Increasing intracellular bioavailable copper selectively targets prostate cancer cells.

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Abstract

The therapeutic efficacy of two bis(thiosemicarbazoneato) copper complexes, glyoxalbis[N(4)-methylthiosemi-carbazonoato]Cu$^{II}$ [Cu$^{II}$(gtsm)] and diacetylbis[N4-methylthiosemicarbazonoato]Cu$^{II}$ [Cu$^{II}$(atsm)] for the treatment of prostate cancer was assessed in cell culture and animal models. Distinctively, copper dissociates intracellularly from Cu$^{II}$(gtsm) but is retained by Cu$^{II}$(atsm). We further demonstrated that intracellular H$_2$gtsm [reduced Cu$^{II}$(gtsm)] continues to redistribute copper into a bioavailable (exchangeable) pool. Both Cu$^{II}$(gtsm) and Cu$^{II}$(atsm) selectively kill transformed (hyperplastic and carcinoma) prostate cell lines, but importantly, do not affect the viability of primary prostate epithelial cells. Increasing extracellular copper concentrations enhanced the therapeutic capacity of both Cu$^{II}$(gtsm) and Cu$^{II}$(atsm) and their ligands (H$_2$gtsm and H$_2$atsm) were only toxic towards cancerous prostate cells when combined with copper. Treatment of the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model with Cu$^{II}$(gtsm) (2.5mg/kg) significantly reduced prostate cancer burden (~70%) and severity (grade), while treatment with Cu$^{II}$(atsm) (30mg/kg) was ineffective at the given dose. However, Cu$^{II}$(gtsm) caused mild kidney toxicity in the mice, associated primarily with interstitial nephritis and luminal distention. Mechanistically, we demonstrated that Cu$^{II}$(gtsm) inhibits proteasomal chymotrypsin-like activity, a feature further established as being common to copper-ionophores that increase intracellular bioavailable copper. We have demonstrated that increasing intracellular bioavailable copper can selectively kill cancerous prostate cells in vitro and in vivo and reveal the potential for bis(thiosemicarbazone) copper complexes to be developed as therapeutics for prostate cancer.
Introduction

Elevated intracellular copper levels predispose cancerous cells to ionophoric-copper sensitivity and several classes of copper coordinating lipophilic compounds (hydroxyquinolines, dithiocarbamates and thiosemicarbazones) are being investigated as potential anticancer therapeutics.\textsuperscript{1-4} Despite reports dating back to the 1970s, which demonstrate that cancerous cells harbor elevated copper\textsuperscript{1}, there is still insufficient information on how cellular transformation drives copper accumulation, or on the mechanism(s) by which cells adapt to tolerate the ensuing oxidative pressure (not mutually exclusive). Nonetheless, ionophoric-copper therapy may be applicable to a range of cancer types including prostate, breast, ovarian, cervical, lung, stomach, and leukaemia, where copper levels are intrinsically augmented (reviewed in\textsuperscript{1}).

In the context of cancer treatment, 5-chloro-7-iodo-8-quinolinol (clioquinol) is the most studied hydroxyquinoline analogue and is selectively toxic towards cancerous cell lines both \textit{in vitro}\textsuperscript{5-7} and \textit{in vivo}.\textsuperscript{6, 8} In preclinical models of hematologic malignancies clioquinol showed considerable therapeutic efficacy.\textsuperscript{5, 6} However, clioquinol failed to elicit pharmacodynamic or clinical activity in a recent human clinical trial in patients with refractory hematologic malignancies.\textsuperscript{3} Similarly, 1-(diethylthiocarbamoyldisulfanyl)-N,N-diethyl-methanethioamide (disulfiram), is selectively toxic towards cancerous cells both \textit{in vitro} and \textit{in vivo}.\textsuperscript{2, 9-11} A patient with metastatic ocular melanoma was successfully treated by disulfiram\textsuperscript{12} and a human clinical trial using disulfiram against recurrent prostate cancer is near completion (Id: NCT01118741). Clioquinol and disulfiram form neutral membrane permeable metal complexes and their anticancer activities are dependent upon their metal-ionophoric properties.\textsuperscript{11, 13-15} Both clioquinol and disulfiram transport copper into mammalian cells and consequently cause death of cancerous cells by inhibiting the proteasomal system and inducing apoptosis.\textsuperscript{5, 8-10, 13, 15, 16} The mechanistic basis of the selective toxicity toward cancerous cells has not been established.

Investigations into the anticancer activity of bis(thiosemicarbazones) began in the late 1950s, originating with the demonstration that glyoxal-bis(thiosemicarbazone) (H\textsubscript{2}gts) inhibited sarcoma 180 tumour growth in Swiss mice.\textsuperscript{17} Subsequently, other bis(thiosemicarbazones) analogues were found to similarly possess anticancer activities, reliant on either coordinated copper or zinc.\textsuperscript{18-20} However, preclinical pharmacological studies demonstrated hepatic toxicity and weight loss in animals treated with these compounds.\textsuperscript{21} Renewed interest in bis(thiosemicarbazones) copper
complexes has focused on developing less toxic analogues and to better understand their mechanisms of action.\textsuperscript{22, 23} Bis(thiosemicarbazone) ligands doubly deprotonate upon coordination with copper(II) to form neutral complexes, with the copper in a square planar N$_2$S$_2$ coordination geometry. Changes to the backbone of the ligand can dramatically alter the biological activity of copper complexes.\textsuperscript{17, 19} This subtle control of biological activity is exemplified by the differing cellular metabolism of glyoxalbis[N(4)-methylthiosemi-carbazonato]Cu$^{II}$ [Cu$^{II}$(gtsm)] and diacetylbis[N4-methylthiosemicarbazonato]Cu$^{II}$ [Cu$^{II}$(atsm)] (Figure 1A). Unlike Cu$^{II}$(atsm), Cu$^{II}$(gtsm) releases its coordinated copper intracellularly under normoxic conditions\textsuperscript{24} and importantly both analogues have been administered to mice at appreciable doses (10-30mg/kg/day for >6 weeks) without reported side effects.\textsuperscript{25, 26} Cu$^{II}$(gtsm) is being investigated as a therapeutic for Alzheimer’s disease\textsuperscript{25}, whilst Cu(atsm) has also shown potential as a therapy for Parkinson’s disease in animal models.\textsuperscript{26} In the present study we evaluated the therapeutic efficacy of both Cu$^{II}$(gtsm) and Cu$^{II}$(atsm) in the treatment of prostate cancer cells \textit{in vitro} and \textit{in vivo}, directly comparing their distinct chemistry and cell metabolism with their anticancer efficacy.
Results

Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm) selectively kill human prostate cancer in vitro.

In comparison to the Cu\textsuperscript{II}(gtsm) complex, the methyl group substituents on Cu\textsuperscript{II}(atsm) do not alter ionophoric capacity but lower the potential for coordinated divalent copper(II) to be reduced (Figure 1A) (Em=0.44 mV for Cu\textsuperscript{II}(gtsm) compared to Em=0.60 mV for Cu\textsuperscript{II}(atsm), versus SCE where Fc/Fc+=0.54 V).\textsuperscript{24} This difference in reduction potential results in Cu\textsuperscript{II}(atsm) retaining coordinated copper better under the reductive intracellular environment, whereas copper dissociates readily from Cu\textsuperscript{II}(gtsm) increasing intracellular bioavailable copper.\textsuperscript{24} Despite this difference, both Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm) proved to be efficient at killing human prostate carcinoma PC3 cells (~LD\textsubscript{50} of 1.5 µM and 7 µM, respectively) (Figure 1B), however, Cu\textsuperscript{II}(gtsm) was more potent. Cell viability was measured by propidium iodide (PI) dye exclusion and flow cytometry analysis. The addition of a physiological concentration of copper to the growth medium (20 µM CuCl\textsubscript{2}) enhanced their capacity to kill PC3 cells, especially for Cu\textsuperscript{II}(gtsm) (~LD\textsubscript{50} of 150 nM) (Figure 1B). Note: that standard growth medium contains <1 µM copper. When compared to clioquinol, a copper-ionophore recently evaluated in patients with advanced haematological malignancies (phase 1), Cu\textsuperscript{II}(gtsm) is more than 100-fold more efficient at killing PC3 cells in the presence of 20 µM copper (Figure 1B). Importantly, the metal-free ligands H\textsubscript{2}gtsm and H\textsubscript{2}atsm are not toxic unless combined with a physiological concentration of copper (20 µM CuCl\textsubscript{2}) (Figure 1C and Supplementary Figure 1). When the ligands are combined with either divalent zinc or iron and administered to PC3 cells there is no effect on cell viability or the percentage of cells in each phase of the cell cycle (Supplementary Figure 1A+B). The concentration of each metal used in this experiment represents the upper limit of their normal physiological range found in the serum of healthy individuals. The glyoxalbis[N(4)-methylthiosemi-carbazonato]Zn\textsuperscript{II} [Zn\textsuperscript{II}(gtsm)] complex likewise does not elicit toxicity towards PC3 cells even at high concentrations (32 µM) (Supplementary Figure 1C+D). Both Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm) complexes further kill human prostate hyperplastic and carcinoma cell lines with differences in p53 status, androgen receptor status and metastatic potential (Figure 1D). In contrast, human primary prostate epithelial cells (PrEC) remained refractory to Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm) treatment (Figure 1E). However, cell cycle analysis indicated that a high concentration of Cu\textsuperscript{II}(gtsm) (>64 µM), while not affecting viability per se (Figure 1E), cause G1 cell cycle arrest in PrECs (Figure 1F). Nevertheless, there is quite a large
therapeutic window for Cu\textsuperscript{II}(gtsm), with the upper range for drug dosage being at least 16 µM (Figure 1F). Cu\textsuperscript{II}(atsm) treatment did not alter the percentage of PC3 cells in each phase of the cell cycle, even when administered at a high concentration (64 µM) (Figure 1F). Together, these results demonstrate that Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm), despite having distinct chemistry and cell metabolism, can both utilize copper to selectively kill transformed (hyperplastic and carcinoma) prostate lines without effecting the viability of primary prostate cells, but Cu\textsuperscript{II}(gtsm) is more potent.

**Cu\textsuperscript{II}(gtsm) redistributes copper making it bioavailable.**

The role of extracellular (medium) copper in enhancing the toxicities of Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm) towards cancerous prostate cells (PC3) (Figure 1B) was further investigated through a competition assay using the copper-chelator tetrathiomolybdate (TTM) (Figure 2A). Cu\textsuperscript{II}(gtsm) when applied to cells alone (4 µM) (~LD80, Figure 1C) was to a small extent dependent on available extracellular copper (medium contains <1 µM copper) for toxicity (Figure 2A). Cu\textsuperscript{II}(gtsm) provided at a lower concentration (250 nM) in combination with a physiological concentration of copper (20 µM CuCl\textsubscript{2}) (~LD80, Figure 1C) was completely reliant on extracellular copper for toxicity (Figure 2A). Therefore, Cu\textsuperscript{II}(gtsm) uses extracellular copper to elicit and enhance the killing of cancerous prostate cells. In contrast, the level of extracellular copper played a minimal role in the toxicity of Cu\textsuperscript{II}(atsm) towards cancerous prostate cells (Figure 2A).

Both Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm) increased intracellular copper when applied to cells (Figure 2B), but distinctively, Cu\textsuperscript{II}(gtsm) undergoes intracellular reduction causing the dissociation of its coordinated copper thereby increasing bioavailable copper.\textsuperscript{24} We therefore investigated whether Cu\textsuperscript{II}(gtsm) can utilize extracellular copper to further increase the level of intracellular bioavailable copper. Intracellular dissociation of copper from Cu\textsuperscript{II}(gtsm) was examined using PC3 cells transfected with a metal-responsive element (MRE)-luciferase reporter, as previously described (Figure 2C).\textsuperscript{24,27} Briefly, an increase in bioavailable copper displaces zinc from endogenous metallothioneins, which consequently activates the metal-responsive transcription factor 1 (MTF-1) promoting the expression of genes that contain MREs in their promoter region.\textsuperscript{27} When PC3 cells transfected with the (MRE)-luciferase reporter were treated with Cu\textsuperscript{II}(gtsm) (500 nM) alone, there was a 3-fold induction of the reporter, indicating an increase in bioavailable intracellular copper (Figure 2C). In contrast, when Cu\textsuperscript{II}(atsm) was used at the same concentration (500 nM), or a
physiological level of copper (20 µM CuCl₂) was added, there was no reportable increase in bioavailable intracellular copper. Consistent with dose dependence, treatment with a lower concentration of Cu²⁺(gtsm) (125 nM) produced a more modest increase in bioavailable intracellular copper (only ~2-fold induction or reporter) (Figure 2D). However, when the same low concentration of Cu²⁺(gtsm) (125 nM) was combined with a physiological level of copper (20 µM CuCl₂) there was a remarkable rise (~5-fold) in bioavailable intracellular copper (Figure 2D). In contrast, the addition of copper had no impact on the inability of Cu²⁺(atsm) to dissociate copper intracellularly and activate the reporter (Figure 2D). Taken together, these results indicate that Cu²⁺(gtsm), but not Cu²⁺(atsm), can exploit extracellular copper to increase bioavailable intracellular copper. We next pre-treated PC3 cells with Cu²⁺(gtsm) (500 nM) for 1 hour, removed the treatment, and further cultured the cells with either fresh basal medium, or medium containing a physiological level of copper (20 µM CuCl₂), for 2 hours (Figure 2E). Thus, following pre-treatment extracellular Cu²⁺(gtsm) was removed leaving reduced H₂gtsm ligands inside the cells. The Cu²⁺(gtsm) pre-treatment in combination with the physiological level of copper (20 µM CuCl₂) profoundly raised bioavailable intracellular copper, as measured by a ~4-fold increase in (MRE)-luciferase reporter activity (Figure 2E). These results indicate that intracellular H₂gtsm [reduced Cu²⁺(gtsm)] can continue to redistribute copper from conventional homeostasis pathways and redistribute this copper into a bioavailable pool.

**Cu²⁺(gtsm) reduces prostate cancer burden and grade in TRAMP mice.**

The therapeutic utility of Cu²⁺(gtsm) and Cu²⁺(atsm) *in vivo* was appraised in the TRAMP transgenic prostate cancer mouse model. Heterozygous male TRAMP mice develop adenocarcinoma of the prostate, under control of the prostate-epithelial-specific probasin promoter driving expression of the SV40 large T Antigen. Disease progression in TRAMP mice is well defined, initiated with low-grade prostate intraepithelial neoplasia (PIN) (6-8 weeks), then proceeding through high-grade PIN (12-18 weeks) to prostate adenocarcinoma (~24 weeks).²⁸ Heterozygous male TRAMP mice 20 weeks of age were treated (oral gavage) daily with vehicle, Cu²⁺(gtsm) (2.5mg/kg/day) or Cu²⁺(atsm) (30mg/kg/day) for 28 days (Figure 3). Genitourinary (GU) tracts (includes prostate, seminal vesicles, testicles, and empty urinary bladder) were subsequently dissected and weighed as an established indicator
of prostate cancer burden.\textsuperscript{28} TRAMP mice treated with Cu\textsuperscript{II}(gtsm) displayed a significant reduction (~70\%) in the weight of their GU tracts, while Cu\textsuperscript{II}(atsm) had no effect at the given dose (Figure 3A). Note: that GU tract weights were normalized against the respective mouse weights. We next evaluated the effect of Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm) treatment on the grade of prostate disease (Figure 3B-D). We employed the scoring system developed by Suttie and colleagues (2003)\textsuperscript{29} and used histological assessment (blinded) of each prostate lobe [anterior prostate (AP), dorsal prostate (DP), lateral prostate (LP) and ventral prostate (VP)] to rank the severity of proliferative lesions (summarized in Supplementary Table 1). The most advanced proliferative lesion in each lobe signified the grade of disease (Figure 3B). In TRAMP mice treated with Cu\textsuperscript{II}(gtsm) there was a significant reduction in the severity of prostate lesions in every lobe (Figure 3B&C). A representative image of the mean histological lesion seen in each lobe for both vehicle and Cu\textsuperscript{II}(gtsm) treated mice is shown (Figure 3C). Consistent with Cu\textsuperscript{II}(atsm) treatment having no effect on tumour burden (Figure 3A), it likewise did not change the severity of prostate disease (Supplementary Figure 3). These data were further adjusted to take into account the degree of lesion distribution throughout the prostate lobes, as previously described by Suttie and colleagues (2003)\textsuperscript{29} (summarized in Supplementary Table 2). The distribution-adjusted (DA) lesion grade in each prostate lobe was also significantly reduced in TRAMP mice treated with Cu\textsuperscript{II}(gtsm) (Figure 3D), but not in mice treated with Cu\textsuperscript{II}(atsm) (Supplementary Figure 3). We estimated the serum levels of Cu\textsuperscript{II}(gtsm) in mice dosed with 2.5mg/kg using the MRE-luciferase bioavailable copper assay in PC3 cells (Figure 3E). The concentration of Cu\textsuperscript{II}(gtsm) exceeded 1.5 µM 1 hour after administration and was mostly cleared from serum after 16 hours. Copper-chelation by TTM abrogated the capacity of Cu\textsuperscript{II}(gtsm)-containing serum to activate the MRE-luciferase reporter assay (Figure 3E), suggesting that indeed Cu\textsuperscript{II}(gtsm) continues to coordinate copper and no other divalent metal (e.g. zinc) \textit{in vivo}. Taken together, these results demonstrate that increasing bioavailable copper through administration of Cu\textsuperscript{II}(gtsm) significantly reduces prostate cancer burden and severity in the TRAMP mouse model.

\textbf{Cu\textsuperscript{II}(gtsm) treatment causes micro-renal toxicity.} TRAMP mice treated with Cu\textsuperscript{II}(gtsm) for the 28 days displayed on average an ~2.5g (~8.7\%) weight loss in comparison to the vehicle treated mice (Figure 4A). These mice
however, displayed no signs of discomfort and actually appeared healthier and more active than those in the other treatment groups. There was no significant weight change in mice treated with Cu$^{II}$(atsm) (Figure 4A). To distinguish if the Cu$^{II}$(gtsm)-induced weight loss was related to the TRAMP genetic background, we repeated the treatment regime using wtC57BL/6 mice (Figure 4B). Again wtC57BL/6 mice treated with Cu$^{II}$(gtsm) (2.5mg/kg/day) displayed weight loss (~2.35 g, 8.3%), indicating an associated toxicity. Post-mortem examination revealed no morphological or color change to any vital organ throughout the mice cohorts. However, further histological examination revealed that all mice treated with Cu$^{II}$(gtsm) displayed kidney toxicity, involving multifocal non-suppurative interstitial nephritis of moderate severity with mild luminal distention and occasional tubular epithelial degeneration/necrosis (Figure 4C (i)+(ii)). There was no apparent toxicity associated with Cu$^{II}$(atsm) treatment.

**Ionophores that increase bioavailable copper inhibit proteasomal activity.**

Lastly, we investigated the mechanism behind Cu$^{II}$(gtsm) toxicity towards cancerous prostate cells (PC3) (Figure 5). Two copper-ionophores, clioquinol and disulfiram, previously were shown to be selectively toxic towards malignant cells and are known proteasome inhibitors. Specifically, they are inhibitors of chymotrypsin-like activity.$^{8, 9, 15}$ Analogous to Cu$^{II}$(gtsm), we demonstrated that clioquinol and disulfiram cause prostate cancer cell death (Figure 5A) and also increase intracellular bioavailable copper (Figure 5B). Note: that in the viability assay, for all three copper-ionophores, Annexin-V positive and PI negative cell populations existed (not shown), indicative of early apoptotic cells. We hypothesized that copper-ionophores that increase intracellular bioavailable copper may commonly cause proteasomal inhibition. To determine whether Cu$^{II}$(gtsm) inhibits the proteasome we initially investigated its effects on the expression and subcellular localization of the X-linked inhibitor of apoptosis protein (XIAP). We and other groups have previously demonstrated that inhibition of chymotrypsin-like activity causes cytoplasmic XIAP to relocate to the nucleus, thereby permitting caspase activation and apoptosis to occur.$^{15, 30, 31}$ In cells treated for 5 hours with Cu$^{II}$(gtsm) (1.5 µM) alone, or at a low concentration (150 nM) in combination with a physiological level of copper (20 µM CuCl$_2$), XIAP levels were decreased in the cytoplasmic fraction and were correspondingly increased in the nuclear-enriched fraction (Figure 5C), indicative of proteasome inhibition. No such response was observed with Cu$^{II}$(atsm) treatments (Figure 5C). We further
demonstrated using a fluorogenic LLVY-7-Amino-4-methylcoumarin (AMC) based assay, that Cu\textsuperscript{II}(gtsm) (16 µM) treatment for 1.5 hours was sufficient to inhibit intracellular proteasomal chymotrypsin-like activity (Figure 5D). This effect was specific to the Cu\textsuperscript{II}(gtsm) complex as the proteasomal activity was not inhibited by Cu\textsuperscript{II}(atsm) (16 µM), gtsm, or atsm free ligands (16 µM), or with a physiological level of copper (20 µM CuCl\textsubscript{2}) (Figure 5D).
Discussion

Differences in backbone substituents on bis(thiosemicarbazonato) copper(II) complexes dictate their cellular metabolism and can dramatically change their biological activity. Earlier studies demonstrated that 2-keto-3-ethoxybutyaldehydebis[thiosemicarbazonato]Cu\(^{II}\) [Cu\(^{II}\)(kts)] dissociates copper intracellularly and then the ligand diffuses back out of the cell\(^{32}\). Copper likewise dissociates from Cu\(^{II}\)(gtsm) following cellular uptake\(^{24}\), however, the ligand (H\(_2\)gtsm) can further redistribute copper and continually reallocate it into an exchangeable (bioavailable) pool (Figure 2E). This property renders Cu\(^{II}\)(gtsm) particularly toxic towards prostate cancerous cells that contain predisposing elevated intracellular copper concentrations (Figure 1B,D).\(^{15}\) Further, it explains how increasing extracellular copper enhances Cu\(^{II}\)(gtsm) toxicity, as additional copper internalized by cells and ordinarily chaperoned is subjected to H\(_2\)gtsm-mediated redistribution (Figure 1B). Addition of two methyl groups to the bis(thiosemicarbazone) ligand backbone [Cu\(^{II}\)(atsm)] renders the complex more resistant to intracellular copper dissociation\(^{24}\) (Figure 2B-D) and reduced overall anticancer activity (Figure 1B). Thus, even small modifications to bis(thiosemicarbazones) copper complexes can dramatically change their cell metabolism and anticancer properties. Previously, we used clioquinol as a proof-of-principle compound to demonstrate that cancerous prostate cells are exquisitely sensitive to ionophoric-copper.\(^{15}\) Here we show that the therapeutic efficacy of Cu\(^{II}\)(gtsm) is far superior to clioquinol (>100-fold) (Figure 1B,5A). Cu\(^{II}\)(gtsm) killed cancerous prostate cells in vitro (Figure 1B) and significantly reduced prostate cancer burden and severity (grade) in the TRAMP mouse model (Figure 3A-D).

Pharmacokinetic analyses performed in mice confirmed that Cu\(^{II}\)(gtsm) does not exchange its coordinated copper with other divalent metals in vivo (Figure 3E). However, the anticancer activity of Cu\(^{II}\)(gtsm) and similar copper-ionophores may involve other metals. We monitored intracellular bioavailable copper indirectly using the MRE-luciferase reporter construct, which is based on copper displacing zinc from endogenous metallothioneins.\(^{27}\) It is plausible that the anticancer activity of Cu\(^{II}\)(gtsm) could therefore involve additional toxic effects from liberated zinc, or conceivably from other displaced intracellular metals. However, while zinc does play a role in apoptosis there are reports suggesting that an increase in bioavailable intracellular zinc can actually suppress cell death.\(^{33, 34}\) Consistent with these reports, Zn\(^{II}\)(gtsm) had no affect on PC3 cell viability even at high concentrations (32 µM) (Supplementary Figure
1). Other ionophores can induce apoptosis by targeting zinc, or copper, to lysosomes.35, 36 The lysosomal-apoptotic pathway may likewise be selectively activated in cancerous cells, with ionophores that presumably retain their coordinated metal (zinc or copper). Three chemically distinct copper-ionophores [Cu(II)(gtsm), clioquinol, and disulfiram] that commonly release their coordinated copper under the reductive intracellular environment (Figure 5B), all inhibit proteasomal chymotrypsin-like activity (Figure 5C+D).35, 36 However, the anticancer activity of clioquinol did not correlate well with its ability to increase bioavailable copper (Figure 5A+B). This may reflect interplay between different modes of inducing cell death, as clioquinol has strong affinities for several additional metals including zinc and iron, unlike H2gtsm, which has relatively weak affinities for these metals. The anticancer activities of both Cu(II)(gtsm) and disulfiram paralleled with their ability to increase bioavailable copper (LD50 = 4 to 5-fold increase) (Figure 5A,B).

There is mounting interest in conventional proteasome inhibitors as anticancer therapeutics. Bortezomib (Velcade) produced significant clinical responses in patients (30%) with relapsed multiple myeloma.37 Multiple myeloma cells produce more mutated/misfolded proteins and are consequently more dependent on proteasomal activity in comparison to normal cells.38 More recently, the proteasome inhibitor Carfilzomib has shown even greater therapeutic efficacy against multiple myeloma in human clinical trials.39 Unfortunately, the use of these agents is restricted to several hematologic malignancies. Copper-ionophore therapy may be applicable to a greater range of cancer types, including prostate, breast, ovarian, cervical, lung, stomach, and haematological malignancies, where copper levels are significantly elevated.1 Using copper-ionophores may also offer enhanced selectivity towards cancerous cells over conventional proteasome inhibitors, through targeting elevated intracellular copper.

Cu(II)(gtsm) had previously been administered (oral gavage) to C3H/HeJ background mice (20-weeks old) at 10mg/kg/day for 6 weeks without reported side effects.25 In C57BL/6 background mice (wt and TRAMP) treated with only 2.5mg/kg/day for 4 weeks, we observed weight loss possibly associated with mild kidney toxicity (Figure 4). Tolerance to copper-ionophores can vary between mice strains as previously observed.7 It is also important to note that renal clearance of therapeutics can vary considerably from rodents to humans. Chemical modification of the ligand is possible and this could reduce side effects whilst maintaining biological activity. Another approach to minimize side effects is to tether a biologically active
molecule to the complex that specifically targets it to cancerous cells. Bis(thiosemicarbazone) compounds conjugated to the cancer targeting peptide bombesin(7-14)-NH2 (BBN) have recently been developed. The receptors for BBN are over-expressed in a number of cancers, including prostate cancer.

In conclusion, we now have the necessary tools to better categorize the different types of copper-ionophores and define the biological features ideal for their anticancer activity. We demonstrated that increasing intracellular bioavailable copper destroys prostate cancer in vitro and in the TRAMP mouse model. Moreover, copper-ionophores offer a degree of therapeutic selectivity towards prostate cancer cells not seen with current therapy options. With recent advances in metallomics we are now in an excellent position to develop bis(thiosemicarbazones) as anticancer therapeutics.
Methods

Cell lines and reagents

Human prostate hyperplastic (BPH-1), carcinoma (PC3, Du145 and LNCaP) and primary epithelial cells (PrECs) were cultured as previously described.\textsuperscript{15} 5-chloro-7-iodo-8-quinolinol (Clioquinol), tetrathiomolybdate (TTM) and carboxymethlcellulose were purchased from Sigma Aldrich. Diacetylbis[N4-methylthiosemicarbazone]Cu\textsuperscript{II} [Cu\textsuperscript{II}(atsm)], glyoxalbis[N(4)-methylthiosemi-carbazone]Cu\textsuperscript{II} [Cu\textsuperscript{II}(gtsm)] and glyoxalbis[N(4)-methylthiosemi-carbazone]Zn\textsuperscript{II} [Zn\textsuperscript{II}(gtsm)] and respective metal-free ligands were synthesized following published procedures.\textsuperscript{40} All ionophores and chelators were dissolved in DMSO to a concentration of 5 mM.

Cell viability

Prostate hyperplastic (BPH-1) and carcinoma (PC3, Du145 and LNCaP) cells were seeded (3 X 10\textsuperscript{5} cells) separately into 24-well plates and cultured for 6-8 h to allow adhesion. Human primary prostate epithelial cells (PrEC) were seeded at 1.5 X 10\textsuperscript{5} into 24-well plates and cultured for 6-8 h. Cells were treated as outlined in the Results section. Cell viability was measured by propidium iodide (Merck) dye exclusion assay and flow cytometry analysis (BD FACSCantotm II), as previously described.\textsuperscript{15} To determine annexinV/PI double positive cells, cells were prepared as outlined above, then 100,000 cells were incubated in 50 µl of binding buffer [10 mM Hepes (pH 7.4), 140 mM NaCl, 5 mM CaCl\textsubscript{2}] containing 0.5 µg/ml (0.74 µM) propidium iodide and AnnexinV-APC conjugated Antibody (diluted 1/100) (BD Pharmigen 550475). Cells were incubation at room temperature for 15 min and then 150 µl of binding buffer was added to each sample. The proportion of cells double positive for AnnexinV and PI staining was ascertained using flow cytometry analysis (BD FACSCantotm II).

Western blot analysis

Prostate carcinoma PC3 cells seeded (2 X 10\textsuperscript{5} cells) into 24-well plates were cultured for 16 h to allow adhesion. Cells were treated as outlined in the Results. Western blot analysis was performed as previously described\textsuperscript{15}.

TRAMP mice experiments

Housing and experimental protocols were reviewed and approved by the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee (AEEC). TRAMP
mice in pure C57BL/6 background originated from Norman Greenberg (Fred Hutchinson Cancer Research Centre, Seattle, WA). Heterozygous male TRAMP mice were obtained from founder homozygous males crossed with wtC57BL/6 females. Genotype was confirmed by PCR-based screening of tail tips as previously described\(^2\), using the forward primer 5'-CCGGTCGACCGGAAGCTTCCACAAGTGCATTTA-3' and reverse primer 5'-CTCCTTTCAAGACCTAGAAGGTCCA-3'. Primers against β-casein were used as an internal PCR control (forward: 5'-GATGTGCTCCAGGCTAAAGTT-3' and reverse: 5'-AGAAACGGAATGTGTTGGAGT-3'). 20-week old heterozygous TRAMP mice were randomly assigned to either vehicle alone (0.9% w/v NaCl, 0.5% w/v carboxymethylcellulose, 0.5% v/v benzyl alcohol, 0.4% v/v tween-80), Cu\(^{2+}\)(gtsm) (2.5mg/kg/day) or Cu\(^{2+}\)(atsm) (30mg/kg/day) groups. Administration was daily through oral gavage (20-gauge bulb-tipped feeding needle) for 28 days. Genitourinary (GU) tracts were then removed, weighed and formalin-fixed before further dissected for lobe histological examination (see below). All GU tract weights were normalized against respective mouse weights. Vital organs, including those involved in copper homeostasis (liver and kidney), were also dissected and analyzed by histological examination.

**Histological examination**

Dissected tissues were fixed in 10% neutral buffered formalin at 4°C for 16-24 h, before being transferred into 70% ethanol and paraffin embedded. Formalin-fixed, paraffin-embedded (FFPE) tissue sections were stained with hematoxylin and eosin (H&E) at the Microscopy and Histology Core Facility at Peter MacCallum Cancer Centre. Individual prostate lobes were dissected from fixed GUT tissue prior to paraffin embedding, to avoid physically damaging the lobes. Multiple step sections of the prostate lobes were stained with H&E and assessed through histological examination (n=7 slides per mouse) using the TRAMP model scoring system previously described\(^2\) and summarized in Supplementary Table 1. The most aggressive histological score observed in each lobe was used to grade each lobe.

**Statistical Analyses**

Statistical analyses were performed using two-tailed unpaired t tests. The means of at least triplicate determinations for each test condition with standard deviations (±S.D.)
were used for comparisons.

The following methods are provided in the Supplementary Information; Cell cycle analysis; Proteasome activity assay; Copper analysis and MRE-luciferase bioavailable copper assay.
Acknowledgments

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Supporting Information

Supplementary methods, results and tables. This material is available free of charge via the Internet at http://pubs.acs.org
References


Figure 1. Cu^{II}(gtsm) and Cu^{II}(atsm) select selectively kill human prostate cancer in vitro. 

(A) Schematic of Cu^{II}(gtsm) and Cu^{II}(atsm) copper-ionophores entering the reductive environment of the cell. (B) Cu^{II}(gtsm) and Cu^{II}(atsm) effectively kill human prostate cancer cells. PC3 cells were treated for 16 hours with either Cu^{II}(gtsm), Cu^{II}(atsm) or clioquinol alone or in combination with 20 µM CuCl$_2$. Ionophore concentrations are shown. Cell viability was determined by the propidium iodide dye-exclusion viability assay. (C) Cu^{II}(gtsm) and Cu^{II}(atsm) require copper for toxicity. PC3 cells were treated for 16 hours with either H$_2$gtsm or H$_2$atsm ligands (metal-free) alone or in combination with 20 µM CuCl$_2$. Ionophore concentrations are shown. Cell viability was determined by the propidium iodide dye-exclusion viability assay. (D) Cu^{II}(gtsm) and Cu^{II}(atsm) kill a variety of human prostate hyperplastic and carcinoma cell lines. Human hyperplastic (BPH-1) and carcinoma (PC3, Du145 and LNCaP) prostate cells were treated for 16 hours with either Cu^{II}(gtsm) or Cu^{II}(atsm) alone or in combination with 20 µM CuCl$_2$. Ionophore concentrations are shown. Cell viability was determined by the propidium iodide dye-exclusion viability assay. (E) Cu^{II}(gtsm) and Cu^{II}(atsm) do not effect viability of primary prostate epithelial cells. PrECs were treated for 16 hours with either Cu^{II}(gtsm) or Cu^{II}(atsm) alone or in combination with 20 µM CuCl$_2$. Ionophore concentrations are shown. Cell viability was determined by the propidium iodide dye-exclusion viability assay. (F) Cell cycle analysis predicts a large therapeutic window for both Cu^{II}(gtsm) and Cu^{II}(atsm). PrECs were treated for 16 hours with either Cu^{II}(gtsm) or Cu^{II}(atsm). Ionophore concentrations are shown. The percentages of cells in each cell cycle phase were analyzed by propidium iodide dye and flow cytometric analysis.
Figure 2. Cu\textsuperscript{II}(gtsm) redistributes intracellular copper. (A) Cu\textsuperscript{II}(gtsm) recruits extracellular copper to enhance toxicity towards cancerous prostate cells. PC3 cells were treated for 16 hours with Cu\textsuperscript{II}(gtsm) or Cu\textsuperscript{II}(atsm) alone or in combination with 20 µM CuCl\textsubscript{2} and/or 20 µM TTM. Ionophore concentrations are shown. Cell viability was determined by the propidium iodide dye-exclusion viability assay. (B) Cu\textsuperscript{II}(gtsm) and Cu\textsuperscript{II}(atsm) increase total intracellular copper. PC3 cells were incubated for 3 hours with either basal medium (control), 20 µM CuCl\textsubscript{2}, Cu\textsuperscript{II}(gtsm) (500 nM) or Cu\textsuperscript{II}(atsm) (500 nM). Total intracellular copper was measured by ICP-MS. (C) Cu\textsuperscript{II}(gtsm) and not Cu\textsuperscript{II}(atsm) increase bioavailable intracellular copper. PC3 cells transfected with a metal-responsive element (MRE)-luciferase reporter constructs, were treated for 3 hours with either with 20 µM CuCl\textsubscript{2}, Cu\textsuperscript{II}(gtsm) or Cu\textsuperscript{II}(atsm). Ionophore concentrations are shown. Firefly and Renilla luciferase activities were measured, normalized and expressed as fold-induction relative to no-treatment control. (D) Cu\textsuperscript{II}(gtsm) and not Cu\textsuperscript{II}(atsm) can exploit extracellular copper to increase bioavailable intracellular copper. PC3 cells transfected with a metal-responsive element (MRE)-luciferase reporter, were treated for 3 hours with either Cu\textsuperscript{II}(gtsm) or Cu\textsuperscript{II}(atsm) alone or in combination with 20 µM CuCl\textsubscript{2}. Ionophore concentrations are shown. Firefly and Renilla luciferase activities were measured, normalized and expressed as fold-induction relative to no-treatment control. (E) Cu\textsuperscript{II}(gtsm) redistributes intracellular copper making it bioavailable. PC3 cells transfected with a metal-responsive element (MRE)-luciferase reporter were pre-treated for 1 hour with either basal medium (control), or Cu\textsuperscript{II}(gtsm) (500 nM), then were further cultured for 2 hours with basal medium or medium containing 20 µM CuCl\textsubscript{2}. As further controls, cells were treated with either Cu\textsuperscript{II}(gtsm) (500 nM), or 200 µM CuCl\textsubscript{2}, for the full 3 hours. Firefly and Renilla luciferase activities were measured, normalized and expressed as fold-induction relative to no-treatment control.
Figure 3. Cu\textsuperscript{II}(gtsm) reduces prostate cancer burden and grade in TRAMP mice.
(A) Cu\textsuperscript{II}(gtsm) significantly reduced the weight (burden) of prostate cancer in TRAMP mice. 20-week old heterozygous TRAMP mice were gavaged with either vehicle, Cu\textsuperscript{II}(gtsm) or Cu\textsuperscript{II}(atsm) daily for 28 days. Genitourinary (GU) tracts were dissected and weighted as an indicator of prostate cancer burden. Control GU tract weights from wtC57BL/6 mice are also shown. Ionophore dose and mouse numbers for each treatment group are shown. (B) Cu\textsuperscript{II}(gtsm) significantly reduced prostate cancer severity in the TRAMP mice. Prostate disease in the above vehicle and Cu\textsuperscript{II}(gtsm) treated mice was evaluated. H&E-stained sections were analyzed and the most advanced proliferative lesion in each prostate lobe was used to grade disease severity for each mouse. (C) Representative H&E-stained sections displaying mean grade of prostate disease for each lobe for both the vehicle and Cu\textsuperscript{II}(gtsm) treated mice. Images are at 20x magnification. (D) Cu\textsuperscript{II}(gtsm) significantly reduced the distribution-adjusted (DA) lesion grade in each prostate lobe in TRAMP mice. Grade data presented in (B) was further adjusted to take into account the degree of lesion distribution throughout the prostate lobes, as summarized in Supplementary Table 2. (E) Serum Cu\textsuperscript{II}(gtsm) evaluation in mice orally gavaged with 2.5mg/kg. Subsequent to 20-week old male wtC57BL/6 mice being administered 2.5mg/kg of Cu\textsuperscript{II}(gtsm), serum was isolated at the indicated time-points and applied to PC3 cells, and the intracellular bioavailable copper was measured using the (MRE)-luciferase reporter assay. Addition of TTM demonstrated the dependence on copper for activation of luciferase by serum containing Cu\textsuperscript{II}(gtsm) (1h time-point only). To estimate the serum Cu\textsuperscript{II}(gtsm) concentration, normal mouse serum was spiked with Cu\textsuperscript{II}(gtsm) at the concentrations shown. (*p<0.05; **p<0.01; ***p<0.001).
Figure 4. Cu\textsuperscript{II}(gtsm) causes weight loss and renal toxicity in C57BL/6 mice. (A) Cu\textsuperscript{II}(gtsm) significantly reduced the weight of TRAMP mice. 20-week old heterozygous TRAMP mice were gavaged with either vehicle, Cu\textsuperscript{II}(gtsm) or Cu\textsuperscript{II}(atsm) daily for 28 days. The weights of the mice were compared after 28 days. (B) Cu\textsuperscript{II}(gtsm) significantly reduced the weight of wtC57BL/6 mice. 20-week old wtC57BL/6 mice were gavaged with Cu\textsuperscript{II}(gtsm) for 28 days. The weights of the mice were compared before and after treatment. (C) Cu\textsuperscript{II}(gtsm) causes kidney toxicity. (i) Representative H&E-stained sections of kidneys from TRAMP mice orally gavaged with either vehicle or Cu\textsuperscript{II}(gtsm) for 28 days. Magnifications are shown. (ii) Representative H&E-stained sections of kidney from wtC57BL/6 mice orally gavaged with Cu\textsuperscript{II}(gtsm) for 28 days. Magnifications are shown. (*p<0.05; **p<0.01; ***p<0.001).
Figure 5. Bioavailable intracellular copper inhibits proteasomal activity. (A) Cu\textsuperscript{II}(gtsm), clioquinol and disulfiram induce apoptotic cell death. PC3 cells treated for 16 hours with either Cu\textsuperscript{II}(gtsm), clioquinol or disulfiram in combination with 20 µM CuCl\textsubscript{2}. Ionophore concentrations are shown. Annexin-V and propidium iodide double staining identified dead cells. (B) Clioquinol and disulfiram increase bioavailable intracellular copper. PC3 cells transfected with a metal-responsive element (MRE)-luciferase reporter constructs, were treated for 3 hours with either with Cu\textsuperscript{II}(gtsm), clioquinol or disulfiram in combination with 20 µM CuCl\textsubscript{2}. Ionophore concentrations are shown. Firefly and Renilla luciferase activities were measured, normalized and expressed as fold-induction relative to no-treatment control. (C) Cu\textsuperscript{II}(gtsm) induces the cytoplasmic clearance of XIAP, indicative of proteasome inhibition. PC3 cells were treated for 5 hours with either Cu\textsuperscript{II}(gtsm) or Cu\textsuperscript{II}(atsm) alone or in combination with 20 µM CuCl\textsubscript{2}. Ionophore concentrations are shown. Cytoplasmic and nuclear enriched fractions were analyzed by western blotting. Endogenous XIAP, Histone H2B (nuclear fraction control) and anti-β-tubulin III (cytoplasmic fraction control), were all immunolabelled as described in the Methods section. (D) Cu\textsuperscript{II}(gtsm) inhibits intracellular proteasomal chymotrypsin-like activity. PC3 cells were treated for 1.5 hours with either Cu\textsuperscript{II}(gtsm), Cu\textsuperscript{II}(atsm), H\textsubscript{2}gtsm or H\textsubscript{2}atsm ligand, alone or in combination with 20 µM CuCl\textsubscript{2}. The proteasome inhibitor epoxomicin and 20 µM CuCl\textsubscript{2} were used as controls. Ionophore concentrations are shown. Conversion of the fluorogenic substrate specific for chymotrypsin-like activity (Suc-LLVY-AMC) was used to measure intracellular proteasomal activity.
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