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Methemoglobin Formation by Triapine, Di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT), and Other Anticancer Thiosemicarbazones: Identification of Novel Thiosemicarbazones and Therapeutics That Prevent This Effect[S]

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ABSTRACT

Thiosemicarbazones are a group of compounds that have received comprehensive investigation as anticancer agents. The antitumor activity of the thiosemicarbazone, 3-amino-2-pyridinecarboxaldehyde thiosemicarbazone (3-AP; triapine), has been extensively assessed in more than 20 phase I and II clinical trials. These studies have demonstrated that 3-AP induces methemoglobin (metHb) formation and hypoxia in patients, limiting its usefulness. Considering this problem, we assessed the mechanism of metHb formation by 3-AP compared with that of more recently developed thiosemicarbazones, including di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT). This was investigated using intact red blood cells (RBCs), RBC lysates, purified oxyhemoglobin, and a mouse model. The chelation of cellular labile iron with the formation of a redox-active thiosemicarbazone-iron complex was found to be crucial for oxyhemoglobin oxidation. This observation was substantiated using a thiosemicarbazone that cannot ligate iron and also by using the chelator, desferrioxamine, that forms a redox-inactive iron complex. Of significance, cellular copper chelation was not important for metHb generation in contrast to its role in preventing tumor cell proliferation. Administration of Dp44mT to mice catalyzed metHb and cardiac metmyoglobin formation. However, ascorbic acid administered together with the drug in vivo significantly decreased metHb levels, providing a potential therapeutic intervention. Moreover, we demonstrated that the structure of the thiosemicarbazone is of importance in terms of metHb generation, because the DpT analog, di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC), does not induce metHb generation in vivo. Hence, DpC represents a next-generation thiosemicarbazone that possesses markedly superior properties. This investigation is important for developing more effective thiosemicarbazone treatment regimens.

Introduction

The antitumor agent, 3-amino-2-pyridinecarboxaldehyde thiosemicarbazone (3-AP; triapine) (Fig. 1A), has been examined in more than 20 phase I and II clinical trials for cancer treatment (Murren et al., 2003; Wadler et al., 2004; Gojo et al., 2007; Knox et al., 2007; Kunos et al., 2010). In general, studies using 3-AP report limited antitumor activity or no response (Murren et al., 2003; Wadler et al., 2004; Gojo et al., 2007; Knox et al., 2007; Kunos et al., 2010). However, other

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ABBREVIATIONS: 3-AP, 3-amino-2-pyridinecarboxaldehyde thiosemicarbazone (triapine); DpT, di-2-pyridylketone thiosemicarbazone; BpT, 2-benzoylpyridine thiosemicarbazone; oxyHb, oxyhemoglobin; metHb, methemoglobin; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; Bp4eT, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone; metMb, metmyoglobin; AA, ascorbic acid; DpC, di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone hydrochloride; Dp4e4mT, di-2-pyridylketone-4-ethyl-4-methyl-3-thiosemicarbazone; Dp4pT, di-2-pyridylketone-4-phenyl-3-thiosemicarbazone; Dp2mT, di-2-pyridylketone-2-methyl-3-thiosemicarbazone; DFO, desferrioxamine; RBC, red blood cells; HBSS, Hanks' balanced salt solution; oxyMb, oxymyoglobin.

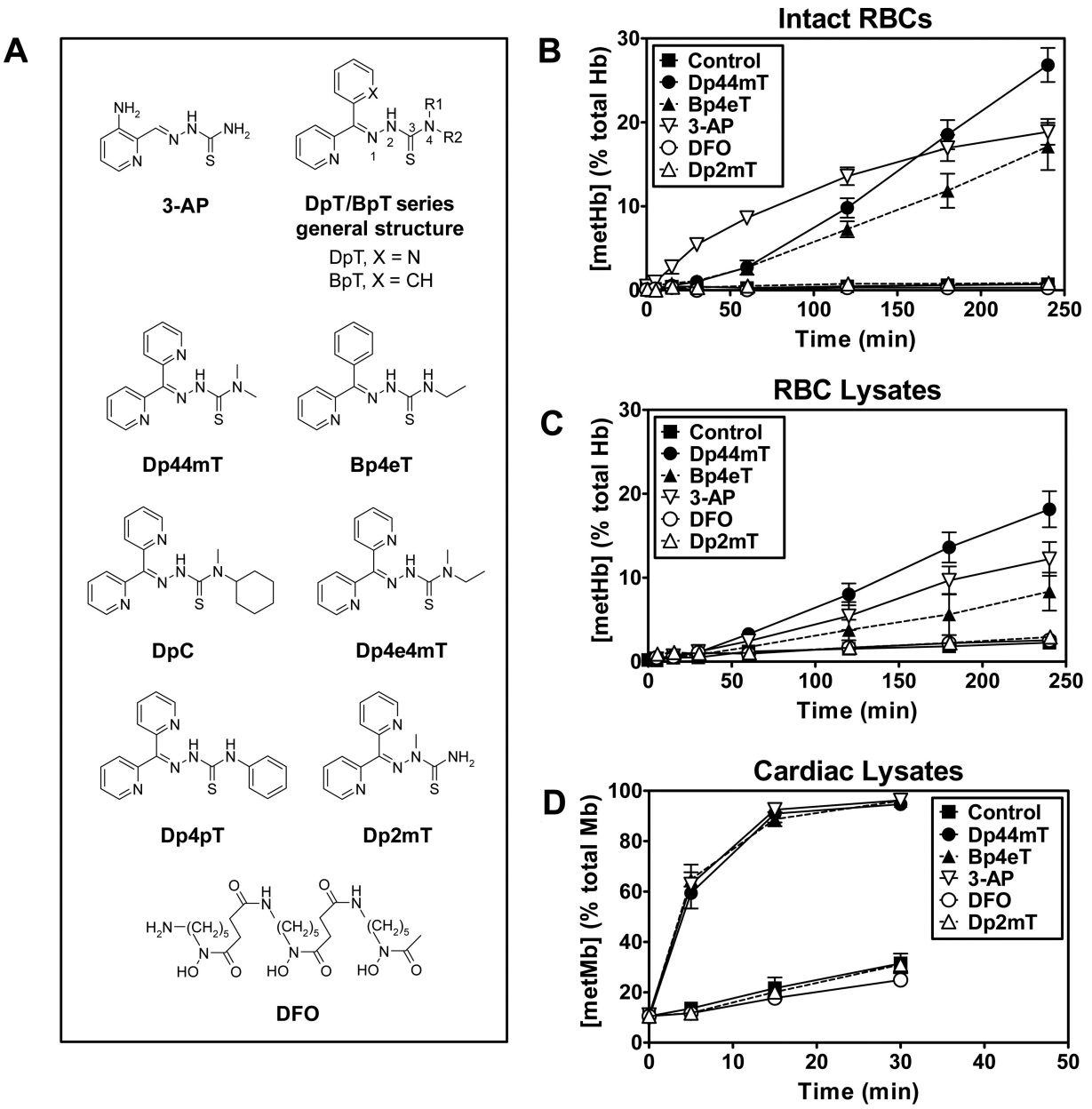

Fig. 1. metHb and metMb formation by thiosemicarbazone chelators. A, chemical structures of the agents assessed. Intact RBCs (B) and RBC lysates (C), respectively, were incubated with Dp44mT, Bp4eT, 3-AP, DFO, and Dp2mT (25 μM) for up to 4 h/37°C. D, mouse heart lysates were incubated similarly with the agents (25 μM at 37°C) and monitored for metMb formation. Results are means ± S.D. (three experiments). Mb, myoglobin.

studies suggest some positive results after 3-AP was coadministered with cisplatin and daily pelvic radiation in patients with locally advanced cervical and vaginal cancer (Kunos et al., 2010). The notable side effects of 3-AP include hypoxia and methemoglobinemia (Yen et al., 2004; Gojo et al., 2007; Knox et al., 2007; Odenike et al., 2008; Traynor et al., 2010), which are major concerns for patients with compromised cardiopulmonary function.

The di-2-pyridylketone thiosemicarbazone (DpT) series (Richardson et al., 2006) and the 2-benzoylpyridine thiosemicarbazone (BpT) group (Kalinowski et al., 2007) of tridentate ligands (Fig. 1A) have been developed and show potent and selective antitumor and antimetastatic activity, being far more active than 3-AP (Richardson et al., 2006; Whitnall et al., 2006; Kalinowski et al., 2007; Rao et al., 2009, 2011; Liu et al., 2012). The DpT-iron and -copper complexes are redox-active and exert their antiproliferative effects via sequestration of intracellular iron and copper and generation of deleterious reactive oxygen species (Chaston et al., 2003; Richardson et al., 2006; Whitnall et al., 2006; Kalinowski et al., 2007; Lovejoy et al., 2011). Like 3-AP, which also forms a redox-active iron complex (Chaston et al., 2003), it is possible the DpT- and BpT-iron and -copper complexes may induce oxidation of oxyHb [iron(II)] to metHb [iron(III)] that could limit their clinical utility.

In this study, we demonstrate that the iron complexes of 3-AP and the lead DpT and BpT chelators, di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT) and 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone (Bp4eT) (Yuan et al., 2004; Whitnall et al., 2006; Kalinowski et al., 2007), induce metHb and metmyoglobin (metMb) formation in vitro and in vivo. We also show that ascorbic acid (AA) can prevent this effect. Of

importance, the recently synthesized DpT analog, di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) (Fig. 1A), which shows greater activity than Dp44mT against pancreatic cancer xenografts (Kovacevic et al., 2011), does not induce significant metHb generation in vivo. Taken together, these findings have important implications for the development of thiosemicarbazones as antitumor agents.

Materials and Methods

Chemicals. 3-AP was synthesized by standard methods (Liu et al., 1992). Dp44mT, Bp4eT, DpC, di-2-pyridylketone-4-ethyl-4-methyl-3-thiosemicarbazone (Dp4e4mT), di-2-pyridylketone-4-phenyl-3-thiosemicarbazone (Dp4pT), and di-2-pyridylketone-2-methyl-3-thiosemicarbazone (Dp2mT) were synthesized and characterized, as described previously (Richardson et al., 2006; Kalinowski et al., 2007; Kovacevic et al., 2011). The iron complexes $[Fe^{III}(Bp4eT)_2](ClO_4)$, $[Fe^{II}(Dp44mT)_2]$, $[Fe^{III}(DpC)_2]ClO_4$, and $[Fe^{III}(Dp44mT)_2](ClO_4)\cdot 1\frac{1}{2}H_2O$ were prepared by established procedures (Richardson et al., 2006; Kalinowski et al., 2007). Desferrioxamine (DFO) was from Novartis (Basel, Switzerland). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

Hemoglobin Preparation. Blood samples were collected from healthy human donors in Vacutainer collection tubes (BD, Plymouth, UK) and used immediately. Red blood cells (RBCs) were isolated by centrifugation (480*g* for 5 min at 4°C) and then were washed in Hanks' balanced salt solution (HBSS). RBCs were resuspended 1:1 in HBSS, and assays using intact RBCs (15% hematocrit) were performed for up to 4 h at 37°C. To prepare RBC lysates, RBCs were lysed with ultra-pure water, the debris was removed by centrifugation (16,000*g* for 30 min at 4°C), and the supernatant was used ([oxyHb] = 1.5 mM; from a 15% hematocrit content). Commercially purified human oxyHb A_0 (97–100% purity; Sigma-Aldrich, St. Louis, MO) was dissolved in HBSS and used at 1.5 mM.

Myoglobin Preparation. Mouse heart tissue was exhaustively perfused with ice-cold HBSS to remove blood and homogenized in ice-cold HBSS containing protease inhibitor cocktail (Roche, Basel, Switzerland). Heart homogenates were centrifuged (16,000*g* for 45 min at 4°C), and the supernatant ([oxyMb] = 50 μM) was used immediately.

Assay Additives. Chelators were freshly prepared in DMSO (final [DMSO] <0.5%). Chelator-iron complexes were prepared by combining the ligand with $FeCl_3\cdot 6H_2O$ in DMSO in a 2:1 ratio, the most stable form of the complex (Richardson et al., 2006). Chelator-copper complexes were formed by combining the ligand with $CuCl_2\cdot 2H_2O$ in DMSO in a 1:1 ratio (Jansson et al., 2010). The AA was freshly prepared as a 20× stock in HBSS.

RBC Lysate Preincubation with Chelex-100 Resin or DFO. RBC lysates and Chelex-100 resin (0.05 g/ml) (Raymond and Weinshilboum, 1975) or DFO (25 μM) were mixed by slow rotation (1 h at 4°C). Chelex-100 resin samples were centrifuged (7000*g* for 10 min at 4°C), and the supernatant was used immediately.

Spectral Analysis of MetHb and MetMb. Spectra (250–700 nm) of RBC lysates were obtained using a Shimadzu UV-visible spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). Concentrations of oxyHb and metHb were determined at 577 and 630 nm (Winterbourn and Carrell, 1977).

Kinetic and Redox Potential Measurements. Kinetic measurements of the reaction of iron complexes with commercially purified oxyHb A_0 (~2 μM) were undertaken in HBSS at 37°C. The iron complexes were used with at least a 5-fold molar excess of oxyHb. Spectra were acquired on a PerkinElmer Lambda 40 spectrophotometer (PerkinElmer Life and Analytical Sciences, Waltham, MA), and the data were analyzed globally with SPECFIT (R. A. Binstead, SPECFIT Global Analysis System; Spectrum Software Associates, Marlborough, MA). Cyclic voltammetry (100 mV/s sweep rate) was used to measure redox potentials of the thiosemicarbazone-iron complexes implementing a BAS100B/W potentiostat (Jansson et al., 2010).

MetHb and MetMb Formation in Mice. C57BL/6 mice (7–8 weeks old) were used under a protocol approved by the University of Sydney Animal Ethics Committee. Dp44mT or DpC (6 mg/kg) alone or in combination with AA (15 or 30 mg/kg) was dissolved in 30% propylene glycol-saline (Whitnall et al., 2006) and administered intravenously via the tail vein. Then, 30 to 240 min after administration, mice were anesthetized with isoflurane, and blood was obtained by cardiac puncture. RBCs were lysed with 2.5 volumes of ultrapure water for metHb estimation. Mice were sacrificed with isoflurane and spinal dislocation, the heart was exhaustively perfused with HBSS, and myoglobin was isolated.

Statistics. Data were compared using Student's *t* test. Results were considered significant when $p < 0.05$. Results are presented as means ± S.D.

Results

Dp44mT, Bp4eT, and 3-AP Induce MetHb Formation in Human RBCs and Their Lysates. The activity of 3-AP, Dp44mT, and Bp4eT on metHb formation was compared with that of the chelator, DFO, which forms an iron(III) complex that is not redox-active (Kalinowski and Richardson, 2005). The thiosemicarbazone, Dp2mT (Fig. 1A), which cannot bind iron was used as a negative control for Dp44mT (Yuan et al., 2004). The agents (25 μM) were incubated for up to 4 h at 37°C with intact RBCs, leading to metHb increasing as a function of time for 3-AP, Dp44mT, and Bp4eT, which form redox-active iron complexes (Fig. 1B) (Chaston et al., 2003; Yuan et al., 2004; Kalinowski et al., 2007). 3-AP induced a significant ($p < 0.001$) 6-fold increase in metHb relative to that in the control after 15 min, whereas Dp44mT and Bp4eT showed a significant ($p < 0.001$) 4-fold increase relative to that in the control only after 60 min at 37°C. After 240 min, Dp44mT was significantly ($p < 0.001$) more effective than Bp4eT or 3-AP in inducing metHb formation with 27 ± 2, 17 ± 3, and 19 ± 2% being generated, respectively, relative to the control value (1 ± 1% metHb) (Fig. 1B). Despite their similar structures (Fig. 1A), Dp44mT was significantly ($p < 0.05$) more effective than Bp4eT in increasing metHb generation at all time points after 120 min (Fig. 1B).

In contrast to the thiosemicarbazones, DFO had no significant effect on metHb formation, demonstrating that iron chelation alone and the formation of a non–redox-active iron complex does not potentiate metHb generation (Fig. 1B). The thiosemicarbazone negative control, Dp2mT, which cannot bind iron (Yuan et al., 2004), had no significant influence on metHb generation, demonstrating that metal binding by Dp44mT was important for oxidation. Studies using intact RBCs showed a significant ($p < 0.001$) dose-dependent (1–25 μM) increase in metHb at all concentrations of Dp44mT, Bp4eT, or 3-AP after 3 h at 37°C (Supplemental Fig. 1). In contrast, no significant increase in metHb was detected at any Dp2mT or DFO concentration examined (Supplemental Fig. 1).

To assess the effect of chelator membrane permeability, the same experiment was performed on RBC lysates (Fig. 1C). Similar to the results for intact RBCs (Fig. 1B), Dp44mT was the most effective ligand for inducing metHb generation, with DFO and Dp2mT showing no significant activity relative to the control.

It was of interest to examine the effect of these ligands on

oxyMb oxidation (George and Stratmann, 1952; Eguchi and Saltman, 1984; Hegetschweiler et al., 1987), because this could be detrimental in muscles including the heart, in which oxyMb plays an important role in oxygen storage/transport (Gunther et al., 1999). In contrast to RBC lysates in which little oxyHb was auto-oxidized in the control samples (Fig. 1C), oxyMb auto-oxidized much faster (Fig. 1D), as reported previously (Mauk and Gray, 1979; Eguchi and Saltman, 1987). However, 3-AP, Dp44mT, and Bp4eT (25 μM) were markedly more effective than was observed for the control, with the three agents inducing metMb formation in mouse cardiac lysates at a similar rate, leading to 96 ± 1% of metMb formation after incubation for 30 min at 37°C (Fig. 1D). In contrast to oxyHb, for which differences between thiosemicarbazones were noted (Fig. 1, B and C), there was no significant difference between the three agents and their effects on oxyMb (Fig. 1D). This finding indicates that protein structure is of importance with regard to the variation in oxidation between oxyHb and oxyMb. Faster oxidation of oxyMb relative to that of oxyHb has been observed with other iron complexes (Mauk and Gray, 1979; Eguchi and Saltman, 1987) and is a consequence of the different redox potentials and shorter separation distance between the iron complex and oxyMb for electron transfer (Eguchi and Saltman, 1987). As observed with oxyHb (Fig. 1, B and C), DFO and Dp2mT had no significant effect on metMb generation (Fig. 1D).

Importance of Labile Cellular Iron in MetHb Formation. Considering that thiosemicarbazones chelate cellular iron and copper (Chaston et al., 2003; Richardson et al., 2006; Whitnall et al., 2006; Kalinowski et al., 2007; Lovejoy et al., 2011), we examined whether depletion of metal ions from RBC lysates preincubated with the chelating resin, Chelex-100 (Raymond and Weinshilboum, 1975), prevented metHb generation when subsequently incubated for 3 h at 37°C with the most effective ligand, Dp44mT (25 μM) (Fig. 2A). Chelex-100 treatment of RBC lysates led to a significant ($p < 0.001$) 5-fold reduction in metHb generation relative to that when Dp44mT was added to the untreated RBC lysate, viz., 10 ± 1 to 2 ± 1%. Of importance, metHb generation by Dp44mT in Chelex-100-treated samples was not significantly different ($p > 0.05$) from that of Chelex-100-treated control samples (Fig. 2A). To demonstrate that this effect was due to Chelex-100-depleting metals, $FeCl_3$ or $CuCl_2$ (12.5 and 25 μM, respectively) was added to the Chelex-100-treated and -untreated lysates, and the effect of Dp44mT (25 μM) was assessed. The stoichiometries of ligand to metal used reflect the coordination modes of these ligands with iron or copper, namely 2:1 or 1:1, respectively (Bernhardt et al., 2009; Jansson et al., 2010). The addition of $FeCl_3$ to the untreated or Chelex-100-treated lysates led to a significant ($p < 0.001$) 4- to 10-fold increase in metHb generation upon incubation with Dp44mT relative to that with Dp44mT alone (Fig. 2A). It is noteworthy that the addition of $CuCl_2$ to Chelex-100-treated and nontreated lysates did not significantly stimulate metHb generation by Dp44mT relative to that with Dp44mT alone (Fig. 2A). Studies using RBC lysates preincubated with the iron chelator, DFO (25 μM), gave results similar to those of lysates treated with Chelex-100, significantly ($p < 0.01$) decreasing metHb generation (Fig. 2B). Moreover, differences in activity between the ligand and metal-ligand complexes were also observed, which indicated that iron complexes of Dp44mT, Bp44mT, and 3-AP were more effective than the ligands or their copper complexes in oxidizing oxyHb in both RBCs and RBC lysates (Fig. 2, C and D). Taken together, these results indicated that iron, rather than copper, was essential for metHb formation by Dp44mT through formation of its redox-active iron complex (Yuan et al., 2004; Richardson et al., 2006).

The possibility of a direct interaction of thiosemicarbazones and their iron complexes with oxyHb to form metHb was examined using commercially purified oxyHb. In these studies, Dp44mT, Bp4eT, and 3-AP (25 μM) induced high levels of metHb after 3 h at 37°C, similar to those of the iron(III) complexes (Fig. 3A), indicating chelatable "free" iron

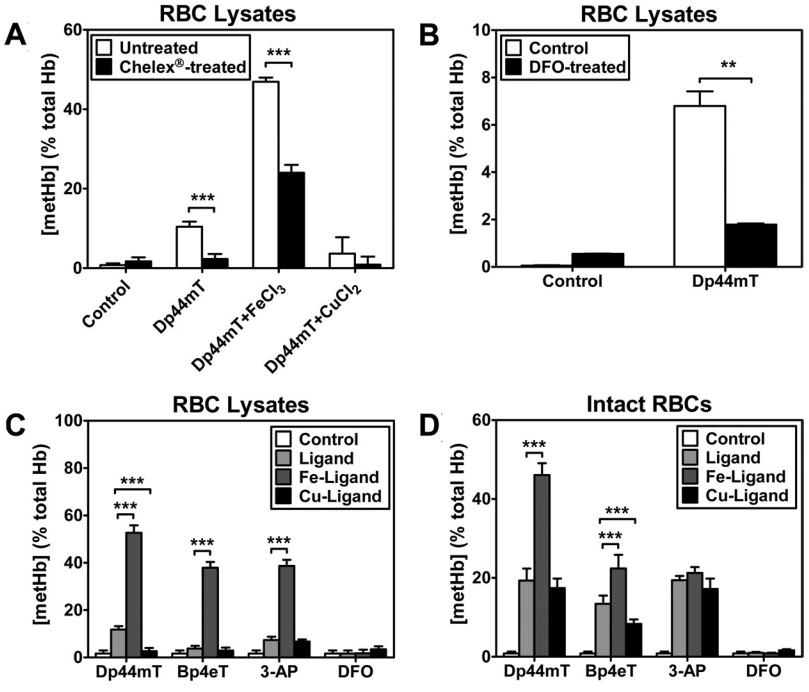

Fig. 2. Evaluation of the role of iron in oxyHb oxidation. "Free" metal ions from RBC lysates were chelated using (A) Chelex-100 resin (0.05 g/ml) or (B) DFO (25 μM). The Chelex-100 resin-treated lysates were then incubated with either Dp44mT (25 μM), Dp44mT (25 μM), and $FeCl_3$ (12.5 μM) or Dp44mT (25 μM) and $CuCl_2$ (25 μM) at 37°C for 3 h. The DFO-treated and untreated lysates were incubated with Dp44mT (25 μM; 37°C for 3 h). Intact RBCs (C) or RBC lysates (D) were incubated with control medium, Dp44mT, Bp4eT, 3-AP, DFO, Dp2mT, or their iron (Fe-Ligand) or copper (Cu-Ligand) complexes (all at 25 μM) for 3 h at 37°C. MetHb was detected spectrophotometrically. Values are means ± S.D. (three experiments). ***, $p < 0.001$; **, $p < 0.01$.

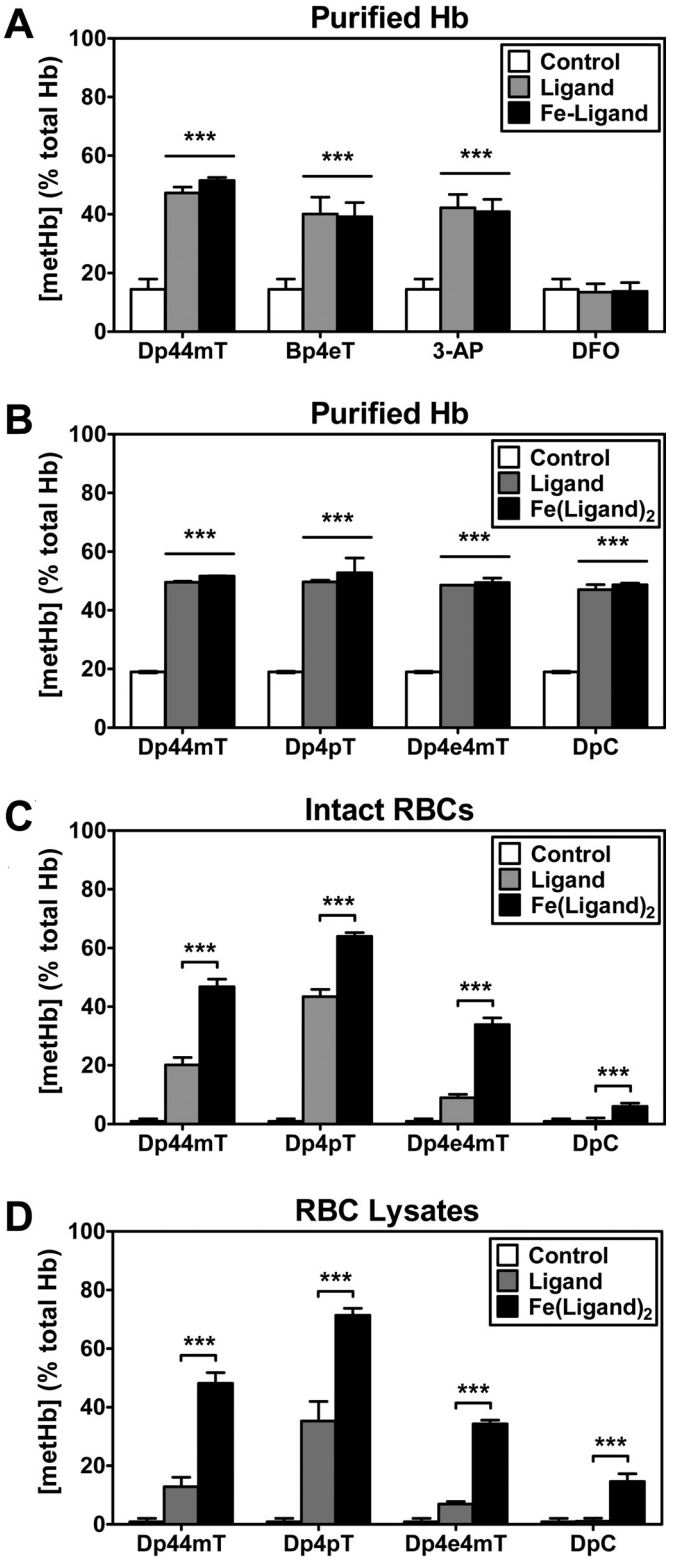

Fig. 3. Investigations of the mechanism of metHb formation with purified oxyHb. A, purified oxyHb was treated with Dp44mT, Bp4eT, 3-AP, or DFO or their iron complexes (iron-ligand); all at 25 μM at 37°C for 3 h. MetHb formation in purified oxyHb (B), intact RBCs (C), or RBC lysates (D) incubated for 37°C for 3 h with Dp44mT, Dp4pT, Dp4e4mT, or DpC or their iron complexes (all at 25 μM). MetHb was detected spectrophotometrically. Results are means ± S.D. (three experiments). Significance levels are relative to the control (A and B) or represent the comparisons in C and D. ***, $p < 0.001$.

in the purified oxyHb. This was confirmed by experiments in which purified oxyHb was pretreated with DFO, which significantly ($p < 0.001$) reduced metHb generation by the thiosemicarbazones (Supplemental Fig. 2). In agreement with the studies using RBC lysates and intact RBCs, DFO or its iron(III) complex did not induce metHb after incubation with purified Hb (Fig. 3A). Thus, these thiosemicarbazone-iron(III) complexes oxidize purified oxyHb to metHb in the absence of other cellular components, suggesting a direct interaction between the complex and oxyHb.

Steric Effects of DpT Chelators and MetHb Formation. Although the size of the thiosemicarbazone-iron(III) complexes (Richardson et al., 2006; Kalinowski et al., 2007) precludes entry to the Hb oxygen-binding pocket (Mouawad et al., 2005), steric effects of the complexes may lead to different interactions with oxyHb and affect their activity. Considering this possibility, ligands of the DpT series with different sized substituents (Fig. 1A) were examined for their ability to induce metHb by incubating purified oxyHb with the chelators and their 2:1 iron(III) complexes at 37°C for 3 h. There was no significant difference in metHb formation between the more bulky (Dp4pT and DpC) and the less bulky (Dp44mT and Dp4e4mT) ligands examined (Fig. 3B). Hence, alterations in the substituents did not play a major role in influencing metHb generation when pure oxyHb was used.

Although no major differences in metHb formation were observed in the reaction between the DpT chelators/complexes and purified Hb (Fig. 3B), significant differences were found with use of intact RBCs (Fig. 3C) or RBC lysates (Fig. 3D). A significant finding was that DpC induced markedly less metHb in intact RBCs and RBC lysates and thus may overcome the side effect of methemoglobinemia caused by other thiosemicarbazones (Yen et al., 2004; Knox et al., 2007; Traynor et al., 2010).

Kinetics of the Reaction of Thiosemicarbazone-Iron Complexes with OxyHb. The $\alpha_2\beta_2$ oxyHb tetramer bears four heme groups; thus, theoretically, four thiosemicarbazone-iron(III) complexes are required to fully oxidize oxyHb to its metHb form. Kinetic studies demonstrated that each separate heme cofactor is oxidized sequentially in a second-order, outer-sphere, electron-transfer reaction with the thiosemicarbazone-iron(III) complexes. The four sequential oxidation reactions could not be elucidated because there was significant overlap between them. However, the second-order rate constants determined for the first oxidation step (k_1; pH 7.4; ionic strength 0.1 M) are presented in Table 1. These rates are similar, although $[Fe(Dp44mT)_2]^+$ seems to be the most reactive oxidant of the four complexes examined. An example of the spectral changes with time is shown in Fig. 4A for a reaction between oxyHb (5.3 μM) and $[Fe(Bp4eT)_2]^+$ (50 μM, pH 7.4). Attenuation of the peaks at 535 and 576 nm

TABLE 1
Second-order rate constants determined for the first oxidation step of oxyHb with various ferric thiosemicarbazone complexes
Results are mean ± S.D. (three experiments).

Complex	Second-Order Rate Constant (k_1)
	$M^{-1} \cdot s^{-1}$
$[Fe(Dp44mT)_2]^+$	$3.1 \pm 0.1 \times 10^2$
$[Fe(Bp4eT)_2]^+$	$8.6 \pm 0.1 \times 10^1$
$[Fe(DpC)_2]^+$	$6.1 \pm 0.1 \times 10^1$
$[Fe(3\text{-}AP)_2]^+$	$1.3 \pm 0.1 \times 10^2$

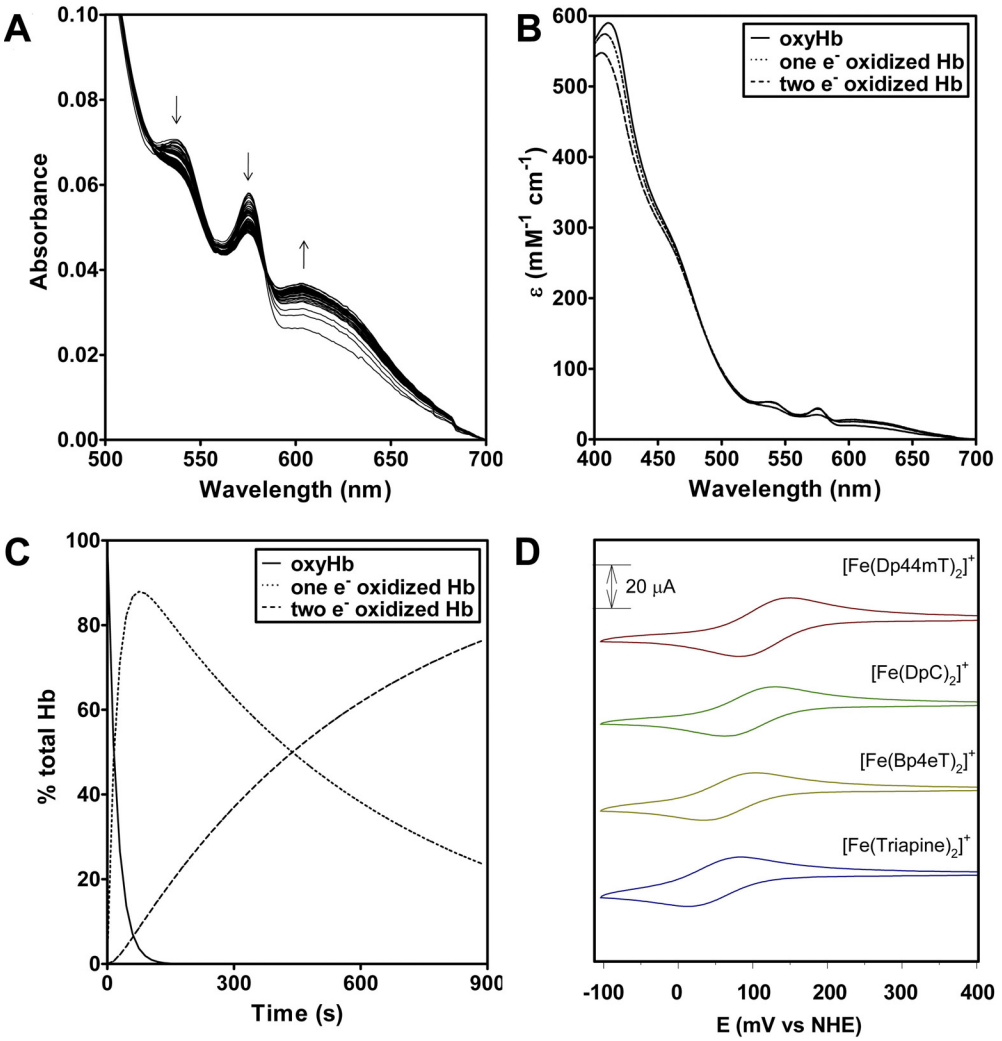

Fig. 4. Interaction of oxyHb with the Dp44mT-iron complex. A, visible spectral changes of oxyHb (5.3 μM, pH 7.4) after reaction with $[Fe(Bp4eT)_2]^+$ (50 μM). Spectra were taken every 15 s for 360 s. B, global analysis (SPECFIT) of the time-dependent changes in the spectrum of oxyHb (5.3 μM) after reaction with $[Fe(Bp4eT)_2]^+$ (50 μM) at pH 7.4. Spectra calculated for oxyHb and its single and two electron-oxidized forms are shown. C, speciation diagram as a function of time showing the rapid initial oxidation (within 3 min) of oxyHb and slower second electron oxidation. D, cyclic voltammograms of the four complexes: $[Fe(Dp44mT)_2]^+$, $[Fe(DpC)_2]^+$, $[Fe(Bp4eT)_2]^+$, and $[Fe(3\text{-}AP)_2]^+$ (all at 1 mM). Results in A, B, C, and D are typical experiments from three. NHE, normal hydrogen electrode.

concomitant with increased absorbance at 602 nm was consistent with the conversion of oxyHb into metHb (Mauk and Gray, 1979; Eguchi and Saltman, 1987). A thorough analysis of all time-dependent spectra was not possible because of protein precipitation at longer time scales (hours) and also reoxidation of the Fe(II)-thiosemicarbazone complex. The reaction between oxyHb and $[Fe(Bp4eT)_2]^+$ under pseudo-first order conditions is shown in Fig. 4. B and C, demonstrating that the second oxidation step is significantly slower than the first.

Correlation between Redox Potentials and OxyHb Oxidation. To examine whether a correlation exists between the redox potentials of thiosemicarbazone-iron complexes and oxyHb oxidation, cyclic voltammetry was used. In all cases, totally reversible iron(III/II) couples were identified, and these fell in a narrow potential range (Fig. 4D). The two complexes derived from di-2-pyridylketone, namely $[Fe(Dp44mT)_2]^{+/0}$ (+113 mV versus normal hydrogen electrode) and $[Fe(DpC)_2]^{+/0}$ (+94 mV versus normal hydrogen electrode), exhibited the highest potential couples as expected from the inductive effect of the dipyridyl group. The ferric 3-AP complex (+40 mV) gave the lowest redox potential, whereas $[Fe(Bp4eT)_2]^+$ (+63 mV) was in the middle of this range. In theory, one may expect a correlation between the redox potential and the oxyHb oxidation rate (k_1 values) (Table 1), but this was not apparent. This result is not surprising given the very narrow potential range spanned by these compounds (73 mV) and modest differences in k_1 values (the first phase of a_2b_2 oxidation) (Table 1).

AA Rescues MetHb Formation by Dp44mT In Vitro. Methylene blue and AA are used clinically for treating methemoglobinemia (Mansouri and Lurie, 1993). However, in our studies, myoglobin interfered with the spectrophotometric method used to detect oxyHb/metHb and also the commonly used CO oximetry technique (Gourlain et al., 1997; Dötsch et al., 1999), and, hence, prevented metHb estimation. Thus, further experiments focused on the ability of AA to reduce metHb generation by Dp44mT using intact RBCs. In these studies, RBCs were incubated (3 h for 37°C) with AA (0.2, 1, or 2 mM) in the presence or absence of Dp44mT (25 μM) (Fig. 5A). In the absence of AA, Dp44mT increased metHb formation to 21.5 ± 0.3% relative to that for the control (1.0 ± 0.5%), whereas the combination of Dp44mT and AA (0.2 mM) significantly ($p < 0.001$) decreased metHb generation to 13 ± 0.7% relative to that for Dp44mT alone (21.5 ± 0.3%) (Fig. 5A). Increasing AA from 0.2 to 1 or 2 mM did not result in a significant further decrease in metHb generation by Dp44mT. In fact, increasing AA to 1 to 2 mM in the absence of Dp44mT exhibited a pro-oxidative effect, with

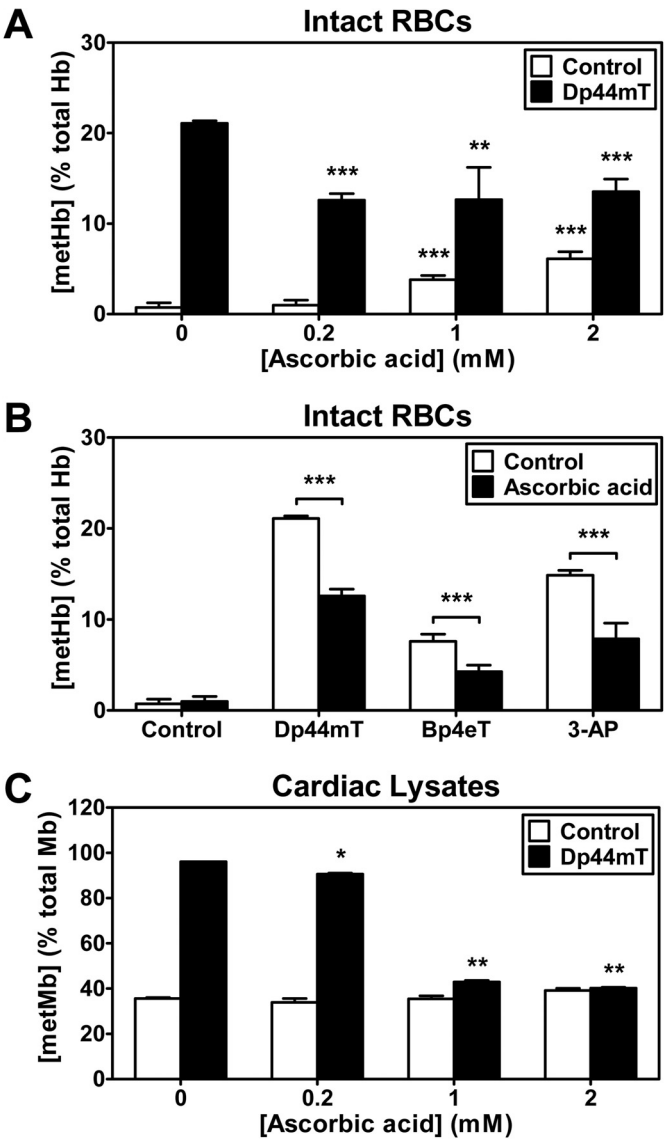

Fig. 5. Effect of AA on metHb and metMb formation in vitro. A, intact RBCs were incubated with Dp44mT (25 μM) in the presence or absence of AA (0.2–2 mM) for 3 h at 37°C. B, intact RBCs were incubated with Dp44mT, Bp4eT, or 3-AP (all at 25 μM) in the presence or absence of AA (0.2 mM) at 37°C for 3 h before determination of metHb. C, mouse cardiac tissue lysates were incubated with Dp44mT (25 μM) in the presence or absence of a range of AA concentrations at 37°C for 30 min. MetHb and metMb were determined spectrophotometrically. Significance levels are relative to the corresponding treatment without AA (A and C) or represent the indicated comparisons in B. Results are the means ± S.D. from three experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Mb, myoglobin.

metHb generation significantly ($p < 0.05$) increasing at 1 and 2 mM AA, respectively (Fig. 5A).

AA (0.2 mM) also significantly ($p < 0.001$) reduced metHb generation during incubation of intact RBCs for 3 h at 37°C with 25 μM Bp4eT or 3-AP (Fig. 5B). Addition of AA (1–2 mM) to Dp44mT (25 μM) also markedly and significantly ($p < 0.001$) reduced oxidation of cardiac oxyMb (50 μM) in mouse cardiac lysates relative to that by Dp44mT alone (25 μM) (Fig. 5C). Increasing AA to 1 and 2 mM was significantly ($p < 0.01$) more effective in suppressing metMb generation than AA at 0.2 mM and almost completely prevented metMb generation. The pro-oxidant effect of increasing AA concentration observed with oxyHb in control samples (Fig. 5A) was not found for oxyMb (Fig. 5C).

Effect of Dp44mT on MetHb and MetMb Formation In Vivo. To assess metHb/metMb generation by Dp44mT in vivo, C57BL/6 mice were treated with a single intravenous injection of Dp44mT (6 mg/kg) or vehicle alone, with metHb in blood and metMb from heart being monitored for up to 4 h. The high dose of Dp44mT (6 mg/kg) was specifically chosen in these short-term studies to ensure marked metHb/metMb generation that could be readily measured. A maximum in metHb and metMb formation was found 30 min after Dp44mT injection (Fig. 6, A and B). After this time point, a gradual decrease in metHb and metMb was identified, which could be due to Dp44mT excretion and the effect of endogenous reducing systems in rescuing the oxidative insult (e.g., NADH metHb reductase) (Weis, 1975).

Effect of AA on MetHb and MetMb Formation by Dp44mT In Vivo. To examine whether the AA-induced inhibition of Dp44mT-mediated oxyHb oxidation observed in vitro could be translated in vivo, C57BL/6 mice were intravenously administered Dp44mT (6 mg/kg) or vehicle in the absence or presence of AA (15 or 30 mg/kg), and blood was taken after 30 min to assess metHb generation. These AA doses correspond approximately to an initial blood AA concentration of 1 and 2 mM used in the in vitro studies (Fig. 5, A and C), assuming a mouse blood volume of 2 ml (Riches et al., 1973). The combination of AA with Dp44mT induced a significant ($p < 0.05$–0.01) decrease in metHb generation compared with Dp44mT alone (Fig. 6C). Lower (3 mg/kg) or higher (60 mg/kg) AA doses did not lead to greater efficacy in decreasing metHb generation (data not shown). This suggests an optimal AA dose-response range for preventing Dp44mT-induced metHb. No significant decrease in mouse heart metMb levels was observed after treatment of mice with AA (15 or 30 mg/kg) in combination with Dp44mT (Fig. 6D). Again, lower (3 mg/kg) or higher (60 mg/kg) AA doses had no effect on reducing metMb levels (data not shown).

DpC Generates Less MetHb Compared with Dp44mT In Vivo. Studies herein demonstrated that DpC was markedly less effective in inducing metHb in RBC lysates or intact RBCs (Fig. 3, C and D), and this chelator has shown greater antitumor activity than Dp44mT in vivo without cardiotoxic effects (Kovacevic et al., 2011). Therefore, we examined the ability of DpC to induce metHb and metMb in vivo. C57BL/6 mice were administered Dp44mT (6 mg/kg) or DpC (6 mg/kg) intravenously, and blood was taken after 30 min to assess metHb and metMb generation (Fig. 6, E and F). As observed previously, Dp44mT induced significant ($p < 0.001$) levels of metHb (6.3 ± 0.8%) relative to the vehicle (1.3 ± 0.9%), whereas metHb levels induced by DpC were comparable with those of the control (Fig. 6E). In addition, although Dp44mT induced significant ($p < 0.001$) levels of metMb (82.3 ± 2.6%), DpC generated significantly ($p < 0.001$) lower levels of metMb (31.7 ± 3.2%). However, DpC-mediated levels of metMb were significantly ($p < 0.001$) higher than those of the control (12.6 ± 2.4%) (Fig. 6F). Taken together, the properties of DpC, in terms of being significantly less effective in inducing metHb and metMb formation but maintaining pronounced antitumor activity (Kovacevic et al., 2011), make it an ideal candidate for further in vivo assessment.

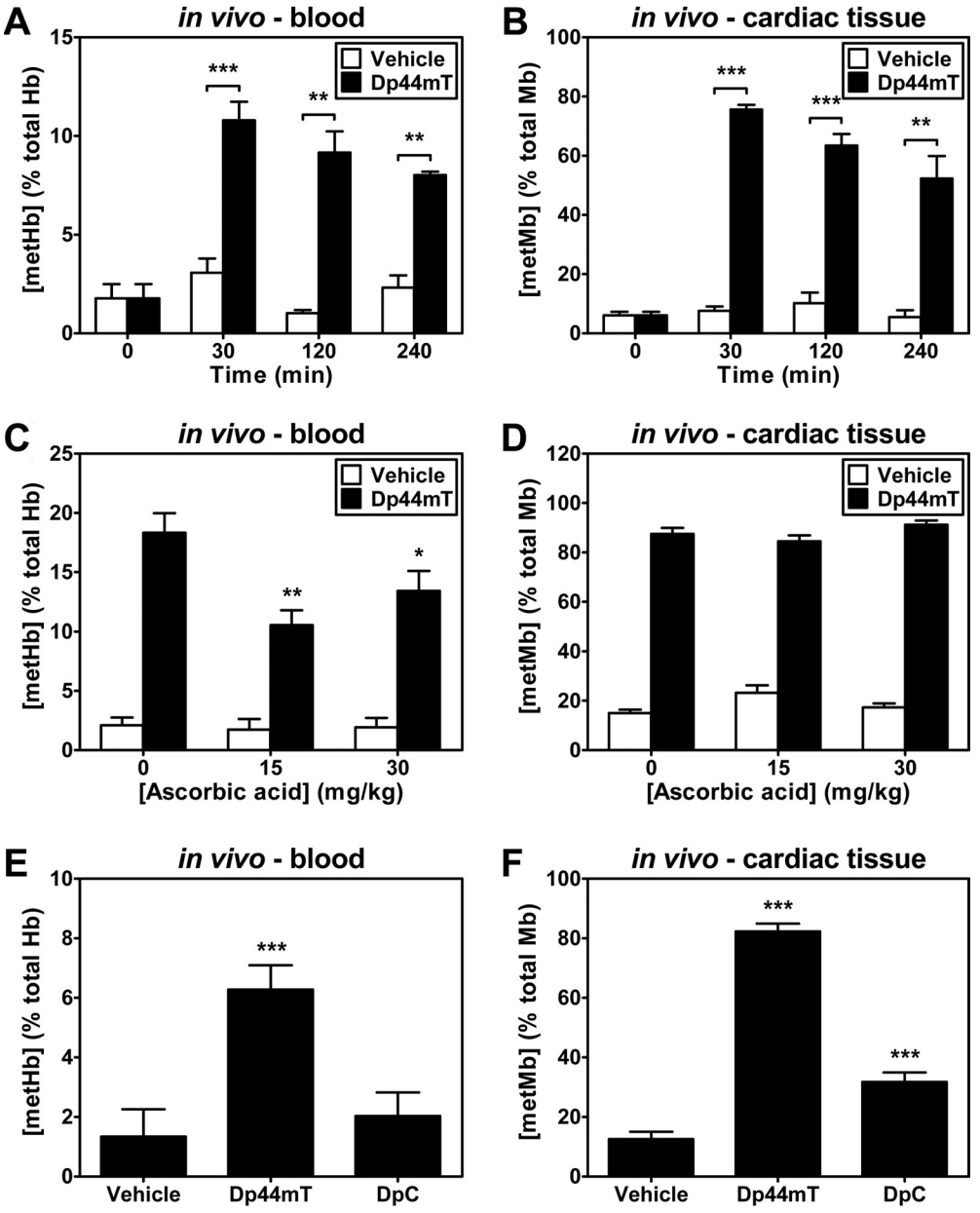

Fig. 6. Effect of AA on metHb and metMb formation in vivo. Vehicle or Dp44mT (6 mg/kg) was administered intravenously to mice. At 30 to 240 min after administration, blood and the heart were collected. MetHb (A) and metMb (B) levels from the RBC lysates and heart tissue lysates, respectively, were assayed spectrophotometrically. MetHb (C) and metMb (D) levels measured 30 min after intravenous administration of the vehicle or Dp44mT (6 mg/kg) alone or in combination with AA (15 or 30 mg/kg). At 30 min after administration, blood and the heart were collected. RBC and cardiac tissue lysates were prepared as in A and B. MetHb (E) and metMb (F) levels measured after vehicle, Dp44mT (6 mg/kg), or DpC (6 mg/kg) was administered to mice. At 30 min after administration, blood and heart were collected (see C and D) for determination of metHb/metMb. Results in A and B are means ± S.E.M. (n = 4 mice/group), and those in C to F are means ± S.E.M. (three experiments; n = 6 mice/group). Significance levels represent indicated comparisons in A and B, relative to Dp44mT without AA (C) or the vehicle control (E and F). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Mb, myoglobin.

Discussion

Hypoxia and methemoglobinemia are major concerns for patients undergoing anticancer therapies who have compromised cardiopulmonary function (Yen et al., 2004; Knox et al., 2007; Odenike et al., 2008; Traynor et al., 2010). Recent studies demonstrated that 3-AP led to metHb generation and hypoxia in patients, limiting its usefulness (Yen et al., 2004; Gojo et al., 2007; Knox et al., 2007; Odenike et al., 2008; Traynor et al., 2010). The mechanism responsible was not known and in consideration of the interest in the development of novel thiosemicarbazones as anticancer agents (Yu et al., 2009), it was crucial to investigate their effect on oxyHb, as this would facilitate the development of these compounds (Yu et al., 2009).

In a previous study, we have shown that copper chelation was important for the potent antitumor activity of Dp44mT (Lovejoy et al., 2011). Herein, we demonstrate that the iron-binding rather than the copper-binding ability of Dp44mT, Bp4eT, and 3-AP was essential in metHb formation. Because Dp44mT, Bp4eT, and 3-AP are not redox-active on their own (Richardson et al., 2006), they must sequester labile iron within normal RBCs (Prus and Fibach, 2008; Szuber et al., 2008; Prus and Fibach, 2010), generating a redox-active iron complex, which leads to metHb formation. The source of this labile iron in RBCs is unclear, but it seems to be present in a nonheme form, which can be chelated. The necessity of iron chelation and the formation of an iron complex was substantiated using the DpT analog, Dp2mT, which cannot bind iron (Yuan et al., 2004) and did not induce metHb formation. Moreover, despite the high affinity of DFO for iron (Kalinowski and Richardson, 2005), it could not induce metHb formation. This is because the iron(III/II) redox potential of the DFO-iron complex was too low to be reduced by oxyHb, whereas the thiosemicarbazone-iron complexes are much stronger oxidants, leading to metHb generation (Kalinowski and Richardson, 2005; Richardson et al., 2006).

In consideration of the mechanism of oxidation of oxyHb, it is notable that the four heme pockets are partially exposed at the surface (Shikama, 2006). In addition, Mouawad et al. (2005) identified a cavity consisting of three tunnels with widths of 2, 5, and 6 Å, spreading from the vicinity of the heme iron to the protein surface. However, the thiosemicarbazone-iron complexes seem sterically unable to enter these tunnels to directly interact with heme iron(II), because their dimensions reach 20 Å (Richardson et al., 2006; Kalinowski et al., 2007). In addition, our kinetic studies demonstrated that each separate heme cofactor was oxidized sequentially in a second-order, outer-sphere, electron-transfer reaction with the thiosemicarbazone-iron(III) complexes (Fig. 4, B and C; Table 1). Taken together, these results support the hypothesis that oxidation of oxyHb by thiosemicarbazone-iron complexes follow an outer-sphere mechanism occurring over the exposed heme edge, as reported for other iron(III) complexes (Eguchi and Saltman, 1984, 1987; Hegetschweiler et al., 1987; Harrington and Hicks, 1994). This is in contrast to a site-specific mechanism that involves direct metal chelate binding to oxyHb, forming an oxyHb-iron-ligand intermediate before electron transfer (Eguchi and Saltman, 1987; Bakan et al., 1991). Furthermore, a site-specific mechanism can be excluded when all six iron coordination sites are occupied by high-affinity ligands, as is the case for the thiosemicarbazones used herein (Kalinowski et al., 2007; Bernhardt et al., 2009; Kowol et al., 2009).

In an attempt to therapeutically prevent Hb oxidation, we demonstrated that coincubation of an optimal AA concentration with Dp44mT, Bp4eT, and 3-AP decreased metHb and metMb formation in vitro (Fig. 5A). The protective effects of AA are in agreement with studies showing it can reduce metHb and metMb to their oxy counterparts (Gibson, 1943; Sullivan and Stern, 1982; Galaris et al., 1989; Giulivi and Cadenas, 1993). Indeed, previous investigations suggest that reduction of metHb to oxyHb by AA occurs by direct interaction with oxyHb (Gibson, 1943) and, also indirectly, with AA acting as an electron donor to NADH-metHb reductase, which then reduces metHb (Weis, 1975).

The rapid oxidation of oxyHb and oxyMb to their Met counterparts was also shown in vivo when Dp44mT was administered at a high dose (6 mg/kg) in mice. Again, coadministration of AA was effective in reducing levels of metHb in C57BL/6 mice treated with Dp44mT and may represent an option for preventing thiosemicarbazone-induced metHb formation and hypoxia.

Of interest, a structurally diverse series of DpT-iron complexes induced metHb formation at similar levels in purified oxyHb (Fig. 3B) but resulted in pronounced differences in oxidation compared with those in intact RBCs (Fig. 3C) and RBC lysates (Fig. 3D). This result indicates that although structural differences between chelators are not important for their ability to directly oxidize purified oxyHb (Fig. 3B), they are crucial in intact RBCs (Fig. 3C), RBC lysates (Fig. 3D), and in vivo in mice (Fig. 6, E and F). Considering that the results obtained for intact RBCs and lysates were similar, variations in chelator RBC membrane permeability cannot explain why these results are different from those with purified oxyHb. Thus, other factors, such as interactions with physiological reductants or oxidants, may play an important role in oxyHb oxidation. Nonetheless, the ability of DpC to induce lower metHb and metMb levels is clearly advantageous over that of Dp44mT in terms of its therapeutic potential. This is of significance considering the greater antitumor efficacy and selectivity of DpC relative to those of Dp44mT, including the fact that DpC does not induce the cardiac fibrosis (Kovacevic et al., 2011) identified with Dp44mT (Whitnall et al., 2006).

In conclusion, several thiosemicarbazone chelators permeate RBCs to generate iron complexes that induce metHb formation. This occurs by a simple outer-sphere mechanism, and AA at appropriate doses can counteract this effect. Furthermore, we show that the structure of the thiosemicarbazones is of great importance in terms of inducing metHb generation. This is demonstrated by our studies using the recently developed DpC, which demonstrates highly selective antitumor efficacy in vivo (Kovacevic et al., 2011) but does not markedly induce metHb generation, overcoming the disadvantages of Dp44mT.

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Authorship Contributions

Participated in research design: Quach, Kalinowski, Bernhardt, Jansson, and Richardson.

Conducted experiments: Quach, Gutierrez, Basha, Sharpe, and Jansson.

Contributed new reagents or analytic tools: Lovejoy, Bernhardt, and Richardson.

Performed data analysis: Quach, Gutierrez, Basha, Sharpe, and Jansson.

Wrote or contributed to the writing of the manuscript: Quach, Gutierrez, Kalinowski, Bernhardt, Jansson, and Richardson.

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