**The Relationship of Thioredoxin-1 and Cisplatin Resistance: Its Impact on ROS and Oxidative Metabolism in Lung Cancer Cells**

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**Abstract**

Elimination of cisplatin-resistant lung cancer cells remains a major obstacle. We have shown that cisplatin-resistant tumors have higher reactive oxygen species (ROS) levels and can be exploited for targeted therapy. Here, we showed that increased secretion of the antioxidant thioredoxin-1 (TRX1) resulted in lowered intracellular TRX1 and contributed to higher ROS in cisplatin-resistant tumors in vivo and in vitro. By reconstituting TRX1 protein in cisplatin-resistant cells, we increased sensitivity to cisplatin but decreased sensitivity to elesclomol (ROS inducer). Conversely, decreased TRX1 protein in parental cells reduced the sensitivity to cisplatin but increased sensitivity to elesclomol. Cisplatin-resistant cells had increased endogenous oxygen consumption and mitochondrial activity but decreased lactic acid production. They also exhibited higher levels of argininosuccinate synthetase (ASS) and fumarase mRNAs, which contributed to oxidative metabolism (OXMET) when compared with parental cells. Restoring intracellular TRX1 protein in cisplatin-resistant cells resulted in lowering ASS and fumarase mRNAs, which in turn sensitized them to arginine deprivation. Interestingly, cisplatin-resistant cells also had significantly higher basal levels of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Overexpressing TRX1 lowered ACC and FAS proteins expressions in cisplatin-resistant cells. Chemical inhibition and short interfering RNA of ACC resulted in significant cell death in cisplatin-resistant compared with parental cells. Conversely, TRX1 overexpressed cisplatin-resistant cells resisted 5-(tetradecyloxy)-2-furoic acid (TOFA)-induced death. Collectively, lowering TRX1 expression through increased secretion leads cisplatin-resistant cells to higher ROS production and increased dependency on OXMET. These changes raise an intriguing therapeutic potential for future therapy in cisplatin-resistant lung cancer. *Mol Cancer Ther;* 11(3); 604–15. ©2012 AACR.

**Introduction**

Cisplatin is a widely used therapeutic agent in the treatment of several types of solid tumors including lung cancer (both small cell and non–small cell), head and neck, and ovarian cancer. The majority of patients with cancer initially respond to cisplatin treatment; however, development of drug resistance is expected resulting in disease progression. Mechanisms of resistance are complex and involve multiple molecular pathways (1), making cisplatin resistance difficult to overcome. Thus far, no drugs can reverse cisplatin resistance or selectively kill cisplatin-resistant cells.

We have previously discovered that elevated reactive oxygen species (ROS) are found in cisplatin-resistant cell lines (2); however, the question remains as to why this occurs. A number of investigators have shown that cisplatin can inhibit thioredoxin reductase1 (TrxR1), a thioredoxin-1 (TRX1) reducing enzyme, which leads to increased ROS, resulting in further damage of DNA and subsequent cell death (3, 4). To avoid cell death caused by cisplatin, cells adapt to survive at high ROS levels and use less TrxR1/TrxX1 but upregulate other antioxidant systems (5). We have reported lower TRX1 in a panel of cisplatin-resistant lung cancer cells (2). However, it is not known whether decreased TRX1 protein was due to transcriptional downregulation or increased secretion. Increased TRX1 secretion has been reported by others as a result of cellular stress and after cisplatin treatment (6–8). We investigated whether decreased TRX1 found in cisplatin-resistant cells is regulated at the mRNA or the protein level and report our results herein.

TRX1, a disulfide-reducing dithiol enzyme, is an important antioxidant protein that facilitates the reduction of...
other enzymes (9). TRX1 also interacts with certain transcription factors, which are known to be redox regulated via the dithiol–disulfide exchange reaction, thereby altering their DNA-binding capacity (10, 11). Interestingly, it has been reported recently that TRX1 is involved in adipogenesis in fibroblast cells (12). This study showed that downregulation of TRX1 can lead to increased lipogenesis. Thus, it is possible that lower TRX1 found in cisplatin-resistant cells may have an impact on the fatty acid synthesis pathway and other pathways that are involved in oxidative metabolism (OXMET).

In this report, we present evidence that decreased TRX1 expression in cisplatin-resistant lung cancer cells is an important mediator of ROS and reprograms lung cancer cells to become more reliant on OXMET. To our knowledge, this is the first report that shows that decreased TRX1 in cisplatin-resistant lung cancer can result in increased ROS and alteration in tumor metabolism. These biochemical changes can be used as a target for future treatment of cisplatin-resistant cells.

**Materials and Methods**

**Cell lines and reagents**

SCLC1 was derived from the bone marrow of a patient with small cell lung carcinoma (SCLC). SR2 is the cisplatin-resistant variant derived from SCLC1 that was generated by intermittent exposure to cisplatin. Non–small cell lung carcinoma (NSCLC) was established from metastatic adenocarcinoma to the brain, and small cell is a cisplatin-resistant variant derived from NSCLC by intermittent exposure to cisplatin. These cell lines have been previously characterized (13–16). Note that SR2 exhibits 20-fold resistance to cisplatin and NSCLC exhibits 7-fold resistance to both cisplatin and carlobinol. Elesclomol was kindly provided by Synta Pharmaceuticals. The 5-(tetradecyloxy)-2-furoic acid (TOFA) was purchased from Sigma.

**Growth inhibition and cytotoxicity assay**

Cells were seeded in 24-well dishes and treated with various concentrations of elesclomol or cisplatin for 72 hours as described previously (13, 17). At 72 hours, the culture mediums as well as the trypsinized cells were collected and this admixture was centrifuged at 400 × g for 5 minutes. The cell pellet was resuspended in 1 mL of Hank’s buffer and assayed for live cells and death cells using trypan blue exclusion method.

**Western blot analysis**

Cells were seeded at 1 × 10^5/mL onto 100-mm dishes, treated, collected, and lysed. Total protein was separated on an SDS-PAGE, transferred onto a nitrocellulose membrane (Amersham Biosciences), and immunoblotted with ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS; Cell Signaling Inc.), TRX1 (BD Bioscience), TRX2 (Santa Cruz), citrate synthase (Sigma), or actin (Sigma; refs. 13, 17). The signal intensity was measured with a molecular imager Chemidoc system with Quality One software (Bio-Rad).

**Assay of intracellular ROS/H_2O_2**

As previously described (2), cells were collected and intracellular H_2O_2 was measured by incubating with 10 μmol/L of acetyl-penta-fluorobenzensulfonyl fluorescein (APFB; EMD) at 37°C for 30 minutes in the dark. Then, the cells were washed once with PBS and centrifuged to remove impermeable reagents. Cells were resuspended in 500 μL of PBS and analyzed in a fluorometer, FLUOstar OPTIMA, BMG Labtech (excitation at 485 nm and emission at 520 nm).

**Assay of mitochondrial membrane potential**

Cells were collected and incubated with 50 nmol/L of tetramethylrhodamine ethyl ester (TMRE; Invitrogen) at 37°C for 30 minutes in the dark. Then, the cells were washed once with PBS and centrifuged to remove impermeable reagents. Cells were resuspended in 500 μL of PBS and analyzed in the Accuri Flow Cytometer (excitation at 544 nm and emission at 590 nm) for the mitochondrial membrane potential (MMP).

**Determination of the concentration of TRX1 in the culture medium or in the plasma**

The concentration of TRX1 was determined by sandwich ELISA with 2 TRX1 antibodies. Tetramethylbenzidine was used as coloring agent (Chromogen). The strength of coloring was proportional to the quantities of human TRX1. Briefly, 100 μL of samples (medium or plasma), standards, or reagent blank were incubated with 96-well precoated plate for 60 minutes at 37°C (IBL, Co., Ltd). The precoated plate was washed vigorously with wash buffer 7 times. One hundred microliters of labeled antibody solution was added to each well and incubated for 30 minutes at 37°C. The precoated plate was then washed with wash buffer 9 times. One hundred microliters of tetramethylbenzidine was added to each well and incubated for 30 minutes at room temperature in the dark. After 30 minutes, stop solution was added into each well, and then subjected to ELISA plate reader at 450 nm against reagent blank. TRX1 concentration was quantified against the standard curve and normalized with the cell number.

**Assay of TrxR activity**

A TrxR kit (Cayman Chemical) was used to measure total cellular TrxR as previously described (2). Briefly, cells were seeded at 4 × 10^5 and cell lysate was prepared by sonication using the conditions recommended by the manufacturer. Total TrxR activity was detected by measuring the reduction of 5,5'-Dithio-bis(2-nitrobenzoic acid) with NADPH to thionitrobenzoate by UV spectrophotometer at 405 nm.

**RNA interference experiments**

A total of 8 × 10^5 cells were seeded in a 60 mm petri dish and incubated for 24 hours. INTERFERin transfection reagent (Polyplus) was then used to transfect following short interfering RNAs (siRNA; Dharmacon) siTRX1 (GAAAAAGUAUUCCAAGUGUA), siTRX2 (GGACG-CUGAGGUGAUAAA), siACC (SMARTpool siRNA,
TRX1 overexpression experiment

TRX1-cDNA with restriction sites SgfI and MluI was cloned in to the pCMV6 expression vector (Origene). The plasmid DNA was transfected into cultured cells ($8 \times 10^5$) by Fugene 6 (Roche). After 24 hours, 0.5 mg/mL of neomycin (G418) was added to the culture media for transfection selection. Two clonal populations (SR2TRX+C1 and SR2TRX+C2) were selected and maintained in media containing 0.2 mg/mL of neomycin.

Assay for lactate production

The lactate assay was carried out with a kit (Biovision). Following the manufacturer’s instruction, 200 μL/well of media was filter through 10-kD molecular weight spin filter. Fifty microliters of supernatant were then added to reaction mix that contained lactate assay buffer, probe, enzyme mix, and incubated for 30 minutes. Lactate levels were measured by ELISA plate reader at 570 nm against reagent blank (media alone) and normalized with the cell number.

Qualitative real-time PCR

Qualitative real-time PCR (qRT-PCR) was carried out as previously described (18). Briefly, 1μg of RNA was used for cDNA synthesis. The primers for qRT-PCR are designed with Beacon Designer for SYBR Green fluorophore. Forty cycle amplification was used. The data were analyzed with iQ5 software from Bio-Rad. To calculate the relative mRNA level, we used the ΔΔCt method. The level of mRNA was corrected with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Assay of oxygen consumption

Live cells were trypsinized and counted. Cells were then resuspended in 1 mL of RPMI supplemented with 5% FBS and 2 mg/mL of glucose. Each cell line was tested 3 or more independent times for a minimum of 6 readings per cell. Oxygen consumption readings were carried out as suggested by the manufacturer (Yellow Springs Instruments model 5300).

Immunohistochemistry staining

Immunohistochemical staining was carried out according to routine methodology with some modification. We used the target retrieval solution and the biotin block system (both from Dako) to enhance the staining. Samples were incubated overnight with primary antibody (1:200 in antibody dilution solution; Dako S3022) and washed. Secondary antibody solution (Dako Link, biotinylated antibody solution) was added for 25 minutes, washed, and streptavidin conjugated to peroxidase (horseradish peroxidase) solution was added for 25 minutes. 3,3'-Diaminobenzidine (DAB) chromogen (Dako) and then hematoxylin were used for staining. Section with only antibody solution was used as control. A modified protocol without the rehydration steps was used for cells.

Statistical analysis

All statistical analyses were conducted from 3 separate measurements using the 2-tailed t-test, and the results were expressed as mean ± SD. A P value of less than 0.05 was considered as statistically significant.

Results

Decreased TRX1 in cisplatin-resistant cells is due to increased secretion

To confirm that cisplatin-resistant cells have higher basal levels of ROS, we assayed for H$_2$O$_2$ production per cell in cisplatin-resistant versus parental cells (Fig. 1A). Our previous results also showed that TRX1 was lower in cisplatin-resistant cells and most likely contributes to higher ROS levels. TRX1 has 2 functional isoforms; TRX1 localized in the cytoplasm and TRX2 in the mitochondria. In this report, we have clarified that the TRX1 isoforms were decreased whereas TRX2 showed no changes in all lung cancer cell lines tested (Fig. 1B). We then investigated whether decreased TRX1 protein was due to a decrease in transcription by evaluating the TRX1 mRNA using RT-PCR. TRX1 mRNA levels were similar between the parental and cisplatin-resistant cells (Fig. 1C), whereas their activities were significantly lower (Fig. 1D). Thus, our data suggest that the decreased TRX1 protein in cisplatin-resistant cells is regulated post-transcription. Because it has been reported that TRX1 can be secreted from cells through the leaderless secretory pathway (19), it is possible that lower TRX1 found in cisplatin-resistant cells may be due to the excessive secretion of this protein. We, therefore, compared the concentration of extracellular TRX1 in culture media from the parental versus cisplatin-resistant cells. Results showed that TRX1 concentrations in culture media from cisplatin-resistant cells were significantly higher (Fig. 1E). To further investigate whether decreased TRX1 protein expression and increased TRX secretion also occur in vitro, we chose SCLC1 (parental) and SR2 (cisplatin resistant) as xenograft models in severe combined immunodeficient (SCID) mice and assayed for TRX1 in tumors via immunohistochemical staining (Fig. 1F). Simultaneously, we assayed mouse serum for TRX1 (Table 1). SR2 tumors clearly expressed less TRX1 intracellular protein consistent with the low intracellular expression we detected in vitro (Fig. 1G). Mice with SR2 xenografts had higher TRX1 in serum than mice with SCLC1 xenografts. These findings were consistent with the increased TRX1 secretion we observed in culture (Fig. 1E). Taken together from the in vitro and in vivo models, our findings suggest that cisplatin-resistant cells have a lower intracellular TRX1 protein level that is not a transcriptional consequence, but rather due to an increase in secretion, ultimately resulting in higher ROS accumulation.

Downregulation of TRX1 correlates with increased ROS production, resistance to cisplatin, and sensitivity to elesclomol in a cisplatin sensitive cell line

To determine whether TRX1 correlates with ROS levels and sensitivity to cisplatin as well as ROS-producing
Figure 1. Cisplatin-resistant (CR) lung cancer cells express lower levels of thioredoxin-1 (TRX1) protein. A, fluorometer analysis of H2O2 in various lung cancer cell lines detected by APFB probe indicates that cisplatin-resistant lung cancer cell lines expressed higher basal levels of ROS (Mean ± SD of 3 experiments). B, immunoblot of TRX1 and TRX2 in lung cancer cell lines showed that variants resistant to lower levels of TRX1, whereas no significant changes in TRX2 occurred. Actin was used as a loading control. C, RT-PCR of TRX1 in lung cancer cell lines indicated that all 4 cell lines have similar levels of TRX1 mRNA expression. D, using TrxR1 activity assay, cisplatin-resistant cells have lower TRX1 activity than their parental cells. * P < 0.001; † † † P < 0.003. E, the concentration of extracellular TRX1 in culture medium. Cisplatin-resistant cells secreted greater levels of TRX1 than their parental counterparts. *, P = 0.001; † † † † P = 0.002. F, immunohistochemistry of TRX1 in mouse xenograft tissue showed that SR2 has lower levels of TRX1 protein. G, immunocytochemistry of TRX1 in lung cancer cell cultures also showed decreased levels of TRX1 in cisplatin-resistant cells (< 200). SC, small cell.

We have previously shown that cisplatin-resistant cells that have high ROS levels are sensitive to elesclomol (2). We anticipate that TRX1 knockdown cells that have high ROS should be more sensitive to elesclomol. Indeed, we have found that both TRX1 knockdown clones (SCLC1[TRX1] and SCLC1[TRX2]) were more sensitive to elesclomol (Fig. 2D, i and ii). At 30 nmol/L of elesclomol, 70% of SCLC1 cells were still viable whereas only 35% of the knocked down cells survived. These data suggest that TRX1 plays a role in determining elesclomol sensitivity via alteration in ROS levels.

Overexpression of TRX1 results in decreased ROS production, increased sensitivity to cisplatin treatment, and resistance to elesclomol in cisplatin-resistant cells

To further verify the role of TRX1 in cisplatin resistance in lung cancer cells, we overexpressed TRX1 protein in SR2, using the pCMV6 vector containing full-length TRX1 cDNA. We were able to generate 2 stably transfected cells (SR2[TRX1] and SR2[TRX2]) that have about 3- to 4-fold increase in TRX1 protein expression when compared with...
SR2 (Fig. 3A, i and ii). These transfectants containing increased levels of TRX1 were used to study ROS production and sensitivity to cisplatin. Results showed that ROS production was significantly reduced in both TRX1-overexpressed clones (Fig. 3B, i and ii) whereas cisplatin sensitivity was increased (Fig. 3C, i and ii). At 2.5 μg/mL of cisplatin, 55% of SR2 cells were still viable whereas about 30% and 40% of SR2TRX1 and SR2TRX1-C2 survived, respectively. Next, we analyzed the sensitivity of both clones to the ROS-producing agent, elesclomol. As predicted, elesclomol had a lesser antitumor effect in both clones to the ROS-producing agent, elesclomol. As a result, elesclomol consumed 2.4 nmol/L of O2/cell/mL of media in the same time period (Fig. 4A). These data indicate that TRX1 levels have a major impact on high ROS levels seen in cisplatin-resistant cells and that manipulation of ROS levels through TRX1 expression can alter cisplatin and elesclomol sensitivity. Although further work in this area is needed, TRX1 may be used as a marker in the future selection of patients for cisplatin and/or elesclomol treatment.

### Cisplatin-resistant cells consume more oxygen and have more mitochondrial activity

Although we have shown that low intracellular TRX1 levels in cisplatin-resistant cells contribute to higher ROS levels, it is also possible that these cisplatin-resistant cells have highly active mitochondria that are known to contribute to elevated ROS. To verify this, we first analyzed the amount of oxygen consumed in SCLC1 versus SR2 cells. SCLC1 consumed 0.6 nmol/L of O2/cell/mL of media, whereas SR2 consumed 2.4 nmol/L of O2/cell/mL of media in the same time period (Fig. 4A). These data indicate that cisplatin-resistant cells use more oxygen (aerobic) than parental cells. In addition, under hypoxic conditions (0.5% O2), cell growth was restricted more in cisplatin-resistant cells than in parental cells (data not shown). This observation led us to assess mitochondrial activity through mitochondrial staining (MitoTracker). SR2 had more intense fluorescence than SCLC1, which is indicative of higher mitochondrial activity (Fig. 4B and Supplementary Fig. 51). To further show that oxidative phosphorylation was used more in SR2, we measured MMP with TMRE staining. Higher MMP correlates to more active oxidative phosphorylation (20). MMP was significantly higher in SR2 cells (Fig. 4C) than SCLC1, suggesting that cisplatin-resistant cells have more highly active mitochondria that can also contribute to increased ROS production. Higher mitochondrial activity implies that cisplatin-resistant cells may use more OXMET and less glycolytic flux. To consider this possibility, we assayed for the lactate production, which is a known indicator of glycolytic metabolism. We found lower amounts of lactic acid (nmol/L/well/cell) in media from cisplatin-resistant cells (Fig. 4D). Thus, our initial findings suggest that cisplatin-resistant cells have higher mitochondrial activity and rely more on OXMET instead of glycolysis.

### Cisplatin-resistant cells preferentially used OXMET, which was partially reversed upon overexpression of TRX1

We further validated the involvement of specific OXMET components as well as the impact of TRX1 on cisplatin-resistant lung cancer metabolism. To investigate the dependence of cisplatin-resistant cells on OXMET and TRX1 as an important factor in this metabolic switching, we studied key proteins in the tricarboxylic acid (TCA) cycle, the urea cycle, and fatty acid synthesis using our cell lines model. Citrate synthase is the first rate-determining enzyme in the TCA cycle (21, 22), whereas fumarase participates in maintaining the equilibrium between succinate and fumarate. This equilibrium impacts the functionality of complex II in the electron transport chain (21). We found no significant changes in citrate synthase protein in all 4 cell lines (data not shown); however, fumarase mRNA was increased in SR2 (1.8-fold) and attenuated in TRX1-transfected cells, SR2TRX1-C1 and SR2TRX1-C2 (Fig. 5A). Fumarase hydrates fumarate to malate, an important intermediate in the TCA cycle; however, the major source of fumarate is from the urea cycle (23). Hence, we investigated 2 important enzymes in the urea cycle, argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), which generate arginine and yield fumarate as by-product. Interestingly, SCLC1 had a negligible amount of endogenous ASS mRNA (Fig. 5B) whereas SR2 had a relatively robust expression (30-fold increase). Both TRX1-overexpressed transfectants showed a decrease in ASS mRNA (Fig. 5B). ASL expression was not significantly different in these 4 cell culture models, so it was not evaluated further (data not shown). These results imply that SCLC1 will require an exogenous arginine supply for their growth whereas SR2 should survive better in arginine-free media. To confirm the functional role of ASS, we compared the growth sensitivity of these 4 cell lines to arginine-free media supplemented with citrulline, as an ASS substrate. SCLC1 could not withstand arginine deprivation due to lack of ASS expression, and hence, only 30% cells were viable after 48 hours in arginine-free media. In contrast, 80% of SR2 were still viable. Importantly, overexpression of TRX1 suppressed ASS in SR2,

#### Table 1. The concentration of extracellular TRX1 in mouse serum

<table>
<thead>
<tr>
<th>Name</th>
<th>Tumor volume, mm³</th>
<th>TRX1, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontumor</td>
<td>NA</td>
<td>&lt;0.62</td>
</tr>
<tr>
<td>SCLC1</td>
<td>270</td>
<td>3.86 ± 0.26</td>
</tr>
<tr>
<td>SR2</td>
<td>248</td>
<td>4.78 ± 0.46</td>
</tr>
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NOTE: Mice-bearing SR2 xenografts were found to have higher levels of TRX1 in the serum. The value (ng/mL) represents the average of 3 mice per group. ($P = 0.03$; SCLC1 vs. SR2).
Figure 2. Downregulation of TRX1 results in increased ROS production, sensitivity to elesclomol, and resistance to cisplatin in a SCLC1 cell line. We selected SCLC1 that had the highest amount of TRX1, whereas its cisplatin-resistant variant (SR2) had the lowest amount of TRX1 to do the study. A, i and ii, immunoblot of TRX1 and TRX2 in SCLC1 scramble (control), SR2, SCLC1siTRXC1, and SCLC1siTRXC2 cells. Two different siRNAs were able to downregulate TRX1 by 80% at 48 hours posttransfection, whereas no effect occurred in TRX2. Actin was used as a loading control. B, i and ii, downregulation of TRX1 resulted in significant ROS production. *, P = 0.02; **, P = 0.035. C, i and ii, growth inhibitory effect of cisplatin for 72 hours showed that downregulation of TRX1 in SCLC1 cells resulted in resistance to cisplatin treatment. *, P = 0.02; **, P = 0.001; ***, P < 0.05; ****, P = 0.04. D, i and ii, growth inhibitory effect of elesclomol for 72 hours showed that downregulation of TRX1 in SCLC1 results in increased sensitivity to elesclomol treatment. *, P = 0.01; **, P = 0.003; ***, P = 0.12; ****, P = 0.04. Mean ± SD of 3 experiments.
which in turn sensitized them to arginine deprivation (Fig. 5C).

Fatty acid synthesis also contributes to OXMET by providing carbon skeleton to β-oxidation in the mitochondria. Recently, it has been reported that overexpression of the Txnip protein, an inhibitor of TRX1, can lead to adipogenesis (12). Thus, it is likely that the relatively lower TRX1 expression found in SR2 may promote fatty

![Diagram](image-url)
The earliest data strongly suggest that SR2 depends on fatty acid synthesis pathway for survival as SR2 expressed 15-fold more ACC and 5-fold more FAS than SCLC1. To further verify that fatty acid synthesis plays a vital role on SR2 survival, first, we treated SR2, SR2TRX C1 and SR2TRX C2, and SCLC1 with TOFA, an allosteric inhibitor of ACC that blocks the synthesis of malonyl CoA (24) and then measured for cell death (Fig. 6B). TOFA induced cell death in a dose responsive manner in all cells with SR2 showing the highest sensitivity to TOFA, with 30% cell death at all doses with about only 30% to 40% cell death at the highest dose (10 μg/mL), which were 2-fold less than SR2. Importantly, knockdown of ACC by about 90% using siRNA (SR2siACC; Fig. 6B) resulted in significant cell death (55%), whereas the control SR2 cells with scramble sequence can survive with only 6% cell death (Fig. 6C). Thus, our data strongly suggest that SR2 relies on fatty acid synthesis for survival and decreased TRX1 impacts fatty acid metabolism dependency.

**Discussion**

We previously reported that cisplatin-resistant cells have more basal ROS levels than parental cell counterparts. Furthermore, increasing ROS using elesclomol in these cisplatin-resistant cells can push them beyond their tolerance limit, which ultimately leads to cell death (2). Elesclomol is an investigational drug that chelates copper (Cu) outside the cells and enters as elesclomol-Cu(II), generating ROS via redox cycling of Cu(II) to Cu(I) inside mitochondria (25, 26). This drug has entered into both phase I and II clinical study, and thus far has been very well tolerated (27, 28).

Here, we showed that lower TRX1 levels were found in all cisplatin-resistant cells tested and this is primarily due to the increase in secretion. High TRX1 levels were found in the media as well as in the serum of the SR2 mouse

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**Figure 4.** Cisplatin-resistant cells consume more oxygen and have higher mitochondrial activity than parental cells. A, SCLC1 and SR2 were assayed for baseline oxygen consumption. Live cells were counted and placed in an oxygen chamber. The amount of oxygen consumed per cell in 10 minutes was measured. The rate of oxygen consumption (O2 nmol/L/mL/cell/min) was 4 times higher in SR2 cells than SCLC1 cells. B, SCLC1 and SR2 were incubated with 100 nmol/L of MitoTracker. Bar graph represents the relative fluorescent units per cell via fluorometer plate reader. SR2 cells have a higher number of active mitochondria than SCLC1. *, P < 0.001. C, flow analysis of MMP in SCLC1 versus SR2, using 50 nmol/L of TMRE. SR2 has significantly higher levels of MMP. *, P < 0.001. D, media from the SCLC1 versus SR2 cells was collected and used in a chromatogenic assay to measure amounts of lactic acid (nmol/L/well/cell). SR2 cells produced less lactic acid than cisplatin-resistant cells. Mean ± SD of 3 experiments; *, P < 0.05.
xenograft model. TRX1, a 12-kD protein, is a member of the leaderless protein family and can be effluxed from the cells through the leaderless pathway (6, 19). It has been shown that increased TRX1 secretion is found when cells are under stress (8, 29, 30). Increased plasma TRX1 levels are found in patients who received cisplatin treatment (31, 32). It is very likely that these cisplatin-resistant cells excrete higher amounts of TRX1 due to continuous cellular stress, which in turn results in lower intracellular accumulation of TRX1 and consequently increased ROS.

To mimic low TRX1 levels observed in cisplatin-resistant cells, we used siRNA to decrease TRX1 levels. SCLCsiTRX cells generated more ROS and became resistant cells, we used siRNA to decrease TRX1 levels. Accumulation of TRX1 and consequently increased ROS.

32). It is very likely that these cisplatin-resistant cells excrete higher amounts of TRX1 due to continuous cellular stress, which in turn results in lower intracellular accumulation of TRX1 and consequently increased ROS.

Overexpression TRX1 protein can partly suppress these fatty acid synthesis pathway. Cisplatin-resistant cells had relative higher levels of expression in all of these proteins than parental cells. Overexpression TRX1 protein can partly suppress these fatty acid synthesis enzymes. Actin was used as a loading control. Bar graph indicates the relative adjusted density of indicated protein expressions in each cell line. SR2TRX yielded similar results (data not shown).

Figure 5. Cisplatin-resistant cells preferentially used OXMET, which partly reversed upon overexpression of TRX1. A and B, relative mRNA levels of fumarase and ASS. Total RNAs extracted from these cells were reverse transcribed and subsequently used as template for qRT-PCR. GAPDH was used as internal control. The results shown in the graph were calculated with the ΔΔCt method by setting the fumarase or ASS mRNA level of SCLC1 as 1. Relative abundance of fumarase and ASS mRNA were higher in cisplatin-resistant cells but decreased with TRX1 overexpression. FH, fumarase. C, comparison of growth inhibition of SCLC1, SR2, SR2TRX−C1, and SR2TRX−C2 in arginine-free media supplement with citrulline. At 72 hours, only 30% of SCLC1 survive compared with 60% to 65% of TRX1-overexpressing clones, and 80% of SR2 were viable. D, immunoblot of key enzymes (ACL, ACC, and FAS) in the fatty acid synthesis pathway. Cisplatin-resistant cells had relative higher levels of expression in all of these proteins than parental cells. Overexpression TRX1 protein can partly suppress these fatty acid synthesis enzymes. Actin was used as a loading control. E, bar graph were calculated with the ΔΔCt method by setting the GAPDH was used as internal control. The results shown in the graph were calculated with the ΔΔCt method by setting the fumarase or ASS mRNA level of SCLC1 as 1. Relative abundance of fumarase and ASS mRNA were higher in cisplatin-resistant cells but decreased with TRX1 overexpression. FH, fumarase. C, comparison of growth inhibition of SCLC1, SR2, SR2TRX−C1, and SR2TRX−C2 in arginine-free media supplement with citrulline. At 72 hours, only 30% of SCLC1 survive compared with 60% to 65% of TRX1-overexpressing clones, and 80% of SR2 were viable. D, immunoblot of key enzymes (ACL, ACC, and FAS) in the fatty acid synthesis pathway. Cisplatin-resistant cells had relative higher levels of expression in all of these proteins than parental cells. Overexpression TRX1 protein can partly suppress these fatty acid synthesis enzymes. Actin was used as a loading control. E, bar graph indicates the relative adjusted density of indicated protein expressions in each cell line. SR2TRX yielded similar results (data not shown).
cisplatin-resistant cells tested (Supplementary Fig. S2). These cisplatin-resistant cells had a lower hexokinase-II level (37) and a lower lactic acid production. Thus, it seems that decreased TRX1 levels have a negative impact on the glycolytic pathway. We have further shown that lower TRX1 found in cisplatin-resistant cells had other consequences on the key enzyme (ASS) involved in the urea cycle. These cisplatin-resistant cells have significantly higher levels of ASS mRNA, whereas the parental cells (high TRX1 and HIF1α) expressed negligible amount of ASS mRNA. Interestingly, we have previously reported that HIF1α is a key transcription factor that negatively regulates ASS (38). Here, we showed that cisplatin-resistant cells that have lower TRX1 and increased HIF1α levels express increased levels of ASS. Consequently, these cisplatin-resistant cells are able to synthesize arginine from citrulline, and therefore, are able to survive in arginine-free media whereas its parental cells could not. In addition, overexpression of TRX1 in cisplatin-resistant cells resulted in lower ASS mRNA and less tolerance to arginine deprivation, which further confirms the role of TRX1 in ASS mRNA expression. This is an important finding that warrants further study as downregulation of ASS has been reported in multiple tumor types such as melanoma, hepatocellular carcinoma, and SCLC (39–42). These tumors are all highly sensitive to arginine deprivation. In fact, arginine deprivation therapy using pegylated arginine deiminase has been shown to have anti-tumor activity in tumors that do not express ASS (18, 41, 43, 44). Thus, alteration of ASS expression by TRX1 could have future clinical implication for treatment.

Figure 6. Downregulation of ACC enhances cell death in cisplatin-resistant cells. A, cells were treated by TOFA to inhibit ACC and cytotoxicity was assessed. Data are shown as percentage of cell death as compared with untreated samples for each cell line. Overexpressing TRX1 protected cisplatin-resistant cells against TOFA-induced cell death. B, downregulation of ACC by siRNA (only 1 nmol/L is used to minimize the off-target effect). Immunoblot of ACC in SR2siACC and SRLACCC cells showed more than 90% decrease in ACC 48 hours posttransfection. Actin was used as a loading control. C, comparison of cell death in SR2scramble and SR2siACC; ACC knockdown resulted in significant increase in cell death. D, a proposed model acquired resistance to cisplatin disrupted the redox system through inhibition of TRX1 system (TrxR1/TRX1) causing TRX1 secretion. Decreased TRX1 resulted in decreased arginine deprivation as well as higher mitochondrial activity. Inhibiting key metabolic enzymes in fatty acid synthesis pathway led to significant cell death in cisplatin-resistant cells.
upregulate the key enzymes (ACL, ACC, and FAS) in the fatty acid synthesis pathway, whereas overexpression of TRX1 resulted in attenuated levels of these enzymes. These data strongly suggest that TRX1 may play an important role in lipid metabolism. Interestingly, Chutkov and Lee have recently reported that inhibition of TRX1 via Txnip can lead to increase in lipogenesis in fibroblast cells (12). Taken together, these findings further support the role of TRX1 in tumor metabolism. Knockdown of ACC in SR2 resulted in significant cell death, which indicates that fatty acid synthesis is essential for cisplatin-resistant cell survival. This is further confirmed by the finding of significant differential sensitivity to ACC inhibitor (TOFA) in SR2 cells and in SR2TRX+c. Although the data presented here are limited to one pair of cisplatin-resistant cell line with different clones of transfectants, our previous data has shown that 4 pairs and 2 additional primary cultures derive from patients who failed cisplatin have higher ROS and low TRX1 levels (2). These cell lines also showed alteration in their tumor metabolism either via TCA cycle or fatty acid or by both. The underlying mechanisms are being investigated and will be forthcoming. We have also established other cisplatin-resistant cells from H69 (SCLC) obtained from American Type Culture Collection, and our initial finding suggested that the extent of decreased TRX1 as well as higher ROS accumulation correlates with the resistant levels. Overall, these findings could have future implication for drug development to selectively kill cisplatin-resistant cells that have high ROS and low TRX1 levels.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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