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 show 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). Overexpression of 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.
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 resistances to cisplatin and NSCLC exhibits 7-fold resistance to both cisplatin and carboplatin. 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-fluorobenzesulfonyl fluorescein (APFB; EMD) at 37°C for 30 minutes in the dark. 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 a color 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 transfet following short interfering RNAs (siRNA; Dharmaco) siTRX1 (GAAAAGUAAUCCACGAGU), siTRX2 (GGACGCUGCAGGUAAGAA), siACC (SMARTpool siRNA,
NM_198836), and siCONTROL (UAGCGACUAACAC-AUCAA; ref. 17).

**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 × 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 (SR2TRX1-C1 and SR2TRX1-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 manufacture’s instruction, 200 μL/well of media was filter through 0.2 μm 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, 1 μg 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 53022) 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 H2O2 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. TRX 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 vivo, 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 (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...
agent (elesclomol), we inhibited TRX1 expression in SCLC1 using 2 different siRNA (SCLC1\textsuperscript{siTRXC1} and SCLC1\textsuperscript{siTRXC2}). TRX1 protein was successfully knocked down (about 80%) in the transfected cells, SCLC1\textsuperscript{siTRXC1} and SCLC1\textsuperscript{siTRXC2} (Fig. 2A, i and ii) and higher levels of ROS were detected relative to SCLC1 (Fig. 2B, i and ii). Nevertheless, the levels of ROS in TRX1-knocked down cells were still lower than in SR2 cells. It is conceivable that mitochondrial ROS may also contribute to high ROS in SR2. Because silencing TRX1 may affect TRX2, we studied TRX2 in SCLC1\textsuperscript{siTRXC1} and found only slightly increased in TRX2 levels (Fig. 2A). The participation of TRX2 in ROS accumulation remains unclear.

Next, we evaluated the viability of these cells under cisplatin treatment. Both siTRX clones were significantly more resistant to cisplatin (Fig. 2C, i and ii). At 0.5 µg/mL of cisplatin, 50–60% of the transfected cells were still viable whereas only 20% of the SCLC1 cells survived. We could not reach the similar cisplatin-resistant levels as in SR2 cells. This may be due to the fact that we cannot knockdown TRX1 down to the same levels as in SR2 and that other mechanisms of resistance in SR2 may be present (see Discussion).

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 knocked down clones (SCLC1\textsuperscript{siTRXC1} and SCLC1\textsuperscript{siTRXC2}) 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\textsuperscript{TRXC1} and SR2\textsuperscript{TRXC2}) 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 SR2TRX2 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 TRX1-overexpressed cells than in SR2 cells. At 10 nmol/L of elesclomol, only 45% of SR2 survived whereas in SR2TRX1 and SR2TRX2 about 80% of the cells were still viable (Fig. 3D, i and ii). Our data clearly 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. 5I). 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 and SR2TRX2 (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 byproduct. 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>
</tbody>
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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 SCLC1scramble (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 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 acid synthesis.
the highest sensitivity to TOFA, with 30% cell death at dose responsive manner in all cells with SR2 showing assured for cell death (Fig. 6A). TOFA induced cell death in a blocks the synthesis of malonyl CoA (24) and then mea-

acid synthesis. To investigate this concept, we assayed 3 important enzymes in fatty acid synthesis ACL, ACC, and FAS. All 3 protein levels were higher in SR2 cells than in SCLC1 (Fig. 5D); ACL (3-fold), ACC (15-fold), and FAS (5-fold) increased (Fig. 5E). TRX1 overexpression (SR2TRX+C1) significantly decreased the expression of fatty acid synthesis proteins. These data support the notion that cisplatin-resistant cells rely on OXMET more than their parental cells. Importantly, the overexpression of TRX1 protein opposes OXMET by downregulating enzymes involved in the urea cycle and fatty acid synthesis. Similarly, SR2TRX+C2 also exhibited a decrease in ACC and FAS when compared with SR2 (data not shown).

Fatty acid synthesis is important for survival of cisplatin-resistant cells

The earlier 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, 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. 6A). 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 2.5 μg/mL and 70% at 10 μg/mL, which was 6- to 7-fold higher than SCLC1. TRX1 overexpression rescued SR2TRX+C1 and SR2TRX+C2 cells from TOFA-induced 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...
SCLCsiTRX cells generated more ROS and became resistant to cisplatin, which in turn results in lower intracellular TRX1 levels. Increased plasma TRX1 levels are found when cells are under stress (8, 29, 30). Whether TRX1 has any impact on hCTR1 is not known and is currently under investigation.

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 to cisplatin but hypersensitive to elesclomol. Likewise, overexpressing TRX1 in cisplatin-resistant cells resulted in decreased ROS and increased sensitivity to cisplatin, but more resistant to elesclomol. Although the differential sensitivity to elesclomol in these transfectants could be explained on the basis of ROS that is influenced by TRX1 levels, it is unclear why TRX1 levels also influence cisplatin sensitivity. It is noteworthy that SR2 has other mechanisms of resistance, such as decreased cisplatin uptake due to decreased copper transport (hCTR1), a known transporter of cisplatin into the cells and is influenced by the redox system (33–35). Whether TRX1 has any impact on hCTR1 is not known and is currently under investigation.

TRX1 has been reported to be involved in tumor angiogenesis. Welsh and colleagues have shown that overexpression of TRX1 increased hypoxia-inducible factor 1α (HIF1α) whereas transfection of nonfunctional TRX1 decreased HIF1α (36). Because HIF1α also regulates enzymes involved in the glycolytic pathway, it is possible that decreased TRX1 found in cisplatin-resistant cells also has an impact on glucose metabolism. In fact, we found that basal HIF1α protein expression was lowered in all
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 decreased 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.

During the generation of arginine, fumarate is produced as a by-product. We have found that fumarate was higher in SR2 cells and attenuated with TRX1 overexpression. Interestingly, it has been reported that fumarate deficiency in renal cell carcinoma results in glycolytic addiction, HIF stabilization, and alteration in ROS (45), which is similar to our parental cells. It is also noteworthy that the majority of renal cells also do not express ASS. Increased ASS and fumarase expression in cisplatin-resistant cells implies that more oxidative phosphorylation is taking place. This is also confirmed by increased oxygen consumption as well as higher mitochondrial activity found in cisplatin-resistant cells. To further support that levels of TRX1 influenced cisplatin-resistant cells to use more OXMET, we showed that cisplatin-resistant cells

![Diagram of TRX1 and Oxidative Metabolism]

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 SR2<sup>scram</sup> and SR2<sup>siACC</sup> cells showed more than 90% decrease in ACC 48 hours posttransfection. Actin was used as a loading control. C, comparison of cell death in SR2<sup>scram</sup> and SR2<sup>siACC</sup>; 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 increased accumulation of cellular ROS. Further increased ROS using elesclomol in these cisplatin-resistant cells can push them beyond their tolerance limit, which ultimately leads to cell death. Decreased TRX1 levels may involve metabolic reprogramming by switching cisplatin-resistant cells from glycolysis toward OXMET. 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, Chutkow 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<sup>+</sup>. 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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