Antitumor activity of metal chelating compound Dp44mT is mediated by formation of a redox-active copper complex that accumulates in lysosomes.

Running title: Dp44mT forms a copper complex that targets lysosomes

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ABSTRACT

The metal chelating compound Dp44mT is a di-2-pyridylketone thiosemicarbazone (DpT) which displays potent and selective anti-tumor 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 re-distribution of cathepsin D to the cytosol with ensuing cleavage of the pro-apoptotic BH3 protein Bid. Redox activity of Cu[Dp44mT] caused cellular depletion of glutathione and lysosomal damage was prevented by co-treatment with the glutathione precursor N-acetylcysteine. Copper binding was essential for the potent anti-tumor activity of Dp44mT, since co-incubation with non-toxic 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.
INTRODUCTION

Neoplastic cells have high requirements for iron (Fe) due to their generally greater 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 anti-cancer strategy (1, 5). Indeed, the chelator, Triapine® (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; Fig. 1), which inhibits tumor growth, has entered clinical trials (1). However, the di-2-pyridylketone thiosemicarbazone (DpT) chelators possess far greater anti-tumor activity and selectivity than Triapine® (6, 7).

One of the most effective DpT chelators is di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 1) (6). Dp44mT demonstrates 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) (6, 8, 9). The potent and anti-tumor activity of Dp44mT has been verified by others (10-12). In particular, Rao and colleagues reported topoisomerase IIα inhibition (10) and broad and specific anti-tumor activity (12). Additional modes of anti-cancer 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, 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 (Basel, Switzerland). Buthionine sulfoximine (BSO), D-penicillamine (D-pen), tetrathiomolybdate (TM) and Trientine (Trien) were purchased from Sigma-Aldrich (St. Louise, MO). Triapine® was from Vion Pharmaceuticals (New Haven, CT).

**Cell culture.** Human SK-N-MC neuroepithelioma cells, MCF-7 breast cancer cells, DMS-53 lung carcinoma cells and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Manassas, VA) and grown as described (17, 18). Cells were used within 2 months of purchase after resuscitation of frozen aliquots. Cell lines were authenticated based on 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 performing 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 $\mu$Ci/mL; $^{64}$CuCl$_2$; ANSTO, Sydney) from cells were done
by standard methods (17). Briefly, cells were prelabeled for 3 h/37°C with $^{64}$Cu (10 µCi) or $^{59}$Fe-transferrin ($^{59}$Fe-Tf; 0.75 µM), washed 4 times on ice and reincubated with medium (control) or medium and chelators (25 µM) for up to 6 h/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 h/37°C, washed 4 times on ice, reincubated for 3 h/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 h/37°C with $^{64}$Cu (10 µCi), washed and reincubated at 37°C with medium (control) or Dp44mT (5 µM) for 3 h/37°C. Cells were added to extraction buffer (0.25 M sucrose, 10 mM 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 1000 x g/15 min/4ºC, the pellet discarded and supernatant centrifuged at 16,000 x g/15 min/4ºC to yield a crude lysosomal/mitochondrial fraction. As a further control, enzyme marker analysis for the lysosomal specific enzyme acid phosphatase (Sigma) showed this fraction was enriched with lysosomes (as determined by spectrophotometric assessment at 405 nm).

**Speciation Studies.** Potentiometric titrations were performed as described (8).
**Assessment of lysosomal membrane permeability.** Distribution of acridine orange (AO; 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 min/37°C with AO (20 μM), washed 3 times with PBS and then incubated for 30 min/37°C with 25 μM chelator or reagent. Cathepsin D release was examined by fluorescence microscopy as previously reported (20) using a pepstatin A-BODIPY FL conjugate (Invitrogen, Carlsbad, CA) that selectively binds to cathepsin D. Lysotracker® red (Invitrogen) was used to confirm co-localization 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, Beverly, MA) 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 TMRE (20 nM) for 0.25 h/37°C and then incubated with Cu[Dp44mT] (5 μM) for 0.5-2 h/37°C. Mitochondrial damage was examined by flow cytometry (21).

**GSH/GSSG assay.** This was performed using a kit (Calbiochem, Darmstadt, Germany). SK-N-MC cells were treated at 25 μM for up to 24 h/37°C with Cu(II), Fe(III), Dp44mT, Cu[Dp44mT] or Fe[Dp44mT]₂. As a control, BSO (100 μM), was added as a glutathione synthesis inhibitor (22).

**Redox studies: Oxidation of H₂DCF.** Studies without cells were performed as described with H₂DCF (5 μM) (23). As a positive control, Fe(III) at 5 μM was
reduced to Fe(II) using cysteine (100 μM) in 150 mM acetate buffer (pH 5.0). Hydrogen peroxide (100 μM) was then added to initiate hydroxyl radical generation. To confirm hydroxyl radical production, DMSO (10% v/v) was used as it has been reported to be an effective hydroxyl radical scavenger (24, 25). Cu[DP44mT] and other reagents (at 5 μM, except DMSO at 10% v/v) were added to examine hydroxyl radical production. Intracellular oxidation of H2DCF studies were performed 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 $^{64}$Cu mobilization, but markedly induce $^{59}$Fe efflux.

To assess the anti-neoplastic mechanisms of the DpT analogues (Fig. 1), their ability to remove $^{64}$Cu from pre-labeled cells was compared to their interaction with $^{59}$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) (1). The DpT chelators, Dp4mT, Dp44mT, Dp4eT, Dp4aT and Dp4pT, were found to be ineffective at 25 μM at inducing $^{64}$Cu release from cells (leading to $^{64}$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 $^{59}$Fe, where the DpT chelators significantly ($p<0.001$) induced a >700% increase in $^{59}$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 $^{64}$Cu- or $^{59}$Fe-efflux (Fig. 2A and B). By design, Dp2mT cannot bind metals (6), demonstrating the importance of metal-binding for DpT chelator activity. The chelators DFO, PIH and 311 were included as positive controls to increase $^{59}$Fe efflux and we also examined their effects on $^{64}$Cu release. Only 311 showed a marked interaction with $^{64}$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 $^{64}$Cu efflux at 25 μM (Fig. 2A). However, the Cu chelator, TM, significantly ($p<0.05$) reduced $^{64}$Cu efflux to 76 ± 2% of the control. The low activity of the Cu chelators, d-pen, Trien and TM, at mobilizing $^{64}$Cu was unexpected based on 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 in order to provide a comparison to tumor cells, normal human umbilical vein endothelial cells (HUVECs; Fig. 2E and F). Generally, the active chelators accumulated $^{64}$Cu similarly across the three cell types (Fig. 2A-F). In terms of the effect of Dp44mT on inhibiting $^{64}$Cu release, its effect was greater in HUVECs and MCF-7 cells than SK-N-MC.

The kinetics of $^{64}$Cu and $^{59}$Fe release were assessed over a range of reincubation times (0.5-6 h) using SK-N-MC cells and HUVECs that were prelabeled with $^{64}$Cu or $^{59}$Fe-Tf for 3 h/37°C (Supplementary Fig. S1A-D). These results demonstrated that Dp44mT and 311 decreased $^{64}$Cu release relative to the control, while both ligands markedly increased $^{59}$Fe efflux as a function of time. In addition, dose-response curves demonstrated that 311 and Dp44mT decreased $^{64}$Cu release compared to the control, leading to $^{64}$Cu accumulation in cells (Supplementary Fig. S1E). With Dp44mT, this effect was reversible as its concentration increased >5 µM, at these higher concentrations, cellular damage results in the release of the Cu-Dp44mT complex from the cells. In contrast, at lower concentrations the cells are still intact preventing $^{64}$Cu release. In terms of $^{59}$Fe efflux, 311 and Dp44mT markedly increased cellular $^{59}$Fe release as a function of concentration (Supplementary Fig. S1F). Collectively, the active DpT group of chelators markedly prevented $^{64}$Cu efflux from all cell types, leading to $^{64}$Cu accumulation.

*Pre-complexation of $^{64}$Cu with 311 and the DpT ligands leads to intracellular accumulation of their $^{64}$Cu-complexes.* The results indicating intracellular accumulation of DpT-$^{64}$Cu complexes, were confirmed by studies where SK-N-MC cells were labeled for 3 h/37°C with pre-formed $^{64}$Cu-chelator complexes. The cells
were then washed and reincubated for 3 h/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, Dp4cT, Dp4aT and Dp4pT) and then reincubated with control medium, showed that 39-68% 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.

**Sub-cellular 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 h/37°C with ⁶⁴Cu, washed and reincubated for 3 h/37°C with Dp44mT (5 µM) 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 due to the poly-protic 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 performed. At pH 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 pH 5, this leads to accumulation of the ligand over time. This occurs due to the process of: (1) the neutral ligand entering the lysosome; (2) the ligand becoming protonated and charged at lysosomal pH (pH 5), preventing its passage out of the organelle; (3) the charged ligand binding copper in the lysosome; and (4) since 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 permeabilisation 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 (AO; Fig. 3D), that accumulates within lysosomes (18). High lysosomal concentrations of AO give a red fluorescence, while lower cytosolic and nuclear concentrations give a green fluorescence (18).

Examining control cells by fluorescence microscopy, a granular red fluorescence consistent with AO concentration in lysosomes was found (Fig. 3D) (18). However, incubation of cells with Cu[Dp44mT] (25 µM) for 0.5 h 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 µM), 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 h) used, which was optimal for detecting the effect of Cu[Dp44mT]. Indeed, Dp44mT and Fe[Dp44mT]₂ only induce significant cytotoxicity after 24 h (Fig. 6A). These data using fluorescence microscopy were
confirmed upon quantification by flow cytometry, where only the Cu[Dp44mT] complex (25 μM) 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 a lysosomal enzyme, cathepsin D (18), utilizing a fluorescent probe (peptatin A-BODIPY FL) that binds to cathepsin D (20). Control cells stained with Lysotracker® red and peptatin A-BODIPY FL showed a granular/vesicular pattern consistent with lysosomes (Fig. 4A). The overlay of Lysotracker® red and peptatin A-BODIPY FL-stained cells demonstrated co-localization. After a 0.5 h incubation with Cu[Dp44mT] (25 μM), 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 AO (Fig. 3D). Neither Cu(II) (as CuCl₂; 25 μM) or Dp44mT (25 μM) alone, had any significant effect on Lysotracker® red or cathepsin D staining (Fig. 4A).

To demonstrate 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 (27) (Fig. 4B). As a positive control, cells were incubated with cisplatin (20 mM), that induces Bid cleavage (27). SK-N-MC cells incubated with Cu[Dp44mT] (5 μM) or cisplatin (20 mM) for 2 h demonstrated 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 if mitochondria were also damaged. Mitochondrial stability was determined by measuring the reduction in mitochondrial membrane potential using TMRE (21), while LMP was assayed using AO via flow cytometry. As in Fig. 4C, the damage induced by Cu[Dp44mT] (5 μM) to lysosomes was significantly (p<0.05-0.001) more apparent than that to mitochondria after 1 or 2 h, indicating lysosomes were more sensitive to Cu[Dp44mT].

**Ability of the Cu- and Fe-Dp44mT complexes to generate ROS.** To understand why lysosomes were so sensitive to Cu[Dp44mT], we assessed if the Fe or Cu complexes of Dp44mT generate oxidative stress that could damage lysosomes. These studies in a cell-free system were performed under lysosomal-like conditions at pH 5.0 and in the presence of cysteine (28) (Fig. 5A and B). Oxidative stress was determined by the oxidation of non-fluorescent H$_2$DCF to fluorescent DCF, a well characterized probe for assessing redox stress (6, 23). The high redox activity of Cu[Dp44mT] (5 μM) was significantly (p<0.01) greater than CuCl$_2$, FeCl$_3$ and Fe[Dp44mT]$_2$ (all at 5 μM) after only 2 min (Fig. 5A). Addition of the Cu chelator TM (5 μM) to Cu[Dp44mT] (5 μM) totally prevented its activity, consistent with the ability of TM to bind Cu from Cu[Dp44mT] (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 H$_2$DCF. At 5 μM, Cu[Dp44mT] caused a significant (p<0.001) increase in intracellular H$_2$DCF oxidation to 273 ± 20% of that found for control cells after a 0.5 h incubation (Fig. 5C). No increase in H$_2$DCF oxidation was observed with Cu(II) (as CuCl$_2$), Fe(III) (as FeCl$_3$), Dp44mT
alone, or Fe[Dp44mT]$_2$; even at a five-fold higher concentration (25 µM; Fig. 5C). Collectively, these experiments demonstrate 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 physiological substrates, levels of the well-described indicator of oxidative stress, glutathione (GSH) and oxidized GSH (GSSG) (22), were determined. Over 0.5-24 h incubations with SK-N-MC cells, Cu[Dp44mT] (25 µM) significantly ($p<0.001$) reduced the GSH/GSSG ratio to 0-7% of the control (Fig. 5D), confirming that Cu[Dp44mT] possesses pronounced redox activity. In comparison, Dp44mT (25 µM), the GSH-synthesis inhibitor, BSO (100 µM), or Fe[Dp44mT]$_2$ (25 µM), also reduced the GSH/GSSG ratio to 4-20% of the control, but only after 24 h (Fig. 5D).

**Cell survival after exposure to Dp44mT and its Cu and Fe complexes.** The studies above indicate 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 h incubation, Cu[Dp44mT] (5 µM) significantly ($p<0.001$) decreased viability to 28 ± 4% of the control. In contrast, Dp44mT or Fe[Dp44mT]$_2$ (both at 5 µM) did not significantly decrease viability within 3 h, but showed activity at 24 h (Fig. 6A). These results demonstrate 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 h/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 μM, whereas the preformed Cu-TM (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 disulphonate (BCS), in identical studies and observed a similar, but less pronounced rescue effect on Dp44mT cytotoxicity (Fig. 6B). Like TM, BCS decreased Dp44mT cytotoxicity, while 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 h (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 anti-tumor efficacy, its Fe chelation efficacy is also important (6, 8).

**GSH levels modulate Cu[Dp44mT] cytotoxicity.** Since GSH plays an important role in buffering redox stress (22), and considering the marked redox activity of Cu[Dp44mT] (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 h incubation of SK-N-MC cells with NAC (5 mM) or BSO (100 μM) 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 mM) with Cu[Dp44mT] significantly (p<0.001) prevented the decrease in viability due to Cu[Dp44mT] after 3 h and 24 h (Fig. 6C) and similar results were observed as a function of concentration over 72 h (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 h (Fig. 6C, D), and thus, decreased their cytotoxicity. Further, morphological studies using AO also showed that NAC preserved lysosomal integrity in the presence of Cu[Dp44mT] (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 h (Fig. 6E). However, the addition of BSO with Cu[Dp44mT] (5 μM) 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 μM), BSO potentiated cytotoxicity of Dp44mT, Fe[Dp44mT]2 and Cu[Dp44mT] after 72 h (Fig. 6F). Hence, at low Cu[Dp44mT] concentrations (i.e., 0.02-0.25 μM; Fig. 6F), BSO clearly potentiated the cytotoxicity of this complex after 72 h, in contrast to higher Cu[Dp44mT] concentrations (i.e., 5 μM; Fig. 6E).
DISCUSSION

In this investigation, we demonstrated 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 physiological pH, Dp44mT is neutral and permeates cell membranes (6, 8). However, in the lysosomal compartment (pH~5) (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 demonstrated 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 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 pro-apoptotic form (Fig. 4B). Indeed, cathepsins can cleave Bid, which migrates to mitochondria and induces outer membrane permeabilization that is dependent on pro-apoptotic 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. 4D) 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 non-toxic 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 lysosomal damage caused by Cu[Dp44mT] was directly due to its redox activity. Based on 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 mono-allelic 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 show 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 demonstrates that targeting lysosomes can lead to potent and selective anti-cancer therapeutics. Knowledge of this mechanism can be used to design more potent cytotoxic agents that affect the lysosomal-apoptosis pathway.
REFERENCES


FIGURE LEGENDS

Fig. 1 Chelator Structures.

Fig. 2 DpT chelators cause significant cellular accumulation of $^{64}$Cu (A), but induce considerable $^{59}$Fe release (B) from pre-labeled SK-N-MC cells, (C,D) MCF-7 cells; and (E,F) HUVECs when incubated as described in Materials and Methods. Results are mean ± SD (3 experiments). * vs. control, $p<0.05$; ** vs. control, $p<0.01$; *** vs. control, $p<0.001$.

Fig. 3 (A) Complexation of DpT chelators with $^{64}$Cu leads to its cellular accumulation. Results represent intracellular $^{64}$Cu (% total $^{64}$Cu) expressed as mean ± SD (3 experiments). (B) Dp44mT causes $^{64}$Cu accumulation in a crude lysosome fraction. Results are mean ± SD (3 experiments). (C) Distribution of ionised species of Dp44mT as a function of pH. Results are typical of 3 experiments. (D) Cu[Dp44mT] (25 μM) disrupts lysosomal integrity after 0.5 h/37°C. Results are a typical of 3 experiments. Scale bar: 50 μm.

Fig. 4 (A) Cu[Dp44mT] (25 μM) causes redistribution of cathepsin D from lysosomes to the cytosol after a 0.5 h/37°C incubation with SK-N-MC cells. Scale bar: 50 μm. Representative images are from 3 experiments. (B) Cu[Dp44mT], but not Dp44mT, Fe[Dp44mT]2, CuCl₂ or FeCl₃, increases cleaved Bid in SK-N-MC cells. The blot is a typical experiment from 3, while the densitometry is mean ± SD (3 experiments). *** vs. control, $p<0.001$. (C) Graph showing lysosomal stability compared to mitochondrial stability after 0.5, 1 and 2 h/37°C in cells treated with Cu[Dp44mT] (5 μM). Lysosomal and mitochondrial stability was shown by staining with AO (20 μM).
or TMRE (20 nM), respectively, via flow cytometry. Results are mean ± SD (3 experiments). *lysosomes vs. mitochondria, $p<0.05$; *** lysosomes vs. mitochondria, $p<0.001$

**Fig. 5 (A)** Hydroxyl radical production by Cu[Dp44mT] relative to FeCl$_3$ and other reagents (at 5 μM, except DMSO which was 10% v/v) as shown by oxidation of non-fluorescent H$_2$DCF to fluorescent DCF *in vitro* (in solution) under lysosomal-like conditions (pH 5; 100 μM cysteine). **(B)** Effect of Cu[Dp44mT] (at 5 μM) on H$_2$DCF oxidation after 12 min in the presence and absence of various reagents, using the conditions in **(A)**. **(C)** Intracellular hydroxyl radical generation by Cu[Dp44mT] (5 μM) following a 0.5 h/37°C incubation as shown by flow cytotometry using DCF in SK-N-MC cells. **(D)** Incubation of SK-N-MC cells for 0.5, 1, 2 or 24 h/37°C with Dp44mT, Cu[Dp44mT], Fe[Dp44mT]$_2$, FeCl$_3$, CuCl$_2$ (all 25 μM) or the GSH-synthesis inhibitor, BSO (100 μM), reduces the GSH/GSSG ratio. Results are mean ± SD (6 experiments). *** vs. control, $p<0.001$

**Fig. 6 (A)** Cytotoxicity of Cu[Dp44mT] is far more rapid than that of Dp44mT or Fe[Dp44mT]$_2$ at 5 μM using SK-N-MC cells. **(B)** The non-toxic Cu chelators, tetramethylmolybdate (TM) and bathocuproine sulfonate (BCS), reduce Dp44mT cytotoxicity after 72 h, suggesting it is mediated by Cu-complexation. Results are mean ± SD (5 experiments). **(C)** The GSH precursor, N-acetylcysteine (NAC; 5 mM), attenuates cytotoxicity of Cu[Dp44mT] (5 μM) and at 72 h reduces Dp44mT (5 μM) cytotoxicity. **(D)** NAC (5 mM) reduces cytotoxicity of Dp44mT, Cu[Dp44mT] and Fe[Dp44mT]$_2$ after 72 h. **(E,F)** BSO (100 μM), potentiates cytotoxicity of Dp44mT,
Cu[Dp44mT] and Fe[Dp44mT]. The data in (F) are after a 72 h incubation. Results are mean ± SD (3 experiments).

**Fig. 7** Lysosomal targeting by Dp44mT. Due to its ionization properties, Dp44mT becomes trapped in acidic lysosomes and binds Cu to form a redox-active complex that causes LMP and subsequently apoptosis.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
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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