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RESEARCH PAPER

Arsenic trioxide and auranofin inhibit selenoprotein synthesis: implications for chemotherapy for acute promyelocytic leukaemia

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Background and purpose: Arsenicals have been used medicinally for decades to treat both infectious disease and cancer. Arsenic trioxide (As_2O_3) is effective for treatment of acute promyelocytic leukaemia (APL), yet the mechanism of action of this drug is still widely debated. Recently, As_2O_3 was shown to inhibit the activity of the selenoenzyme thioredoxin reductase (TrxR). TrxR has been proposed to be required for selenium metabolism. The effect of inhibitors of TrxR on selenium metabolism has yet to be assessed. This study aims to determine whether chemotherapeutics that target selenocysteine within selenoenzymes may also affect the metabolism of selenium.
Experimental approach: A lung cell line, A549, was used to assess the effect of TrxR inhibitors on selenium metabolism, using ^{75}Se-selenite. The level of mRNA encoding cytosolic TrxR (TrxR1) was determined using real-time reverse transcriptase-PCR. TrxR activity was determined in whole-cell extracts.
Key results: Exposure of cells to As_2O_3, arsenite or auranofin led to a concentration-dependent reduction of selenium metabolism into selenoproteins. Knockdown of TrxR1, using small inhibitory RNA, did not affect selenium metabolism. Exposure of cells to monomethylarsonic acid, a potent inhibitor of TrxR, did not alter selenium metabolism but did inhibit enzyme activity.
Conclusions and implications: As_2O_3 and auranofin block the metabolism of selenium in A549 cells. Because As_2O_3 is used to treat APL, our findings may reveal the mechanism of this therapeutic action and lead to further research targeting selenium metabolism to find novel chemotherapeutic agents for the treatment of APL.
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Keywords: arsenic trioxide; arsenite; auranofin; thioredoxin reductase; selenium; selenite; selenoprotein; acute promyelocytic leukaemia

Abbreviations: APL, acute promyelocytic leukaemia; MMA^{III}, monomethylarsonic acid; TrxR, thioredoxin reductase

Introduction

Environmental exposure to arsenic has been associated with the development of cancer of the lung, bladder, liver, kidney and skin (Hughes, 2002). The molecular mechanisms by which arsenicals contribute to carcinogenesis have been elusive and many hypotheses are being tested (Hughes, 2002; Kitchin and Ahmad, 2003). Trivalent arsenicals have been shown to induce oxidative stress in cell culture and animal models, and this oxidative damage is now seen as a critical link between arsenic exposure and cancer (Kitchin and Ahmad, 2003). Hydrogen peroxide has been implicated as the primary oxidant that increases upon exposure to arsenite (Pi *et al*., 2003); however, there is also evidence that superoxide and hydroxyl radicals increase (Shi *et al*., 2004). The binding of trivalent arsenicals to vicinal thiols has long been a hallmark of these compounds and indeed is likely to be one of the possible mechanisms for this oxidative stress (Hughes, 2002). Even the classic inhibition of pyruvate dehydrogenase has now been proposed to be due to oxidative stress (Samikkannu *et al*., 2003).

Arsenic is most often studied for its contribution to carcinogenesis, although arsenicals have been used medicinally for centuries (Waxman and Anderson, 2001; Dilda and Hogg, 2007). The trivalent form of arsenic, arsenic trioxide (As_2O_3), remains a vital tool in the treatment of acute promyelocytic leukaemia (APL) (Miller *et al*., 2002; Woo *et al*., 2002). Although arsenicals have been used medicinally for treating both APL and infectious diseases, the molecular

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mechanism(s) of these metalloid-based drugs remains poorly understood (Waxman and Anderson, 2001). Several clinical trials are ongoing to test the efficacy of As_2O_3 against solid tumours either in conjunction with all *trans*-retinoic acid or alone. It is well established that As_2O_3 reacts with thiols in the cell to form glutathione conjugates (Rey *et al*., 2004) and subsequently will also interact with closely spaced protein thiols (Woo *et al*., 2002; Samikkannu *et al*., 2003). A recent report has demonstrated that As_2O_3 can inhibit the active site of the selenoenzyme thioredoxin reductase (TrxR) (Lu *et al*., 2007). Arsenite and the methylated trivalent arsenical monomethylarsonic acid (MMA^{III}) have also been shown to be potent inhibitors of TrxR both *in vitro* and *in vivo* (Lin *et al*., 1999, 2001; Chouchane and Snow, 2001).

Selenium, also a toxic metalloid, has been studied in recent years more for its need as a critical micronutrient in the mammalian diet (Stadtman, 2000). Selenium is required for the synthesis of 25 selenoproteins that contain selenocysteine (Gromer *et al*., 2005). The two best-studied mammalian selenoenzymes are TrxR and glutathione peroxidase. Several isoenzymes exist for both, and selenocysteine is required for activity in both enzymes. The primary role of cytosolic TrxR (TrxR1) is to reduce thioredoxin (Trx). Reduced Trx is the source of reducing potential for DNA synthesis, methionine sulphoxide reductases and peroxiredoxins (Arner and Holmgren, 2000). Glutathione peroxidase isoenzymes play a critical role in reducing both hydrogen peroxides and lipid peroxides throughout the cell (Flohe *et al*., 1973, 2000; Ursini *et al*., 1995). Because of its presence in these enzymes, selenium is a critical micronutrient both for defending against oxidative stress and in cellular proliferation.

As TrxR has such a key role in proliferation and in defence against oxidative stress, several recent studies have attempted to validate inhibition of TrxR as a novel cancer therapy (Urig and Becker, 2006). Both Trx and TrxR were found to be upregulated in tumour-derived cell lines, helping to validate TrxR as a potential target (Berggren *et al*., 1996). Both novel natural products and platinum compounds have been found to inhibit TrxR1 (Becker *et al*., 2001; Wipf *et al*., 2001). The combination of oxidative stress and a lack of DNA synthesis could indeed be a potent weapon for cancer chemotherapy, as indicated by more recent studies using a gadolinium compound that shows promise in clinical trials (Hashemy *et al*., 2006). Gold-based drugs have been established inhibitors of TrxR enzymes *in vitro* for several years (Hill *et al*., 1997; Gromer *et al*., 1998; Rigobello *et al*., 2002; Engman *et al*., 2006; Omata *et al*., 2006). The gold compound, auranofin, used to treat rheumatoid arthritis, has been shown to be the most potent inhibitor of this enzyme activity *in vitro* and *in vivo* (Rigobello *et al*., 2002; Omata *et al*., 2006). Both have a high affinity for the active site of selenol (reduced form of selenocysteine) in the penultimate CXU (cysteine, any amino acid, selenocysteine) motif (Gladyshev *et al*., 1996).

Selenocysteine is inserted into the polypeptide chain by a series of specific elongation factors and through the use of an RNA stem–loop structure known as a SECIS (selenocysteine insertion sequence) element (Berry *et al*., 2001; Carlson *et al*., 2004; Hoffmann and Berry, 2005; de Jesus *et al*., 2006). In both eukaryotes and prokaryotes, selenium must first be reduced to hydrogen selenide (Se^{2-}) before activation by an enzyme termed selenophosphate synthetase (Ehrenreich *et al*., 1992; Low *et al*., 1995; Yuan *et al*., 2006). The mechanisms by which selenium is taken up into cells and subsequently reduced are poorly understood, yet a chemical reaction known as the Painter reaction has been shown to efficiently reduce selenite to selenide in the presence of excess thiols (Ganther, 1968, 1971, 1999). Because of the need for a strong reducing potential to maintain selenium in the form of selenide, it has been suggested that TrxR plays a role in selenium metabolism (Arner and Holmgren, 2000). Indeed, it has been shown that TrxR can efficiently reduce selenite to selenide *in vitro* (Bjornstedt *et al*., 1997). A putative role for TrxR in selenium metabolism has been postulated based on this study, but no evidence for this occurring *in vivo* has been reported.

In addition to affecting TrxR activity, trivalent arsenicals have also been shown to affect the metabolism of selenium. The initial report by Moxon (1938) demonstrated a reduction in toxicity from selenium poisoning by administration of arsenite. This mutual sparing effect was later named the Moxon effect and has been studied primarily in animal models over several decades (Levander and Baumann, 1966; Levander, 1977). A metabolic link between arsenic and selenium was established in a key finding by Gailer *et al*. (2000b, 2002). A stable compound, seleno-bis(*S*-glutathionyl) arsinium ion, is formed upon reaction of arsenite with reduced glutathione, followed by reaction with hydrogen selenide. Similarly, studies have shown that administration of selenium to animals can affect the metabolism and distribution of mercury (Cikrt and Bencko, 1989; Urano *et al*., 1997; Gailer *et al*., 2000a; Gregus *et al*., 2001), and evidence has been shown that the coexcretion of mercury and selenium is tied to the formation of thiol conjugates. Platinum and gold drugs have been shown to affect selenium metabolism in animal models as well (Gregus *et al*., 2000). Therefore, there is a growing body of evidence that many of the metals that function to inhibit the reduced selenol of TrxR may also react with other reduced forms of selenium. Given the surge of interest in targeting TrxR, essentially through its reduced selenol residue, one must both determine the role that this enzyme plays in selenium metabolism and also appreciate the potential chemistry that can occur between reactive metals and selenium metabolic intermediates.

In this study, we have determined the impact of two clinically relevant inhibitors of TrxR, As_2O_3 and auranofin, on selenium metabolism in a cultured lung cell line. We have also evaluated the role that TrxR plays in the reduction and metabolism of selenium in the same culture model.

Methods

Cell culture

A549 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with L-glutamine, sodium pyruvate, $4.5\,g\,L^{-1}$

glucose, 100 μg mL^{-1} streptomycin, 100 IU mL^{-1} penicillin (Mediatech, Herndon, VA, USA) and 10% fetal bovine serum (Equi-tech Bio, Kerrville, TX, USA). Cells were maintained in a humidified incubator at a temperature of 37 °C under an atmosphere of 5% CO_2.

Radioisotope labelling

To follow incorporation of selenium into selenoproteins, A549 cells were cultured in six-well plates and grown to approximately 70% confluence. Radioisotope ^{75}Se (2 μCi), carried with 10 nM of unlabelled sodium selenite, was added to each culture. Exposure to arsenite (0, 2, 6 or 10 μM), As_2O_3 (0, 1, 2.5 or 5 μM) or MMA^{III} (0, 2 or 6 μM) immediately preceded the addition of the radioisotope. In studies following the effects of auranofin on selenium metabolism, the addition of auranofin (0.1, 0.25, 1 or 3 μM) to cells was performed 4 h before addition of either ^{75}Se or ^{35}S. The cells were incubated in the presence of radioisotope selenium for 24 h before harvesting. To monitor effects on protein synthesis, 30 μCi of ^{35}S, in the form of a cysteine/methionine mixture, was added to each culture under the same conditions described above for the labelling of selenoproteins. In these experiments, cells were cultured in a cysteine/methionine-depleted DMEM (Mediatech).

Cells were harvested by first washing with Dulbecco's phosphate-buffered saline, and then the monolayer was disrupted by the addition of trypsin-EDTA (Mediatech) for 4 min at 37 °C. Cells were collected by centrifugation and subsequently washed with Dulbecco's phosphate-buffered saline. The resulting cell pellets were resuspended in 200 μL of lysis buffer (50 mM tricine, pH 8.0, 0.1 mM benzamidine, 0.5 mM EDTA and 1 mM dithiothreitol) and lysed by sonication for 6–8 s using a model 100 sonic dismembrator (Thermo Fisher Scientific, Pittsburgh, PA, USA) at a power setting of 4 W. Crude lysates were clarified by centrifugation at 15 700 *g* for 7 min at 4 °C. The amount of ^{75}Se was determined in extracts using a Wallac Wizard Gamma Counter, model 1470 (Perkin Elmer, Waltham, MA, USA) and ^{35}S by liquid scintillation with a Packard TriCarb 2900TR counter (Perkin Elmer). Protein concentration was quantified by the method of Bradford (1976) using BSA as a standard. To confirm that selenium incorporation into specific selenoproteins occurred, 25 μg of protein was separated using a 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, dried and then exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA). Selenoproteins were visualized using a Storm Phosphoimager and analysed using ImageQuant software (Molecular Dynamics).

Transient small inhibitory RNA knockdowns

A549 cells were seeded in six-well plates and grown to approximately 60% confluence. Small inhibitory RNA (siRNA) duplex molecules specifically targeting the mRNA encoding p42 microtubule-associated protein kinase (Erk2 (extracellular signal-regulated kinase)), Trx and TrxR1 were obtained from Qiagen (Valencia, CA, USA). The sequences (sense and antisense RNA) used are as follows (5′ to 3′): Erk2-UGCUGACUCCAAAGCUCUG, CAGAGCUUUGGAGUCAG CA; Trx-CCAUUAAUGAAUUAGUCUA, UAGACUAAUUCAU UAAUGG; TrxR1-GCAAGACUCUCGAAAUUAU, AUAAUUU CGAGAGUCUUGC. A non-silencing fluorescent control oligonucleotide duplex, labelled with Alexa Fluor 488, was used to determine siRNA transfection efficiency. The efficiency of transfection with this fluorescent siRNA was determined to be 66% (s.d.: ± 9%) by directly counting fluorescent cells using an inverted fluorescence microscope (Zeiss, Thornwood, NY, USA).

Transfection complexes were prepared by a mixture of serum-free media, HiPerfect transfection reagent (Qiagen) and 5 nM siRNA. These reagents were incubated for 10 min at room temperature to allow transfection complexes to form and were subsequently added dropwise to cells for optimal transfection efficiency. Immediately following the addition of 5 nM siRNA, cells were treated with 3 μM auranofin to inhibit existing TrxR. Cells were incubated for 24 h with siRNA and auranofin before removing media and labelling with ^{75}Se, as described in the previous section.

Real-time reverse transcriptase-PCR analysis

For semiquantitative analysis of mRNA, cells were cultured in 25 cm^2 flasks and treated with arsenite, As_2O_3 or auranofin for 24 h at the appropriate concentrations. Cells were harvested by washing with Dulbecco's phosphate-buffered saline and subsequently removed from the monolayer using trypsin-EDTA. The cells were then washed with Dulbecco's phosphate-buffered saline treated with 0.1% diethylpyrocarbonate. RNA was isolated using a ChargeSwitch Total RNA kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and the RNA concentration was determined by UV-visible spectrophotometry at 260 nm using an Agilent 8453 UV-visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). cDNA was produced from 0.5 μg of RNA as a template using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Primers used for real-time reverse transcriptase-PCR (RT-PCR) included (listed forward then reverse) TrxR1: 5′-AGCTCAGTCCACCAATAGTGA-3′ and 5′-GGTATTTTTCCAG TCTTTTCAT-3′; β-actin: 5′-CATGTACGTTGCTATCCA-3′ and 5′-CTCCTTAATGTCACGCACGAT-3′; Trx: 5′-GCAGATCGAG AGCAAGACTG-3′ and 5′-CTCCAGAAAATTCACCCACC-3′. All real-time RT-PCR was carried out in a Bio-Rad I-Cycler (Bio-Rad), and reactions (total volume of 25 μL) consisted of SYBRgreen supermix (Bio-Rad), four oligonucleotides at a final concentration of 0.2 mM each, and forward and reverse primers at a final concentration of 0.2 μM and 5 μL of 1:100 diluted cDNA. Reaction conditions were as follows: one cycle at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 55 °C for 45 s. Melt curves were used to determine the formation of a single product. Efficiency of amplification of each primer pair was calculated with a 10-fold dilution series of control cDNA. The relative expression (fold change in expression versus control) for each of the mRNAs of interest using β-actin as an internal mRNA standard was determined using a previously described method (Pfaffl, 2001).

TrxR activity assays

A549 cells were cultured in 75 cm^2 flasks in DMEM with 10% serum as described above. Cells were treated with arsenite, MMA^{III} or auranofin for 48 h before to harvesting with the appropriate concentrations of each compound. Cell pellets were resuspended in lysis buffer (5 mM potassium phosphate (pH 7.4) and 0.5 mM EDTA) and sonicated using a model 100 sonic dismembranator (Thermo Fisher Scientific) with a power of 4 W. TrxR assays were performed as described previously with minor modifications (Smith and Levander, 2002). To measure the gold-inhibited activity to differentiate TrxR from glutathione reductase, cell extracts were preincubated with 1 μM auranofin for 20 min at room temperature to allow for complete inhibition of TrxR. A 50 μg portion of protein was used to initiate the activity assay in reactions in a 96-well plate. Reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) was followed at 412 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Reactions were carried out at 37 °C, taking readings every 15 s for 3 min. TrxR activity is reported as previously described (Smith and Levander, 2002).

Statistical analysis

All statistical analyses were performed using Microsoft Excel, and ANOVA was performed to test for statistical significance within data sets.

Materials

Sodium selenite, sodium arsenite and As_2O_3 were obtained from Acros Organics (Geel, Belgium). MMA^{III} was from the laboratory of Dr. William Cullen (University of British Columbia, Vancouver, BC, Canada). The ^{75}Se radioisotope was obtained from the University of Missouri Research Reactor (MURR, Columbia, MO, USA) in the form of selenious acid. ^{35}S-methionine/cysteine label was obtained either from Amersham BioSciences (Piscataway, NJ, USA) or from Perkin Elmer Life Sciences (Boston, MA, USA). Auranofin was from Axxora LLC (San Diego, CA, USA).

Results

Arsenite inhibits selenoprotein synthesis in lung adenocarcinoma cells (A549)

Addition of arsenite at concentrations between 2 and 10 μM resulted in a concentration-dependent decrease in selenoprotein synthesis in A549 cells. We separately tested for the cytotoxicity of arsenite after incubation with A549 for 24 h using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (data not shown). A 50% reduction in cell viability was observed when cells were incubated with 100 μM arsenite. At concentrations of arsenite at or below 10 μM, no significant changes in cell viability were observed. Exposure of cells to arsenite did slightly reduce protein synthesis in cells treated at all three concentrations (Figure 1b); however, there was no dose-dependent decrease as is seen for selenoprotein synthesis.

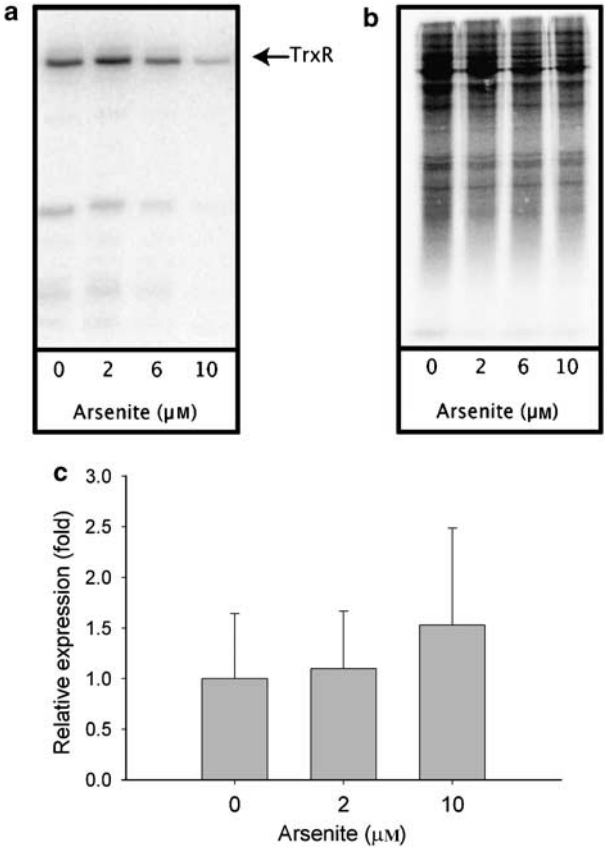

Figure 1 Treatment of lung adenocarcinoma cells (A549) with arsenite inhibits selenoprotein synthesis. A549 cells were cultured in DMEM and then exposed to arsenite (0, 2, 6 and 10 μM), immediately followed by radiolabelling with ^{75}Se (selenite) (**a**) or ^{35}S (**b**) for 24 h. Cells were subsequently harvested and 30 μg of protein from cell extracts was separated by 15% SDS-PAGE. Proteins were visualized by autoradiography. TrxR was identified as the predominant labelled selenoprotein by its size using a standard protein marker (data not shown). Cells were treated for 24 h with 0, 2 or 10 μM arsenite followed by isolation of RNA for real-time RT-PCR analysis. β-Actin was used as an internal standard. Mean relative expression (fold) is plotted from multiple experiments with cultures grown in triplicate in each experiment. Error bars represent the s.d. No statistical significance was found between TrxR1 transcript levels in cells treated with arsenite, as determined by one-way ANOVA ($P > 0.05$) (**c**). DMEM, Dulbecco's modification of Eagle's medium; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TrxR, thioredoxin reductase; RT, reverse transcriptase.

As overall selenoprotein synthesis is reduced in cells treated with arsenite, we determined whether this treatment was significantly affecting the stability of mRNAs encoding selenoproteins. TrxR1 is the most abundant selenoprotein expressed in A549 (Figure 1a), hence we chose its mRNA for this analysis. Using semiquantitative real-time RT-PCR, we evaluated the impact of treating cells with arsenite on TrxR1 mRNA levels. Figure 1c shows that no statistically significant changes in TrxR1 mRNA levels were observed, yet a slight increase was seen in cells treated with 10 μM arsenite.

These results, when combined with radiolabelling data in Figure 1a, clearly show that subtoxic levels of arsenite significantly reduce the incorporation of selenium into selenoproteins in lung adenocarcinoma cells. Two mechanisms could account for this effect: the direct formation of

seleno-bis(*S*-glutathionyl) arsinium ion, as described by Gailer *et al*. (2000b), or the inhibition of TrxR, as implicated in selenoprotein synthesis, as described by Holmgren (Bjornstedt *et al*., 1997; Arner and Holmgren, 2000). As the impact of trivalent inorganic arsenicals on human cells has implications both in carcinogenesis and chemotherapy, we designed experiments to determine which of the above mechanisms are most likely to account for the observed inhibition of selenium metabolism.

As_2O_3 blocks selenium incorporation into selenoproteins

Similar to the effects of arsenite, As_2O_3 treatment of A549 cells led to decreases in selenoprotein synthesis as demonstrated by ^{75}Se radiolabelling (Figure 2a). This inhibition was observed in cells treated with As_2O_3 at a concentration of 2.5 μM and continues in a concentration-dependent manner.

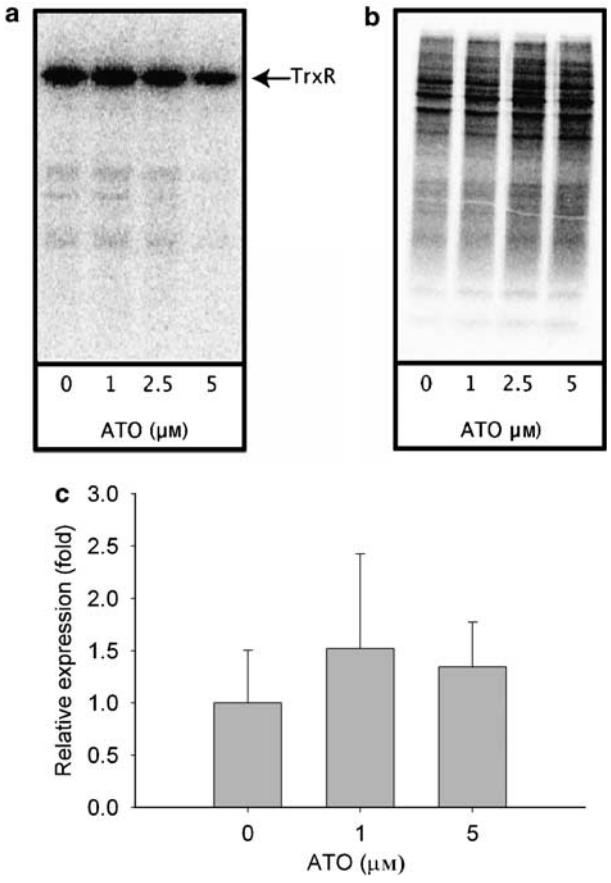

Figure 2 As_2O_3 (ATO) inhibits selenoprotein synthesis but not general protein synthesis in A549 cells. A549 cells were cultured in DMEM and treated with either 0, 1, 2.5 or 5 μM ATO and labelled with 2 μCi of ^{75}Se (**a**) or ^{35}S (**b**) for 48 h. Cells were harvested and 15 μg of protein from extracts was separated by SDS-PAGE (15%). Selenoproteins (^{75}Se) and general protein synthesis (^{35}S) were visualized by phosphorimage analysis. For RT-PCR, (**c**) cells were exposed to 0, 1 or 5 μM ATO for 48 h. Mean relative expression (fold) is plotted from multiple experiments with cultures grown in triplicate in each experiment. Error bars represent the s.d. No statistical significance was found between TrxR1 transcript levels in cells treated with arsenite, as determined by one-way ANOVA ($P>0.05$) (**c**). DMEM, Dulbecco's modification of Eagle's medium; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; RT, reverse transcriptase.

In addition, As_2O_3 did not affect general protein synthesis, as determined by ^{35}S labelling studies (Figure 2b). Likewise, as with treatment with arsenite, mRNA levels encoding TrxR1 do not significantly change with increasing exposure to As_2O_3 (Figure 2c). Given that two trivalent arsenicals, known inhibitors of TrxR1, decrease selenium incorporation into selenoproteins, the next step was to see if this phenotype was again present with a separate inhibitor of TrxR1 that is not arsenic based. For this, we chose the gold-containing compound, auranofin.

Auranofin treatment of A549 cells inhibits selenoprotein synthesis

Auranofin has been shown to inhibit TrxR both *in vitro* and in cultured cells (Gromer *et al*., 1998, 2002; Rigobello *et al*., 2002, 2005; Omata *et al*., 2006). Treatment of A549 cells with auranofin at concentrations ranging from 0.1 to 3 μM resulted in dramatic decreases in selenoprotein synthesis (Figure 3a). Treatment with as little as 1 μM auranofin led to a near complete cessation of selenium incorporation into newly synthesized selenoproteins. To confirm that this effect was not due to the inhibition of protein synthesis in general,

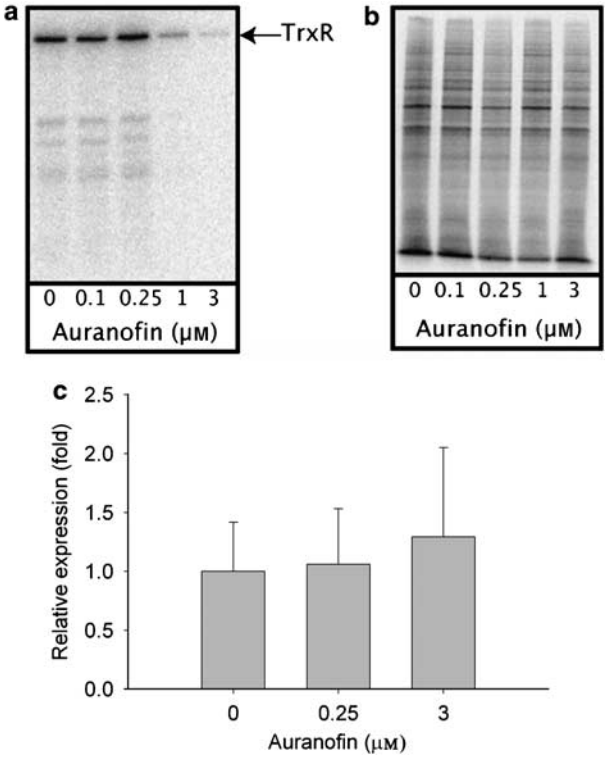

Figure 3 Auranofin treatment results in inhibition of incorporation of selenium into selenoproteins. A549 cells were treated with varying concentrations of auranofin (0, 0.1, 0.25, 1 and 3 μM) and protein synthesis was followed with either ^{75}Se (**a**) or ^{35}S (**b**) for 24 h. Cell extracts were separated by 15% SDS-PAGE and visualized by autoradiography. For RT-PCR, (**c**) cells were exposed to 0, 0.25 or 3 μM auranofin for 24 h. Mean relative expression (fold) was plotted from multiple experiments from cultures grown in triplicate in each experiment. Error bars represent the s.d. No statistical significance was found between the TrxR1 transcript levels in cells treated with arsenite as determined by one-way ANOVA ($P>0.05$) (**c**). SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; RT, reverse transcriptase.

Table 1 TrxR activity in cells treated with auranofin

Concentration of auranofin (μM)	*TrxR activity (nmol min^{-1} mg^{-1})*	*s.d.*
0	10.4	1.86
0.25	10.3	1.83
1	7.82	1.19
3	1.99	0.81

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TrxR, thioredoxin reductase.
TrxR activities were determined by DTNB assay, as previously described. A549 cells were cultured for 24 h in the presence of 0, 0.25, 1 or 3 μM auranofin before harvesting. The average TrxR activity is derived from six independent experiments with duplicate cultures, and the s.d. from the mean is shown.

we followed incorporation of ^{35}S into proteins under the same experimental conditions (Figure 3b). No significant changes in protein synthesis occurred, demonstrating that the impact of auranofin was specific to selenium metabolism. The treatment of cells with auranofin also did not affect mRNA levels encoding for TrxR1 (Figure 3c). These results further support the hypothesis that the mode of action of arsenite and As_2O_3 is through a direct inhibition of TrxR.

Auranofin and other gold compounds have been previously shown to inhibit TrxR1 in cultured cells (Omata *et al*., 2006). We wanted to confirm that inhibition of TrxR1 was indeed occurring under our treatment conditions in A549. Auranofin treatment of A549 cells did result in inhibition of TrxR activity (Table 1); however, the residual TrxR activity present in cells treated with 1 and 3 μM auranofin did not directly correlate with the potency observed in the selenium labelling experiment (Figure 3a). When TrxR activity was reduced only slightly, specifically in cells treated with 1 μM auranofin (Table 1), the incorporation of selenium into selenoproteins was dramatically reduced (Figure 3a). This finding suggests that TrxR1 may not be the primary target of arsenite and also suggests that auranofin may inhibit selenium metabolism through an alternative, as yet undescribed, mechanism.

Knockdown of Trx or TrxR1 does not result in changes in selenoprotein synthesis

To directly assess the role of TrxR1 on selenoprotein synthesis, we utilized transient transfection of siRNA molecules that specifically target TrxR1, Trx or a negative control (microtubule-associated protein kinase-1/Erk2) to critically evaluate the role of the TrxR/Trx system on selenium metabolism. Because of the need for potent reducing potential for the efficient conversion of selenite to selenide, it had been previously suggested that this system was required for selenium metabolism to selenide (Bjornstedt *et al*., 1997; Arner and Holmgren, 2000). There is good biochemical evidence that TrxR can efficiently reduce selenite to selenide *in vitro*, but there is no evidence for the reduction of selenite to selenide in cell culture or animal studies.

As expected, knockdown of microtubule-associated protein kinase-1 did not affect selenoprotein synthesis (Figure 4, lane 2). Knockdown of the mRNA encoding Trx was confirmed by real-time RT-PCR (Figure 5a), but it did not affect the incorporation of selenium into other selenoproteins (Figure 4a, lane 3). Surprisingly, knockdown of TrxR1 did not affect overall selenoprotein synthesis, although no new synthesis of TrxR1 was taking place based on selenium ^{75}Se incorporation (Figure 4, lane 4). For the experiment shown in Figure 4, cells were pretreated with auranofin at 3 μM to efficiently reduce TrxR1 activity (residual) before and during the early phases of the RNA interference transfection. This was performed in an attempt to remove existing TrxR1, so that a critical assessment of the role of TrxR1 could be accomplished. Omitting the pretreatment with auranofin did not alter the results obtained (data not shown). Knockdown of Trx and TrxR1 was confirmed by RT-PCR (Figure 5). These results strongly suggest that Trx and TrxR1 are not directly required for the reduction of selenium under these conditions.

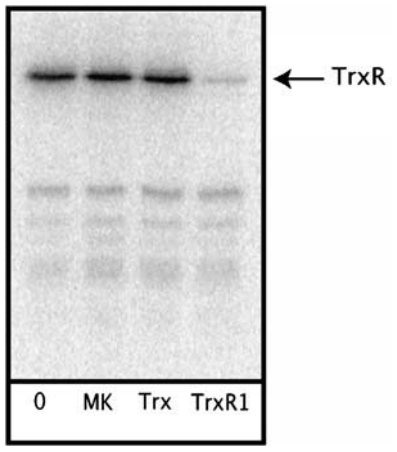

Figure 4 Knockdown of Trx or TrxR1 does not affect incorporation of selenium into other selenoproteins. A549 cells were treated with 5 nM siRNA targeting the mRNA encoding microtubule-associated protein kinase-1 (MK), Trx or TrxR1. Cells were also treated with 3 μM auranofin to inhibit the activity of existing TrxR. After 24 h, the media were removed, replaced with fresh media without auranofin and ^{75}Se was then added. The cells were then incubated for an additional 24 h to assess selenium incorporation. A 20 μg portion of protein from cell lysates was separated by 15% SDS-PAGE, and radiolabelled selenoproteins were visualized by ^{75}Se autoradiography. Trx, thioredoxin; TrxR1, thioredoxin reductase 1; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

MMA^{III} treatment of cells does not inhibit selenium incorporation into selenoproteins

Monomethylarsonic acid is known to be a potent inhibitor of TrxR and is probably the most potent inhibitor of TrxR1 of all trivalent arsenicals (Lin *et al*., 1999). If TrxR is involved in selenoprotein synthesis, then exposure of cells to MMA^{III} should generate the same phenotype as arsenite, As_2O_3 and auranofin. However, in cells treated with increasing concentrations of MMA^{III} and subsequently labelled with ^{75}Se, there was no change in selenium incorporation into selenoproteins (Figure 6). Cells were exposed to a high enough concentration to inhibit the enzyme as determined by the 5,5′-dithiobis-(2-nitrobenzoic acid) assays (Table 2). Upon exposure to 6 μM MMA^{III}, TrxR is significantly inhibited and the level of selenium incorporation into

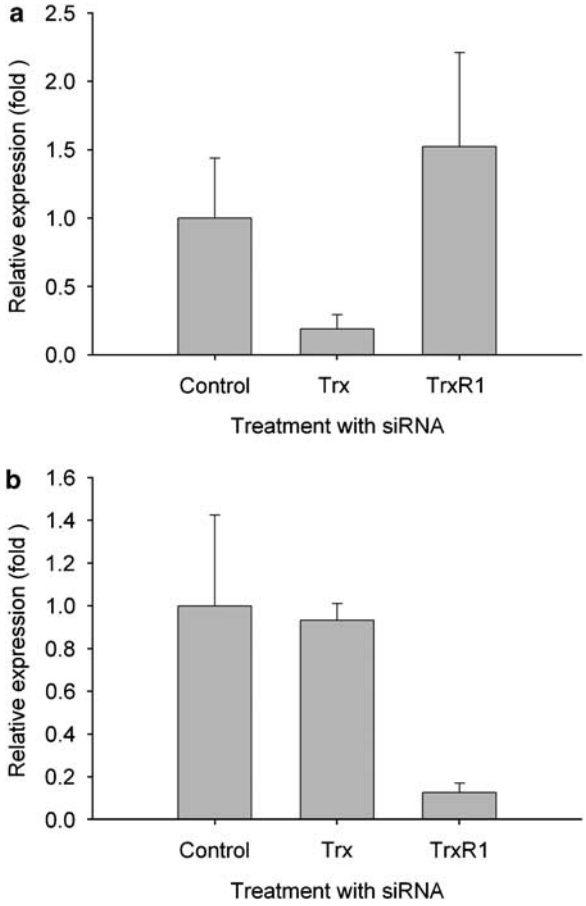

Figure 5 Confirmation of Trx and TrxR1 knockdowns by RT-PCR. Real-time RT-PCR (**a** and **b**) was used to monitor efficiency of the Trx and TrxR1 knockdowns by analysing mRNA levels. β-Actin was used as an internal standard for analysis. Mean relative expression (fold change) in (**a** and **b**) is derived from a representative experiment with duplicate cultures that were analysed in triplicate. Error bars represent the s.d. Trx, thioredoxin; TrxR1, thioredoxin reductase 1; RT, reverse transcriptase.

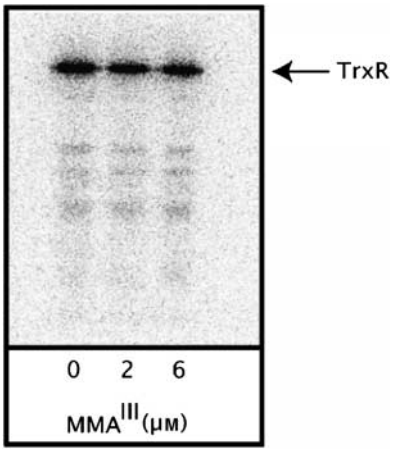

Figure 6 MMA^{III} does not affect selenium incorporation into selenoproteins. Cells were exposed to 0, 2 or 6 μM MMA^{III} and immediately labelled with ^{75}Se to follow selenium incorporation into selenoproteins for 24 h before harvesting. Approximately 20 μg of protein was separated by 15% SDS-PAGE. Selenoproteins were visualized by autoradiography. MMA^{III}, monomethylarsonic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Table 2 TrxR activity in A549 cells exposed to MMA^{III}

Concentration of MMA^{III} (μM)	*TrxR activity (nmol min^{-1} mg^{-1})*	*s.d.*
0	8.05	0.290
2	6.41	3.46
6	0.103	0.226

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); MMA^{III}, monomethylarsonic acid; TrxR, thioredoxin reductase.
TrxR activities were determined by the DTNB assay, as described in the Methods section. Cells were cultured (six independent cultures) in duplicate with 0, 2 or 6 μM MMA^{III} for 48 h before harvesting.

selenoproteins was unchanged (Table 2 and Figure 5, lane 3). These results clearly show that inhibition of TrxR did not lead to inhibition of selenium metabolism.

Discussion and conclusion

Arsenic trioxide is currently approved for the treatment of APL; however, the mechanism of its carcinostatic action is not well understood. In a previous study, 2.5 and 5 μM As_2O_3 were found to inhibit TrxR1 in MCF-7 breast cancer cells by 40% (Lu *et al.*, 2007). Here, we demonstrate that treatment of cells with As_2O_3 decreased selenium incorporation into selenoproteins. Our results show that arsenite or auranofin can also inhibit the incorporation of selenium into selenoproteins in lung cells, possibly through forming a complex as described by Gailer *et al.* (2002). Indeed, a recent study has shown that incubation of red blood cells with arsenite and selenite *in vitro* results in intracellular accumulation of this complex (Manley *et al.*, 2006). The sole chemical requirement for synthesis of this complex is reduced glutathione, present in millimolar concentrations in nearly all mammalian cells. Our results may have implications for the mode of action of As_2O_3 in chemotherapy. With the decreased capacity to produce selenoproteins, the cell loses some of its antioxidant capabilities, and this could lead to increased oxidative stress. Oxidative stress has been suggested to be part of the mechanism of how As_2O_3 works in the treatment of APL. Our results would indicate that a metabolic inhibition of selenium metabolism could lead to this observed oxidative stress.

The mechanism of auranofin in the treatment of rheumatoid arthritis is also poorly understood. It has been reported to function by inhibiting nuclear factor-κB translocation to the nucleus (Yamashita *et al.*, 2003). Several cell culture studies have also suggested that auranofin could be used for chemotherapy of tumours. In one such study, it was found that auranofin induced apoptosis in cisplatin-resistant ovarian cancer cells, mediated through the release of cytochrome *c* (Marzano *et al.*, 2007). Auranofin was also shown to induce apoptosis in APL-derived cells when combined with retinoic acid (Kim *et al.*, 2004). As our results show that auranofin reduces selenium incorporation into selenoproteins when administered to cells at a subtoxic level, this may suggest that both As_2O_3 and auranofin act to generate oxidative stress and arrest DNA synthesis through inhibition of selenium metabolism. This would then indicate

that these results represent a 'proof of principle' that blocking selenium metabolism could be a valid target for the development of novel chemotherapeutic agents.

There has been some speculation on the role of TrxR in selenoprotein synthesis (Ganther, 1999; Papp *et al*., 2006). Hydrogen selenide is required as the substrate for selenophosphate synthetase 2 to produce selenophosphate (Xu *et al*., 2006). We have recently shown that cells can transport selenide with high affinity (Ganyc and Self, 2008). This suggests that selenide metabolism is the critical link to selenium metabolism from uptake to initial activation by phosphorylation. In this study, we have demonstrated that TrxR is not involved in selenoprotein synthesis. Even though three known inhibitors of TrxR decreased selenium incorporation into selenoproteins, knockdown of TrxR1 expression resulted in no change in incorporation of selenium into selenoproteins. The targeted siRNA experiments suggest that TrxR1 is not involved in selenoprotein synthesis. However, exposure of cells to MMA^{III}, which blocked all TrxR activity, resulted in no change in incorporation of selenium into selenoproteins. It then becomes important to understand the possible molecular mechanisms of the inhibition of selenium metabolism induced by auranofin and As_2O_3.

A well-established metabolic inhibitor of selenium metabolism is arsenite (Gailer *et al*., 2000b, 2002). This metabolic inhibition occurs via the formation of seleno-bis(*S*-glutathionyl) arsinium ion (Gailer *et al*., 2002). We propose that inhibition of selenium metabolism is also occurring when cells are treated with As_2O_3 and auranofin, but not with MMA^{III}. The chemical nature of complexes formed between As_2O_3 or auranofin and selenide has not as yet been determined. Insoluble arsenic selenides have been studied for years as semiconductors as glasses (Chen *et al*., 2006); however, it is unclear as to whether an insoluble complex of arsenic and selenium is formed *in vivo*. Likewise, it is not yet known whether auranofin and selenide will form a stable conjugate. Studies are ongoing to define the metal/metalloid complexes formed between As_2O_3 or auranofin and selenide, and the results of this analysis should lead to an understanding of the inhibition of selenoprotein synthesis observed in this study. Indeed, we believe that the results shown in this report may lead to novel drug discovery efforts to target selenium metabolism as a means to treat APL.

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Conflict of interest

The authors state no conflict of interest.

References

Arner ES, Holmgren A (2000). Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* **267**: 6102–6109.

Becker K, Herold-Mende C, Park JJ, Lowe G, Schirmer RH (2001). Human thioredoxin reductase is efficiently inhibited by (2,2′:6′,2″-terpyridine)platinum(II) complexes. Possible implications for a novel antitumor strategy. *J Med Chem* **44**: 2784–2792.

Berggren M, Gallegos A, Gasdaska JR, Gasdaska PY, Warneke J, Powis G (1996). Thioredoxin and thioredoxin reductase gene expression in human tumors and cell lines, and the effects of serum stimulation and hypoxia. *Anticancer Res* **16**: 3459–3466.

Berry MJ, Tujebajeva RM, Copeland PR, Xu XM, Carlson BA, Martin III GW *et al*. (2001). Selenocysteine incorporation directed from the 3′UTR: characterization of eukaryotic EFsec and mechanistic implications. *Biofactors* **14**: 17–24.

Bjornstedt M, Kumar S, Bjorkhem L, Spyrou G, Holmgren A (1997). Selenium and the thioredoxin and glutaredoxin systems. *Biomed Environ Sci* **10**: 271–279.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**: 248–254.

Carlson BA, Xu XM, Kryukov GV, Rao M, Berry MJ, Gladyshev VN *et al*. (2004). Identification and characterization of phosphoseryl-tRNA[Ser]Sec kinase. *Proc Natl Acad Sci USA* **101**: 12848–12853.

Chen G, Jain H, Vlcek M, Ganjoo A (2006). Photoinduced volume change in arsenic chalcogenides by band-gap light. *Phys Rev B* **74**: 174203.

Chouchane S, Snow ET (2001). *In vitro* effect of arsenical compounds on glutathione-related enzymes. *Chem Res Toxicol* **14**: 517–522.

Cikrt M, Bencko V (1989). Mercury–selenium interaction: distribution and excretion of 203Hg2+ in rats after simultaneous administration of selenite or selenate. *Toxicol Lett* **48**: 159–164.

de Jesus LA, Hoffmann PR, Michaud T, Forry EP, Small-Howard A, Stillwell RJ *et al*. (2006). Nuclear assembly of UGA decoding complexes on selenoprotein mRNAs: a mechanism for eluding nonsense-mediated decay? *Mol Cell Biol* **26**: 1795–1805.

Dilda PJ, Hogg PJ (2007). Arsenical-based cancer drugs. *Cancer Treat Rev* **33**: 542–564.

Ehrenreich A, Forchhammer K, Tormay P, Veprek B, Bock A (1992). Selenoprotein synthesis in *E. coli*. Purification and characterisation of the enzyme catalysing selenium activation. *Eur J Biochem* **206**: 767–773.

Engman L, McNaughton M, Gajewska M, Kumar S, Birmingham A, Powis G (2006). Thioredoxin reductase and cancer cell growth inhibition by organogold(III) compounds. *Anticancer Drugs* **17**: 539–544.

Flohe L, Andreesen JR, Brigelius-Flohe R, Maiorino M, Ursini F (2000). Selenium, the element of the moon, in life on earth. *IUBMB Life* **49**: 411–420.

Flohe L, Gunzler WA, Schock HH (1973). Glutathione peroxidase: a selenoenzyme. *FEBS Lett* **32**: 132–134.

Gailer J, George GN, Pickering IJ, Madden S, Prince RC, Yu EY *et al*. (2000a). Structural basis of the antagonism between inorganic mercury and selenium in mammals. *Chem Res Toxicol* **13**: 1135–1142.

Gailer J, George GN, Pickering IJ, Prince RC, Ringwald SC, Pemberton JE *et al*. (2000b). A metabolic link between arsenite and selenite: the seleno-bis(*S*-glutathionyl) arsinium ion. *J Am Chem Soc* **122**: 4637–4639.

Gailer J, George GN, Pickering IJ, Prince RC, Younis HS, Winzerling JJ (2002). Biliary excretion of $[(GS)_{(2)}AsSe](-)$ after intravenous injection of rabbits with arsenite and selenate. *Chem Res Toxicol* **15**: 1466–1471.

Ganther HE (1968). Selenotrisulfides. Formation by the reaction of thiols with selenious acid. *Biochemistry* **7**: 2898–2905.

Ganther HE (1971). Reduction of the selenotrisulfide derivative of glutathione to a persulfide analog by glutathione reductase. *Biochemistry* **10**: 4089–4098.

Ganther HE (1999). Selenium metabolism, selenoproteins and mechanisms of cancer prevention: complexities with thioredoxin reductase. *Carcinogenesis* **20**: 1657–1666.

Ganyc D, Self WT (2008). High affinity selenium uptake in a keratinocyte model. *FEBS Lett* **582**: 299–304.

Gladyshev VN, Jeang KT, Stadtman TC (1996). Selenocysteine, identified as the penultimate C-terminal residue in human T-cell thioredoxin reductase, corresponds to TGA in the human placental gene. *Proc Natl Acad Sci USA* **93**: 6146–6151.

Gregus Z, Gyurasics A, Csanaky I (2000). Effects of arsenic-, platinum-, and gold-containing drugs on the disposition of exogenous selenium in rats. *Toxicol Sci* **57**: 22–31.

Gregus Z, Gyurasics A, Csanaky I, Pinter Z (2001). Effects of methylmercury and organic acid mercurials on the disposition of exogenous selenium in rats. *Toxicol Appl Pharmacol* **174**: 177–187.

Gromer S, Arscott LD, Williams Jr CH, Schirmer RH, Becker K (1998). Human placenta thioredoxin reductase. Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. *J Biol Chem* **273**: 20096–20101.

Gromer S, Eubel JK, Lee BL, Jacob J (2005). Human selenoproteins at a glance. *Cell Mol Life Sci* **62**: 2414–2437.

Gromer S, Merkle H, Schirmer RH, Becker K (2002). Human placenta thioredoxin reductase: preparation and inhibitor studies. *Methods Enzymol* **347**: 382–394.

Hashemy SI, Ungerstedt JS, Avval FZ, Holmgren A (2006). Motexafin gadolinium, a tumor-selective drug targeting thioredoxin reductase and ribonucleotide reductase. *J Biol Chem* **281**: 10691–10697.

Hill KE, McCollum GW, Boeglin ME, Burk RF (1997). Thioredoxin reductase activity is decreased by selenium deficiency. *Biochem Biophys Res Commun* **234**: 293–295.

Hoffmann PR, Berry MJ (2005). Selenoprotein synthesis: a unique translational mechanism used by a diverse family of proteins. *Thyroid* **15**: 769–775.

Hughes MF (2002). Arsenic toxicity and potential mechanisms of action. *Toxicol Lett* **133**: 1–16.

Kim IS, Jin JY, Lee IH, Park SJ (2004). Auranofin induces apoptosis and when combined with retinoic acid enhances differentiation of acute promyelocytic leukaemia cells *in vitro*. *Br J Pharmacol* **142**: 749–755.

Kitchin KT, Ahmad S (2003). Oxidative stress as a possible mode of action for arsenic carcinogenesis. *Toxicol Lett* **137**: 3–13.

Levander OA (1977). Metabolic interrelationships between arsenic and selenium. *Environ Health Perspect* **19**: 159–164.

Levander OA, Baumann CA (1966). Selenium metabolism. VI. Effect of arsenic on the excretion of selenium in the bile. *Toxicol Appl Pharmacol* **9**: 106–115.

Lin S, Cullen WR, Thomas DJ (1999). Methylarsenicals and arsinothiols are potent inhibitors of mouse liver thioredoxin reductase. *Chem Res Toxicol* **12**: 924–930.

Lin S, Del Razo LM, Styblo M, Wang C, Cullen WR, Thomas DJ (2001). Arsenicals inhibit thioredoxin reductase in cultured rat hepatocytes. *Chem Res Toxicol* **14**: 305–311.

Low SC, Harney JW, Berry MJ (1995). Cloning and functional characterization of human selenophosphate synthetase, an essential component of selenoprotein synthesis. *J Biol Chem* **270**: 21659–21664.

Lu J, Chew EH, Holmgren A (2007). Targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide. *Proc Natl Acad Sci USA* **104**: 12288–12293.

Manley SA, George GN, Pickering IJ, Glass RS, Prenner EJ, Yamdagni R *et al*. (2006). The seleno bis(*S*-glutathionyl) arsinium ion is assembled in erythrocyte lysate. *Chem Res Toxicol* **19**: 601–607.

Marzano C, Gandin V, Folda A, Scutari G, Bindoli A, Rigobello MP (2007). Inhibition of thioredoxin reductase by auranofin induces apoptosis in cisplatin-resistant human ovarian cancer cells. *Free Radic Biol Med* **42**: 872–881.

Miller Jr WH, Schipper HM, Lee JS, Singer J, Waxman S (2002). Mechanisms of action of arsenic trioxide. *Cancer Res* **62**: 3893–3903.

Moxon AL (1938). The influence of arsenic on the toxicity of seleniforous grains. *Science* **88**: 81.

Omata Y, Folan M, Shaw M, Messer RL, Lockwood PE, Hobbs D *et al*. (2006). Sublethal concentrations of diverse gold compounds inhibit mammalian cytosolic thioredoxin reductase (TrxR1). *Toxicol In vitro* **20**: 882–890.

Papp LV, Lu J, Striebel F, Kennedy D, Holmgren A, Khanna KK (2006). The redox state of SECIS binding protein 2 controls its localization and selenocysteine incorporation function. *Mol Cell Biol* **26**: 4895–4910.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Pi J, Qu W, Reece JM, Kumagai Y, Waalkes MP (2003). Transcription factor Nrf2 activation by inorganic arsenic in cultured keratinocytes: involvement of hydrogen peroxide. *Exp Cell Res* **290**: 234–245.

Rey NA, Howarth OW, Pereira-Maia EC (2004). Equilibrium characterization of the As(III)-cysteine and the As(III)-glutathione systems in aqueous solution. *J Inorg Biochem* **98**: 1151–1159.

Rigobello MP, Folda A, Baldoin MC, Scutari G, Bindoli A (2005). Effect of auranofin on the mitochondrial generation of hydrogen peroxide. Role of thioredoxin reductase. *Free Radic Res* **39**: 687–695.

Rigobello MP, Scutari G, Boscolo R, Bindoli A (2002). Induction of mitochondrial permeability transition by auranofin, a gold(I)-phosphine derivative. *Br J Pharmacol* **136**: 1162–1168.

Samikkannu T, Chen CH, Yih LH, Wang AS, Lin SY, Chen TC *et al*. (2003). Reactive oxygen species are involved in arsenic trioxide inhibition of pyruvate dehydrogenase activity. *Chem Res Toxicol* **16**: 409–414.

Shi H, Hudson LG, Ding W, Wang S, Cooper KL, Liu S *et al*. (2004). Arsenite causes DNA damage in keratinocytes via generation of hydroxyl radicals. *Chem Res Toxicol* **17**: 871–878.

Smith AD, Levander OA (2002). High-throughput 96-well microplate assays for determining specific activities of glutathione peroxidase and thioredoxin reductase. *Methods Enzymol* **347**: 113–121.

Stadtman TC (2000). Selenium biochemistry. Mammalian selenoenzymes. *Ann N Y Acad Sci* **899**: 399–402.

Urano T, Imura N, Naganuma A (1997). Inhibitory effect of selenium on biliary secretion of methyl mercury in rats. *Biochem Biophys Res Commun* **239**: 862–867.

Urig S, Becker K (2006). On the potential of thioredoxin reductase inhibitors for cancer therapy. *Semin Cancer Biol* **16**: 452–465.

Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D *et al*. (1995). Diversity of glutathione peroxidases. *Methods Enzymol* **252**: 38–53.

Waxman S, Anderson KC (2001). History of the development of arsenic derivatives in cancer therapy. *Oncologist* **6** (Suppl 2): 3–10.

Wipf P, Hopkins TD, Jung JK, Rodriguez S, Birmingham A, Southwick EC *et al*. (2001). New inhibitors of the thioredoxin–thioredoxin reductase system based on a naphthoquinone spiroketal natural product lead. *Bioorg Med Chem Lett* **11**: 2637–2641.

Woo SH, Park IC, Park MJ, Lee HC, Lee SJ, Chun YJ *et al*. (2002). Arsenic trioxide induces apoptosis through a reactive oxygen species-dependent pathway and loss of mitochondrial membrane potential in HeLa cells. *Int J Oncol* **21**: 57–63.

Xu XM, Carlson BA, Mix H, Zhang Y, Saira K, Glass RS *et al*. (2006). Biosynthesis of selenocysteine on its tRNA in eukaryotes. *PLoS Biol* **5**: e4.

Yamashita M, Ashino S, Oshima Y, Kawamura S, Ohuchi K, Takayanagi M (2003). Inhibition of TPA-induced NF-kappaB nuclear translocation and production of NO and PGE2 by the anti-rheumatic gold compounds. *J Pharm Pharmacol* **55**: 245–251.

Yuan J, Palioura S, Salazar JC, Su D, O'Donoghue P, Hohn MJ *et al*. (2006). RNA-dependent conversion of phosphoserine forms selenocysteine in eukaryotes and archaea. *Proc Natl Acad Sci USA* **103**: 18923–18927.