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Treatment of lung cancer cells with cytotoxic levels of sodium selenite: Effects on the thioredoxin system

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ABSTRACT

Selenium at subtoxic doses has been shown to have tumor specific cytotoxic effects. In this work, viability measurements in different lung cancer cell lines showed that selenite was more effective compared to three different conventional cytotoxic drugs. In addition, the cell line most sensitive to selenite toxicity comprised the highest level of thioredoxin reductase 1 (TrxR1). The human selenoenzyme TrxR1 is a central enzyme for cell growth, differentiation, and the protection against oxidative stress. TrxR1, which in several studies has been shown to be up-regulated in various tumor cells, is also a target for many anticancer drugs. In this study, inhibition of TrxR resulted in enhanced selenite cytotoxicity, clearly connecting the thioredoxin system to the toxic effects mediated by selenite. The complex regulation of TrxR1, involving the expression of many different transcript forms of mRNA, was investigated by real-time qPCR in lung cancer cell lines following treatment with toxic doses (2.5–10 μM) of sodium selenite. Selenium treatment resulted in increased expression of almost all TrxR1 mRNA variants with increasing concentrations of selenite. On the contrary, the TrxR protein level and activity, increased at low to moderate doses followed by a decrease at higher doses, indicating impairment of protein synthesis by selenite.

1. Introduction

The mammalian thioredoxin system consists of the two redoxproteins thioredoxin (Trx) and thioredoxin reductase (TrxR) with NADPH as the electron donor (for review, see [1,2] and references therein). Trx is a small ubiquitous redox-active protein functioning as a general protein disulfide reductant and the active site contains a redox-active disulfide (Cys–Gly–Pro–Cys). Trx, which was first identified as a hydrogen donor to ribonucleotide reductase in *E. coli*, has several additional physiological functions. These include reduction of reactive oxygen species [3], regeneration of other antioxidant enzymes, involvement in regulation of transcription factors and inhibition of apoptosis [4]. The active site disulfide is recycled through reduction by TrxR. Mammalian thioredoxin reductases are selenocystein-containing oxidoreductase flavoproteins, with a C-terminal active site including a conserved GCUG tetrapeptide [5] necessary for catalytic activity [6]. Three different mammalian genes for TrxR have previously been identified. All three forms have an identical C-terminal selenocysteine-containing motif and similar overall structure. TrxR reduces not only oxidized Trx, but also a broad spectrum

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of other substrates including granulysin [7], NK-lysin [8], ubiquinone [9], hydroperoxides [10] and different selenium compounds [11]. The regulation of the cytosolic TrxR1 is very complex (for review, see [12]); in addition to post-translational modifications it also involves regulation on DNA and RNA levels with extensive alternative splicing and special AU-rich sequences in the 3′-untranslated region. Transcriptional regulation of human TrxR1 comprises at least 21 different mRNA forms, all differing in the 5′-end and encoding five possible protein forms [13–15]. TrxR is one of 25 different selenoproteins in human [16] and is known to be involved in numerous regulatory cellular pathways. The importance of this protein is demonstrated by the embryonic lethality of TrxR knock-outs [17]. Moreover, several reports have shown that TrxR1 is induced up to 20 times in tumor cells [18–21] and several commonly used antitumor drugs interact with the enzyme [22]. TrxR1 is also a key enzyme in the metabolism of selenium being able to reduce several selenium compounds [11,23] and thereby contributing to its own synthesis. Selenium compounds in turn have been demonstrated to exhibit selective toxicity on tumor cells [24,25]. Selenium has a broad and concentration dependent biological spectrum, ranging from being an essential micronutrient necessary for synthesis of selenoproteins, including TrxR, to causative agent of oxidative stress [26]. Selenium has also been shown to have modulating effects on cell growth [27], and previous studies have demonstrated tumor selective apoptosis inducing properties on therapy resistant cancer cells.

In the present study five different human lung cancer cell lines have been used to investigate viability and influence on the patterns of mRNA and protein expression of the Trx system following treatment with cytotoxic doses of sodium selenite.

2. Material and methods

2.1. Cell culture

Five different cell lines from therapy resistant lung tumors were used for measuring effects of toxic doses of selenite, three non-small cell lung cancer cell lines (H157, U1810 and H611) and two small cell lung cancer cell lines (U1906E and U1906L). U1906L (late) is a more radioresistant subline of U1906E (early) that spontaneously develops from U1906E after some months of culture. Cells were grown under conditions of 37 °C and 5% CO_2 in 75 cm^2 culture flasks with RPMI 1640 medium (Invitrogen) supplemented with 10% foetal calf serum.

2.2. Cell viability assay

Susceptibility of the cells to treatment of selenite (SeO_3^{2-}) or cytostatic drugs was measured using the Cell Proliferation Kit II XTT (Roche Molecular Biochemicals). The viability assay was performed in 96-well flat-bottomed culture dishes with 100 μl medium/well with approximately 10% cell confluence. Pre-incubation time was 4–8 h depending on the cell line. All cell lines were subjected to treatment with selenite and doxorubicin for 20 h. Absorbance was measured at 490 nm (with a reference wavelength of 650 nm subtracted) on a SpectraMax 250 (Molecular Devices). Viability was measured by normalizing samples to untreated controls. The concentrations used ranged from 2.5 to 50 μM (for cell line H611 two further concentrations for selenite of 75 and 100 μM were included). Two cell lines, H157 and U1810, were further subjected to treatment with three different cytostatic drugs (cisplatin, docetaxel and doxorubicin) either alone or in combination with toxic doses of selenite (5, 7.5 and 10 μM). Cell line H157, with the highest levels of TrxR1 (Fig. 1), was also measured in comparison with 1 h pre-treatment with 0.5 μM of the TrxR specific inhibitor auranofin ($C_{20}H_{34}AuO_9PS$) (Alexis Biochemicals) (Fig. 2). All samples were measured in quadruplicates and the entire assay was repeated three times or more for each cell line.

2.3. Real-time qPCR

Before treatment, cell confluence was assessed by light microscopy to approximately 75–85%. Culture medium was exchanged with either new medium or medium prepared with 2.5, 5.0 or 10 μM sodium selenite (Na_2SeO_3). Cells were treated for 20 h before harvesting. Total RNA was isolated using the Rneasy Mini Kit (Qiagen), with optional on column Dnase digestion according to the manufacturers protocol. RNA quantification was performed using the Ribogreen RNA Quantification Kit (Molecular probes) according to the supplied High range protocol. Synthesis of cDNA was performed through reverse transcription on 2 μg of RNA using the Omniscript Reverse Transcription Kit (Qiagen) with oligo$(dT)_{12-18}$ as primer (final concentration 0.1 μg/μl). Quantative RT-PCR was then used to compare the total levels of TrxR1 mRNA and 10 of the different transcript forms (these are called α1, 2, 6, 7, 8, 10, 11, 13, β1 and γ2, 3, 4) (for nomenclature, see [13]). Some of the forms displayed too small variation to be successfully discriminated in the PCR-reaction, which resulted in a total of eight different primer sets for the analysis (see [15] for primer sequences). The reaction was performed on a BIO-RAD ICycler with 30 ng of cDNA/reaction in triplicates on 96-well plates using Platinum SYBR Green qPCR super mix (Invitrogen). The final volume for each reaction was 25 μl with a primer concentration of 300 nM. The PCR-program used was: 50° 2 min, 95° 2 min, 95° 15 s, 60° 1 min (50 cycles) or 50° 2 min, 95° 2 min, 95° 15 s, 64° 1 min (50 cycles) for the γ-primer. β-actin was used as endogenous control. Results were analysed using the $2^{-\Delta\Delta C_T}$ method. C_T-value cut-off was set at 32 cycles. PCR reactions were repeated three times for totals levels of TrxR1, α1, 2, 7, 8 and 13. Single experiments with α6, 10, 11, β- and γ-forms were also included.

2.4. Western blotting

Samples were subjected to a 7.5% SDS–PAGE at 20 mA followed by electroblotting to a nitrocellulose membrane for 1 h at 100 V (BioRad). Membranes were probed with anti-TrxR1 (1:2000) (AbCam) or anti-actin (1:2000) (Sigma) antibody and incubated for 1 h at RT. The membranes were further incubated with a horseradish peroxidase-conjugated secondary antibody (1:15,000 (DakoCytomation) or 1:20,000

(DakoCytomation), respectively). Bound antibodies were detected using chemiluminescence Western-Lightning-kit (PerkinElmer) according to the manufacturers instructions.

2.5. TrxR activity assay

The activity of TrxR was determined using insulin as substrate, essentially as described by Holmgren and Björnstedt [28], with minor modifications. In order to measure the activity in a 96-well plate, a standard curve was prepared in the range of 0–580 ng/ml TrxR1.

2.6. ELISA

The assay for Trx1 was performed according to Pekkari et al. [29], with some minor modifications. All steps were performed in a volume of 50 μl/well. The primary monoclonal antibody (5 μg/ml 2G11, kindly provided by Professor Anders Rosén) was coated over-night at 4 °C in carbonate buffer, pH 9.6. The secondary polyclonal biotinylated antibody (5 μg/ml, IMCO Corp.) was incubated for 2 h at room temperature. Absorbance at 405 nm was measured using a microplate reader (SpectraMax 250 from Molecular Devices).

2.7. Statistical analysis

Statistical analysis was performed using the Wilcoxon matched pair test, with a significance level at $p < 0.05$.

3. Results

3.1. Effects of selenite and cytostatic drugs on cell viability

Five cell lines were subjected to treatment with increasing concentrations of sodium selenite and doxorubicin for 20 h. The IC_{50} values were determined in order to compare the degree of sensitivity. For selenite, cell lines H157, U1906E and U1906L displayed an IC_{50} value of approximately 5 μM followed by U1810 with an IC_{50} value of 7.5 μM, while cell line H611 in comparison had an exceptionally high IC_{50} value of 50 μM. High degree of sensitivity towards selenite seemed to correlate to strong resistance to doxorubicin, in a reciprocal manner (data not shown). Consequently, H611 was very susceptible to doxorubicin treatment while the IC_{50} value was much higher for the other cell lines. Two cell lines, H157 and U1810, with different basal levels of TrxR (Fig. 1), were further treated with two additional cytostatic drugs, cisplatin and docetaxel. The results from the treatment of H157 with either the cytotoxic agent alone or in combination with a single high dose of selenite are shown in Table 1. Cell line U1810 showed a similar pattern (data not shown). Results show that selenite alone was a superior cytotoxic agent.

In order to examine the role of TrxR in selenite toxicity, H157, with the highest level of TrxR, was further studied. Pretreatment of cells with the TrxR specific inhibitor auranofin ($C_{20}H_{34}AuO_9PS$) for 1 h at 0.5 μM decreased the activity of TrxR (Fig. 2), without any effect on cell viability (data not shown). TrxR inhibition further resulted in a pronounced sensitization to selenite with a decreased viability (Fig. 2).

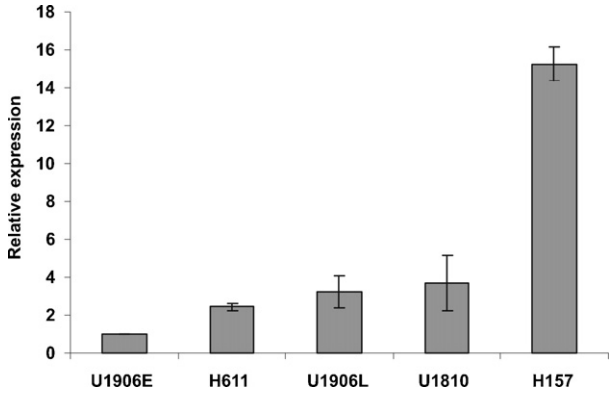

Fig. 1 – Total levels of TrxR1 mRNA in the different cell lines. Comparison of the total TrxR1 mRNA levels in five different lung cancer cell lines. Untreated controls were normalized against U1906E (highest C_T-value). Bars denote standard deviation of three different experiments.

3.2. Regulation of TrxR1 mRNA expression by selenite treatment

TrxR1 is an antioxidant selenoprotein that also has a central role in selenium metabolism. Hence, the effect on expression of this enzyme in response to cytotoxic doses of selenite is of special interest. Real-time qPCR was used to study the effect on TrxR1 with selenite treatment. Comparisons of total levels of TrxR1 mRNA transcripts between the five cell lines showed that the basal level in H157 was several fold higher than the other cell lines (Fig. 1). U1906E had the lowest, while U1906L, U1810 and H611 were intermediates with comparable levels. Treatment with sodium selenite resulted in an evident up-regulation of TrxR1 mRNA levels (Fig. 3a). The highest concentration of selenite (10 μM) resulted in a significant up-regulation of total

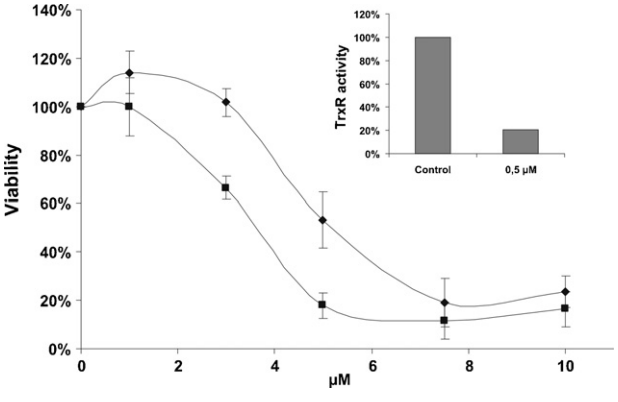

Fig. 2 – Cell viability and inhibition of TrxR1. Comparison of viability of cell line H157 with selenite treatment for 20 h with (■) and without (◆) the TrxR1 specific inhibitor auranofin ($C_{20}H_{34}AuO_9PS$). The Y-axis shows the viability in percent as compared to untreated control cells. Cells were pre-incubated for 1 h with 0.5 μM auranofin. This dose did not affect cell viability by itself. The upper right graph shows TrxR inhibition by auranofin at 0.5 μM. Bars denote standard deviation of three different experiments.

Table 1 – Viability measurements with selenite and cytostatic drugs

Cell line H157	2.5 μM	5.0 μM	7.5 μM	10 μM	20 μM	30 μM	50 μM
Selenite	0.91	0.55	0.44	0.39	0.35	0.34	0.31
Cisplatin	0.98	0.97	1.01	1.03	1.01	1.05	0.8
Docetaxel	0.89	0.89	0.89	0.85	0.88	0.85	0.79
Doxorubicin	0.89	0.88	0.9	0.93	0.89	0.91	0.88
Cisplatin/selenite 5 μM	0.63	0.7	0.77	0.76	0.94	0.96	0.84
Cisplatin/selenite 7.5 μM	0.51	0.53	0.58	0.64	0.8	0.91	0.88
Cisplatin/selenite 10 μM	0.45	0.45	0.48	0.49	0.7	0.83	0.8
Docetaxel/selenite 5 μM	0.5	0.48	0.5	0.52	0.51	0.53	0.54
Docetaxel/selenite 7.5 μM	0.45	0.44	0.43	0.46	0.42	0.4	0.43
Docetaxel/selenite 10 μM	0.42	0.41	0.42	0.39	0.37	0.35	0.4
Doxorubicin/selenite 5 μM	0.61	0.53	0.56	0.57	0.53	0.56	0.53
Doxorubicin/selenite 7.5 μM	0.52	0.56	0.55	0.53	0.54	0.54	0.54
Doxorubicin/selenite 10 μM	0.47	0.51	0.53	0.53	0.54	0.53	0.52

Cell line H157 was treated with sodium selenite and three different cytostatic drugs for 20 h. Cells were treated in the dose range 2.5–50 μM with selenite or cytostatic drugs alone or cytostatic drugs in combination with a fixed dose of selenite at 5, 7.5 or 10 μM. Numbers denote the surviving fraction of cells normalized to an untreated control.

TrxR1 levels in all tested cell lines, ranging from minor (H611) to very high (U1810). At the intermediate concentration, all apart from H157, demonstrated a strong increase of expression, most pronounced in U1810 and U1906E. The TrxR1 total levels were affected even at the lowest concentration (2.5 μM) with U1810 and U1906L showing significant 6-fold and 3-fold increase, respectively. The cell line H157 expressed a pattern of low to moderate changes in mRNA levels after selenite treatment, with α13 exhibiting the strongest response (Fig. 3d). The α6- and β-forms were below detection limit at 10 μM (Fig. 3e and g). Overall, U1810 displayed an outline of high up-regulation of splice variants (Fig. 3a–e, f and h) on mRNA level with the exception of the β-form (Fig. 3g) that was below the cut-off value, and α10/11 (Fig. 3f) at 10 μM selenite where no significant data were obtained. U1906E also demonstrated a general high response to selenite treatment in particular for the forms α6 and α10/11. In contrast, the radiation-resistant subline U1906L showed an expression pattern with low response for all transcript variants except for the β- and γ-forms (Fig. 3a–h). The selenite resistant H611 showed a general low response to selenite in comparison to the other cell lines.

3.3. *Effects on TrxR protein level and activity by selenite treatment*

Evaluations of TrxR expression at mRNA level were followed by measurements of the enzymatic activity and protein level. The overall tendency after selenite treatment was an increase in TrxR activity, peaking at 2.5 or 5.0 μM selenite followed by a drop to levels slightly higher or close to the untreated control at the highest doses (Fig. 4). Cell line U1810 showed significant change in activity at all doses, with an approximate 3-fold increase at 2.5 μM selenite followed by a slightly less effect at 5 and 10 μM, compared to untreated cells. A similar pattern was observed for U1906L. There was a clear elevation in the TrxR enzyme activity in U1906E but peaking at 5.0 μM. In contrary, H157, with the highest basal TrxR level, was not able to further induce the TrxR activity.

To further analyse the TrxR1 expression pattern following selenite treatment, the protein levels were analysed in U1810. The results indicate that the protein level changed in the same manner as the activity, but with only minor effects, with a slight increase at low doses of sodium selenite followed by a drop at higher levels (Fig. 5). In contrast, the levels of total TrxR1 mRNA were strongly up-regulated.

3.4. *Trx1 levels after selenite treatment*

The protein level of Trx1 following selenite treatment was measured by ELISA. No significant changes in Trx1 levels in response to selenite treatment were detected (data not shown).

4. Discussion

Selenite can function as a redox modulating factor with regulation of cell growth and differentiation and with a high potential as a tumor selective drug [30]. Selenite in moderate concentrations (0.01 mM) has growth inhibitory effects, especially for tumor cells, and leads to S-phase arrest [27]. Selenium compounds may also efficiently and selectively induce apoptosis and inhibit transcription factors, *e.g.* AP-1 [31]. Our previous studies have shown that drug resistant cells are significantly more sensitive to selenite compared to drug sensitive cells and that selenium induces apoptosis at concentrations that do not affect the growth and viability of normal, benign cells [25]. The high degree of selectivity of selenium for tumor cells is also supported by a recent study where pronounced selenium cytotoxicity was shown in prostate cancer cells while the benign prostate epithelial cells from the same patients remained unaffected [32]. However, despite a high degree of tumor specificity, selenium is a multi-target drug affecting several enzymes, receptors and transcription factors important for cell growth, differentiation and viability. It has previously been shown that the sensitivity of a tumor cell to selenite is not dependent on the basal levels, but rather the capacity of the cells to induce TrxR in response to selenite [24,25]. The human selenoenzyme TrxR1 is part of a major biological anti-oxidative defence system and is also an important factor for cell growth and differentiation. In this

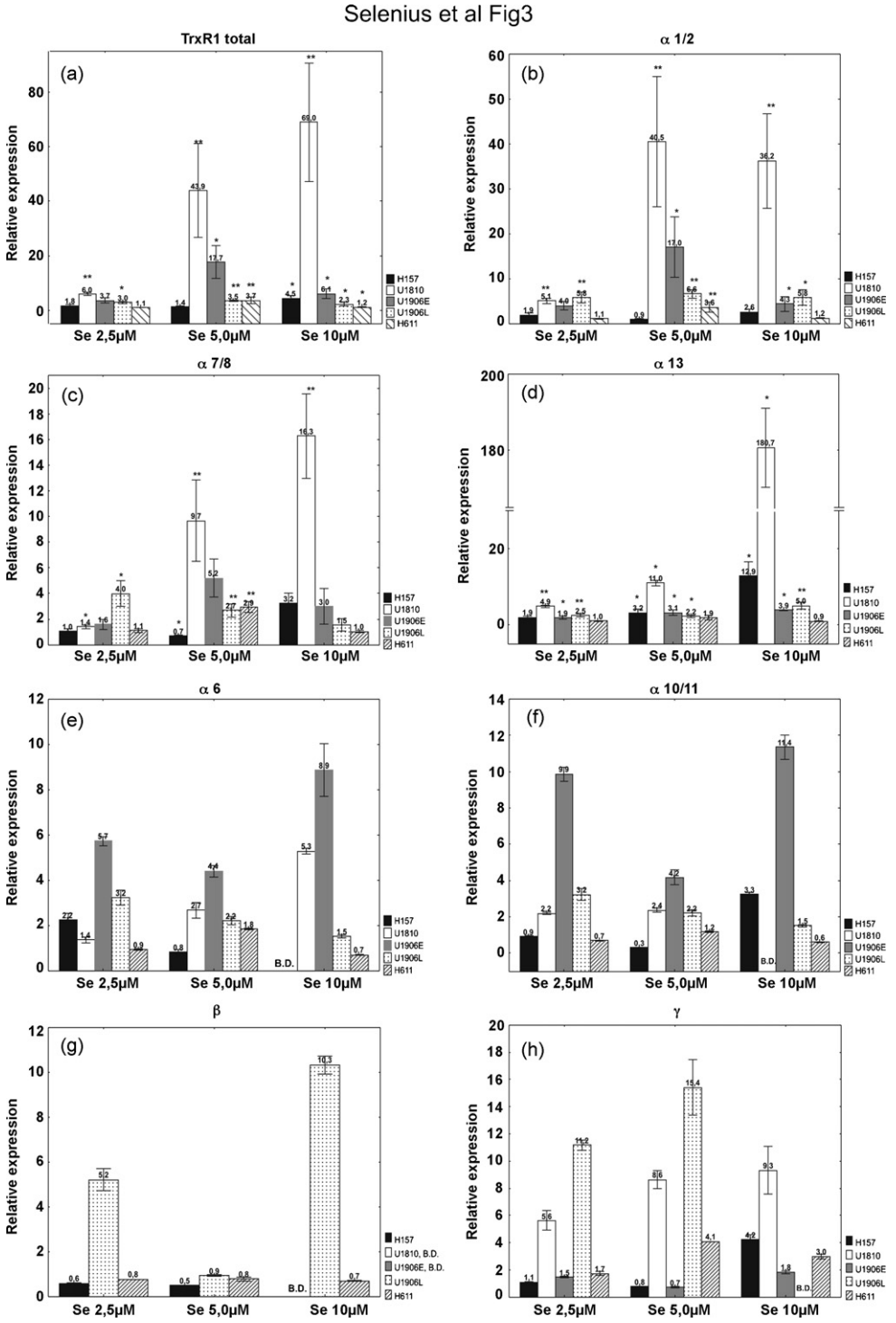

Fig. 3 – (a–h) TrxR1 mRNA patterns with selenite treatment. Relative amount of thioredoxin reductase splice variants after treatment with selenite. Five cell lines were treated with 2.5–5.0 or 10 μM selenite for 20 h before harvesting. Quantitative reverse transcriptase-polymerase chain reaction was performed and relative expressions of different mRNA variants were measured with real-time qPCR. The data was analysed using the $2^{-\Delta\Delta C_T}$ method with β-actin as an endogenous control and the C_T-value cut-off set to 32 cycles. Results are expressed as relative amounts with untreated cells set to 1 (B.D. – below detection level). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Bars denote standard error of the mean.

paper we studied the cytotoxicity and effect of sodium selenite on the regulation of TrxR in cell lines derived from human lung cancer. The IC_{50} doses of selenium used in the cell lines were at levels that could readily be obtained in human plasma and which would not cause side effects [33,34]. The cell line that was most sensitive towards selenite (H157) also showed the highest resistance to doxorubicin. This inverse sensitivity towards selenite and doxorubicin indicates essentially differ-

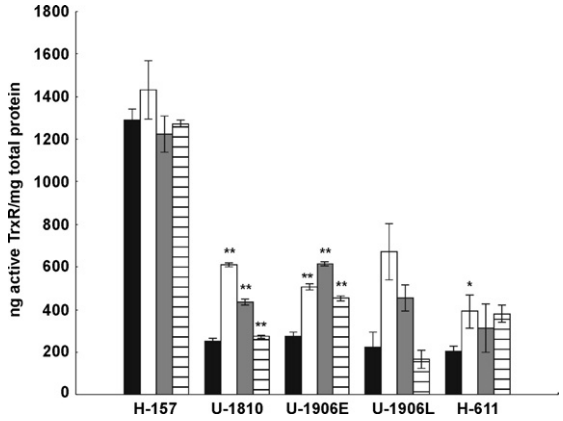

Fig. 4 – TrxR-activity assay. The enzymatic activity of TrxR in cell lysates after treatment with selenite for 20 h. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Bars denote standard error of the mean. Black bars show untreated control samples. White, grey and hatched bars show samples treated with 2.5, 5 and 10 μM selenite, respectively.

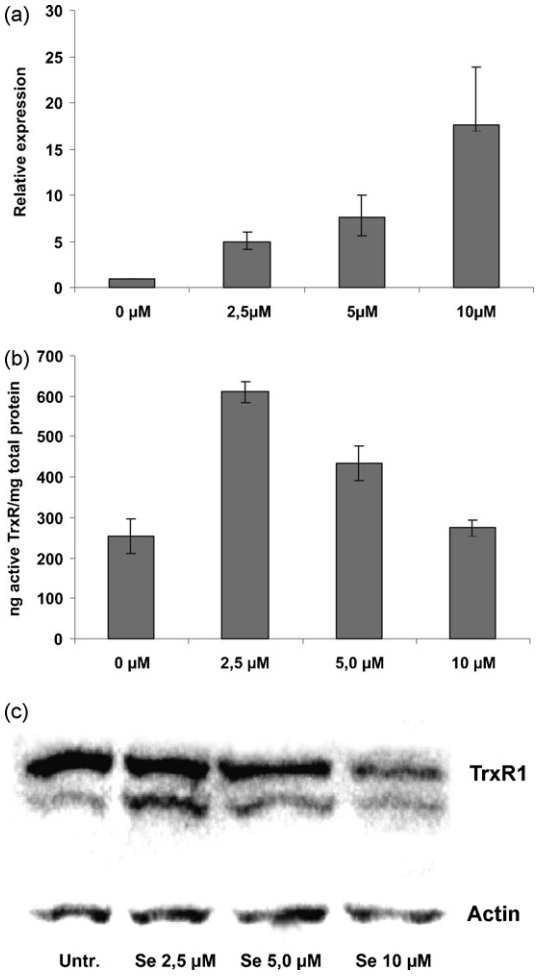

Fig. 5 – (a–c) Levels of TrxR1 mRNA, activity and protein levels. Cell line U1810 treated with increasing concentrations of sodium selenite. (a) TrxR1 mRNA levels with 0 μM selenite set to 1, bars denote standard deviation of three different experiments. (b) TrxR specific activity, bars denote standard deviation of three different experiments. (c) Protein levels of TrxR1 determined by Western blot performed on cell line U1810. Bands show untreated control, 2.5, 5 and 10 μM selenite treated samples from left to right. The densiometric quantities of the immunoreactive bands normalized to actin were the following: Control: 1.65; Se 2.5 μM: 1.93; Se 5 μM: 1.36; Se 10 μM: 1.31.

ent mechanisms of cytotoxicity, which suggests a possible way to circumvent drug resistance. Combination experiments on two cell lines with different levels of TrxR expression using both selenite and cytostatic drugs show that selenite alone was the most effective cytotoxic agent.

Several studies have shown that TrxR1 is induced up to 20 times in tumor cells [18–21] and several commonly used cytostatic drugs interact with the enzyme [22] and it has thus been proposed as a drug target (for reviews see [35–37]). Moreover, in a previous study by Yoo et al. [38] the TrxR1 expression was down-regulated by siRNA in a lung tumor cell line, which reverted the tumor phenotype to normal morphology. In the same study several cancer-related proteins were decreased and the metastasis and tumor progression capacity was greatly reduced in the cells with lowered TrxR1 expression. Specific inhibition of TrxR activity by gold compounds (auranofin) has also been reported to be an effective cytotoxic drug in ovary carcinoma cells that are cisplatin resistant [39]. Here we studied inhibition of TrxR with the specific inhibitor auranofin, at non-toxic doses, which resulted in enhanced selenite cytotoxicity demonstrating a clear role of TrxR in selenite toxicity. This observation is further supported by previously published data where efficient induction of TrxR activity, and not the basal level, was correlated to increased resistance to selenite [40].

The regulation of TrxR1 is very complex and involves the expression of different transcript forms of mRNA [13]. We have, by real-time PCR, investigated the total expression of TrxR1 mRNA and quantified the expression of alternative transcripts in five different human lung cancer cell lines following treatment with cytotoxic doses of sodium selenite for 20 h. An interesting finding was the different TrxR1 mRNA expression pattern for the two sublines U1906E and U1906L, where U1906L is radiation resistant, which was proposed to be dependent on differences in the glutathione level [41,42]. Toxic doses of sodium selenite elicit a response of TrxR1 on mRNA level, on both total levels of TrxR1 mRNA as well as alternative transcripts. The response was dose dependent, with stronger up-regulation at higher concentrations. Unexpectedly, the enzyme activity and protein levels showed a different pattern, where activity of TrxR and total protein level from treated cell lysates showed an increase at lower doses of selenite, which then declined at higher doses. This suggests that the selenite treatment results in impairment of protein synthesis. The synthesis of selenoproteins is very complex and involves a unique mechanism where selenocysteine is encoded by the stop codon UGA [43,44]. Selenocysteine incorporation requires several *cis*-, and *trans*-activating factors such as SECIS-binding protein 2 (SBP2), which is essential for selenoprotein synth-

esis. Recently, SBP2 was shown to be redox regulated by the Trx system and a down-regulation of SBP2 resulted in an enhanced sensitivity against oxidative stress [45]. Since selenite treatment at higher concentrations will cause an increased ROS production it could possibly lead to an impaired function of SBP2 and, consequently, a lowered TrxR activity.

The selenocysteine in TrxR is necessary for catalytic efficiency; inactivation of it causes downstream accumulation of oxidized Trx thereby immobilizing one of the most important cellular defence systems. Several chemotherapeutic drugs act through generation of ROS and downstream cellular damage; therefore, a principal component of defence such as TrxR can be seen as an important potential target for anticancer drugs. This is further strengthened by the fact that up-regulated levels of TrxR protein have been demonstrated in tumor cells [18–20]. Various electrophilic compounds that target the selenocysteine moiety in TrxR have been shown to both reduce enzymatic activity and to have apoptosis inducing capabilities [35,46]. Among these are gold compounds such as auranofin [39]. The fact that TrxR1 is a selenoprotein whose activity can be modulated by selenium in combination with its cancer therapeutic effect is an interesting connection.

Selenite has, at moderate concentrations, a great potential as a chemotherapeutic drug, which can aim at populations of tumor cells that could not be targeted by other cytostatic drugs. This study also clearly indicates that TrxR1 has important functions in neoplastic growth and thus, TrxR1 remains an interesting target in treatment of resistant tumor diseases.

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