Antitumor Activity of Metal-Chelating Compound Dp44mT Is Mediated by Formation of a Redox-Active Copper Complex That Accumulates in Lysosomes

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Abstract

The metal-chelating compound Dp44mT is a di-2-pyridylketone thiosemicarbazone (DpT) which displays potent and selective antitumor activity. This compound is receiving translational attention, but its mechanism is poorly understood. Here, we report that Dp44mT targets lysosome integrity through copper binding. Studies using the lysosomotropic fluorochrome acridine orange established that the copper–Dp44mT complex (Cu[Dp44mT]) disrupted lysosomes. This targeting was confirmed with pepstatin A–BODIPY FL, which showed redistribution of cathepsin D to the cytosol with ensuing cleavage of the proapoptotic BH3 protein Bid. Redox activity of Cu[Dp44mT] caused cellular depletion of glutathione, and lysosomal damage was prevented by cotreatment with the glutathione precursor N-acetylcysteine. Copper binding was essential for the potent antitumor activity of Dp44mT, as coincubation with nontoxic copper chelators markedly attenuated its cytotoxicity. Taken together, our studies show how the lysosomal apoptotic pathway can be selectively activated in cancer cells by sequestration of redox-active copper. Our findings define a novel generalized strategy to selectively target lysosome function for chemotherapeutic intervention against cancer. Cancer Res; 71(17); 1–10. ©2011 AACR.

Introduction

Neoplastic cells have high requirements for iron (Fe) due to their generally higher rates of proliferation than normal cells (1). In fact, neoplastic cells express enhanced transferrin receptor 1 (TfR1) levels relative to their normal counterparts (2) and take up Fe from transferrin (Tf) at a rapid rate (3), making them selectively sensitive to Fe chelation. Cancer cells also take up more copper (Cu) than their normal counterparts, as this metal is essential for angiogenesis and metastasis (4). Considering the crucial roles of these metals, development of novel Fe and Cu chelators has become a promising anticancer strategy (1, 5). Indeed, the chelator, Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; Fig. 1), which inhibits topoisomerase IIα (6) and inhibits tumor growth, has entered clinical trials (1). In particular, Rao and colleagues reported topoisomerase IIα inhibition (10) and broad and specific antitumor activity (12). Additional modes of anticancer activity reported for Dp44mT include inhibition of the metastasis suppressor protein, Ndrg-1 (1), and modulation of the cell-cycle control proteins of the cyclin family (A, B, D1, D2, and D3) as well as cyclin-dependent kinase 2 (1, 5).

One of the most effective DpT chelators is di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 1; ref. 6). Dp44mT shows marked and selective activity against tumor xenografts in mice (6, 7). The proposed mechanism of action of Dp44mT involves Fe chelation and redox cycling of its Fe complex to generate reactive oxygen species (ROS; refs. 6, 8, 9). The potent and antitumor activity of Dp44mT has been verified by others (10–12). In particular, Rao and colleagues reported topoisomerase IIα inhibition (10) and broad and specific antitumor activity (12). Additional modes of anticancer activity reported for Dp44mT include inhibition of the metastasis suppressor protein, Ndrg-1 (1), and modulation of the cell-cycle control proteins of the cyclin family (A, B, D1, D2, and D3) as well as cyclin-dependent kinase 2 (1, 5).

Dp44mT not only binds Fe, but also Cu, and both complexes are redox active, contributing to their marked cytotoxicity (13). Considering: (i) the potential of lysosomes as a therapeutic target; (ii) their key role in metal metabolism (14, 15); and (iii) their pronounced susceptibility to ROS (15, 16), our goals were to examine the effects of the DpT compounds on Cu and Fe and their interaction with lysosomes. Our work has elucidated a novel strategy for the design of new therapeutics that activate the lysosomal apoptotic pathway by binding redox-active Cu.

Materials and Methods

Chemicals

2-Hydroxy-1-naphthaldehyde isonicotinoyl hydrazone (311), di-2-pyridylketone-2-methyl-3-thiosemicarbazone (Dp2mT), di-
2-pyridylketone-4-ethyl-3-thiosemicarbazone (Dp4eT), di-2-pyridylketone-4-allyl-3-thiosemicarbazone (Dp4aT), di-2-pyridylketone-4-methyl-3-thiosemicarbazone (Dp4mT), Dp44mT, di-2-pyridylketone-4-phenyl-3-thiosemicarbazone (Dp4pT), and pyridoxal isonicotinoyl hydrazone (PIH) were synthesized as described (6, 8). Desferrioxamine (DFO) was from Novartis. Buthionine sulfoximine (BSO), D-penicillamine (D-pen), tetra-thiomolybdate (TM), and Trientine (Trien) were purchased from Sigma-Aldrich. Triapine was from Vion Pharmaceuticals.

**Cell culture**

Human SK-N-MC neuroepithelioma cells, MCF-7 breast cancer cells, DMS-53 lung carcinoma cells, and human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection and grown as described (17, 18). Cells were used within 2 months of purchase after resuscitation of frozen aliquots. Cell lines were authenticated on the basis of viability, recovery, growth, morphology, and also cytogenetic analysis, antigen expression, DNA profile, and isoenzymology by the provider.

Procedures involved in preparing $^{59}$Fe-transferrin and conducting cytotoxicity assays have been described previously (17, 18).

**$^{64}$Cu and $^{59}$Fe efflux assays**

Efflux studies examining the ability of chelators to mobilize $^{59}$Fe or $^{64}$Cu (10 μCi/ml; $^{64}$CuCl$_2$; ANSTO) from cells were done by standard methods (17). Briefly, cells were prelabeled for 3 hours at 37°C with $^{64}$Cu (10 μCi) or $^{59}$Fe-transferrin ($^{59}$Fe-Tf; 0.75 μmol/L), washed 4 times on ice and reincubated with medium (control) or Dp4mT (5 μmol/L) for up to 6 hours at 37°C and then harvested (17). Radioactivity was measured in the pellet and supernatant using a γ-scintillation counter (Wallac Wizard 3; Perkin Elmer).

**Cellular retention of $^{64}$Cu complexes**

Complexes were prepared by adding equimolar $^{64}$Cu and chelator. SK-N-MC cells were incubated with the complexes for 3 hours at 37°C, washed 4 times on ice, reincubated for 3 hours at 37°C in control media, and the percentage of $^{64}$Cu remaining cell associated assessed (17).

**Subcellular fractionation**

SK-N-MC cells (3 x T175 flasks, 80% confluent) were prelabeled for 3 hours at 37°C with $^{64}$Cu (10 μCi), washed and reincubated at 37°C with medium (control) or Dp4mT (5 μmol/L) for 3 hours at 37°C. Cells were added to extraction buffer (0.25 mol/L sucrose, 10 mmol/L Tris-HCl) and disrupted using a Dounce “B” homogenizer on ice. To prevent damage to the released lysosomes, disruption of cells was discontinued when approximately 50% of the intact cells were disrupted. The suspension was centrifuged 1,000 × g for 15 minutes at 4°C, the pellet discarded, and supernatant centrifuged at 16,000 × g for 15 minutes at 4°C to yield a crude lysosomal/mitochondrial fraction. As a further control, enzyme marker analysis for the lysosomal specific enzyme acid phosphatase (Sigma) showed that this fraction was enriched with lysosomes (as determined by spectrophotometric assessment at 405 nm).

**Speciation studies**

Potentiometric titrations were conducted as described (8).

**Assessment of lysosomal membrane permeability**

Distribution of acridine orange (Sigma) was used to determine lysosomal membrane permeability (LMP) as previously reported (18) and was quantified by flow cytometry (19). Briefly, cells were incubated for 15 minutes at 37°C with acridine orange (20 μmol/L), washed 3 times with PBS, and then incubated for 30 minutes at 37°C with 25 μmol/L...
chelator or reagent. Cathepsin D release was examined by fluorescence microscopy as previously reported (20) using a pepstatin A–BODIPY FL conjugate (Invitrogen) that selectively binds to cathepsin D. LysoTracker red (Invitrogen) was used to confirm colocalization of cathepsin D with lysosomes. For details, see Supplementary Methods.

**Western blot analysis**

Assessment of Bid cleavage was assessed by Western blotting by standard methods (6) using antibodies to Bid (Cell Signaling Technology) and β-actin (clone AC-1; Sigma).

**Mitochondrial stability assay**

Tetramethylrhodamine ethyl ester (TMRE; Invitrogen) partitions to the mitochondrial matrix (21). SK-N-MC cells were incubated with 20 μmol/L of TMRE for 0.25 hours at 37°C and then incubated with 5 μmol/L of Cu[DP4HmT] for 0.5 to 2 hours at 37°C. Mitochondrial damage was examined by flow cytometry (21).

**GSH/GSSG assay**

This assay was conducted using a kit (Calbiochem). SK-N-MC cells were treated at 25 μmol/L for up to 24 hours at 37°C with Cu(II), Fe(III), DP4mT, Cu[DP4mT], or Fe[DP4mT]2. As a control, BSO (100 μmol/L was added) as was a glutathione synthesis inhibitor (22).

**Redox studies: oxidation of H2DCF**

Studies without cells were conducted as described with H2DCF (5 μmol/L; ref. 23). As a positive control, Fe(III) at 5 μmol/L was reduced to Fe(II) using cysteine (100 μmol/L) in 150 mmol/L acetate buffer (pH 5.0). Hydrogen peroxide (100 μmol/L) was then added to initiate hydroxyl radical generation. To confirm hydroxyl radical production, dimethyl sulfoxide (DMSO; 10% v/v) was used, as it has been reported to be an effective hydroxyl radical scavenger (24, 25). Cu[DP4HmT] and other reagents (at 5 μmol/L except DMSO at 10% v/v) were added to examine hydroxyl radical production. Intracellular oxidation of H2DCF studies were conducted as described in previous studies (6, 23).

**Statistics**

Data were compared using Student’s t test. Results were expressed as mean ± SD (number of experiments) and considered to be statistically significant when \( P < 0.05 \).

**Results**

**Active DpT chelators prevent \(^{64}\text{Cu} \) mobilization but markedly induce \(^{59}\text{Fe} \) efflux**

To assess the antineoplastic mechanisms of the DpT analogues (Fig. 1), their ability to remove \(^{64}\text{Cu} \) from prelabeled cells was compared with their interaction with \(^{59}\text{Fe} \). The activity of DpT chelators was compared with the Fe chelators, DFO, PIH, and 311 (17), and Cu chelators, d-pen, Trien, and TM (Fig. 1; ref. 1). The DpT chelators, DP4mT, DP4mT, Dp4eT, Dp4HmT, and Dp4pT, were found to be ineffective at 25 μmol/L at inducing \(^{64}\text{Cu} \) release from cells (leading to \(^{64}\text{Cu} \) retention) relative to when cells were incubated with control medium (Fig. 2A). In these studies, SK-N-MC cells were used as their response to chelators is well characterized (6, 17).

The results above were in contrast to their ability to mobilize \(^{59}\text{Fe} \), where the DpT chelators significantly (\( P < 0.001 \)) induced a more than 700% increase in \(^{59}\text{Fe} \) release relative to control cells (Fig. 2B). Hence, there was a difference in the ability of DpT chelators to mobilize these metals. The only exception was the negative control, Dp2mT, which failed to induce significant \(^{64}\text{Cu} \) or \(^{59}\text{Fe} \) efflux (Fig. 2A and B). By design, Dp2mT cannot bind metals (6), showing the importance of metal binding for DpT chelator activity. The chelators DFO, PIH, and 311 were included as positive controls to increase \(^{59}\text{Fe} \) efflux, and we also examined their effects on \(^{64}\text{Cu} \) release. Only 311 showed a marked interaction with \(^{64}\text{Cu} \), significantly (\( P < 0.001 \)) reducing its efflux to 43% ± 10% of the control (Fig. 2A). The Cu chelators, d-pen and Trien, were ineffective at inducing \(^{64}\text{Cu} \) efflux at 25 μmol/L (Fig. 2A). However, the Cu chelator, TM, significantly (\( P < 0.05 \)) reduced \(^{64}\text{Cu} \) efflux to 76% ± 2% of the control. The low activity of the Cu chelators, d-pen, Trien, and TM, at mobilizing \(^{64}\text{Cu} \) was unexpected on the basis of their in vivo efficacy (1). However, their mechanism of action in cell culture is unclear, and the efficacy in vivo could relate to extracellular rather than intracellular chelation (26). These efflux experiments were repeated using MCF-7 (Fig. 2C and D), and to provide a comparison with tumor cells, normal HUVECs were used (Fig. 2E and F). Generally, the active chelators accumulated \(^{64}\text{Cu} \) similarly across the 3 cell types (Fig. 2A–F). In terms of the effect of DP4mT on inhibiting \(^{64}\text{Cu} \) release, its effect was greater in HUVECs and MCF-7 cells than SK-N-MC.

The kinetics of \(^{64}\text{Cu} \) and \(^{59}\text{Fe} \) release were assessed over a range of reincubation times (0.5–6 hours) using SK-N-MC cells and HUVECs that were prelabeled with \(^{64}\text{Cu} \) or \(^{59}\text{Fe}-\text{Tf} \) for 3 hours at 37°C (Supplementary Fig. S1A–D). These results showed that DP4mT and 311 decreased \(^{64}\text{Cu} \) release relative to the control while both ligands markedly increased \(^{59}\text{Fe} \) efflux as a function of time. In addition, dose–response curves showed that 311 and DP4mT decreased \(^{64}\text{Cu} \) release compared with the control, leading to \(^{64}\text{Cu} \) accumulation in cells (Supplementary Fig. S1E). With DP4mT, this effect was reversible as its concentration increased to more than 5 μmol/L, and at these higher concentrations, cellular damage results in the release of the Cu–DP4mT complex from the cells. In contrast, at lower concentrations, the cells are still intact preventing \(^{64}\text{Cu} \) release. In terms of \(^{59}\text{Fe} \) efflux, 311 and DP4mT markedly increased \(^{59}\text{Fe} \) release as a function of concentration (Supplementary Fig. S1F). Collectively, the active DpT group of chelators markedly prevented \(^{64}\text{Cu} \) efflux from all cell types, leading to \(^{64}\text{Cu} \) accumulation.

**Precomplexation of \(^{64}\text{Cu} \) with 311 and the DpT ligands leads to intracellular accumulation of their \(^{64}\text{Cu} \) complexes**

The results indicating intracellular accumulation of DpT–\(^{64}\text{Cu} \) complexes were confirmed by studies where
SK-N-MC cells were labeled for 3 hours at 37°C with preformed ⁶⁴Cu–chelator complexes. The cells were then washed and reincubated for 3 hours at 37°C in control media and the percentage of ⁶⁴Cu remaining cell associated then quantified. In control cells incubated with ⁶⁴Cu only, only 4% ± 1% of ⁶⁴Cu remained intracellular (Fig. 3A). Hence, most ⁶⁴Cu had been released during the reincubation. Cells labeled with the ⁶⁴Cu complexes of the active DpT analogues (Dp4mT, Dp44mT, Dp4eT, Dp4aT, and Dp4pT) and then reincubated with control medium, showed that 39% to 68% of ⁶⁴Cu remained cell associated (Fig. 3A). In contrast, ⁶⁴Cu complexes of Dp2mT, D-pen, and Trien were no more effective than the control at retaining intracellular ⁶⁴Cu (Fig. 3A). The ⁶⁴Cu–TM complex also led to intracellular ⁶⁴Cu accumulation relative to the control. These results explain the low efflux of ⁶⁴Cu observed with the active DpT analogues (Fig. 2A), as their ⁶⁴Cu complexes become “trapped” within cells.

Subcellular fractionation indicates ⁶⁴Cu[Dp44mT] accumulates within a crude lysosomal/mitochondrial fraction

To examine the subcellular localization of the accumulated ⁶⁴Cu, SK-N-MC cells were labeled for 3 hours at 37°C with ⁶⁴Cu, washed, and reincubated for 3 hours at 37°C with Dp44mT (5 µmol/L) or control media. The distribution of ⁶⁴Cu in the cytosol and crude lysosomal/mitochondrial fraction was examined by differential centrifugation. In cells reincubated with Dp44mT, a significantly (P < 0.001) greater proportion of ⁶⁴Cu accumulated in the lysosomal/mitochondrial fraction (Fig. 3B).

Considering this observation, we hypothesized that because of the polyprotic nature of Dp44mT (8), it was trapped in acidic lysosomes as it became positively charged. To assess this, speciation studies of the chelator as a function of pH were conducted. At a pH of 7.4, 100% of the ligand is in its neutral
state (Dp44mT), allowing facile transport through membranes, whereas at a lysosomal pH of 5, 16% is charged leading to lysosomal accumulation (Dp44mT⁺; Fig. 3C). It should be noted that while 16% of the ligand is protonated at a pH of 5, this leads to accumulation of the ligand over time. This occurs due to the process of: (i) the neutral ligand entering the lysosome; (ii) the ligand becoming protonated and charged at lysosomal pH (pH = 5), preventing its passage out of the organelle; (iii) the charged ligand-binding copper in the lysosome; and (iv), as the so formed copper complex is probably positively charged, it also cannot escape the lysosome accounting for the ⁶⁴Cu accumulation in this compartment (see Fig. 3B).

**Acridine orange indicates lysosomal permeabilization by Cu[Dp44mT]**

To further explore the potential effect of Dp44mT and its Cu complex (Cu[Dp44mT]) on the lysosome, we implemented the lysosomotropic fluorophore, acridine orange (Fig. 3D) that accumulates within lysosomes (18). High lysosomal concentrations of acridine orange give a red fluorescence, whereas lower cytosolic and nuclear concentrations give a green fluorescence (18).

Examining control cells by fluorescence microscopy, a granular red fluorescence consistent with acridine orange concentration in lysosomes was found (Fig. 3D; ref. 18). However, incubation of cells with 25 µmol/L of Cu[Dp44mT] for 0.5 hours resulted in a marked loss of red fluorescence and the disappearance of red vesicles consistent with increased LMP, as well as the appearance of apoptotic bodies (Fig. 3D). In contrast, the Fe[Dp44mT]₂ complex, Dp44mT, CuCl₂, or FeCl₃ (at 25 µmol/L), had no significant effect relative to the control (Fig. 3D). The lack of activity of Dp44mT or Fe[Dp44mT]₂ is due to the short incubation period (0.5 hours) used, which was optimal for detecting the effect of Cu[Dp44mT]. Indeed, Dp44mT and Fe[Dp44mT]₂ only induce significant cytotoxicity after 24 hours (Fig. 6A). These data using fluorescence microscopy were confirmed upon quantification by flow cytometry, where only the Cu[Dp44mT] complex (25 µmol/L) significantly (P < 0.001) reduced red fluorescence (Supplementary Fig. S2).

To further examine the effect of Cu[Dp44mT] on the lysosome, we examined the intracellular distribution of LysoTracker red and pepstatin A–BODIPY FL that binds to cathepsin D (20). Control cells stained with LysoTracker red and pepstatin A–BODIPY FL showed a granular/vesicular pattern consistent with lysosomes (Fig. 4A). The overlay of LysoTracker red and pepstatin A–BODIPY FL–stained cells showed colocalization. After a 0.5-hour incubation with 25 µmol/L of Cu[Dp44mT], the granular, lysosomal type pattern disappeared, with the...
fluorescence becoming evenly distributed within the cytosol (Fig. 4A). This observation was consistent with Cu[Dp44mT]-induced LMP, confirming results with acridine orange (Fig. 3D). Neither Cu(II) as CuCl₂ (25 μmol/L) or Dp44mT (25 μmol/L) alone had any significant effect on LysoTracker red or cathepsin D staining (Fig. 4A).

To show a relationship between altered LMP and apoptosis induction, Western blotting assessed cleavage of the pro-apoptotic Bcl-2 family member, BH3-interacting domain death agonist (Bid), by lysosomal proteases (ref. 27; Fig. 4B). As a positive control, cells were incubated with cisplatin (20 mmol/L) that induces Bid cleavage (27). SK-N-MC cells incubated with 5 μmol/L of Cu[Dp44mT] or 20 mmol/L of cisplatin for 2 hours showed significant (P < 0.001) cleavage of Bid (Fig. 4B). This confirmed redistribution of lysosomal proteases to the cytosol (Fig. 4A) leading to Bid cleavage and provides a direct link between lysosomal damage and apoptosis induction by Cu[Dp44mT].

**Cu[Dp44mT] induces damage to lysosomes earlier than mitochondria**

We next examined whether mitochondria were also damaged. Mitochondrial stability was determined by measuring the reduction in mitochondrial membrane potential using TMRE (21), whereas LMP was assayed using acridine orange via flow cytometry. As in Fig. 4C, the damage induced by 5 μmol/L of Cu[Dp44mT] to lysosomes was significantly (P < 0.05–0.001) more apparent than that to mitochondria after 1 or 2 hours, indicating that lysosomes were more sensitive to Cu[Dp44mT].
Ability of the Cu-Dp44mT and Fe–Dp44mT complexes to generate ROS

To understand why lysosomes were so sensitive to Cu[Dp44mT], we assessed whether the Fe or Cu complexes of Dp44mT generate oxidative stress that could damage lysosomes. These studies in a cell-free system were conducted under lysosomal like conditions at a pH of 5.0 and in the presence of cysteine (ref. 28; Fig. 5A and B). Oxidative stress was determined by the oxidation of nonfluorescent H2DCF to fluorescent DCF, a well-characterized probe for assessing redox stress (6, 23). The high redox activity of 5 mMol/L of Cu[Dp44mT] was significantly (P < 0.01) greater than 5 mMol/L of CuCl2, FeCl3, and Fe[Dp44mT]2 after only 2 minutes (Fig. 5A). Addition of the 5 mMol/L of Cu chelator TM to 5 mMol/L of Cu[Dp44mT] totally prevented its activity, consistent with the ability of TM to bind Cu from Cu[Dp44mT], as shown in Fig. 5A and B.

Considering these results, the potential of Cu[Dp44mT] to induce intracellular ROS in SK-N-MC cells was also assessed using H2DCF. At 5 mMol/L, Cu[Dp44mT] caused a significant (P < 0.001) increase in intracellular H2DCF oxidation to 273% /C6 20% of that found for control cells after a 0.5-hour incubation (Fig. 5C). No increase in H2DCF oxidation was observed with Cu(II) as CuCl2, Fe(III) as FeCl3, Dp44mT alone, or Fe[Dp44mT]2, even at a 5-fold higher concentration (25 mMol/L; Fig. 5C). Collectively, these experiments show the marked redox activity of Cu[Dp44mT].

Effect of Cu[Dp44mT] on GSH and GSSG levels

To examine the redox activity of Cu[Dp44mT] on physiologic substrates, levels of the well-described indicator of oxidative stress, glutathione (GSH), and oxidized GSH (GSSG; ref. 22) were determined. Over 0.5 to 24 hours incubations with SK-N-MC cells, 25 mMol/L of Cu[Dp44mT] significantly reduced the GSH/GSSG ratio. Results are mean ± SD (6 experiments). ***, versus control, P < 0.001. a.u., arbitrary units.
In contrast, Dp44mT or Fe[Dp44mT]2 (both at 5 µmol/L) also reduced the GSH/GSSG ratio to 4% to 20% of the control but only after 24 hours (Fig. 5D).

Cell survival after exposure to Dp44mT and its Cu and Fe complexes

The studies above indicate that Dp44mT enters cells and forms a redox-active Cu complex that damages lysosomes. Considering this, we examined the cytotoxicity of Dp44mT and its Cu and Fe complexes. As shown in Fig. 6A, after a 3-hour incubation, 5 µmol/L of Cu[Dp44mT] significantly (P < 0.001) decreased viability to 28% ± 4% of the control. In contrast, Dp44mT or Fe[Dp44mT]2 (both at 5 µmol/L) did not significantly decrease viability within 3 hours but showed activity at 24 hours (Fig. 6A). These results show the pronounced cytotoxicity of Cu[Dp44mT] relative to Fe[Dp44mT]2 or Dp44mT.

Copper chelators, TM and BCS, prevent Dp44mT cytotoxicity

To determine the importance of Cu chelation to the cytotoxicity, we incubated cells for 72 hours at 37°C with increasing Dp44mT concentrations, Dp44mT in the presence of the Cu chelator, TM, or the preformed TM–Cu complex, which blocks the ability of TM to bind cellular Cu (Fig. 6B). The addition of TM to Dp44mT markedly prevented the ability of the latter to reduce cellular viability up to a Dp44mT concentration of 2.5 µmol/L, whereas the preformed Cu-TM complex (which cannot bind cellular Cu) had no significant rescue effect on Dp44mT cytotoxicity (Fig. 6B).

To confirm that the ability of TM to rescue Dp44mT cytotoxicity was due to Cu chelation, we used the structurally unrelated Cu chelator, bathocuproine disulfonate (BCS), in identical studies and observed a similar, but less pronounced, rescue effect on Dp44mT cytotoxicity (Fig. 6B). Like TM, BCS decreased Dp44mT cytotoxicity, whereas its Cu complex (BCS-Cu) had no significant influence (Fig. 6B), as its binding site was saturated with Cu. Notably, BCS or TM alone or their Cu complexes had no significant effect on viability (Supplementary Fig. S3).

As Dp44mT concentration increased, the ability of the Cu chelators BCS or TM to prevent cytotoxicity was markedly reduced after 72 hours (Fig. 6B). This may reflect chelation of Fe by Dp44mT which becomes significant at these higher concentrations and leads to Fe deprivation and cytotoxicity (6). Indeed, while the binding of Cu by Dp44mT is crucial for its antitumor efficacy, its Fe chelation efficacy is also important (6, 8).
GSH levels modulate Cu[DP44mT] cytotoxicity

Because GSH plays an important role in buffering redox stress (22) and considering the marked redox activity of Cu[DP44mT], as shown in Fig. 5A–C, further studies examined its effect on GSH (Fig. 6C–F). To investigate this, the effect of GSH supplementation or depletion on viability was assessed by incubating cells in the presence of N-acetylcysteine (NAC) that enhances GSH levels (22), or the GSH synthesis inhibitor, BSO, that decreases GSH (22). Indeed, a 24-hour incubation of SK-N-MC cells with NAC (5 mmol/L) or BSO (100 μmol/L) significantly \( P < 0.001 \) increased and decreased the GSH/GSSG ratio to 155% ± 5% and 4% ± 2% \((n = 3)\) of the control, respectively.

The addition of NAC (5 mmol/L) with Cu[DP44mT] significantly \( P < 0.001 \) prevented the decrease in viability due to Cu[DP44mT] after 3 and 24 hours (Fig. 6C), and similar results were observed as a function of concentration over 72 hours (Fig. 6D). Supplementation with NAC also significantly \( P < 0.01 \) protected against the decreased viability observed with Fe[DP44mT] \(_2\) or Dp44mT alone after 72 hours (Fig. 6C and D), and thus decreased their cytotoxicity. Further, morphologic studies using acridine orange also showed that NAC preserved lysosomal integrity in the presence of Cu[DP44mT], as shown in Supplementary Fig. S4.

In contrast to NAC, the addition of BSO with Dp44mT and Fe[DP44mT] \(_2\) significantly \( P < 0.001 \) enhanced their cytotoxicity particularly at 24 hours (Fig. 6E). However, the addition of BSO with 5 μmol/L of Cu[DP44mT] did not increase its cytotoxicity (Fig. 6E). This may be due to the marked redox activity of Cu[DP44mT] relative to Dp44mT and Fe[DP44mT] \(_2\) at high concentrations (Fig. 5A), which rapidly depresses GSH levels (Fig. 5D).

As a function of chelator or complex concentration (0.02–0.25 μmol/L), BSO potentiated cytotoxicity of Dp44mT, Fe[DP44mT] \(_2\), and Cu[DP44mT] after 72 hours (Fig. 6F). Hence, at low Cu[DP44mT] concentrations (i.e., 0.02–0.25 μmol/L; Fig. 6F), BSO clearly potentiated the cytotoxicity of this complex after 72 hours, in contrast to higher Cu[DP44mT] concentrations (i.e., 5 μmol/L; Fig. 6E).

Discussion

In this investigation, we showed that incubation of cells with Dp44mT leads to retention of its \(^{64}\text{Cu}\) complex. The probable reason for this is the ionization characteristics of Dp44mT (8). At physiologic pH, Dp44mT is neutral and permeates cell membranes (6, 8). However, in the lysosomal compartment (pH ~5; ref. 29), an increased proportion of Dp44mT becomes positively charged leading to accumulation, ROS formation, and LMP.

Dp44mT induces apoptosis using several cell types and a tumor model in vivo (6). Moreover, we showed that apoptosis occurred via the mitochondrial pathway, where decreased Bcl-2 and increased Bax expression occurred along with holo-cytochrome c (h-cytc) release and caspase activation (6). Our study suggests that these apoptotic events could be caused by Cu[DP44mT]-induced redox stress that results in LMP, causing redistribution of lysosomal cathepsins to the cytosol (Fig. 4A) and concomitant cleavage of Bid into its proapoptotic form (Fig. 4B). Indeed, cathepsins can cleave Bid, which migrates to mitochondria and induces outer membrane permeabilization that is dependent on proapoptotic Bax (30). Bax plays a role in inducing release of mitochondrial h-cytc, thereby activating the caspase cascade (31), which we showed occurs after Dp44mT treatment (6). Hence, LMP by redox-active Cu[DP44mT] could result in downstream effects on mitochondria that lead to h-cytc release.

The Dp44mT–Cu complex showed far greater cytotoxicity as a function of time than either the Fe complex or the ligand alone. This was also reflected in the faster kinetics of lysosomal rupture (Fig. 3D), cathepsin D release (Fig. 4A), and decrease in GSH/GSSG ratio (Fig. 5D) induced by Cu[DP44mT]. As these events all occur over the same time scale, they suggest a coherent and coordinated series of events that ultimately induces significant cytotoxicity. Intriguingly, the importance of Cu in mediating the cytotoxicity of the free ligand was shown by the rescue effect of the nontoxic Cu chelators, TM and BCS. This indicated that formation of a redox-active Cu[DP44mT] complex is important for Dp44mT activity. Moreover, it was shown that Cu[DP44mT] results in a marked decrease in GSH and the cytotoxicity of this complex can be reduced by NAC, which enhances GSH levels (22). Conversely, cytotoxicity can be potentiated by the GSH inhibitor, BSO. These results indicate that lysosomal damage caused by Cu[DP44mT] was directly due to its redox activity. On the basis of these data, we propose a model of the mechanism of action of Dp44mT (Fig. 7).

Another aspect of the mechanism of action of Dp44mT is that it shows selectivity against tumor cells in vitro and in vivo (7). The basis for this relates, in part, to the greater uptake and metabolism of Cu and Fe for essential processes in cancer cells relative to normal cells (5). Under such conditions, the lysosome could be more active in terms of its metal metabolism, and hence, more susceptible to Dp44mT. It is also known that lysosomal autophagic pathways in cancer cells are abnormal due to the monoallelic deletion of the essential autophagy.
regulator, beclin1 (32). As such, metal recycling due to autophagy could be disturbed and lead to differences between neoplastic and normal cells in their response to agents such as Dp44mT.

In summary, we have dissected the mechanism of action of Dp44mT and showed that it accumulates in lysosomes due to its unique ionization characteristics. Our investigation reveals the marked redox activity of Cu[Dp44mT] leads to LMP that induces cell death. Moreover, this study shows that targeting lysosomes can lead to potent and selective anticancer therapeutics. Knowledge of this mechanism can be used to design more potent cytotoxic agents that affect the lysosomal apoptosis pathway.

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

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References

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