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The Potent and Novel Thiosemicarbazone Chelators, Dp44mT and Bp44mT, Affect Crucial Thiol Systems Required for Ribonucleotide Reductase Activity.

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Running title: Thiosemicarbazones affect thiol-containing systems

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Abbreviations: ATO, arsenic trioxide; BCNU, bis-chloronitrosourea; Bp44mT, 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone; Bp4eT, 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone; BpT, 2-benzoylpyridine thiosemicarbazone; BSO, buthionine sulfoximine; DFO, desferrioxamine; Dp44mT, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; DTT, dithiothreitol; EPR, electron paramagnetic resonance; GR, glutathione reductase; Grx, glutaredoxin; GSH, glutathione; GSSG, oxidized GSH; HED, 2-hydroxyethyl disulfide; MTT, 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrabromide; NAC, *N*-acetylcysteine; RIPA, radioimmunoprecipitation; ROS, reactive oxygen species; RR, ribonucleotide reductase; SOD, superoxide dismutase; TfR1, transferrin receptor-1; TNB, thionitrobenzoate; Trx, thioredoxin; TxR, thioredoxin reductase.

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ABSTRACT

Di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT) possesses potent and selective anti-tumor activity. Its cytotoxicity has been attributed to iron chelation leading to inhibition of the iron-containing enzyme, ribonucleotide reductase (RR). Recently, thiosemicarbazone iron complexes were shown to be redox-active, although their effect on cellular anti-oxidant systems is unclear. Using a variety of anti-oxidants, we found only *N*-acetylcysteine significantly inhibited thiosemicarbazone-induced anti-proliferative 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. Surprisingly, 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 ten-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, while the $NADPH/NADP_{total}$ ratio was not reduced. This was important to consider since NADPH is required for thiol reduction. Thus, thiosemicarbazones could have an additional mechanism of RR inhibition *via* their effects on major thiol-containing systems.

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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 anti-cancer 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 (Richardson et al., 2006; Yuan et al., 2004). When the redox potentials of the so-formed chelator-iron complexes are outside 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 which avoids the reduction of iron(III) under physiological conditions (Boukhalfa and Crumbliss, 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 anti-neoplastic 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).

Recently, the potent and selective anti-cancer 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 (Jansson et al., 2010b; Kalinowski et al., 2007; Rao et al., 2009; Richardson et al., 2006; Whitnall et al., 2006). In addition, these chelators have been demonstrated to redox cycle after complexation with iron or copper, leading to cytotoxicity (Kalinowski et al., 2007; Richardson et al., 2006). In fact, we showed that Dp44mT and

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Bp44mT demonstrate pronounced iron chelation efficacy (Kalinowski et al., 2007; Yuan et al., 2004). Dp44mT markedly inhibits tumor growth *in vivo* and is well-tolerated at optimal doses (Whitnall et al., 2006; Yuan et al., 2004). Moreover, the 2-benzoylpyridine thiosemicarbazone (BpT) chelators, including 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone (Bp4eT; Fig. 1B), also showed potent and comparable anti-proliferative activity to Dp44mT (Kalinowski et al., 2007). These thiosemicarbazones are markedly more stable in plasma compared to 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 the iron complexes were redox active (Kalinowski et al., 2007; Richardson et al., 2006). Dp44mT also increased intracellular ROS (Richardson et al., 2006; Yuan et al., 2004), probably *via* complexation with iron or copper (Jansson et al., 2010b; Kalinowski et al., 2007; Richardson et al., 2006) and recent electron paramagnetic resonance (EPR) spectroscopy studies confirmed the redox activity of the Dp44mT-iron complex (Jansson et al., 2010a).

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

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and is known to be inhibited by iron chelators (Avval and Holmgren, 2009; Cooper et al., 1996).

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

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MATERIALS AND METHODS

Chelators

All thiosemicarbazone and aroylhydrazone chelators were synthesized and characterized using standard procedures, as described (Kalinowski et al., 2007; Richardson et al., 2006). DFO was obtained from Novartis (Basel, Switzerland). Thiosemicarbazone and aroylhydrazone chelators were dissolved in DMSO at a stock concentration of 10 mM and were used at the concentrations indicated by dilution in culture media containing 10% FCS. 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 (MTT) 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 (Cat.#: 371757; Calbiochem, Gibbstown, NJ) according to the manufacturer's instructions. Briefly, cells were seeded in 100 mm dishes for experimental treatments. After a

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24 h/37°C incubation with the chelators, the cells were washed with ice-cold PBS and lysed in 50 μL PBS by three freeze-thaw cycles. The lysates were then acidified with 5% metaphosphoric acid and the supernatant 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 (GR) activity

The GR activity was assayed by following the rate of decrease in NADPH absorbance at 340 nm due to 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 re-suspended 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 4ºC and supernatant collected for determination of GR activity. The assay mixture contained supernatant (40 μg), BSA (1 mg/mL) and NADPH (0.4 mM) (Sigma-Aldrich). 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) were incubated with the chelators for 30 min/37°C prior to the assay. Background non-enzymatic oxidation of NADPH was used as the baseline.

Western Blotting

Cells were lysed using radioimmunoprecipitation (RIPA) buffer containing protease inhibitor (Roche Diagnostics, Indianapolis, IN). The protein concentration was determined using the Bradford assay reagent (BioRad, Hercules, CA). Protein samples (50 μg/lane) were separated on a 4-12% NuPage Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to a PVDF membrane (Invitrogen) according to the manufacturer's protocol. Mouse monoclonal anti-

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human GR (Cat.#: ab55075), Trx1 (Cat.#: ab16845), TrxR (Cat.#: ab16847) and Grx1 (Cat.#: ab55059) antibodies (Abcam, Cambridge, MA) were incubated at a 1:1000 dilution. Mouse monoclonal anti-human TfR1 (Invitrogen; Cat.#: 136800) 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 (BioRad, Hercules, CA).

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, 1 mM EDTA) containing 60 mM iodoacetamide (Lu et al., 2007). After 2 h at 4ºC, the cells 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 4-12% Bis-Tris gel (Invitrogen). The same samples were incubated in the presence of DTT (100 mM) in SDS-loading buffer and heated at 60ºC/30 min before separation. Trx was detected with an anti-mouse Trx1 antibody (Abcam) at a 1:1000 dilution.

Measurement of thioredoxin reductase (TxR) activity

TrxR activity in the cell-free system was determined based on the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by NADPH to yield thionitrobenzoate (TNB) 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

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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 (HED; 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 non-enzymatic 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 (Cat. #: ab65349) 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±standard error of the mean (SEM). Data were compared against the respective control in each experiment using Student's *t*-test. Results were considered statistically significant when $p<0.05$.

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RESULTS

Thiosemicarbazones reduce proliferation by affecting the GSH system

Since thiosemicarbazone metal complexes can redox cycle (Jansson et al., 2010b; Kalinowski et al., 2007; Richardson et al., 2006), the effects of various anti-oxidants on their anti-proliferative activities were evaluated using lung carcinoma cells because of the high anti-tumor efficacy of Dp44mT and Bp44mT against this cell-type (Whitnall et al., 2006; Yuan et al., 2004). Initially, the combination of these agents with various anti-oxidants did not significantly (p>0.05) affect their anti-proliferative activity (Supplemental Fig. S1). For example, unlike the inhibitory effect of catalase (1000 U/mL) on the anti-proliferative activity of the earlier generation chelator, pyridylketone isonicotinoyl hydrazone (PKIH) (Chaston et al., 2004), this enzyme or membrane-permeable, pegylated-catalase (1000 U/mL), did not affect the anti-proliferative activity of thiosemicarbazones (Supplemental Fig. S1A and Jansson, P., Richardson, D.R. 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 anti-proliferative activity. The concentrations of anti-oxidants used were the same as those shown to be previously effective 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 anti-proliferative mechanism(s) and/or these anti-oxidants do not access appropriate compartments to prevent oxidative damage.

As GSH is a major anti-oxidant in the cell (Balendiran et al., 2004), its supplementation using NAC was then evaluated (Schafer and Buettner, 2001). The co-incubation with NAC

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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). Conversely, 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, co-incubation of Dp44mT or Bp44mT with BSO enhanced their anti-proliferative activity, significantly (p<0.001) reducing the IC_{90} from >0.25 μM to 0.022±0.009 μM 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 anti-proliferative 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 μM and 25 μM) of Dp44mT and Bp44mT were utilized 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 anti-proliferative 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 when compared to 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 utilized to prevent the confounding effects of cytotoxicity mediated by these ligands that could affect the results obtained.

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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, when compared to control cells (391±36; n=3; Fig. 2D). The ratio observed was within the range described in the literature, where GSH levels are reported to be 100-500 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 (Jansson et al., 2010b; Kalinowski et al., 2007; Richardson et al., 2006; Yuan et al., 2004) affect its metabolism, in contrast to the non-redox-active chelator, DFO (Kalinowski et al., 2007; Richardson et al., 2006). 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 utilizes 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 due to 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

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inhibited the activity of this enzyme, while 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 if the decrease of cellular GR activity was due to an alteration in GR protein expression, western blot analysis was performed (Fig. 3C). All chelators slightly decreased GR protein expression and while 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, since 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 anti-oxidant 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 as 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

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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). Similarly, 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). Interestingly, 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 17-30% (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 kDa and could represent oligomers or complexes with other proteins (Lu et al., 2007). To confirm these bands were disulfide-bridged oxidized Trx, we incubated the lysate with the reducing agent, dithiothreitol (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)

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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 (ATM; (Arner, 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 employed cell-free studies to elucidate if chelators were direct inhibitors of TrxR. However, DFO, Dp44mT or Bp44mT did not significantly inhibit TrxR activity in the cell-free system (Supplemental Fig. S2A-C). Interestingly, $FeCl_3$ 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. Collectively, these results suggested that these chelators were not direct TrxR inhibitors.

Thiosemicarbazones reduce glutaredoxin activity in cells

Glutaredoxin (Grx) (Holmgren, 1989) uses GSH for supplying reducing equivalents to RR (Avval and Holmgren, 2009) and also catalyzes other thiol-disulfide interchange reactions and repairs glutathionylated proteins (Lillig et al., 2008). Due to 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). Similarly, Bp44mT ($p<0.01$) and Dp44mT ($p<0.05$) significantly 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

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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), since the reduction of Grx requires GSH (Holmgren, 1989), emphasizing the effect of thiosemicarbazones on modulating GSH and Grx thiols. The reduction in Grx expression was unlikely due to chelator-induced translational repression, as the expression of an iron-regulated protein, the transferrin receptor-1 (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, as compared to 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). Interestingly, 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 *vs* 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.

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Chelators inhibit ribonucleotide reductase activity in cells

DFO and some types of thiosemicarbazones are known to inhibit RR (Cooper et al., 1996) due to 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 (Avval and Holmgren, 2009), we examined the effect of these chelators on RR activity. EPR was employed as 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 due to the formation of a chelator-derived radical in cells, which confounded analysis. Hence, RR activity was determined using another BpT-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.

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DISCUSSION

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

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Interestingly, GSH levels are elevated in many tumor types and have been associated with resistance to chemo- and radio-therapy (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 anti-cancer 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

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activity. For example, this occurs with arsenic compounds where bio-methylation 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 due to the residual capacity of the enzyme (Eriksson et al., 2009). It is known that mammalian TrxR has broad specificity (Arner, 2009) and many electrophilic compounds can affect TrxR activity (Chew et al., 2008). Other anti-cancer 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 (Kalinowski et al., 2007; Richardson et al., 2006; Yuan et al., 2004). It should be considered that the effects of thiosemicarbazones on these thiol-containing molecules may be important in explaining their selectivity against neoplastic cells

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(Kalinowski et al., 2007; Richardson et al., 2006). Indeed, the latter are known to possess a different redox profile when compared to 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 0.1-0.3 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 (Richardson et al., 2006; Yuan et al., 2004), 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, longer incubations 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

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oxidized Trx to the same level as the well characterized inhibitor, ATO (Lu et al., 2007) (Fig. 4B). Furthermore, in studies examining TrxR activity, where 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), where the known inhibitor of this protein, ($CdCl_2$) (Chrestensen et al., 2000), elicited a similar effect as 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: **(1)** the ability of GSH supplementation using NAC which significantly inhibited their anti-proliferative activity over a 72 h incubation (Fig. 2A) and **(2)** 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 anti-tumor activity. Prior to 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 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 (Avval and Holmgren,

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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 anti-proliferative 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 (Kalinowski et al., 2007; Yuan et al., 2004), as described previously (Richardson et al., 2006).

Collectively, as shown in Fig. 6B, incubation of cells with thiosemicarbazones leads to redox-active metal complexes (Jansson et al., 2010a; Jansson et al., 2010b; Kalinowski et al., 2007; Richardson et al., 2006; Yuan et al., 2004) which induce: **(1)** a decreased GSH/GSSG ratio; **(2)** increased oxidation of Trx-$(SH)_2$ and **(3)** 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 inhibited and this probably dys-regulates multiple processes (Avval and Holmgren, 2009; Balendiran et al., 2004) (Fig. 6B). Finally, it should be noted that thiosemicarbazones such as Dp44mT have various mechanisms of anti-proliferative activity (*i.e.*, generation of cytotoxic radicals, up-regulation of the growth and metastasis suppressor NDRG1 *etc*; (Whitnall et al., 2006; Yuan et al., 2004)). 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

Y.Y. designed studies, performed experiments and wrote the manuscript; Y.S.R. designed studies and wrote the manuscript; C.L.H designed study and performed experiment; and D.R.R. designed studies, obtained grant funding and wrote the manuscript.

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FOOTNOTES

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FIGURE LEGENDS

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

Fig. 2. The effect of modulating glutathione (GSH) on anti-proliferative activity and the effect of chelators on the GSH/oxidized glutathione (GSSG) ratio and GSH levels. The effect of the anti-oxidant *N*-acetylcysteine (NAC; 5 mM) on the anti-proliferative activity of **(A)** thiosemicarbazones (Dp44mT and Bp44mT) and **(B)** PKIH and DFO, determined using the MTT assay in DMS-53 lung cancer cells over 72 h/37^{o}C. **(C)** The effect of buthionine sulfoximine (BSO; 100 μM) on the anti-proliferative activity of thiosemicarbazones (Dp44mT and Bp44mT) using the procedure in Fig. 2A-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 mean±SEM (3-5 experiments). *p<0.05; **p<0.01; ***p<0.001

Fig. 3. The effect of chelators on glutathione reductase (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 anti-mouse GR antibody (1:1000). Results are mean±SEM (3-5 experiments). *p<0.05; **p<0.01

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Fig. 4. The effect of chelators on the thioredoxin (Trx) system. **(A)** The Trx and thioredoxin reductase (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 a SDS-PAGE gel in the **(B)** absence or **(C)** presence of DTT (100 mM). The membranes were probed using anti-mouse Trx antibody (1:1000). Arsenic trioxide (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 end-point insulin assay. Sodium aurothiomalate (ATM) was included as a positive control. Results are mean±SEM (3-4 experiments). *p<0.05; **p<0.01; ***p<0.001

Fig. 5. The effect of chelators on glutaredoxin (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 transferrin receptor 1 (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 anti-mouse Grx1 and TfR1 (1:1000). **(C)** The cellular $NADP_{total}$ and **(D)** $NADPH/NADP_{total}$ ratio after a 24 h/37°C incubation of DFO, Dp44mT or Bp44mT with DMS-53 lung cancer cells. Results are mean±SEM (4 experiments). *p<0.05; **p<0.01; ***p<0.001

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Fig. 6. The effect of chelators on ribonucleotide reductase (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 334-336 mT as shown in the table and expressed as percentage of control in the graph. Results are mean±SEM (3-4 experiments). *$p<0.05$; ***$p<0.001$. **(B)** Schematic summary of the effects of thiosemicarbazones on thiol systems: glutathione (GSH), thioredoxin (Trx) and glutaredoxin (Grx). Thiosemicarbazone chelators enter cells and bind iron or copper (Jansson et al., 2010b; Yuan et al., 2004), forming redox-active complexes (Jansson et al., 2010a; Jansson et al., 2010b; Kalinowski et al., 2007; Richardson et al., 2006; Yuan et al., 2004) 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, anti-oxidant/xenobiotic protection, apoptosis and redox-mediated signaling pathways.

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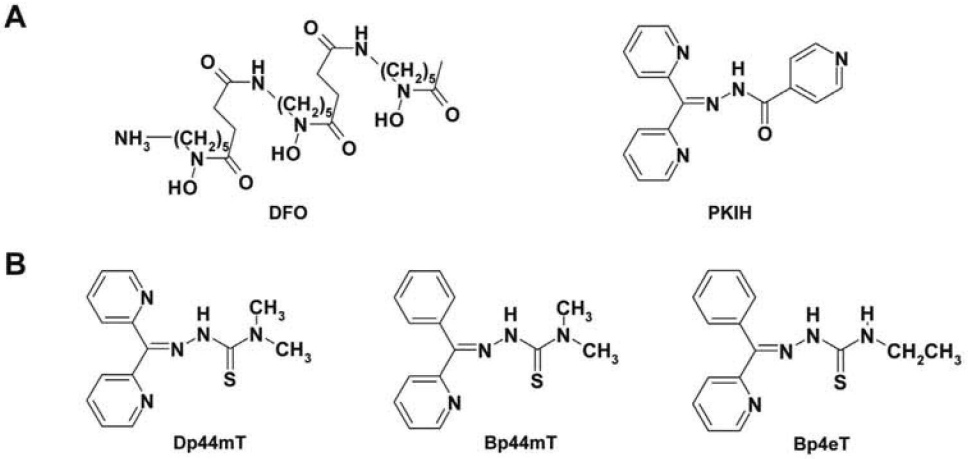

Figure 1

Molecular Pharmacology Fast Forward. Published on March 9, 2011 as DOI: 10.1124/mol.111.071324
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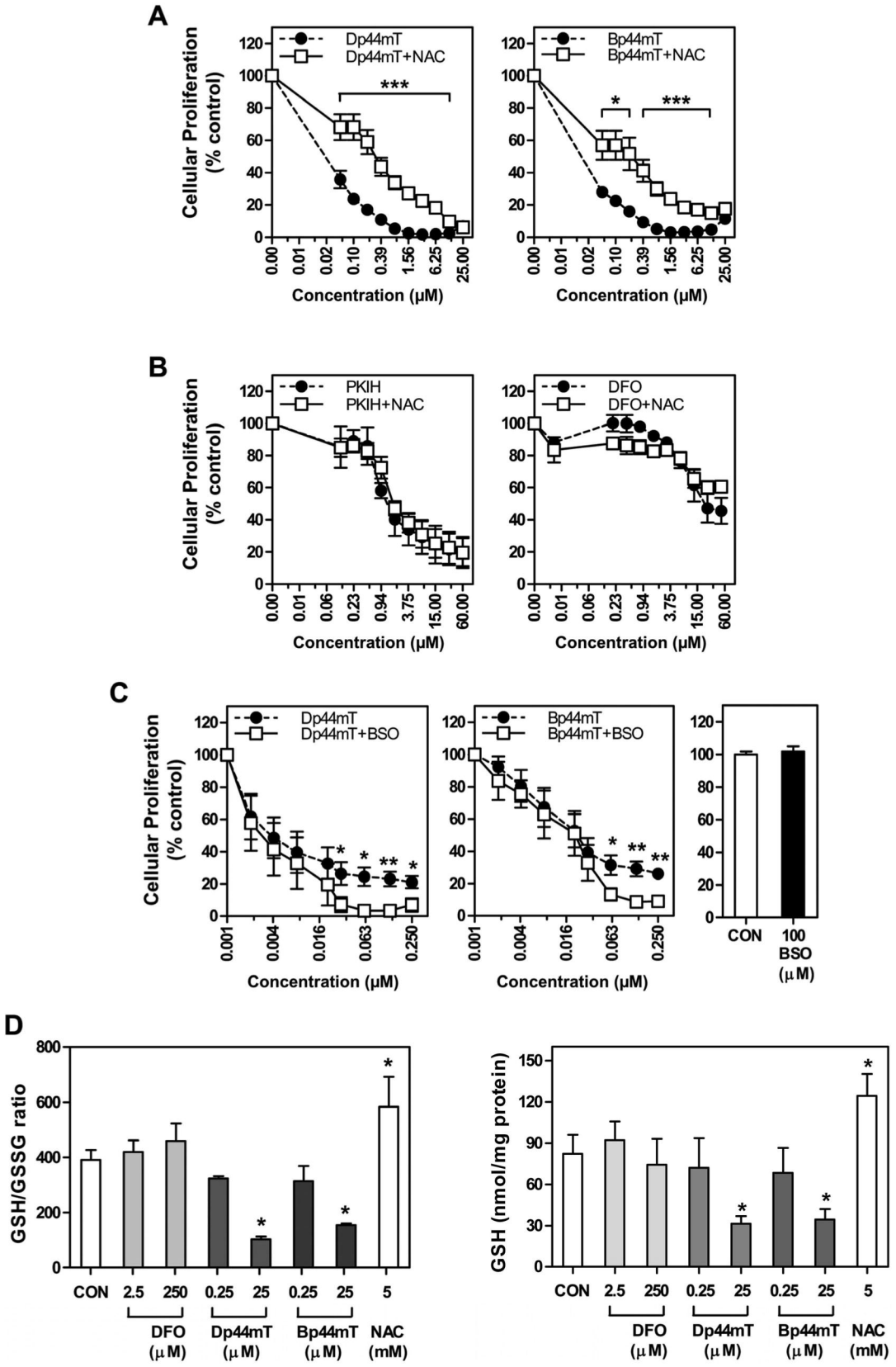

Figure 2

Molecular Pharmacology Fast Forward. Published on March 9, 2011 as DOI: 10.1124/mol.111.071324
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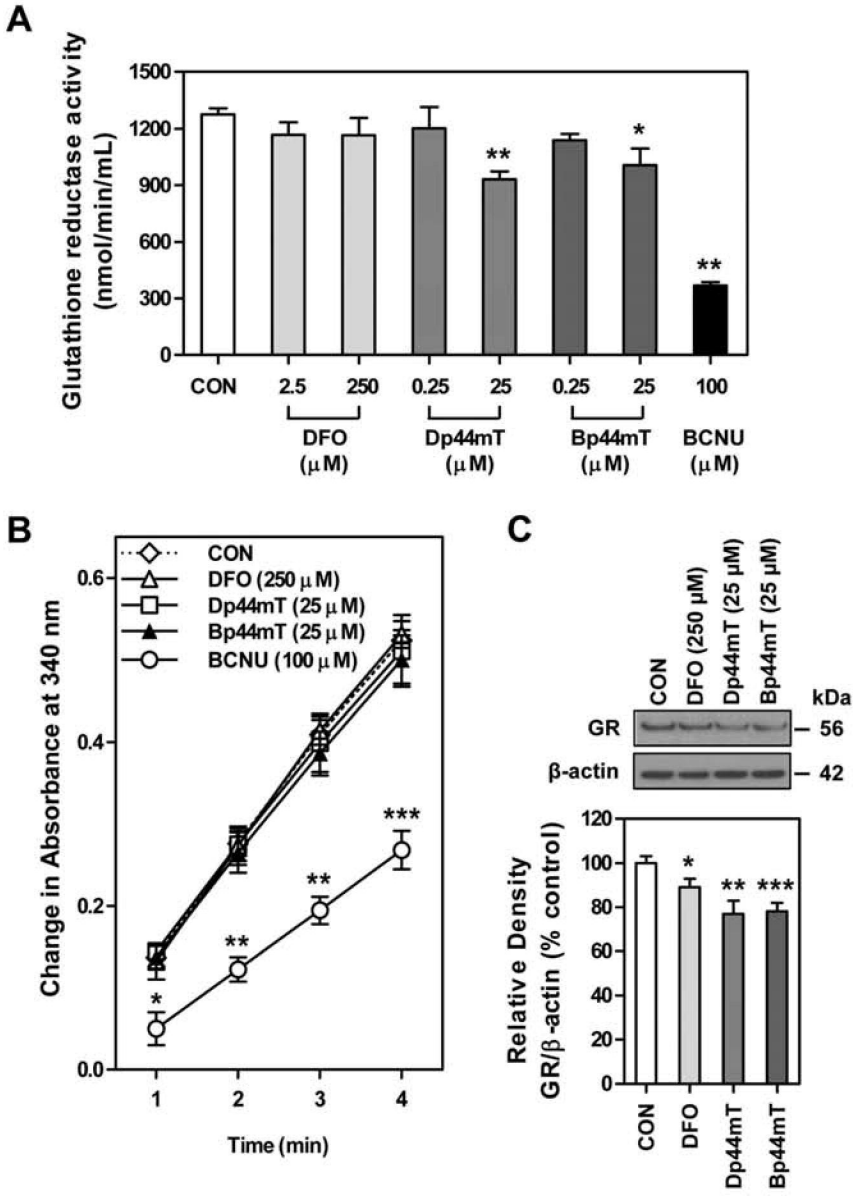

Figure 3

Molecular Pharmacology Fast Forward. Published on March 9, 2011 as DOI: 10.1124/mol.111.071324
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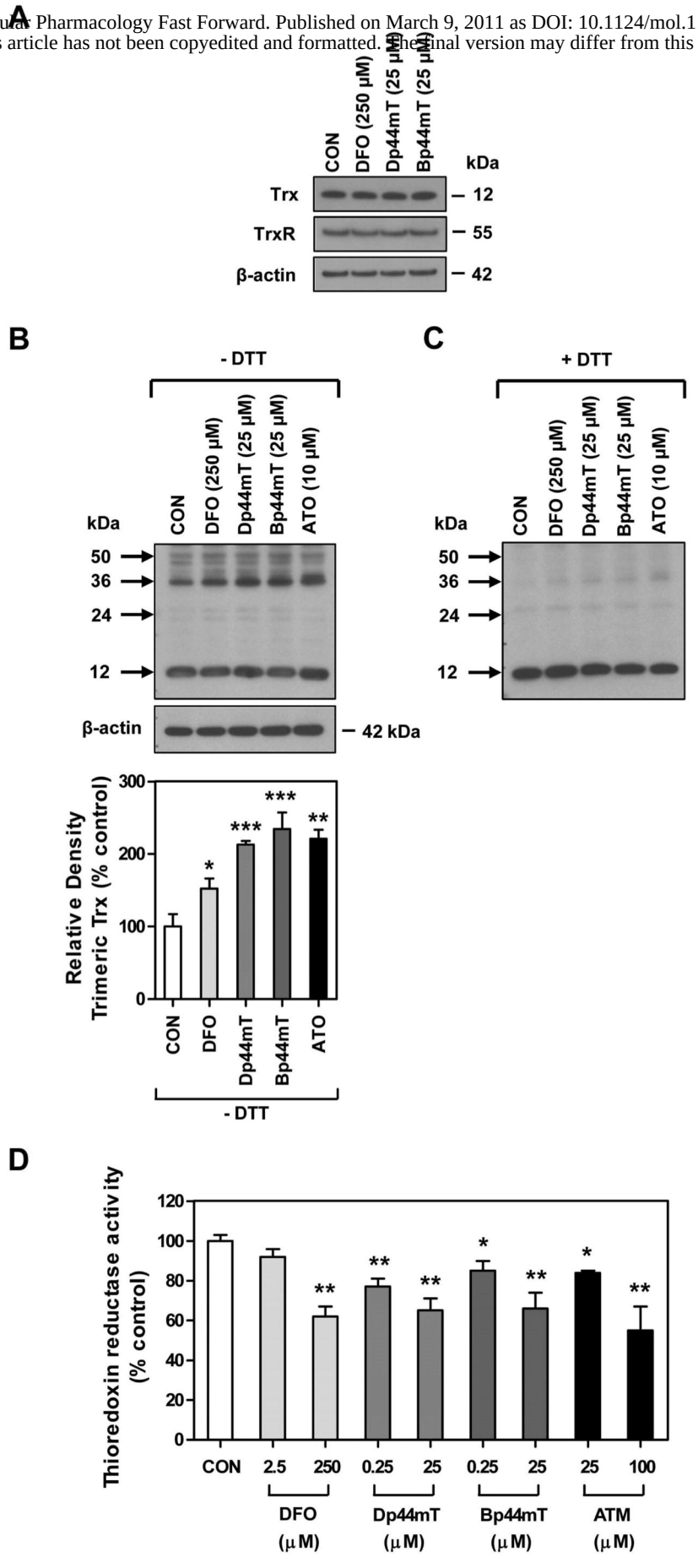

Figure 4

Molecular Pharmacology Fast Forward. Published on March 9, 2011 as DOI: 10.1124/mol.111.071324
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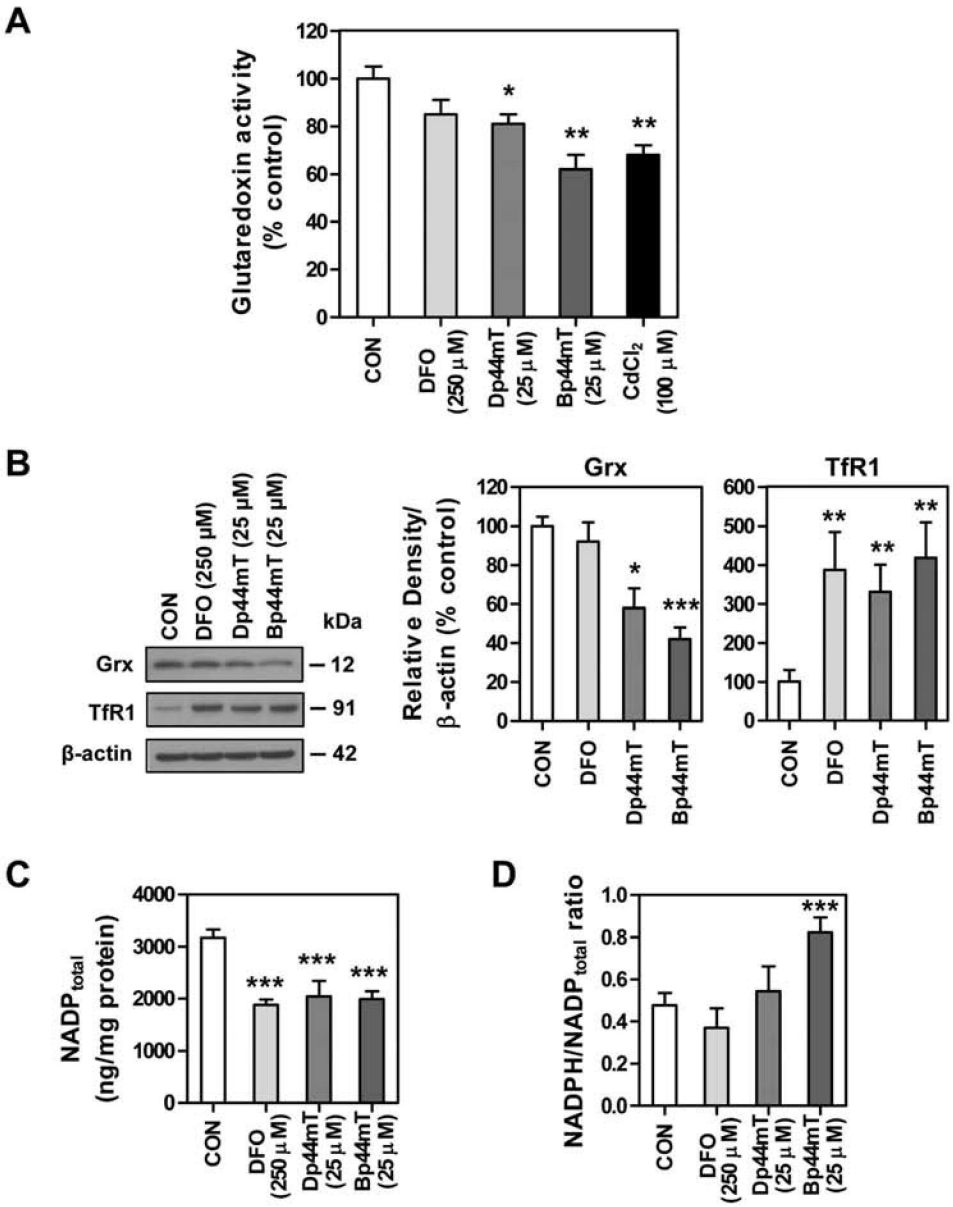

Figure 5

Molecular Pharmacology Fast Forward. Published on March 9, 2011 as DOI: 10.1124/mol.111.071324
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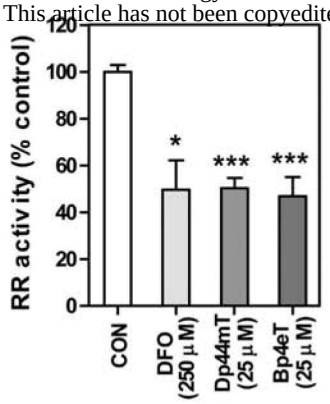
A



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

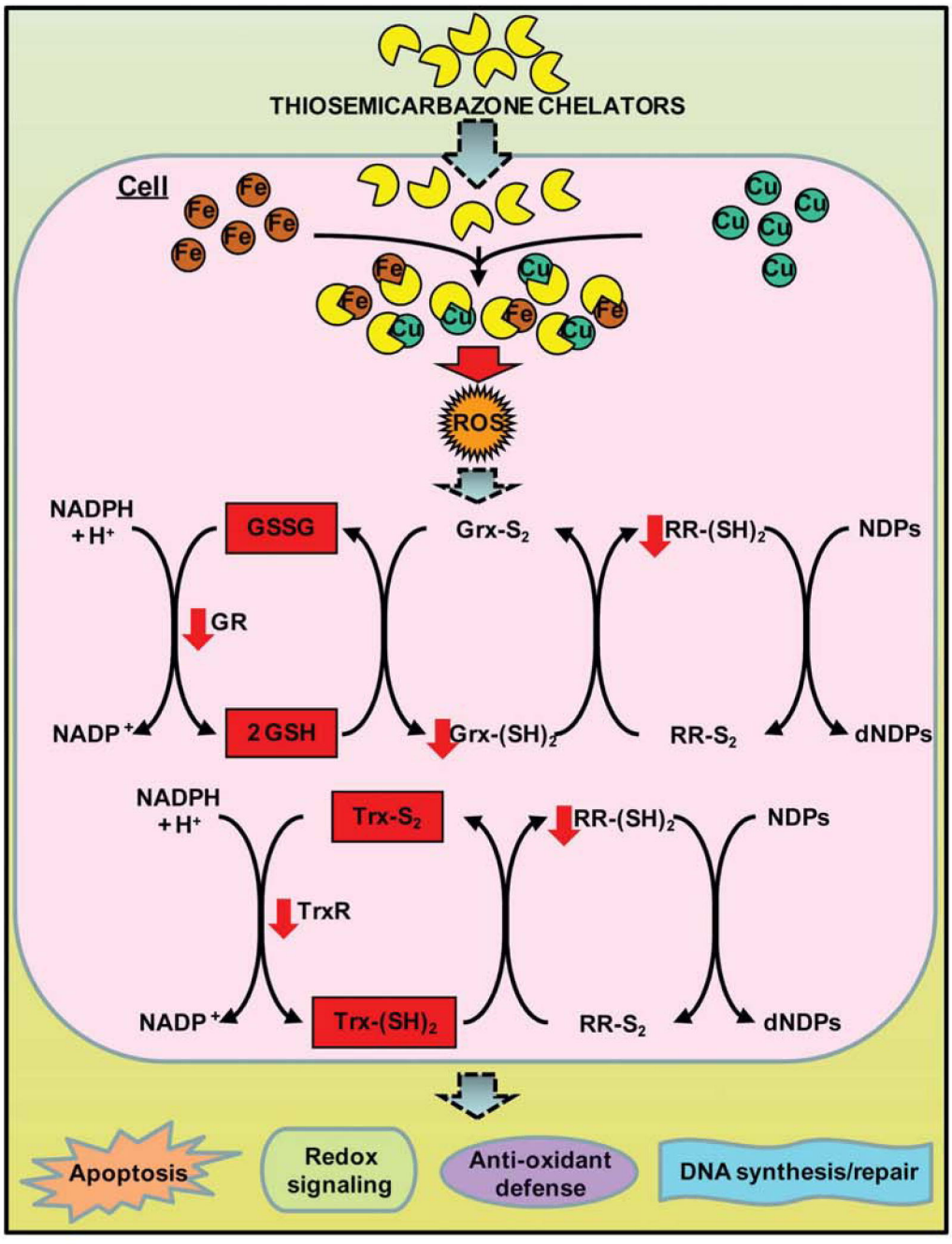


Figure 6