenite, selenodiglutathione, lipid peroxides (*17*−*20*), and proteins, including thioredoxin peroxidase and protein disulfide isomerase (*21*, *22*). Inhibition of TR by methylated arsenicals that contain As^{III} could prevent the regeneration of reduced Trx from Trx disulfide and allow the persistence of oxidized (and inactive) proteins in cells. The actions of these modified macromolecules might underlie the toxic and carcinogenic effects of As.

The work reported here examines the linkage between the metabolism of arsenicals in cultured primary rat hepatocytes and the inhibition of TR activity in these cells. Because primary rat hepatocytes are efficient methylators of iAs^{III} (*23*), it is possible to investigate the temporal association between change in the concentration of various arsenicals and the activity of TR in the cells. The results of this study suggest that the methylation of iAs^{III} yields products that are at least as potent as iAs^{III} as inhibitors of TR. Because exogenously added MAs^{III} was found to be more potent than iAs^{III} as an inhibitor of TR in these cells, it is possible that MAs^{III} formed in the course of metabolism is responsible for the inhibition of TR activity. These findings are consistent with the hypothesis that the methylation of As can be regarded as a mechanism of activation, not a means for its detoxification, and that the conventional view that the methylation of As is a mechanism for the detoxification of this metalloid (*24*) may be incorrect.

Experimental Procedures

Caution: *Inorganic arsenic is classified as a human carcinogen (25). The toxicities of trivalent methylated arsenicals have not been systematically examined in intact organisms. These arsenicals should be handled as highly toxic and potentially carcinogenic compounds.*

Arsenicals. Sodium *m*-arsenite ($NaAs^{III}O_2$) was purchased from Sigma (St. Louis, MO). The trivalent methylated arsenicals, methylarsine oxide ($MAs^{III}O$) and dimethylarsine iodide ($DMAs^{III}I$), were synthesized and characterized as previously described (*13*). The preparation of [^{73}As]iAs^{III} from [^{73}As]iAs^{V} (Los Alamos Meson Production Facility, Los Alamos, NM) has been described previously (*26*). The efficacy of reduction was confirmed by TLC (*9*).

Preparation and Culture of Rat Hepatocytes. Hepatocytes were prepared from livers of adult male Fischer 344 rats at the Advanced Cell Technologies and Tissue Engineering Facility, School of Medicine, University of North Carolina at Chapel Hill, using a previously described two-step perfusion technique (*27*). Initial cell viability determined by trypan blue exclusion ranged from 90 to 97%. Cells (5×10^5 per well) were plated in 12-well plates that were coated with collagen-I (Becton Dickinson, Bedford, MA) and were cultured in William's medium E supplemented with 2 mM glutamine (Sigma), 100 units/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite (Sigma), and 0.5 μM dexamethasone (Sigma). Cultures were maintained at 37 °C in a humidified chamber purged with a mixture of 95% air and 5% CO_2. Media were changed daily.

Treatment of Hepatocytes with Arsenicals and Aurothioglucose. The stock solution of 100 mM $DMAs^{III}I$ was prepared in 50% ethanol. The stock solutions of other arsenicals were prepared in sterile phosphate-buffered saline (PBS) [10 mM KPO_4, 138 mM NaCl, and 2.7 mM KCl (pH 7.4); from Sigma] and stored at 0 °C before addition to cultured cells. Arsenicals were added to culture media at final concentrations of 0.1−50 μM. A stock solution of aurothioglucose (ATG, Sigma) was prepared in PBS and was used to obtain final concentrations of 1−100 μM ATG in cultured cells. Hepatocytes treated with arsenicals or ATG were maintained for up to 24 h. For comparison, hepatocytes that received neither arsenicals nor ATG were prepared, maintained, and harvested in parallel with arsenical- or ATG-treated cultures. These untreated cell cultures were designated control cultures and provided data on TR activity in cells maintained under standard culture conditions.

Analysis of Radiolabeled Metabolites. Arsenicals in cells exposed to [^{73}As]iAs^{III} were analyzed by TLC. Here, medium was removed, and cells were harvested by trypsinization or scraping. Protein-bound arsenicals in cells were released by treatment with 0.2 M CuCl (Sigma) (pH 1) and heating to 100 °C for 5 min (*28*). Denatured proteins were removed by centrifugation, and supernatants were oxidized with H_2O_2 (Sigma) to facilitate further analysis. Aliquots of oxidized supernatants were analyzed by TLC on PEI-F cellulose-coated plates (Fisher Chemical, Fair Lawn, NJ) by previously described methods (*29*). The distribution of the radioactivity on the developed TLC plates was analyzed with an AMBIS 4000 imaging detector (Ambis, San Diego, CA).

Analysis of Stable Arsenicals. For analysis of arsenic metabolites in MAs^{III}-treated cells, hepatocytes were harvested by trypsinization. Cells were wet digested in 2 M HCl at 80 °C for 3 h. Arsenicals were analyzed by hydride generation/atomic absorption spectrophotometry by the method of Crecelius and associates (*30*) using a Perkin-Elmer 5100 atomic absorption spectrometer. Arsenicals in the digested samples were converted to the corresponding arsines by addition of sodium borohydride (EM Science, Gibbstown, NJ) at pH 1−2. Arsines were flushed by a continuous flow of He (150 mL/min) into a trap cooled with liquid N. The warming of the trap to room temperature separated arsines by boiling points. An air/H_2 flame atomized arsines in the atomic absorption detector. The reliability of the analysis was assessed by spiking samples with known amounts of various arsenicals. Recoveries ranged from 92 to 107% with coefficients of variation between 3 and 11%. Because the analytical procedures used in this study cannot determine the original oxidation states of arsenicals in biological samples, metabolites are generically designated as iAs, MAs, and DMAs.

Assay for TR Activity. Hepatocytes were harvested from arsenical- or ATG-treated cultures or from control cultures by scraping of the cell layer in 100 mM Na, K phosphate buffer with 0.5% Triton X-100 (pH 7.4). Cells were lysed by one cycle of freezing and thawing followed by centrifugation at 20000*g* at 4 °C for 10 min. The lysate was used immediately for determination of TR activity by the DTNB reductase assay described by Lin and co-workers (*14*). This assay monitored the NADPH-dependent reduction of DTNB to 2-nitro-5-thiobenzoic

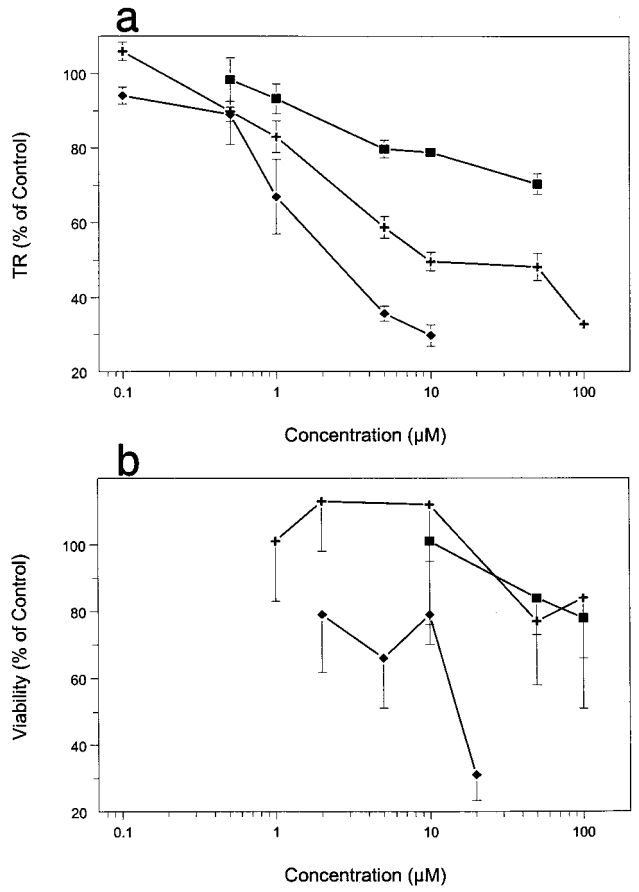

Figure 1. Concentration-dependent changes in thioredoxin reductase activity and viability in rat hepatocytes exposed to inorganic iAs^{III}, methyl As^{III}, and aurothioglucose for 30 min. (a) Cells were exposed to 1−50 μM inorganic iAs^{III} (■), 0.1−10 μM methyl As^{III} (◆), or 0.1−100 μM aurothioglucose (+) for 30 min. Activity of thioredoxin reductase measured by the DTNB reductase assay and expressed as a percentage of activity found in rat hepatocytes concurrently cultured without addition of these agents. (b) Cells were exposed to 10−100 μM inorganic iAs^{III} (■), 2−20 μM methyl As^{III} (◆), or 1−100 μM aurothioglucose (+) for 60 min. Cell viability was measured by the MTT assay ($n = 3$, $\bar{x} \pm$ SD shown).

acid at 412 nm and 37 °C. Each assay replicate used lysate prepared from 2.5×10^5 cells. DTNB reductase activities in arsenical- or ATG-treated cells were expressed as the percentages of activity found in control cells cultured concurrently with treated cells.

Evaluation of Cytotoxicity. An assay based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to an insoluble purple formazan was used to assess the viability of hepatocytes exposed to arsenicals or ATG (*31*). After a 60 min exposure to arsenicals or ATG, the medium was removed and the cells were washed with PBS. A phenol red-free William's medium E containing 5 mg of MTT/mL was added, and the cells were incubated for an additional 3 h. Cells were washed with PBS, dissolved in acidic 2-propanol, and the absorbance by the formazan product was measured at 570 nm with background subtraction at 630 nm.

Data Analysis. Data on the relationship between intracellular concentrations of arsenicals and TR activity were analyzed by linear regression analysis, and the statistical significance of the association between these variables was evaluated (*32*).

Results

The concentration dependencies of the effects of iAs^{III}, MAs^{III}, $DMAs^{III}$, and ATG on TR activity and on cellular viability were examined in cells exposed to these agents.

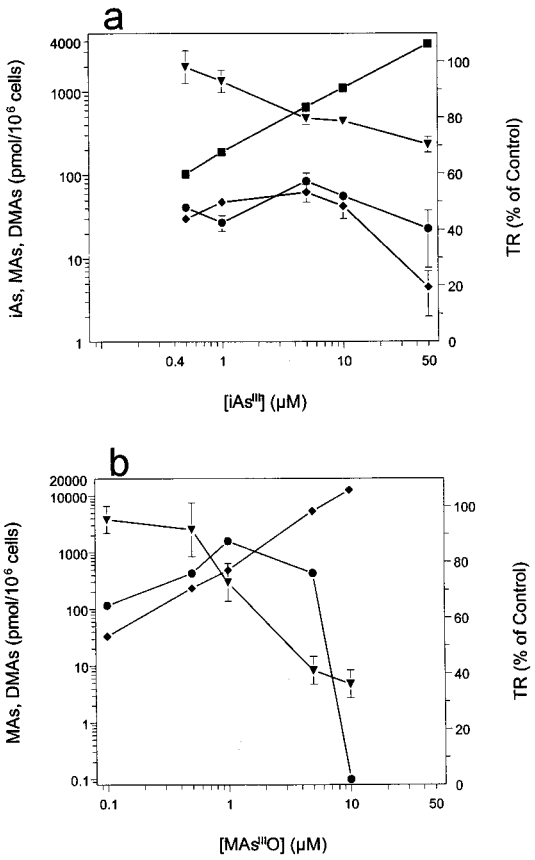

Figure 2. Relationships between the intracellular concentration of arsenicals and thioredoxin reductase activity in rat hepatocytes. Concentrations of inorganic As (■), methyl As (◆), and dimethyl As (●) were determined in cells exposed for 30 min to 1−50 μM inorganic iAs^{III} (a) or 0.1−10 μM methyl As^{III} (b). Activity of thioredoxin reductase (▼) measured by the DTNB reductase assay and expressed as a percentage of activity found in rat hepatocytes concurrently cultured without addition of these agents ($n = 3$, $\bar{x} \pm$ SD shown).

Figure 1 illustrates the effects of these agents following a 30 min exposure. MAs^{III} was the most potent inhibitor of TR ($IC_{50} \sim 3$ μM). For ATG, the IC_{50} was estimated to be ~10 μM. iAs^{III} was a less potent inhibitor of TR than either MAs^{III} or ATG, and its IC_{50} was not determined. However, 100 μM iAs^{III} inhibited TR activity about 30%. Addition of up to 50 μM $DMAs^{III}$ did not alter TR activity (data not shown). Following a 60 min exposure to these agents, MAs^{III} was the most potent cytotoxin with an LC_{50} between 10 and 20 μM. By comparison, the estimated LC_{50} for either iAs^{III} or ATG was greater than 100 μM.

The relation between the metabolism of iAs^{III} and MAs^{III} and the extent of TR inhibition was examined by determination of the amounts of arsenical metabolites in cells after exposure to these agents for 30 min. Following exposure to 0.4−50 μM iAs^{III}, there was a strong correlation between the concentration of iAs in cells and the concentration of iAs^{III} in the culture (Figure 2a). Because the incubation time was relatively short, the amounts of metabolites (MAs and DMAs) found in cells were relatively low. Concentrations of both MAs and DMAs rose in cells as the concentration of iAs^{III} in the medium rose to 4 μM. At higher concentrations of iAs^{III} in the medium, the concentrations of both metabolites declined. TR activity in cells declined as both the concentrations of iAs^{III} in the medium and of iAs in cells increased. At 50 μM iAs^{III}, TR activity in cells was reduced to about 70% of that found in control hepatocytes.

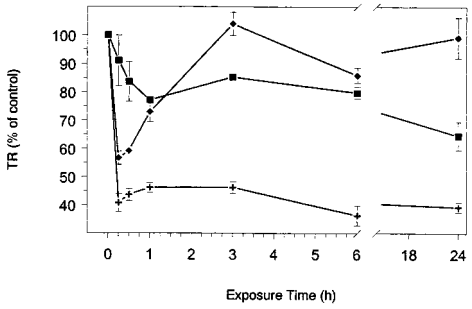

Figure 3. Changes in thioredoxin reductase activity in rat hepatocytes exposed to inorganic iAs^{III}, methyl As^{III}, and aurothioglucose for 24 h. Cells were exposed to 10 μM inorganic iAs^{III} (■), 1 μM methyl As^{III} (◆), or 10 μM aurothioglucose (+) for intervals of up to 24 h. At selected times, cells were harvested for determination of thioredoxin reductase activity by the DTNB reductase assay. Thioredoxin reductase activity expressed as a percentage of activity found in rat hepatocytes concurrently cultured without addition of these agents ($n = 3$, $\bar{x} \pm$ SD shown).

Exposure of cells to 0.1−10 μM MAs^{III} produced a concentration-dependent increase in the concentration of MAs in cells (Figure 2b). For medium concentrations up to 1 μM, exposure to MAs^{III} resulted in an increase in the concentration of DMAs in cells. At higher MAs^{III} concentrations, the concentration of DMAs in cells declined. This decline likely reflected inhibition of the dimethylation reaction by MAs^{III}. TR activity declined over this 100-fold range of MAs^{III} concentrations. At 10 μM MAs^{III} in the medium, TR activity was about 35% of that found in untreated hepatocytes.

Figure 3 illustrates the time dependencies of the degree of inhibition of TR activity in cells exposed for up to 24 h to 10 μM iAs^{III}, 1 μM MAs^{III}, or 10 μM ATG. Notably, none of these agents was cytotoxic at the tested concentration. Treatment with ATG quickly reduced TR activity of cells to about 40% of the activity found in control cultures; this inhibition was sustained through the experimental period. Over the first hour of exposure to iAs^{III}, cellular TR activity declined to about 75% of that found in control cultures. The inhibition of cellular TR activity in iAs^{III}-exposed cells was persistent. After exposure for 24 h, TR activity in iAs^{III}-treated cells was about 65% of that found in control cultures. In MAs^{III}-treated cells, TR activity was reduced after exposure for 15 min to about 55% of that found in control cultures but quickly rebounded. After exposure to MAs^{III} for 3 h, TR activity returned to a level equal to that found in control cultures. Throughout the remainder of the experimental period, TR activity in MAs^{III}-exposed cells paralleled those in control cultures.

Because substantial metabolism of iAs^{III} and MAs^{III} occurred in cells exposed to these arsenicals for 24 h, the relations between the concentrations of various arsenicals present in cells and the degree of inhibition of TR activity were examined. Figure 4a illustrates the time-dependent changes in the concentrations of iAs, MAs, and DMAs and in TR activity in cells exposed to 10 μM iAs^{III} for up to 24 h. The concentration of iAs in cells peaked early in the course of exposure and declined slowly throughout the remainder of the experimental period. The concentrations of both MAs and DMAs rose steadily over the first 12 h of exposure. At this time point, the concentration of MAs in cells was about 50% of that of iAs and the concentration of DMAs was about 10% of that of iAs. As noted above, TR activity in cells exposed to 10 μM iAs^{III}

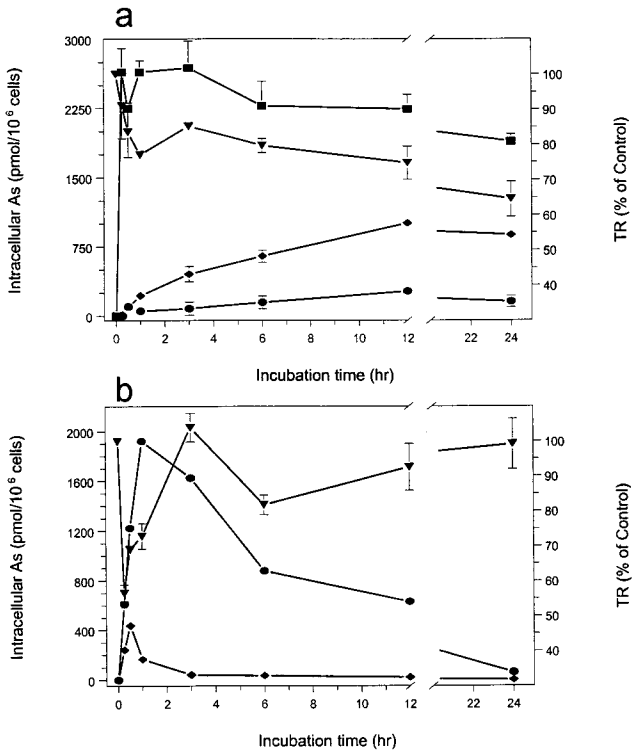

Figure 4. Relationships between the intracellular concentration of arsenicals and thioredoxin reductase activity in rat hepatocytes exposed to inorganic iAs^{III}, methyl As^{III}, and aurothioglucose for 24 h. (a) Cells exposed to 10 μM inorganic iAs^{III} were collected at various times for the determination of inorganic As (■), methyl As (◆), and dimethyl As (●) contents and for determination of thioredoxin reductase activity (▼) by the DTNB reductase assay. Thioredoxin reductase activity expressed as a percentage of activity found in rat hepatocytes concurrently cultured without addition of these agents. ($n = 3$, $\bar{x} \pm$ SD shown). (b) Cells exposed to1 μM methyl As^{III} were collected at various times for the determination of inorganic As (■), methyl As (◆), and dimethyl As (●) contents and for determination of thioredoxin reductase activity by the DTNB reductase assay. Thioredoxin reductase activity (▼) expressed as a percentage of activity found in rat hepatocytes concurrently cultured without addition of these agents ($n = 3$, $\bar{x} \pm$ SD shown).

declined quickly over the first hour of exposure and continued to decline over the remainder of the experimental period. In cells exposed to 1 μM MAs^{III}, the concentration of MAs peaked after exposure for 30 min and declined rapidly thereafter (Figure 4b). The concentration of DMAs in these cells rose quickly, peaked after 1 h of exposure, and declined over the remainder of the experimental period. At each time point, the cellular concentration of DMAs exceeded that of MAs. In these cells, the maximal inhibition of TR activity occurred after exposure to MAs^{III} for 15 min, and inhibition was fully reversed after exposure for 3 h. Hence, the level of inhibition of TR was greatest while the cellular concentration of MAs was maximal.

The relation between the concentration of arsenicals in cells and the activity of TR was examined by plotting the enzyme activities as a function of the intracellular concentrations of specific arsenicals following exposure to either iAs^{III} or MAs^{III} (Figure 5a). This analysis did not distinguish between the source of an arsenical (accumulated from medium or formed by cellular metabolism) or the duration of exposure (30 min or 24 h) to the arsenicals. For iAs and MAs, there was a significant negative correlation ($p < 0.025$) between its intracellular concentration and TR activity expressed as a percentage

of the activity found in matched untreated cultured cells. For DMAs, the correlation between these variables was not significant ($p > 0.1$). For a parallel analysis, the concentrations of all arsenicals in cells after treatment with iAs or MAs for 30 min or 24 h were summed to provide a measure of total cellular As contents (Figure 5b). Here, cells exposed to iAs^{III} contained iAs, MAs, and DMAs and cells exposed to MAs^{III} contained MAs and DMAs. There were significant negative correlations ($p < 0.025$) between the concentration of arsenicals in cells exposed to either iAs^{III} or MAs^{III} and TR activity expressed as a percentage of the activity found in matched and untreated cultured cells.

Discussion

Kinetics of TR Inhibition in Hepatocytes Exposed to Arsenicals. Exposure of cultured primary rat hepatocytes to iAs^{III}, MAs^{III}, or ATG resulted in a concentration-dependent reduction in TR activity and cell viability. With short-term (30 min) exposure to these agents, MAs^{III} was the most potent inhibitor of TR activity ($IC_{50} \sim 3\ \mu M$). The lower potency of iAs^{III} as an inhibitor of TR activity in cultured hepatocytes was consistent with its relatively low potency as an inhibitor of purified TR (*14*). ATG, a competitive inhibitor of several enzymes that, like TR, contain catalytically active selenocysteine residues (*33*, *34*), was less potent as an inhibitor of TR in rat hepatocytes than MAs^{III}. ATG has been reported to be a competitive inhibitor of purified TR (*35*), and treatment of mice with ATG reduces TR activity in a variety of tissues, including liver (*36*). Cell viability was assessed after exposure to iAs^{III}, MAs^{III}, or ATG for 60 min. The choice of a longer exposure interval before assessment of viability was to ensure that cells that might have been irreversibly injured after exposure to these agents for 30 min would be detected. Notably, the inhibitory effect of these agents on TR activity occurred at concentrations that were at least several-fold below those that were associated with significant cytotoxicity. Therefore, the decrease in TR activity produced by short-term exposure to these agents was not a general agonal event but rather a specific toxic effect. The linkage between the inhibition of TR by arsenicals and the induction of cell death that occurs following exposure to these agents has not been established.

In cells exposed to iAs^{III} or MAs^{III} for 30 min, the level of accumulation of either arsenical increased as a function of its concentration in the medium. For both agents, the parent arsenical and its methylated metabolites were found in cells. Thus, iAs, MAs, and DMAs were found in iAs^{III}-exposed cells; MAs and DMAs were found in MAs^{III}-exposed cells. The low concentrations of MAs and DMAs in iAs^{III}-exposed cells partly reflected the short exposure time. Consistent with previous findings (*12*, *23*), lower cellular contents of MAs and DMAs at the higher concentrations of iAs^{III} reflected inhibition of methylation in highly exposed cells. At lower concentrations of MAs^{III} in medium, DMAs concentrations exceeded those of MAs and resulted from the rapid methylation of MAs^{III} in rat hepatocytes (*12*). Because MAs^{III} is a noncompetitive inhibitor of arsenic methyltransferase (*10*), higher concentrations of MAs^{III} in medium inhibited the dimethylation reaction, reducing the percentage of DMAs in the cellular burden of As. In either iAs^{III}- or MAs^{III}-exposed cells, TR activity declined as the concentrations of arsenicals rose in cells.

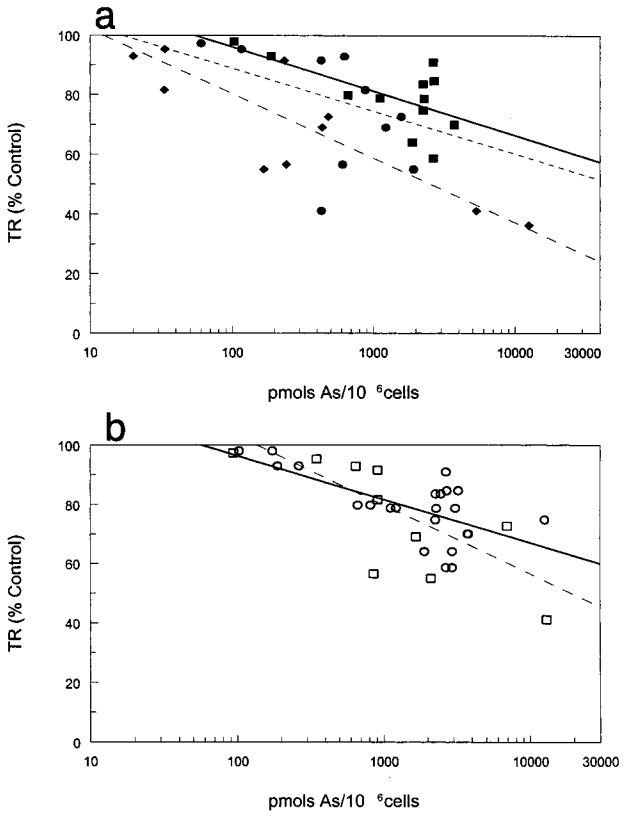

Figure 5. Relationship between the concentration of arsenicals in rat hepatocytes and thioredoxin reductase activity. (a) Relation between the intracellular concentration of inorganic As (■), methyl As (◆), or dimethyl As (●) and the DTNB reductase activity of thioredoxin reductase after exposure to inorganic As^{III} or methyl As^{III}. DTNB reductase activity normalized as a percentage of activity found in untreated hepatocytes prepared and cultured concurrently. Regression line for cellular inorganic As: $y = -14.9x + 125.9$, $r = 0.63$, $p < 0.025$. Regression line for cellular methyl As: $y = -21.6x + 123.5$, $r = 0.85$, $p < 0.01$. Regression line for cellular dimethyl As: $y = -14.4x + 117.6$, $r = 0.33$, $p > 0.1$. (b) Relation between the total intracellular concentration of arsenicals after exposure to inorganic As^{III} (○) or methyl As^{III} (□) and the DTNB reductase activity of thioredoxin reductase. For inorganic As^{III}-exposed cells, total arsenicals are the sum of inorganic As, methyl As, and dimethyl As (picomoles of As per 10^6 cells). For methyl As^{III}-exposed cells, total arsenicals are the sum of methyl As and dimethyl As (picomoles of As per 10^6 cells). DTNB reductase activity normalized as a percentage of activity found in untreated hepatocytes prepared and cultured concurrently. Regression line for inorganic As^{III}-exposed cells: $y = -14.7x + 125.7$, $r = 0.65$, $p < 0.001$. Regression line for MAs^{III}-exposed cells: $y = -23.3x + 149.7$, $r = 0.66$, $p < 0.025$.

In 24 h exposures to iAs^{III} or MAs^{III}, the relation between the cellular accumulation of these arsenicals and their metabolites and changes in TR activity was investigated. In iAs^{III}-exposed cells, TR activity fell sharply over the first hour of exposure and more slowly over the remaining 23 h of exposure. The cellular concentration of iAs in these cells rose quickly and declined over the period of exposure. MAs was the predominant methylated arsenical in these cells. The lower concentration of DMAs in these cells resulted both from the diminished rate of the dimethylation reaction in cells exposed to a relatively high concentration of iAs^{III} and from the efflux of DMAs (*23*). In MAs^{III}-exposed cells, peak concentrations of MAs occurred after 15 min and corresponded temporally to the maximal inhibition of TR activity. The concentration of MAs declined sharply after 15 min, and the inhibition of TR was reversed within the first 3 h of exposure. The

predominance of DMAs in MAs^{III}-exposed cells reflected the rapidity of the dimethylation reaction. The decline of DMAs in these cells after the first hour of exposure was due to its rapid efflux from cells. Exposure of cells to ATG produced a decline in TR activity that persisted over the entire experimental period. However, because the kinetics of ATG accumulation and metabolism in cultured hepatocytes were not examined, it was unknown whether high cellular concentrations of ATG persisted throughout the experimental period.

Dose−Response Relationships for Arsenicals and TR Activity. This study examined the relationships among the accumulation of arsenicals, the metabolic transformation of arsenicals, and inhibition of TR activity. MAs^{III} was hypothesized to be a potent inhibitor of TR activity in cultured hepatocytes. In short-term exposures, MAs^{III} proved to be a more potent inhibitor of TR activity than did iAs^{III}. However, the substantial metabolism of MAs^{III} that occurred over the 30 min time course of the short-term exposure study made it difficult to ascertain which arsenical species (MAs or DMAs) accounted for the inhibitory effect. In longer-term exposures, TR activity was only transiently reduced in MAs^{III}-treated cells. The time-dependent decrease in cellular MAs contents was associated with the recovery of TR activity. In contrast, the persistence of inhibition of TR in iAs^{III}-exposed cells was correlated with an increased concentration of MAs in cells. Taken together, this evidence suggested that MAs is the species that inhibits TR in cultured hepatocytes. Given the high avidity with which iAs^{III} and MAs^{III} are bound to proteins in rat liver cytosol (*37*), it was likely that arsenicals containing trivalent As accounted for much of the As that is retained in cells and which binds to and inhibits the catalytic function of TR. In addition, the analyses of the relationship between intracellular concentrations of arsenicals and the alteration of TR activity also suggested that MAs, among all arsenicals examined, is the most potent inhibitor of TR activity. The first analysis examined the relationship between the concentration of each arsenical metabolite and TR activity. This analysis considered neither the effect of duration of exposure to the metabolite nor its origin (i.e., added exogenously or generated by metabolism). This analysis indicated that MAs is most potent inhibitor of TR activity in cultured hepatocytes. Statistically significant correlations ($p < 0.025$) were found between the cellular concentrations of iAs and MAs and the extent of inhibition of TR activity. In a second and more conservative analysis, the relationship between the total concentration of arsenicals in cells and the extent of TR inhibition was examined. For iAs^{III}- and MAs^{III}-exposed cells, this analysis found similar statistically significant correlations ($p < 0.025$) between the total concentration of arsenicals and the extent of inhibition of TR activity. At a minimum, this analysis demonstrated that MAs^{III} was not less potent than iAs^{III} as an inhibitor of TR activity in cells that efficiently methylate trivalent arsenicals. Thus, the methylation of iAs^{III} in these cells did not detoxify the metalloid; in fact, it yielded a metabolite of equal or perhaps greater potency.

Consequences of TR Inhibition by Arsenicals. The functional consequences of inhibition of TR activity in arsenical-exposed cells are unknown. As noted above, loss of TR activity could reduce the availability of Trx needed to reduce critical macromolecules. Exposure to iAs has been reported to induce a prooxidant state in cultured aortic endothelial cells and to activate transcription factor NF-κB (*38*). Because Trx enhances the transcriptional activities of NF-κB by enhancing its binding to DNA (*15*), the inhibition of TR in As-exposed cells could alter gene expression by reducing the regeneration of Trx from Trx disulfide. The inhibition of TR activity and reduction of Trx levels have been associated with altered cell dynamics (*39*) and the induction of apoptosis (*40*−*42*). Exposure to iAs^{III} has also been associated with the induction of apoptosis in a variety of cell types (*43*−*46*), and it is possible that this effect is mediated through the loss of TR activity and a consequent reduction in cellular Trx levels. It is possible that methylated arsenicals containing As^{III} could also induce apoptosis through inhibition of TR activity.

Acknowledgment. S.L. is a postdoctoral fellow supported by Training Grant T901915 from the U.S. Environmental Protection Agency-University of North Carolina Toxicology Research Program with the Curriculum in Toxicology, University of North Carolina at Chapel Hill. L.M.D.R. was a visiting scientist at The Center for Environmental Medicine and Lung Biology at the School of Medicine, University of North Carolina at Chapel Hill (CR 824915) which is sponsored by the U.S. Environmental Protection Agency. A Drinking Water STAR Grant (R826136-01-0) from the U.S. Environmental Protection Agency and Clinical Nutrition Research Center Grant (DK 56350) from the National Institutes of Health to M.S. supported part of this work. We thank Felecia Walton of the University of North Carolina at Chapel Hill and Karen M. Herbin-Davis of the U.S. Environmental Protection Agency for excellent technical assistance. This paper has been reviewed in accordance with the policy of the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

References

(1) Smith, T. J., Crecelius, E. A., and Reading, J. C. (1977) Airborne arsenic exposure and excretion of methylated arsenic compounds. *Environ. Health Perspect.* **19**, 89−93.

(2) Crecelius, E. A. (1977) Changes in the chemical speciation of arsenic following ingestion by man. *Environ. Health Perspect.* **19**, 147−150.

(3) Yamauchi, H., and Yamamura, Y. (1979) Dynamic changes of inorganic and methylarsenic compounds in human urine after oral intake of arsenic trioxide. *Ind. Hyg.* **17**, 79−83.

(4) Cullen, W. R., McBride, B. C., and Reglinski, J. (1984) The reduction of trimethylarsine oxide to trimethylarsine by thiols: a mechanistic model for the biological reduction of arsenicals. *J. Inorg. Biochem.* **21**, 45−60.

(5) Delnomdedieu, M., Basti, M. M., Styblo, M., Otvos, J. D., and Thomas, D. J. (1994) Complexation of arsenic species in rabbit erythrocytes. *Chem. Res. Toxicol.* **7**, 621−627.

(6) Delnomdedieu, M., Styblo, M., and Thomas, D. J. (1995) Time dependence of accumulation and binding of inorganic and organic arsenic species in rabbit erythrocytes. *Chem.-Biol. Interact.* **98**, 69−83.

(7) Zakharyan, R. A., and Aposhian, H. V. (1999) Enzymatic reduction of arsenic compounds in mammalian systems: the rate-limiting enzyme of rabbit liver arsenic biotransformation is MMA(V) reductase. *Chem. Res. Toxicol.* **12**, 1278−1283.

(8) Radabaugh, T. R., and Aposhian, H. V. (2000) Enzymatic reduction of arsenic compounds in mammalian systems: reduction of arsenate to arsenite by human liver arsenate reductase. *Chem. Res. Toxicol.* **13**, 26−30.

(9) Styblo, M., Yamauchi, H., and Thomas, D. J. (1995) Comparative *in vitro* methylation of trivalent and pentavalent arsenic species. *Toxicol. Appl. Pharmacol.* **135**, 172−178.
(10) Zakharyan, R. A., Ayala-Fierro, F., Cullen, W. R., Carter, D. M., and Aposhian, H. V. (1999) Enzymatic methylation of arsenic compounds. VII. Monomethylarsonous acid (MMAIII) is the substrate for MMA methyltransferase of rabbit liver and human hepatocytes. *Toxicol. Appl. Pharmacol.* **158**, 9−15.
(11) Styblo, M., Vega, L., Germolic, D. R., Luster, M. I., Del Razo, L. M., Wang, C., Cullen, W. R., and Thomas, D. J. (1999) Metabolism and toxicity of arsenicals in cultured cells. In *Arsenic Exposure and Health Effects* (Chappell, W. R., Abernathy, C. O., and Calderon, R. L., Eds.) pp 311−323, Elsevier, Amsterdam.
(12) Petrick, J. S., Ayala-Fierro, F., Cullen, W. R., Carter, D. E., and Aposhian, H. V. (2000) Monomethylarsonous acid (MMAIII) is more toxic than arsenite in Chang human hepatocytes. *Toxicol. Appl. Pharmacol.* **163**, 203−207.
(13) Styblo, M., Serves, S. V., Cullen, W. R., and Thomas, D. J. (1997) Comparative inhibition of yeast glutathione reductase by arsenicals and arsenothiols. *Chem. Res. Toxicol.* **10**, 27−33.
(14) Lin, S., Cullen, W. R., and Thomas, D. J. (1999) Methylarsenicals and arsinothiols are potent inhibitors of mouse liver thioredoxin reductase. *Chem. Res. Toxicol.* **12**, 924−930.
(15) Hirota, K., Murata, M., Sachi, Y., Nakamura, H., Takeuchi, J., Mori, K., and Yodoi, J. (1999) Distinct roles of thioredoxin in the cytoplasm and the nucleus. A two-step mechanism of redox regulation of transcription factor NF-κB. *J. Biol. Chem.* **274**, 27891−27897.
(16) Ueno, M., Masutani, H., Arai, R. J., Yamauchi, A., Hirota, K., Sakai, T., Inamoto, T., Yamaoka, Y., Yodoi, J., and Nikaido, T. (1999) Thioredoxin-dependent redox regulation of p53-mediated p21 activation. *J. Biol. Chem.* **274**, 35809−35815.
(17) May, J. M., Mendiratta, S., Hill, K. F., and Burk, R. F. (1997) Reduction of dehydroascorbate to ascorbate by the selenoenzyme thioredoxin reductase. *J. Biol. Chem.* **272**, 22607−22610.
(18) Savini, I., Duflot, S., and Avigliano, L. (2000) Dehydroascorbic acid uptake in a human keratinocyte cell line (HaCaT) is glutathione-independent. *Biochem. J.* **345**, 665−672.
(19) Bjornstedt, M., Kumar, S., and Holmgren, A. (1992) Selenodiglutathione is a highly efficient oxidant of reduced thioredoxin and a substrate for mammalian thioredoxin reductase. *J. Biol. Chem.* **267**, 8030−8034.
(20) Bjornstedt, M., Hamberg, M., Kumar, S., Xue, J., and Holmgren, A. (1995) Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocysteine strongly stimulates the reaction via catalytically generated selenols. *J. Biol. Chem.* **270**, 11761−11764.
(21) Chae, H. Z., Chung, S. J., and Rhee, S. G. (1994) Thioredoxin-dependent peroxide reductase from yeast. *J. Biol. Chem.* **269**, 27670−27678.
(22) Lundström, J., and Holmgren, A. (1990) Protein disulfide-isomerase is a substrate for thioredoxin reductase and has thioredoxin-like activity. *J. Biol. Chem.* **265**, 9114−9120.
(23) Styblo, M., Del Razo, L. M., LeCluyse, E. L., Hamilton, G. A., Wang, C., Cullen, W. R., and Thomas, D. J. (1999) Metabolism of arsenic in primary cultures of human and rat hepatocytes. *Chem. Res. Toxicol.* **12**, 560−565.
(24) Yamauchi, H., and Fowler, B. A. (1994) Toxicity and metabolism of inorganic and methylated arsenicals. In *Arsenic in the Environment, Part II: Human Health and Ecosystem Effects* (Nriagu, J. O., Ed.) pp 35−43, Wiley, New York.
(25) International Agency for Research on Cancer (1987) Arsenic and arsenic compounds. In *IARC Monograph on the Evaluation of Carcinogenic Risks to Humans-Overall Evaluations of Carcinogenicity: An Update of IARC Monographs 1 to 42*, Suppl. 7, p 100, International Agency for Research on Cancer, Lyon, France.
(26) Reay, P. F., and Asher, C. J. (1977) Preparation and purification of ^{74}As-labeled arsenate and arsenite for use in biological experiments. *Anal. Biochem.* **78**, 557−560.
(27) Seglen, P. O. (1973) Preparation of rat liver cells. *Methods Cell Biol.* **13**, 29−83.
(28) Styblo, M., Hughes, M. F., and Thomas, D. J. (1996) Liberation and analysis of protein-bound arsenicals. *J. Chromatogr., B* **677**, 161−166.
(29) Styblo, M., Delnomdedieu, M., Hughes, M. F., and Thomas, D. J. (1995) Identification of methylated metabolites of inorganic arsenic by thin-layer chromatography. *J. Chromatogr., B* **668**, 21−29.
(30) Crecelius, E. A., Bloom, N. S., Cowan, C. E., and Jenne, E. A. (1986) Determination of arsenic species in limnological samples by hydride generation atomic absorption spectroscopy. In *Speciation of Selenium and Arsenic in Natural Waters and Sediments. Volume 2: Arsenic Speciation*, pp 1−28, Electric Power Research Institute, Palo Alto, CA (EA-4641, Project 2020-2).
(31) Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* **47**, 936−942.
(32) Kleinbaum, D. G., Kupper, L. L., and Muller, K. E. (1988) *Applied Regression Analysis and Other Multivariable Methods*, 2nd ed., pp 80−95, Duxbury Press, Belmont, CA.
(33) Chaudiere, J., and Tappel, A. L. (1984) Interaction of gold(I) with the active site of selenium glutathione reductase. *J. Inorg. Biochem.* **20**, 313−325.
(34) Berry, M. J., Kieffer, J. D., and Larsen, P. R. (1991) Evidence that cysteine, not selenocysteine, is in the catalytic site of type II iodothyronine deiodinase. *Endocrinology* **129**, 550−552.
(35) Gromer, S., Arscott, L. D., Williams, C. H., Jr., Schirmer, R. H., and Becker, K. (1998) Human thioredoxin reductase. Isolation of the selenoenzyme, steady-state kinetics, and inhibition by therapeutic gold compounds. *J. Biol. Chem.* **273**, 20096−20101.
(36) Smith, A. D., Guidry, C. A., Morris, V. C., and Levander, O. A. (1999) Aurothioglucose inhibits murine thioredoxin reductase activity in vivo. *J. Nutr.* **129**, 194−198.
(37) Styblo, M., and Thomas, D. J. (1997) Binding of arsenicals to proteins in an *in vitro* methylation system. *Toxicol. Appl. Pharmacol.* **147**, 1−8.
(38) Barchowsky, A., Dudek, E. J., Treadwell, M. D., and Wetterhahn, K. E. (1996) Arsenic induces oxidant stress and NF-κB activation in cultured aortic endothelial cells. *Free Radical Biol. Med.* **21**, 783−790.
(39) Casso, D., and Beach., D. (1996) A mutation in a thioredoxin reductase homolog suppresses p53-induced growth inhibition in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **252**, 518−529.
(40) Lindner, D. J., Hofmann, R., Karra, S., and Kalvakolanu, D. V. (2000) The interferon-beta and tamoxifen combination induces apoptosis using thioredoxin reductase. *Biochim. Biophys. Acta* **1496**, 196−206.
(41) Powis, G., Mustacich, D., and Koon, A. (2000) The role of the redox protein thioredoxin in cell growth and cancer. *Free Radical Biol. Med.* **29**, 312−322.
(42) Arner, E. S., and Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **267**, 6102−6109.
(43) McCabe, M. J., Jr., Singh, K. P., Reddy, S. A., Chelladurai, B., Pounds, J. G., Reiners, J. J., Jr., and States, J. C. (2000) Sensitivity of myelomonocytic leukemia cells to arsenite-induced cell cycle disruption, apoptosis, and enhanced differentiation is dependent on the inter-relationship between arsenic concentration, duration of treatment, and cell cycle phase. *J. Pharmacol. Exp. Ther.* **295**, 724−733.
(44) Ora, I., Bondesson, L., Jonsson, C., Ljungberg, J., Porn-Ares, I., Garwicz, S., and Pahlman, S. (2000) Arsenic trioxide inhibits neuroblastoma growth in vivo and promotes apoptotic cell death in vitro. *Biochem. Biophys. Res. Commun.* **277**, 179−185.
(45) Roboz, G. J., Dias, S., Lam, G., Lane, W. J., Soignet, S. L., Warrell, R. P., Jr., and Rafii, S. (2000) Arsenic trioxide induces dose- and time-dependent apoptosis of endothelium and may exert an antileukemic effect via inhibition of angiogenesis. *Blood* **96**, 1525−1530.
(46) Huang, S., Huang, C. F., and Lee, T. (2000) Induction of mitosis-mediated apoptosis by sodium arsenite in HeLa S3 cells. *Biochem. Pharmacol.* **60**, 771−780.

TX0001878