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The Potent and Novel Thiosemicarbazone Chelators Di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone and 2-Benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone Affect Crucial Thiol Systems Required for Ribonucleotide Reductase Activity[S]

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ABSTRACT

Di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone possesses potent and selective antitumor activity. Its cytotoxicity has been attributed to iron chelation leading to inhibition of the iron-containing enzyme ribonucleotide reductase (RR). Thiosemicarbazone iron complexes have been shown to be redox-active, although their effect on cellular antioxidant systems is unclear. Using a variety of antioxidants, we found that only *N*-acetylcysteine significantly inhibited thiosemicarbazone-induced antiproliferative activity. Thus, we examined the effects of thiosemicarbazones on major thiol-containing systems considering their key involvement in providing reducing equivalents for RR. Thiosemicarbazones significantly ($p < 0.001$) elevated oxidized trimeric thioredoxin levels to 213 ± 5% ($n = 3$) of the control. This was most likely due to a significant ($p < 0.01$) decrease in thioredoxin reductase activity to 65 ± 6% ($n = 4$) of the control. We were surprised to find that the non–redox-active chelator desferrioxamine increased thioredoxin oxidation to a lower extent (152 ± 9%; $n = 3$) and inhibited thioredoxin reductase activity (62 ± 5%; $n = 4$), but at a 10-fold higher concentration than thiosemicarbazones. In contrast, only the thiosemicarbazones significantly ($p < 0.05$) reduced the glutathione/oxidized-glutathione ratio and the activity of glutaredoxin that requires glutathione as a reductant. All chelators significantly decreased RR activity, whereas the NADPH/$NADP_{total}$ ratio was not reduced. This was important to consider because NADPH is required for thiol reduction. Thus, thiosemicarbazones could have an additional mechanism of RR inhibition via their effects on major thiol-containing systems.

Introduction

Iron is essential for cancer cell proliferation and can also participate in the Fenton reaction to generate reactive oxygen species (ROS) (Dunn et al., 2007). Iron has been shown to be a molecular target for the inhibition of tumor cell growth, and several iron chelators show pronounced anticancer activity (Boukhalfa and Crumbliss, 2002). This can occur by mechanisms involving cellular iron depletion and the formation of redox-active iron complexes that generate cytotoxic ROS (Yuan et al., 2004; Richardson et al., 2006). When the redox potentials of the so-formed chelator-iron complexes are outside of the accessible range for redox cycling, then generation of cytotoxic radicals via the Fenton reaction are not possible (Boukhalfa and Crumbliss, 2002). For example, the iron complex of the chelator desferrioxamine (DFO) (Fig. 1A) has a redox potential that avoids the reduction of iron(III) under physiological conditions (Boukhalfa and Crumbliss,

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ABBREVIATIONS: ROS, reactive oxygen species; ATO, arsenic trioxide; BCNU, bis-chloronitrosourea; Bp44mT, 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone; Bp4eT, 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone; BSO, buthionine sulfoximine; DFO, desferrioxamine; Dp44mT, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone; DTT, dithiothreitol; EPR, electron paramagnetic resonance; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, oxidized glutathione; NAC, *N*-acetylcysteine; RR, ribonucleotide reductase; SOD, superoxide dismutase; TfR1, transferrin receptor-1; Trx, thioredoxin; TrxR, thioredoxin reductase; Trx, thioredoxin; PKIH, pyridylketone isonicotinoyl hydrazone.

2002). In contrast, other iron complexes can promote redox cycling and ROS generation when their redox potentials lie in a range accessible to cellular reductants (Richardson et al., 2006). Agents that form such complexes demonstrate antineoplastic activity, such as the iron complex of the anthracycline doxorubicin, although the activity of anthracyclines also involves DNA intercalation and topoisomerase II inhibition (Xu et al., 2005).

The potent and selective anticancer efficacy of the thiosemicarbazone iron chelators di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT) and 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone (Bp44mT) (Fig. 1B) have been reported by several laboratories (Richardson et al., 2006; Whitnall et al., 2006; Kalinowski et al., 2007; Rao et al., 2009; Jansson et al., 2010b). In addition, these chelators have been demonstrated to redox cycle after complexation with iron or copper, leading to cytotoxicity (Richardson et al., 2006; Kalinowski et al., 2007). In fact, we showed that Dp44mT and Bp44mT demonstrate pronounced iron chelation efficacy (Yuan et al., 2004; Kalinowski et al., 2007). Dp44mT markedly inhibits tumor growth in vivo and is well tolerated at optimal doses (Yuan et al., 2004; Whitnall et al., 2006). Moreover, the 2-benzoylpyridine thiosemicarbazone chelators, including 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone (Bp4eT; Fig. 1B), also showed potent and comparable antiproliferative activity to Dp44mT (Kalinowski et al., 2007). These thiosemicarbazones are markedly more stable in plasma compared with their aroylhydrazone forerunners, potentially increasing their therapeutic utility (Stariat et al., 2009). Examination of their electrochemistry and ability to oxidize ascorbate and hydroxylate benzoate indicated that the iron complexes were redox-active (Richardson et al., 2006; Kalinowski et al., 2007). Dp44mT also increased intracellular ROS (Yuan et al., 2004; Richardson et al., 2006), probably via complexation with iron or copper (Richardson et al., 2006; Kalinowski et al., 2007; Jansson et al., 2010b), and recent electron paramagnetic resonance (EPR) spectroscopy studies confirmed the redox activity of the Dp44mT-iron complex (Jansson et al., 2010a).

Considering the anticancer mechanism of action of chelators, the aim of this study was to evaluate the effects of thiosemicarbazones on vital antioxidant systems in comparison with DFO, which possesses markedly less anticancer activity and forms redox-inactive iron complexes (Boukhalfa and Crumbliss, 2002). Initially, we explored the antioxidant activity of *N*-acetylcysteine (NAC), which attenuated the antiproliferative effect of thiosemicarbazones. Thus, we hypothesized that these chelators affect thiol-related antioxidant systems including GSH, thioredoxin (Trx), and glutaredoxin (Grx). These thiol systems play crucial roles in redox reactions and regulate protein disulfide composition (Holmgren, 1989; Balendiran et al., 2004). One such example is the disulfide bond in ribonucleotide reductase (RR). This enzyme catalyzes the rate-limiting step in DNA synthesis and is known to be inhibited by iron chelators (Cooper et al., 1996; Zahedi Avval and Holmgren, 2009).

In this study, we showed that thiosemicarbazones alter multiple cellular thiol systems, including GSH, Trx, and Grx. These results indicate that the inhibitory effect of thiosemicarbazones on RR can be mediated by mechanisms involving the modulation of crucial thiol systems essential for its function in addition to their known effect on binding iron (Richardson et al., 2006; Kalinowski et al., 2007). Hence, the effects of thiosemicarbazones on thiol-containing molecules probably mediate, in part, their potent antiproliferative activity.

Materials and Methods

Chelators. All thiosemicarbazone and aroylhydrazone chelators were synthesized and characterized using standard procedures as described previously (Richardson et al., 2006; Kalinowski et al., 2007). DFO was obtained from Novartis (Basel, Switzerland). Thiosemicarbazone and aroylhydrazone chelators were dissolved in dimethyl sulfoxide at a stock concentration of 10 mM and were used at the concentrations indicated by dilution in culture media containing 10% fetal calf serum. DFO was dissolved directly in this latter medium.

Cell Culture. The human DMS-53 small cell lung carcinoma and SK-N-MC neuroepithelioma cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI using standard techniques as described previously (Whitnall et al., 2006). Experiments were performed when the cultures were approximately 80% confluent.

Cellular Proliferation Assay. Cell proliferation was assessed using the well established 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrabromide assay, as described previously, after 72 h/37°C incubation with the chelators (Whitnall et al., 2006). Formazan product formation was shown to be directly proportional to viable cell counts (Whitnall et al., 2006).

Measurement of Glutathione and Oxidized Glutathione. Intracellular GSH and oxidized GSH (GSSG) were determined using a GSH/GSSG ratio assay kit (Calbiochem, Gibbstown, NJ) according to the manufacturer's instructions. In brief, cells were seeded in 100-mm dishes for experimental treatments. After a 24-h/37°C incu-

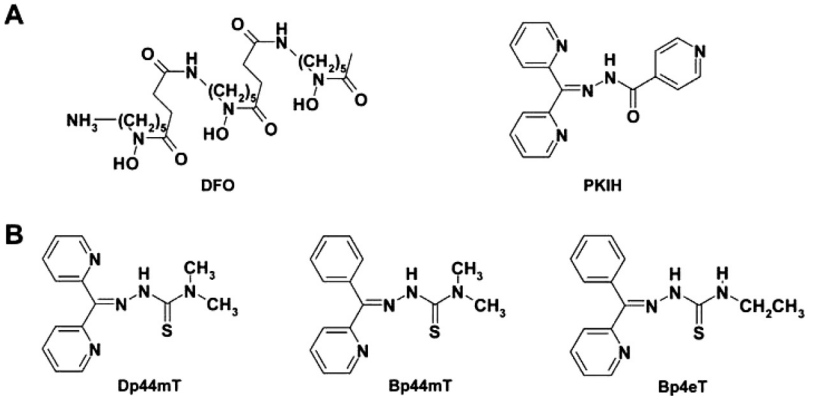


Fig. 1. The chemical structures of DFO and PKIH (A) and Dp44mT, Bp44mT, and Bp4eT (B).

bation with the chelators, the cells were washed with ice-cold PBS and lysed in 50 μl of PBS by three freeze-thaw cycles. The lysates were then acidified with 5% metaphosphoric acid, and the supernatant was separated by centrifugation at 10,000*g* for 10 min at 4°C. The GSH/GSSG ratio was calculated according to (GSH − 2GSSG)/GSSG (Tietze, 1969).

Measurement of Glutathione Reductase Activity. The glutathione reductase (GR) activity was assayed by following the rate of decrease in NADPH absorbance at 340 nm as a result of the reduction of GSSG (Seefeldt et al., 2009). After a 24-h/37°C incubation with the chelators, cells were washed with ice-cold PBS and resuspended in sample buffer (50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA) and then lysed by three freeze-thaw cycles. The homogenate was centrifuged at 10,000*g* for 15 min at 4°C, and supernatant was collected for the determination of GR activity. The assay mixture contained supernatant (40 μg), bovine serum albumin (1 mg/ml), and NADPH (0.4 mM; Sigma-Aldrich, St. Louis, MO). The reaction was initiated by the addition of GSSG (1 mM; Sigma-Aldrich). The same assay was used to determine GR activity in a cell-free system, in which 22 U of GR (Sigma-Aldrich) was incubated with the chelators for 30 min/37°C before the assay. Background nonenzymatic oxidation of NADPH was used as the baseline.

Western Blotting. Cells were lysed using radioimmunoprecipitation buffer containing protease inhibitor (Roche Diagnostics, Indianapolis, IN). The protein concentration was determined using the Bradford assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples (50 μg/lane) were separated on a 4 to 12% NuPage Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a polyvinylidene difluoride membrane (Invitrogen) according to the manufacturer's protocol. Mouse monoclonal anti-human GR, Trx1, thioredoxin reductase (TrxR), and Grx1 antibodies (Abcam, Cambridge, MA) were incubated at a 1:1000 dilution. Mouse monoclonal anti-human transferrin receptor-1 (TfR1) (Invitrogen) was incubated at 1:1000. As an internal control for protein-loading, membranes were also probed for β-actin. Densitometric analysis was performed using Quantity One software (Bio-Rad Laboratories).

Determination of Cellular Thioredoxin Redox State. After a 24-h/37°C incubation with the chelators, the cells were washed with PBS and lysed in guanidine lysis buffer (6 M guanidine hydrochloride, 50 mM Tris/HCl, pH 7.5, and 1 mM EDTA) containing 60 mM iodoacetamide (Lu et al., 2007). After 2 h at 4°C, the cell debris was removed by centrifugation at 10,000*g*/5 min/4°C. The iodoacetamide was removed using a desalting column (GE Healthcare, Sydney, Australia). Proteins (10–20 μg) were incubated with SDS-loading buffer and separated on 4 to 12% Bis-Tris gel (Invitrogen). The same samples were incubated in the presence of dithiothreitol (DTT) (100 mM) in SDS-loading buffer and heated at 60°C/30 min before separation. Trx was detected with an anti-mouse Trx1 antibody (Abcam) at a 1:1000 dilution.

Measurement of TrxR Activity. TrxR activity in the cell-free system was determined based on the reduction of 5,5′-dithiobis-2-nitrobenzoic acid by NADPH to yield thionitrobenzoate that is detectable at 412 nm (Arnér and Holmgren, 2001; Chew et al., 2008). Cell lysates were extracted after 24-h/37°C incubation with the chelators as described in the GR activity protocol above. TrxR activity was determined using the insulin reduction assay (Arnér and Holmgren, 2001). The absorbance was measured at 412 nm against the reagent blank to determine background content of thiol activity in the samples or the activity generated spontaneously by the action of endogenous Trx in the presence of insulin.

Measurement of Glutaredoxin Activity. Glutaredoxin activity in cell lysates was determined by monitoring NADPH-dependent reduction of 2-hydroxyethyl disulfide (Sigma-Aldrich) at 340 nm in the presence of GR (Holmgren and Aslund, 1995). After 24-h/37°C incubation with chelators, cell lysates were prepared in the same manner as for the GR experiment above. The background nonenzymatic oxidation of NADPH was used as a baseline. The enzyme activity was calculated from the linear net change in absorbance at 340 nm.

$NADP_{total}$ and $NADPH/NADP_{total}$ Assay. $NADP_{total}$ and $NADPH/NADP_{total}$ were measured using a kit from Abcam according to the manufacturer's instructions.

Measurement of Ribonucleotide Reductase Activity via EPR Spectroscopy. RR activity was measured using EPR spectroscopy by monitoring the tyrosyl radical using a Bruker EMX X-band spectrometer with 100-kHz field modulation (Cooper et al., 1996). EPR spectra were recorded at −196°C using a liquid nitrogen dewar.

Statistical Analysis. Data are expressed as mean ± S.E.M. Data were compared against the respective control in each experiment using Student's *t* test. Results were considered statistically significant when $p < 0.05$.

Results

Thiosemicarbazones Reduce Proliferation by Affecting the GSH System. Because thiosemicarbazone metal complexes can redox cycle (Richardson et al., 2006; Kalinowski et al., 2007; Jansson et al., 2010b), the effects of various antioxidants on their antiproliferative activities were evaluated using lung carcinoma cells because of the high antitumor efficacy of Dp44mT and Bp44mT against this cell type (Yuan et al., 2004; Whitnall et al., 2006). Initially, the combination of these agents with various antioxidants did not significantly ($p > 0.05$) affect their antiproliferative activity (Supplemental Fig. S1). For example, unlike the inhibitory effect of catalase (1000 U/ml) on the antiproliferative activity of the earlier generation chelator, pyridylketone isonicotinyl hydrazone (PKIH) (Chaston et al., 2004), this enzyme or membrane-permeable, pegylated-catalase (1000 U/ml) did not affect the antiproliferative activity of thiosemicarbazones (Supplemental Fig. S1A; P. Jansson, D. R. Richardson, unpublished results). In addition, the combination of PKIH or the thiosemicarbazones with either superoxide dismutase (SOD; Supplemental Fig. S1B), the glutathione peroxidase mimetic ebselen (Supplemental Fig. S1C), or the cell-permeable SOD mimetic MnTBAP (Supplemental Fig. S1D) did not significantly affect their antiproliferative activity. The concentrations of antioxidants used were the same as those shown to be effective previously in culture systems (Konorev et al., 1999). The inability of SOD, ebselen and MnTBAP to rescue the effect of PKIH, Dp44mT, or Bp44mT suggests superoxide and hydrogen peroxide are not markedly involved in their antiproliferative mechanism(s) and/or these antioxidants do not access appropriate compartments to prevent oxidative damage.

Because GSH is a major antioxidant in the cell (Balendiran et al., 2004), its supplementation using NAC was then evaluated (Schafer and Buettner, 2001). The coincubation with NAC significantly ($p < 0.05$) increased the IC_{50} values of Dp44mT and Bp44mT to 5.8- and 9.5-fold of their respective IC_{50} values in the absence of NAC (Fig. 2A). On the other hand, NAC did not significantly ($p > 0.05$) affect the IC_{50} values of PKIH or DFO (Fig. 2B), and it was of interest that depletion of cellular GSH using buthionine sulfoximine (BSO) (Schafer and Buettner, 2001) did not significantly affect the sensitivity of tumor cells to PKIH treatment (data not shown). However, coincubation of Dp44mT or Bp44mT with BSO enhanced their antiproliferative activity, significantly ($p < 0.001$) reducing the IC_{90} from >0.25 μM to 0.022 ± 0.009 and 0.08 ± 0.03 μM ($n = 3$), respectively (Fig.

2C). The treatment with BSO alone did not affect proliferation relative to the control (Fig. 2C). Therefore, the antiproliferative activity of thiosemicarbazones can be attenuated or enhanced through modulation of GSH levels.

Measurement of GSH or the GSH/GSSG ratio provides a good indication of the cellular redox environment (Schafer and Buettner, 2001). To determine whether chelators affect the GSH/GSSG ratio, two concentrations (2.5 and 25 μM) of Dp44mT and Bp44mT were used over a 24-h/37°C incubation. DFO was used at a 10-fold higher concentration because of its low membrane permeability (Olivieri and Brittenham, 1997) and relatively poor antiproliferative activity (Whitnall et al., 2006) (Fig. 2B). At 25 μM, the thiosemicarbazones are known to modulate cellular iron levels and iron-responsive molecules, as demonstrated previously (Yuan et al., 2004). Furthermore, after a 24-h incubation with the chelators, there was no marked alteration in cellular viability compared with cells in control medium, as determined by trypan blue staining (data not shown). It is important to note that this 24-h incubation period was specifically used to prevent the confounding effects of cytotoxicity mediated by these ligands that could affect the results obtained.

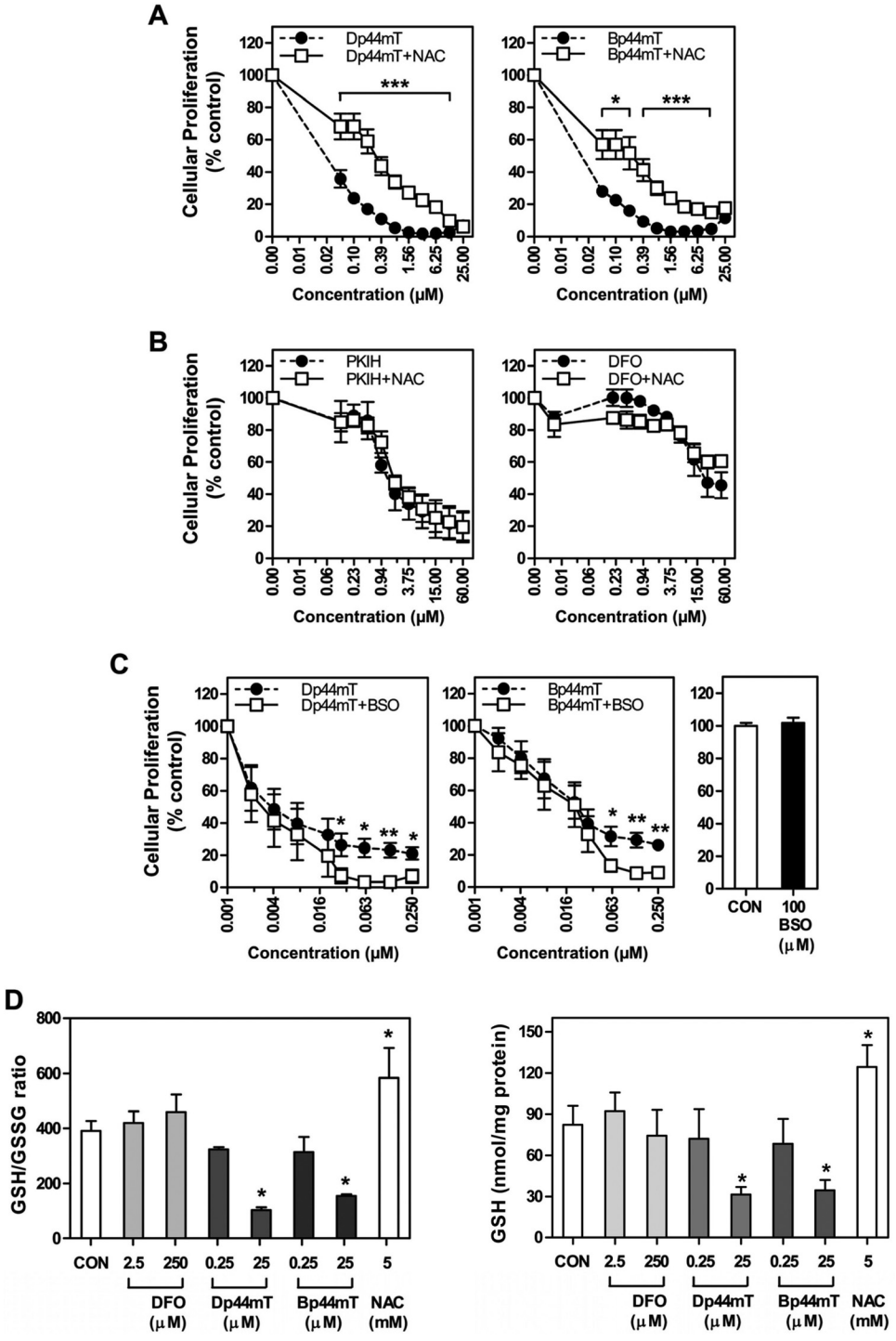


Fig. 2. The effect of modulating GSH on antiproliferative activity and the effect of chelators on the GSH/GSSG ratio and GSH levels. The effect of the anti-oxidant NAC (5 mM) on the antiproliferative activity of thiosemicarbazones (Dp44mT and Bp44mT) (A) and PKIH and DFO (B) determined using the 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrabromide assay in DMS-53 lung cancer cells over 72 h/37°C. C, the effect of BSO (100 μM) on the antiproliferative activity of thiosemicarbazones (Dp44mT and Bp44mT) using the procedure described in A and B. D, the GSH/GSSG ratio and the GSH levels after DMS-53 lung cancer cells were incubated with either DFO or thiosemicarbazones (Dp44mT or Bp44mT) for 24 h/37°C. NAC was included as a positive control to increase cellular GSH levels. The level of GSH and GSSG in the supernatant was determined using 5,5′-dithiobis-2-nitrobenzoic acid. Results are the mean ± S.E.M. (three to five experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Both DFO concentrations did not significantly alter the GSH/GSSG ratio (Fig. 2D). In contrast, Dp44mT and Bp44mT at 25 μM significantly ($p < 0.05$) reduced the GSH/GSSG ratio to 160 ± 56 ($n = 3$) and 235 ± 78 ($n = 3$), respectively, compared with control cells (391 ± 36; $n = 3$; Fig. 2D). The ratio observed was within the range described in the literature, in which GSH levels are reported to be 100 to 500 times higher than GSSG (Kosower and Kosower, 1978). The levels of cellular GSH after incubation with chelators also reflected the GSH/GSSG ratio (Fig. 2D). In these studies, NAC was included as a positive control for increasing the GSH/GSSG ratio. In summary, these results examining the effects of chelators on GSH show that redox-active thiosemicarbazones (Yuan et al., 2004; Richardson et al., 2006; Kalinowski et al., 2007; Jansson et al., 2010b) affect its metabolism in contrast to the nonredox-active chelator DFO (Richardson et al., 2006; Kalinowski et al., 2007). Similar effects of these chelators on GSH levels were also obtained using other cell types (e.g., SK-N-MC neuroepithelioma cells; data not shown).

Thiosemicarbazones Reduce Glutathione Reductase Activity in Cells. The conversion of GSSG to GSH requires GR, which uses NADPH (Balendiran et al., 2004). Thus, the ability of thiosemicarbazones to reduce the GSH/GSSG ratio could be a result of compromised GR activity, and this was then examined. As a positive control, a known GR inhibitor, bis-chloronitrosourea (BCNU; 100 μM), was included (Seefeldt et al., 2009) (Fig. 3A). Both Dp44mT and Bp44mT at 25 μM were less effective than BCNU but significantly ($p < 0.05$) reduced GR activity to 73 and 79% of the control, respectively. Higher concentrations of the thiosemicarbazones could not be used because of cytotoxicity. Consistent with its lack of effect on the GSH/GSSG ratio (Fig. 2D), DFO did not affect GR activity in cells (Fig. 3A). Considering these results, it was important to establish whether Dp44mT and Bp44mT were direct GR inhibitors in a cell-free system. Again, BCNU clearly inhibited the activity of this enzyme, whereas Dp44mT, Bp44mT, or DFO did not (Fig. 3B). We also tested GR activity after incubation with chelator/iron(III) complexes (i.e., 1:1 ligand-to-iron ratios for all chelators or a 2:1 ligand-to-metal ratio for Dp44mT or Bp44mT). However, these complexes also did not inhibit GR (data not shown).

To examine whether the decrease of cellular GR activity was

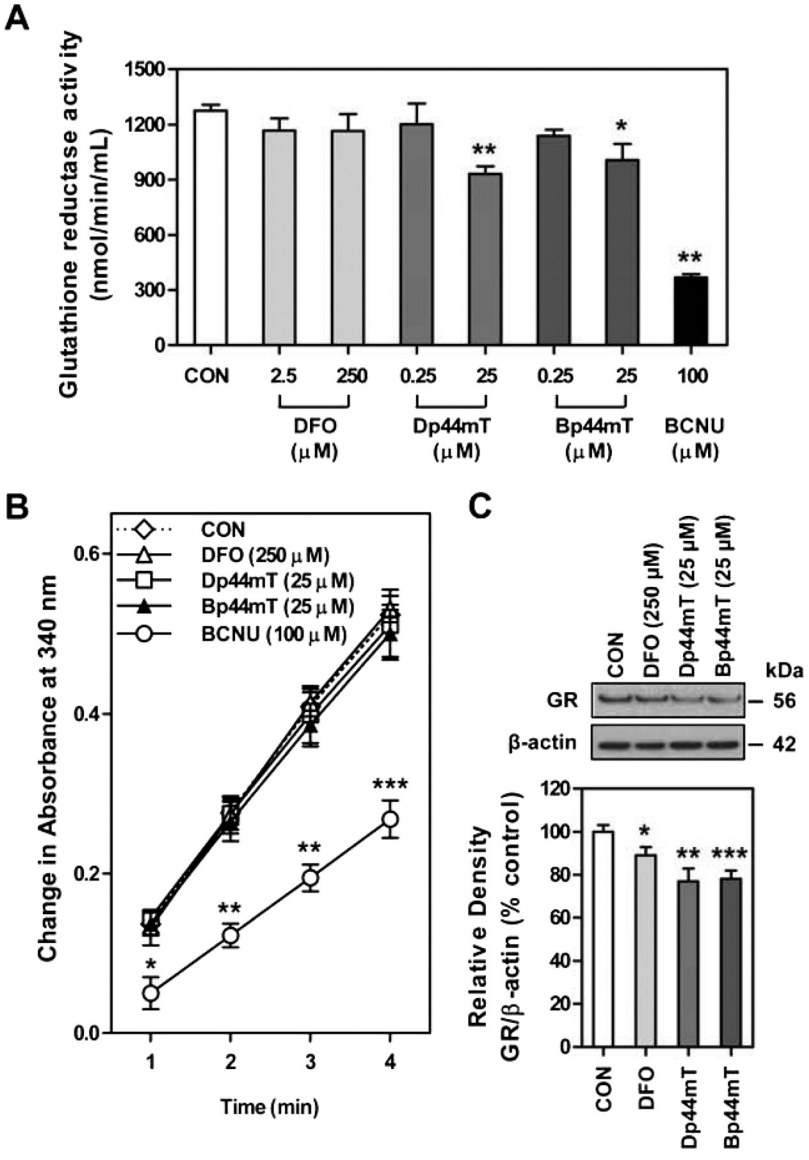


Fig. 3. The effect of chelators on GR activity. A, the cellular activity of GR was determined after a 24-h/37°C incubation of DMS-53 lung cancer cells with either DFO or the thiosemicarbazones (Dp44mT or Bp44mT). B, the GR activity in a cell-free system after a 30-min/37°C incubation with DFO or the thiosemicarbazones (Dp44mT or Bp44mT; 25 μM). BCNU (bis-chloronitrosourea) was included as a positive control. C, the GR protein expression after DMS-53 lung cancer cells were incubated with either DFO or thiosemicarbazones (Dp44mT or Bp44mT) for 24 h/37°C as determined by Western blot using mouse anti-human GR antibody (1:1000). Results are mean± S.E.M. (three to five experiments). *, $p < 0.05$; **, $p < 0.01$.

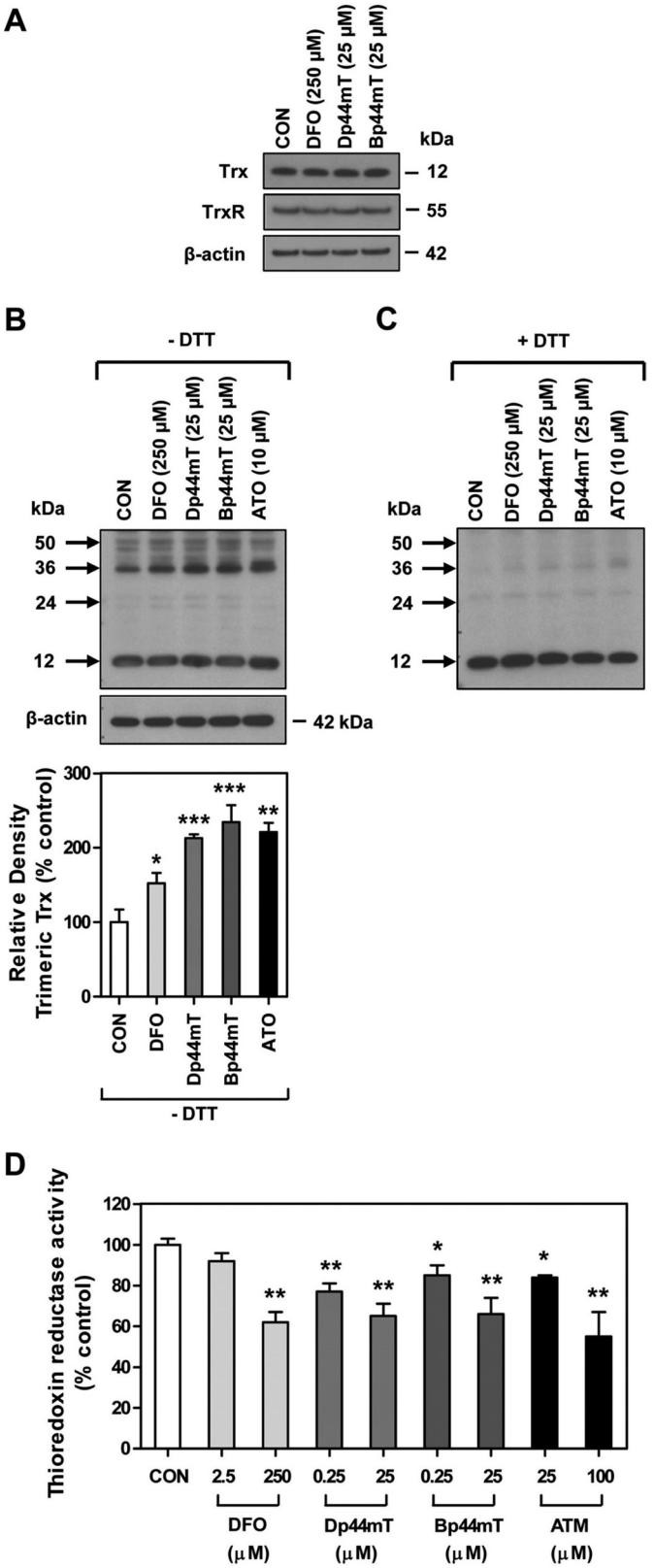


Fig. 4. The effect of chelators on the Trx system. A, the Trx and TrxR protein levels determined by western blot after DMS-53 cells were incubated with DFO or thiosemicarbazones (Dp44mT or Bp44mT) for 24 h/37°C. B, thioredoxin redox state in DMS-53 lung cancer cells after a 24-h/37°C incubation with DFO, Dp44mT, or Bp44mT when resolved on an SDS-polyacrylamide gel electrophoresis gel in the absence (B) or presence (C) of DTT (100 mM). The membranes were probed using mouse anti-human Trx antibody (1:1000). ATO was included as a positive control for Trx oxidation. D, TrxR activity was determined after a 24-h/37°C incubation with DFO, Dp44mT, or Bp44mT using DMS-53 lung cancer cells and implementing the endpoint insulin assay. Sodium aurothiomalate was included as a positive control. Results are mean ± S.E.M. (three to four experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

due to an alteration in GR protein expression, Western blot analysis was performed (Fig. 3C). All chelators slightly decreased GR protein expression, and although DFO had no effect on cellular GR activity (Fig. 3A), it significantly ($p < 0.05$) decreased GR protein levels to 89 ± 4% of the control (Fig. 3C). Nevertheless, the effects of Dp44mT and Bp44mT were more pronounced, significantly ($p<0.01$) reducing GR protein expression to 77 ± 6 and 78 ± 4% ($n = 3$) of the control, respectively (Fig. 3C). Hence, the reduced GSH/GSSG ratio observed (Fig. 2D) could be explained by the decreased cellular GR activity. However, because the decrease in GR activity after incubation with thiosemicarbazones was relatively mild (Fig. 3A), the reduction in GSH/GSSG ratio may also be due to a loss of GSH via other mechanisms, including glutathionylation, cellular efflux, or oxidation to sulfinic or sulfonic acid (Paget and Buttner, 2003).

Iron Chelators Increase Oxidation of Thioredoxin. Another important thiol-based antioxidant mechanism for redox control is the Trx system (Holmgren, 1989). Western blot analysis demonstrated that Trx and TrxR protein expression was not affected by DFO, Dp44mT, or Bp44mT (Fig. 4A), and thus, these compounds did not globally decrease protein levels. The effect of chelators on the redox status of Trx in cells was then examined because this molecule forms dimers and trimers when oxidized (Lu et al., 2007; Sun and Rigas, 2008). As a positive control, arsenic trioxide (ATO; 10 μM) was included (Lu et al., 2007) and led to increased levels of the trimeric oxidized Trx at 36 kDa to 221 ± 12% ($n = 3$) of the control (Fig. 4B). Likewise, Dp44mT or Bp44mT significantly ($p < 0.001$) increased oxidized trimeric Trx to 213 ± 5 ($n = 3$) and 234 ± 23% ($n = 3$) of the control (100%), respectively (Fig. 4B). It is noteworthy that DFO also significantly ($p < 0.05$) increased Trx trimer, but to a lower extent (152 ± 9%; $n = 3$) than thiosemicarbazones.

Dimeric Trx at 24 kDa was a very minor form in these cells (Fig. 4B), and its detection required long exposure times. However, it was also increased after incubation with DFO, Dp44mT, or Bp44mT by 17 to 30% (data not shown). In contrast to other investigations (Lu et al., 2007; Sun and Rigas, 2008), which showed a reduction of monomeric Trx (12 kDa) concomitant with increased Trx oxidation, we detected no significant change in the monomer level when there was a significant increase of Trx dimer/trimer. This is probably because the expression of the reduced monomer of Trx (12 kDa) was extremely high (>15-fold greater) relative to the alteration in the expression of the trimer (36 kDa) between the control and treatments. Higher M_r species of Trx were also evident at >36 and could represent oligomers or complexes with other proteins (Lu et al., 2007). To confirm that these bands were disulfide-bridged oxidized Trx, we incubated the lysate with the reducing agent DTT, which markedly reduced the intensity of these bands, leaving primarily the monomer (Fig. 4C).

Iron Chelators Reduce Thioredoxin Reductase Activity in Cells. The only known enzyme to reduce oxidized

Trx is TrxR (Holmgren, 1989). All chelators significantly ($p < 0.05$) decreased TrxR activity even when cells were incubated with a low concentration of Dp44mT or Bp44mT (0.25 μM; Fig. 4D). At a 100-fold higher concentration, Dp44mT and Bp44mT further inhibited ($p < 0.01$) the enzyme to 65 ± 6% ($n = 4$) and 66 ± 8% ($n = 4$) of the control, respectively. Moreover, at the same concentration (25 μM), the activity of these thiosemicarbazones was more pronounced than the well known TrxR inhibitor, sodium aurothiomalate (Arnér, 2009), which reduced TrxR activity to 84 ± 1% ($n = 4$) of the control (Fig. 4D). DFO (250 μM) also significantly ($p < 0.01$) inhibited the TrxR activity to approximately the same level as thiosemicarbazones at 25 μM. These results show that both types of chelators affect the Trx system, although DFO is less effective.

We used cell-free studies to elucidate whether chelators were direct inhibitors of TrxR. However, DFO, Dp44mT, or Bp44mT did not significantly inhibit TrxR activity in the cell-free system (Supplemental Fig. S2, A–C). It is noteworthy that $FeCl_3$ alone at 250 μM (a control for the DFO-iron complex), significantly ($p < 0.001$) inhibited TrxR (Supplemental Fig. S2A), although all chelator/iron(III) complexes (i.e., DFO/iron, Dp44mT/iron, and Bp44mT/iron) were ineffective. Together, these results suggested that these chelators were not direct TrxR inhibitors.

Thiosemicarbazones Reduce Glutaredoxin Activity in Cells. Grx (Holmgren, 1989) uses GSH for supplying reducing equivalents to RR (Zahedi Avval and Holmgren, 2009) and catalyzes other thiol-disulfide interchange reactions and repairs glutathionylated proteins (Lillig et al., 2008). Because of the effect of thiosemicarbazones on the GSH/GSSG ratio (Fig. 2D), we determined the Grx activity. Cadmium chloride ($CdCl_2$) was included as a positive control (Chrestensen et al., 2000) and significantly inhibited ($p < 0.01$) Grx activity to 68 ± 4% of the control (Fig. 5A). Likewise, Bp44mT ($p < 0.01$) and Dp44mT ($p < 0.05$) signifi-

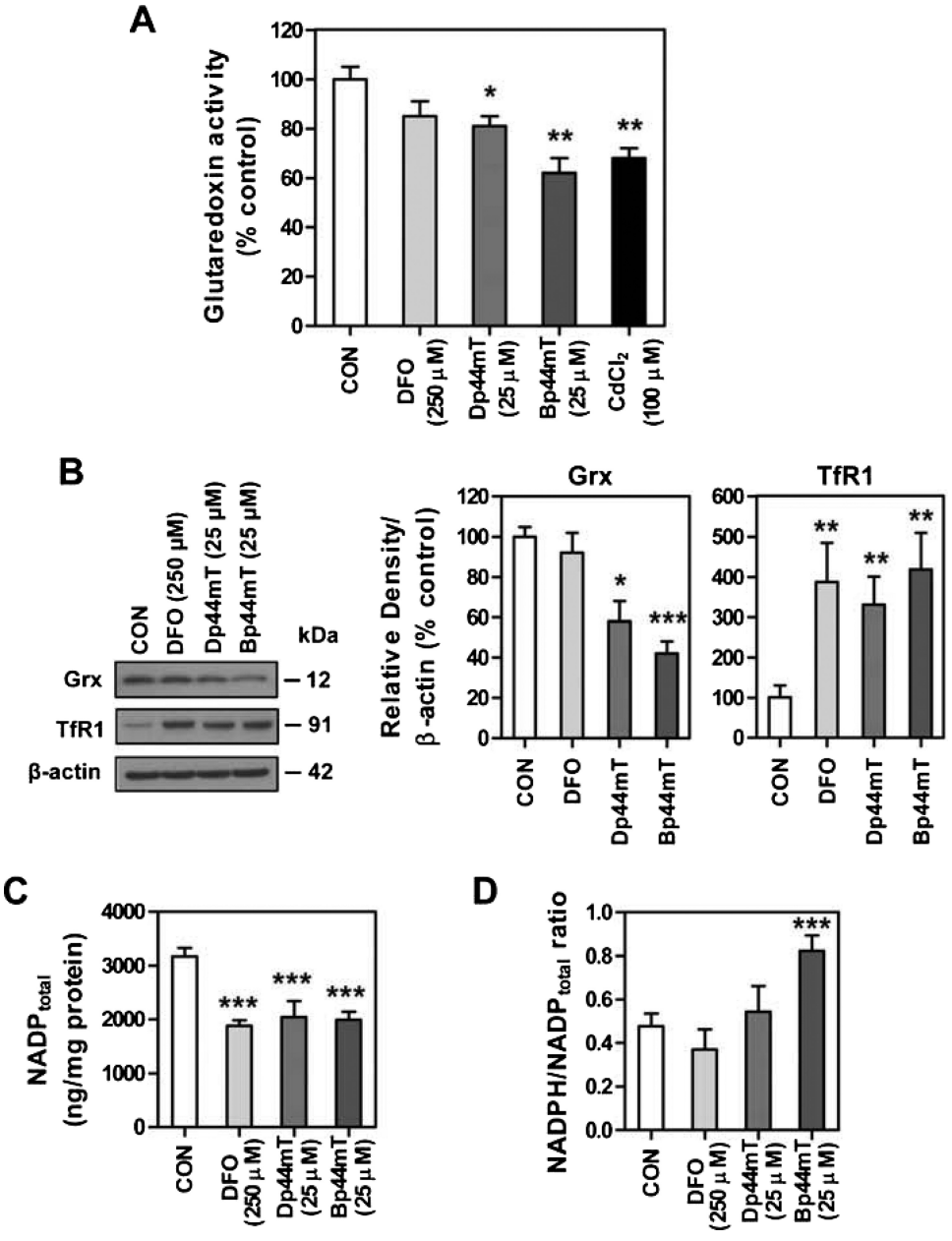


Fig. 5. The effect of chelators on Grx and the NADP pool. A, Grx activity after DMS-53 lung cancer cells were incubated with DFO, Dp44mT, or Bp44mT for 24 h/37°C as determined by NADPH-dependent reduction of 2-hydroxyethyl disulfide. Incubation of cells with cadmium chloride ($CdCl_2$) for 1 h/37°C was included as positive control. B, the cellular Grx and TfR1 protein expression in DMS-53 lung cancer cells after incubation with DFO, Dp44mT, or Bp44mT for 24 h/37°C as determined by Western blot using mouse anti-human Grx1 and TfR1 (1:1000). The cellular $NADP_{total}$ (C) and $NADPH/NADP_{total}$ ratio (D) after a 24-h/37°C incubation of DFO, Dp44mT, or Bp44mT with DMS-53 lung cancer cells. Results are mean± S.E.M. (four experiments). *, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$.

cantly inhibited the Grx activity to 62 ± 6 (n = 4) and 81 ± 4% (n = 4) of the control, respectively. Assessment of Grx protein expression demonstrated that Dp44mT and Bp44mT significantly ($p < 0.05$) decreased Grx to 58 ± 10% (n = 3) and 42 ± 6% (n = 3) of the control, respectively (Fig. 5B). Hence, the reduction in Grx activity could be caused by the decrease in Grx protein expression. These observations on Grx activity agree with our previous studies examining the GSH system (Fig. 2D), because the reduction of Grx requires GSH (Holmgren, 1989), emphasizing the effect of thiosemicarbazones on modulating GSH and Grx. The reduction in Grx expression was unlikely because of chelator-induced translational repression, because the expression of an iron-regulated protein, the TfR1 (Dunn et al., 2007), was markedly up-regulated to 331 ± 70 and 418 ± 91% (n = 3), respectively (Fig. 5B). In addition, there was no alteration in the expression of Trx or TrxR (Fig. 4A), suggesting that the decrease in Grx expression was not due to general depression of translation by the agents.

Chelators Reduce Total NADP but Do Not Decrease Regeneration of Cellular NADPH. The sum of NADPH and $NADP^+$ (the $NADP_{total}$) and the cellular NADPH/$NADP_{total}$ ratio were assessed as NADPH supplies reducing equivalents for GSH and Trx (Holmgren, 1989). In these studies, DFO, Dp44mT, and Bp44mT significantly ($p < 0.001$) reduced $NADP_{total}$ to 1881 ± 110, 2043 ± 297, and 1995 ± 147 ng/mg protein (n = 5), respectively, compared with the control (3173 ± 157 ng/mg protein; n = 5; Fig. 5C). The decrease of $NADP_{total}$ suggested a concomitant decrease in NADPH. However, there was no significant decrease in the NADPH/$NADP_{total}$ ratio (Fig. 5D). It is noteworthy that there was a significant ($p < 0.001$) increase of the NADPH/$NADP_{total}$ ratio in Bp44mT-treated cells relative to the control (i.e., 0.8 ± 0.07 versus 0.5 ± 0.06; n = 5). The decrease of $NADP_{total}$ after chelator treatment suggested a reduction of NAD kinase activity (Pollak et al., 2007) or depletion of NAD or NADP pools (Pollak et al., 2007). Nonetheless, these chelators did not reduce the ability of cells to regenerate NADPH needed for thiol reduction, as shown by the NADPH/$NADP_{total}$ ratio.

Chelators Inhibit Ribonucleotide Reductase Activity in Cells. DFO and some types of thiosemicarbazones are known to inhibit RR (Cooper et al., 1996) as a result of binding of cellular iron (Finch et al., 1999). Considering the results above and the fact that the Trx and Grx systems are hydrogen donors for RR (Zahedi Avval and Holmgren, 2009), we examined the effect of these chelators on RR activity. EPR was used because it is a direct method of examining RR activity in intact cells (Cooper et al., 1996). The tyrosyl radical of RR showed a characteristic EPR signal at g = 2.0049 ± 0.0002 (n = 3) (Cooper et al., 1996). Dp44mT significantly ($p < 0.001$) inhibited RR activity to 50 ± 5% of the control (Fig. 6A). At a 10-fold higher concentration, DFO also significantly ($p < 0.05$) inhibited RR activity to 50 ± 13% (n = 3). The RR activity of Bp44mT was not measurable because of the formation of a chelator-derived radical in cells, which confounded analysis. Hence, RR activity was determined using another 2-benzoylpyridine thiosemicarbazone-based iron chelator, Bp4eT, which shares structural similarity to Bp44mT (Fig. 1B) (Kalinowski et al., 2007) and significantly ($p < 0.001$) inhibited RR activity to 47 ± 5% of the control. After recording EPR spectra, the samples were thawed, leading to no signal, consistent with the rapid decay of the RR tyrosyl radical.

Discussion

Thiosemicarbazones show potent and selective anticancer activity and affect a wide variety of molecular targets (Yuan et al., 2004; Whitnall et al., 2006; Kalinowski et al., 2007; Rao et al., 2009). In the current study, the ability of thiosemicarbazones to also perturb cellular redox systems and induce antiproliferative activity is demonstrated by the ability of NAC (which supplements cellular GSH) to significantly reduce the antiproliferative activity of these ligands (Fig. 2A). Furthermore, the effect of the GSH inhibitor BSO to significantly enhance thiosemicarbazone antiproliferative activity (Fig. 2C) again supports the argument that these agents target thiols. In addition, thiol-containing systems provide crucial reducing equivalents for RR whose enzymatic activity could not function in their absence (Zahedi Avval and Holmgren, 2009). Hence, any perturbation of these reducing systems will affect RR activity.

It is noteworthy that GSH levels are elevated in many tumor types and have been associated with resistance to chemo- and radiotherapy (Balendiran et al., 2004). Cellular GSH content has also been shown to correlate with metastatic activity (Carretero et al., 1999), and its depletion sensitizes cells to ionizing radiation (Balendiran et al., 2004). Hence, the ability of thiosemicarbazones to decrease GSH may be important for their anticancer activity and may account for their marked activity against chemotherapy-resistant cells (Whitnall et al., 2006).

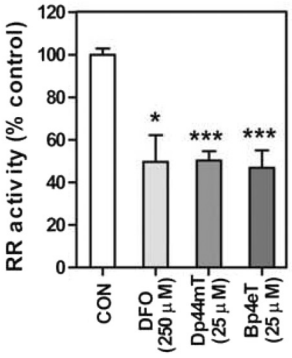
Apart from their effect on GSH levels, cellular GR activity was also slightly decreased by thiosemicarbazones (Fig. 3A), although this was not observed in a cell-free system. The reduction of GR activity in cells could be due to the observed decrease in its protein level and/or generation of intracellular metal complexes or a metabolite that affects enzymatic activity. For example, this occurs with arsenic compounds in which biomethylation generates potent GR inhibitors (Miller et al., 2002).

We also showed that both thiosemicarbazones and DFO (at a 10-fold higher concentration) were able to inhibit TrxR activity (Fig. 4D) and cause Trx oxidation (Fig. 4B). Furthermore, both thiosemicarbazones and DFO (at a 10-fold higher concentration) were able to inhibit TrxR activity (Fig. 4D) and cause Trx oxidation (Fig. 4B). Thus, cellular oxidation of Trx may be related to the reduced capacity of TrxR under our experimental conditions. However, direct oxidation of Trx in cells by the metal complexes of the thiosemicarbazones cannot be excluded. This is suggested considering that previous studies have shown that even with a 90% knockdown of thioredoxin reductase, there was little effect on downstream thioredoxin and thioredoxin-dependent functions because of the residual capacity of the enzyme (Eriksson et al., 2009). It is known that mammalian TrxR has broad specificity (Arnér, 2009), and many electrophilic compounds can affect TrxR activity (Chew et al., 2008). Other anticancer agents can suppress TrxR activity in addition to some metal complexes (e.g., gold) that are also able to inhibit this enzyme (Bragadin et al., 2004).

A less commonly studied thiol system in terms of understanding the response of cancer cells to chemotherapy is the

Grx system. Increased Grx expression is implicated in cancer (Lillig et al., 2008), and Grx activity was significantly reduced in cells after incubation with thiosemicarbazones, but not DFO (Fig. 5A). Again, this demonstrates the difference in the biological activity of these ligands (Yuan et al., 2004; Richardson et al., 2006; Kalinowski et al., 2007). It should be considered that the effects of thiosemicarbazones on these thiol-containing molecules may be important in explaining

A



Treatment	EPR signal area§
CON	3.12 ± 0.31
DFO (250 μM)	1.56 ± 0.52 *
Dp44mT (25 μM)	1.62 ± 0.20 ***
Bp4eT (25 μM)	1.78 ± 0.18 ***

§ The values were calculated by double integration of the tyrosyl radical from 334 - 336 mT (n = 3, mean ± SEM)

B

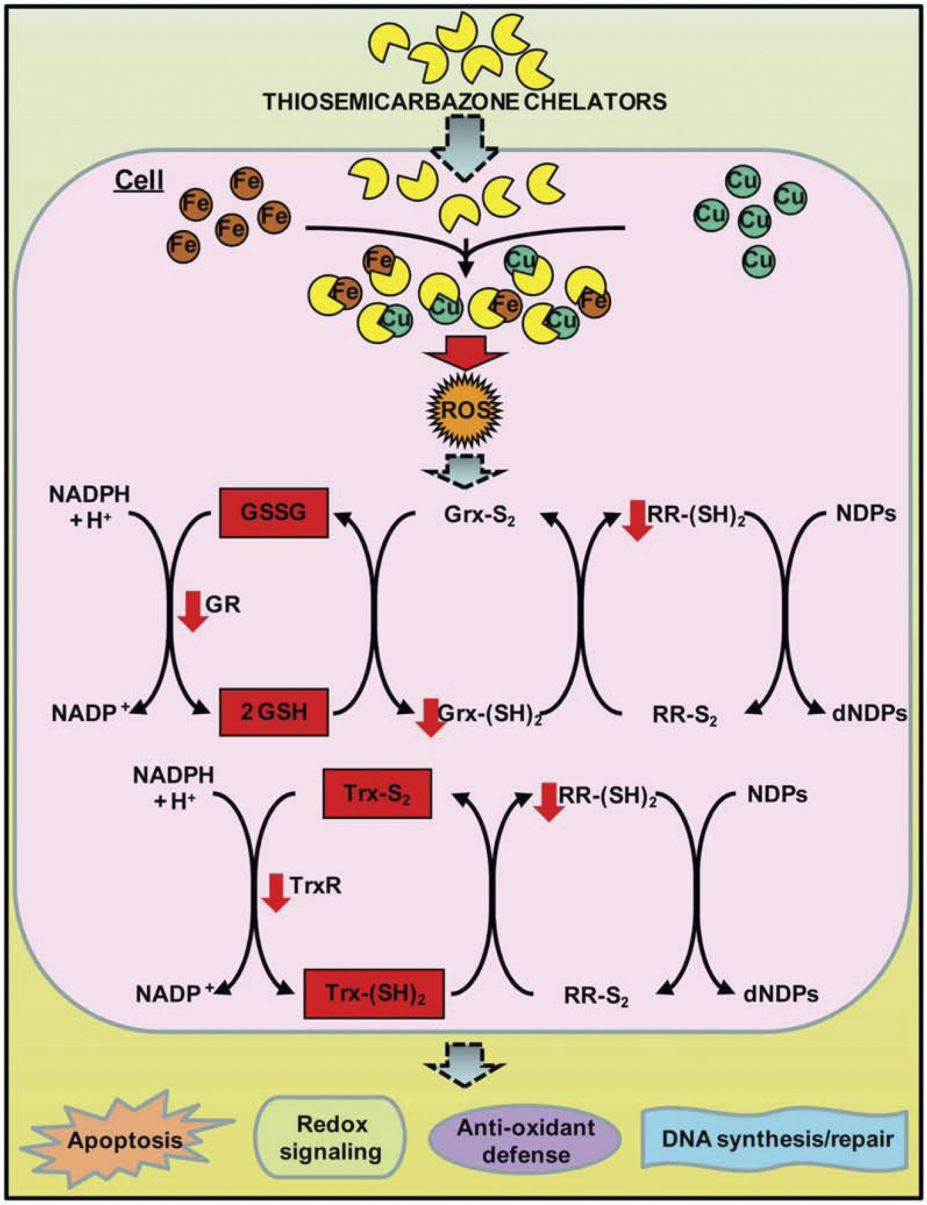


Fig. 6. The effect of chelators on RR activity and summary of the effects of thiosemicarbazones on thiol systems. A, cellular RR activity after a 24-h/37°C incubation with DFO, Dp44mT, or Bp4eT in DMS-53 lung cancer cells as determined using EPR spectroscopy. The RR activity was calculated based on the change in signal area on double integration of the spectra from 334 to 336 mT, as shown in the table and expressed as percentage of control in the graph. Results are mean ± S.E.M. (three to four experiments). *, $p < 0.05$; ***, $p < 0.001$. B, schematic summary of the effects of thiosemicarbazones on thiol systems: GSH, Trx, and Grx. Thiosemicarbazone chelators enter cells and bind iron or copper (Yuan et al., 2004; Jansson et al., 2010b), forming redox-active complexes (Yuan et al., 2004; Richardson et al., 2006; Kalinowski et al., 2007; Jansson et al., 2010a,b), which decrease the GSH/GSSG ratio, glutathione reductase (GR) and Grx activity. These complexes also decrease thioredoxin reductase (TrxR) activity, causing an increase in Trx oxidation. Both the GSH-Grx and Trx systems are crucial for the reduction of RR, which generates deoxyribonucleoside 5′-diphosphates (dNDPs) for DNA synthesis. Some possible consequences of affecting these vital thiol systems include alterations in DNA synthesis/repair, antioxidant/xenobiotic protection, apoptosis, and redox-mediated signaling pathways.

their selectivity against neoplastic cells (Richardson et al., 2006; Kalinowski et al., 2007). Indeed, the latter are known to possess a different redox profile compared with their normal counterparts (Giles, 2006).

It is noteworthy that some effects elicited by thiosemicarbazones required relatively high (25 μM) concentrations of the ligands, such as those observed on GSH (Fig. 2D), GR (Fig. 3A), and Grx (Fig. 5A). In contrast, the inhibitory effect of these agents on TrxR was elicited at a much lower concentration (0.25 μM). These results may reflect the different levels of these redox systems in cells. For instance, the physiological concentration of GSH in mammalian cells is in the millimolar range, providing a marked buffering capacity to prevent redox stress. In contrast, the levels of TrxR in cells have been estimated to be approximately 0.1 to 0.3 μM (Holmgren, 1977). Thus, this enzyme may be more sensitive to much lower concentrations of the thiosemicarbazones. Other potential reasons for the relative insensitivity of some systems could relate to their locations in cells, which may not be accessed by the ligands and the relatively short incubation periods used. It should be noted that for all assays examining redox systems, the cells were only incubated for 24 h with the chelators to prevent cytotoxicity that would markedly perturb these systems. Considering the marked redox activity of these thiosemicarbazones (Yuan et al., 2004; Richardson et al., 2006), it is likely that at later time points, far greater disturbances in these redox systems would become apparent. However, these changes would be perturbed by the cytotoxicity itself, making interpretation difficult. Hence, incubations longer than 24 h were not used.

Acknowledging that only relatively short incubations (24 h) were used in redox assays to prevent cytotoxicity of the ligands, it must be noted that when we compare the effects of the thiosemicarbazones to well characterized inhibitors of these redox systems, we observe similar inhibitory effects. For example, in Trx redox-state studies, thiosemicarbazones oxidized Trx to the same level as the well characterized inhibitor ATO (Lu et al., 2007) (Fig. 4B). Furthermore, in studies examining TrxR activity, in which the known TrxR inhibitor sodium aurothiomalate (Bragadin et al., 2004) was implemented as a positive control, it is notable that it was less effective than thiosemicarbazones at inhibiting the activity of this enzyme at the same concentration (Fig. 4D). This is also the case with Grx studies (Fig. 5A), in which the known inhibitor of this protein, $CdCl_2$ (Chrestensen et al., 2000), elicited an effect similar to the thiosemicarbazone chelators. Hence, the effects of the thiosemicarbazones are similar to well described inhibitors of these redox systems. Finally, the importance of these redox alterations in terms of contributing to the potent activity of the thiosemicarbazones can be clearly observed from the ability of GSH supplementation using NAC, which significantly inhibited their antiproliferative activity over a 72-h incubation (Fig. 2A), and from the effect of GSH depletion via BSO to potentiate their efficacy (Fig. 2C).

The result that thiosemicarbazones affect the GSH/Grx and Trx systems is probably important for understanding their antitumor activity. Before this study, the effect of these chelators on cellular thiol systems had not been comprehensively characterized. However, the ability of chelators to inhibit RR activity is well documented (Cooper et al., 1996). From previous studies (Finch et al., 1999; Kolesar et al., 2008), it remains unclear whether chelation alone is totally responsible for inhibiting RR. For example, the thiosemicarbazone-based chelator, Triapine, is a more active RR inhibitor when added to cells as an iron or copper complex than the ligand alone (Finch et al., 1999). These observations suggest that cellular iron-depletion is not the only mechanism of how thiosemicarbazones inhibit RR.

In this investigation, thiosemicarbazones affected GSH, Grx, and Trx that are hydrogen donors for RR, which involves disulfide bond reduction in its R1 subunit (Zahedi Avval and Holmgren, 2009). Hence, thiosemicarbazone-mediated RR inhibition could be due to alterations in thiol systems as a result of redox-active iron and copper complexes (Jansson et al., 2010b) and is not solely mediated via iron depletion. In contrast, DFO forms a redox-inactive iron complex that abolishes its antiproliferative activity (Boukhalfa and Crumbliss, 2002) and was far less effective at modulating these thiol systems. Hence, although DFO has lower membrane permeability than many thiosemicarbazones (Yuan et al., 2004), it still penetrates cells to inhibit RR mainly by iron depletion but does not markedly influence thiol systems. Furthermore, the fact that PKIH can readily enter cells, but its potency is lower than thiosemicarbazones, probably reflects the additional and marked redox activity of thiosemicarbazones (Yuan et al., 2004; Kalinowski et al., 2007), as described previously (Richardson et al., 2006).

Together, as shown in Fig. 6B, incubation of cells with thiosemicarbazones leads to redox-active metal complexes (Yuan et al., 2004; Richardson et al., 2006; Kalinowski et al., 2007; Jansson et al., 2010a; Jansson et al., 2010b), which induce a decreased GSH/GSSG ratio, increased oxidation of Trx-$(SH)_2$, and compromised Grx activity. These effects could be due to the decrease in GR and TrxR activity. Together, this disrupts the ability to catalyze protein thiol-disulfide exchange and glutathionylation (Holmgren, 1989; Lillig et al., 2008). As a consequence, RR activity is reduced and DNA synthesis is inhibited, and this probably dysregulates multiple processes (Balendiran et al., 2004; Zahedi Avval and Holmgren, 2009) (Fig. 6B). Finally, it should be noted that thiosemicarbazones such as Dp44mT have various mechanisms of antiproliferative activity (i.e., generation of cytotoxic radicals, up-regulation of the growth and metastasis suppressor NDRG1, and so forth (Yuan et al., 2004; Whitnall et al., 2006). Hence, the effects of the thiosemicarbazones on the redox systems demonstrated here illustrate another facet of their mechanism of action.

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Authorship Contributions

Participated in research design: Yu, Suryo Rahmanto, Hawkins, and Richardson.

Conducted experiments: Yu and Hawkins.

Wrote or contributed to the writing of the manuscript: Yu, Suryo Rahmanto, and Richardson.

Other: Richardson obtained grant funding.

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