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# Increasing Intracellular Bioavailable Copper Selectively Targets Prostate Cancer Cells

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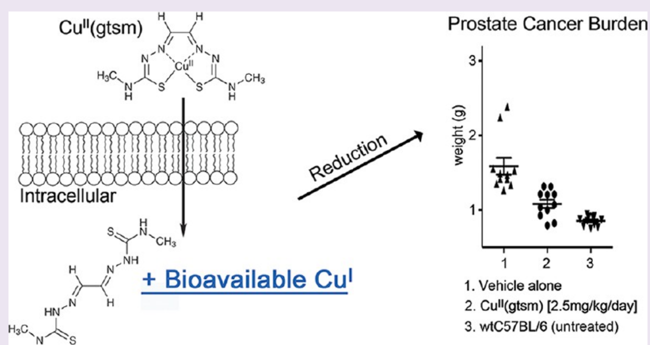
*Supporting Information*

**ABSTRACT:** The therapeutic efficacy of two bis(thiosemicarbazonato) copper complexes, glyoxalbis[*N*4-methylthiosemicarbazonato]$Cu^{II}$ [$Cu^{II}$(gtsm)] and diacetylbis-[*N*4-methylthiosemicarbazonato]$Cu^{II}$ [$Cu^{II}$(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 toxic only toward cancerous prostate cells when combined with copper. Treatment of the Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) model with $Cu^{II}$(gtsm) (2.5 mg/kg) significantly reduced prostate cancer burden (~70%) and severity (grade), while treatment with $Cu^{II}$(atsm) (30 mg/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 have revealed the potential for bis(thiosemicarbazone) copper complexes to be developed as therapeutics for prostate cancer.




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.[1−4] Despite reports dating back to the 1970s, which demonstrate that cancerous cells harbor elevated copper,[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 leukemia, where copper levels are intrinsically augmented (reviewed in ref 1).

In the context of cancer treatment, 5-chloro-7-iodo-8-quinolinol (clioquinol) is the most studied hydroxyquinoline analogue and is selectively toxic toward cancerous cell lines both *in vitro*[5−7] and *in vivo*.[6,8] In preclinical models of hematologic malignancies clioquinol showed considerable therapeutic efficacy.[5,6] However, clioquinol failed to elicit pharmacodynamic or clinical activity in a recent human clinical trial in patients with refractory hematologic malignancies.[3] Similarly, 1-(diethylthiocarbamoyldisulfanyl)-*N*,*N*-diethyl-methanethioamide (disulfiram) is selectively toxic toward

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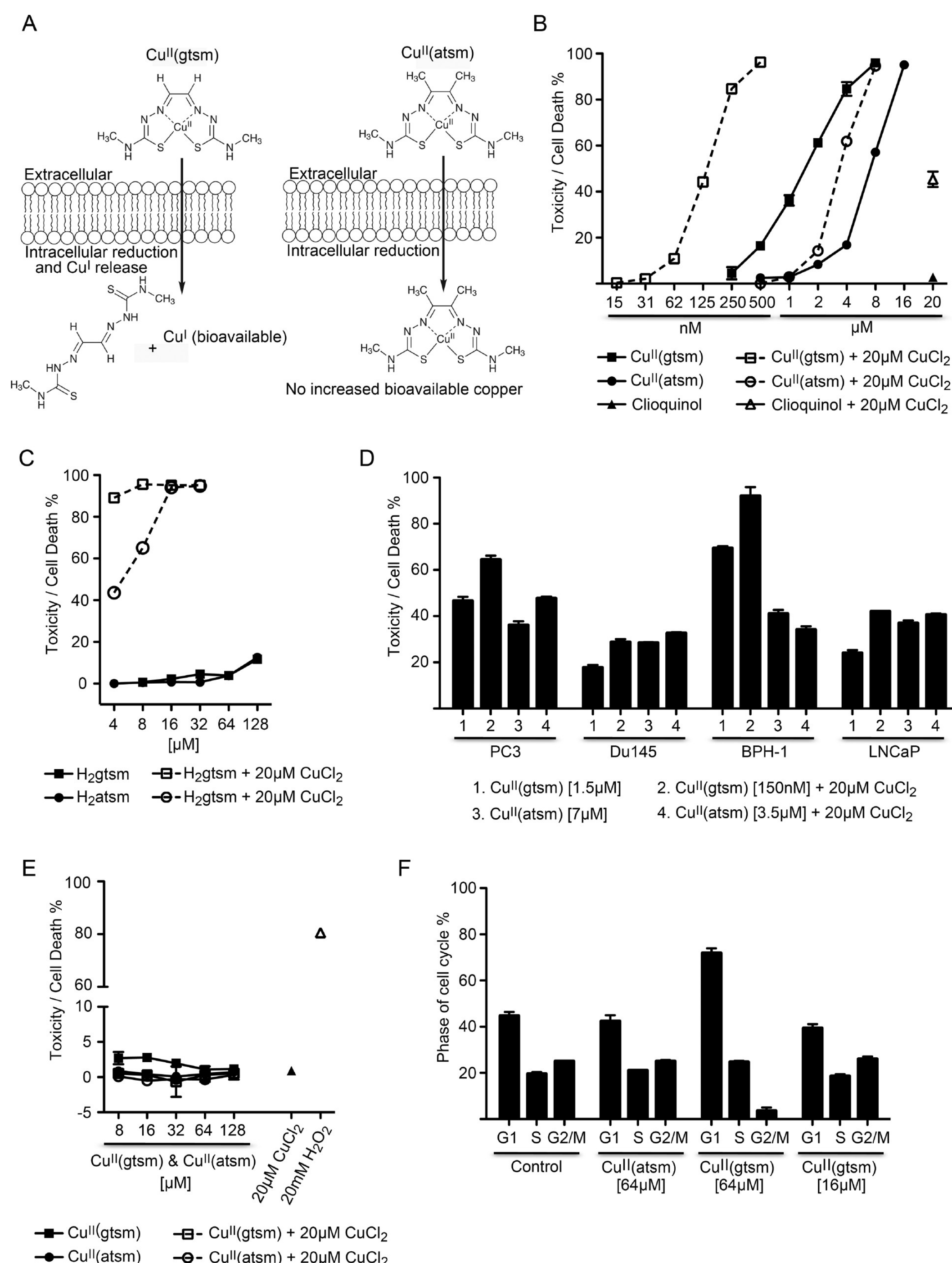


**Figure 1.** $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) selectively kill human prostate cancer *in vitro*. (A) Schematic of $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) copper-ionophores entering the reductive environment of the cell. (B) $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) effectively kill human prostate cancer cells. PC3 cells were treated for 16 h with either $Cu^{II}$(gtsm), $Cu^{II}$(atsm), or clioquinol alone or in combination with 20 $\mu$M $CuCl_2$. 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 h with either $H_2$gtsm or $H_2$atsm ligands (metal-free) alone or in combination with 20 $\mu$M $CuCl_2$. 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 h with either $Cu^{II}$(gtsm) or $Cu^{II}$(atsm) alone or in combination with 20 $\mu$M $CuCl_2$. 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 h with either $Cu^{II}$(gtsm) or $Cu^{II}$(atsm) alone or in combination with 20 $\mu$M $CuCl_2$. 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 h 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.

cancerous cells both *in vitro* and *in vivo*.[2,9−11] A patient with metastatic ocular melanoma was successfully treated by disulfiram,[12] and a human clinical trial using disulfiram against recurrent prostate cancer is near completion (Id:

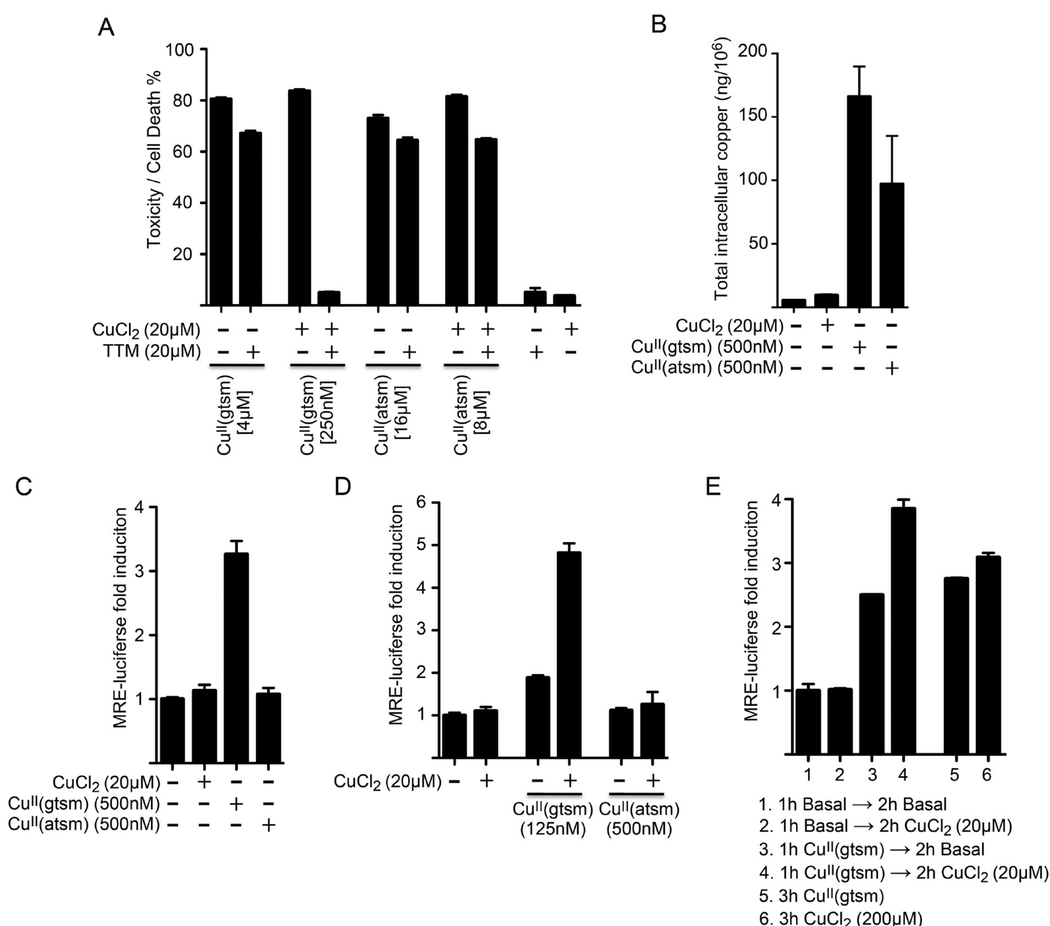


**Figure 2.** $Cu^{II}$(gtsm) redistributes intracellular copper. (A) $Cu^{II}$(gtsm) recruits extracellular copper to enhance toxicity toward cancerous prostate cells. PC3 cells were treated for 16 h with $Cu^{II}$(gtsm) or $Cu^{II}$(atsm) alone or in combination with 20 $\mu$M $CuCl_2$ and/or 20 $\mu$M TTM. Ionophore concentrations are shown. Cell viability was determined by the propidium iodide dye-exclusion viability assay. (B) $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) increase total intracellular copper. PC3 cells were incubated for 3 h with either basal medium (control), 20 $\mu$M $CuCl_2$, $Cu^{II}$(gtsm) (500 nM) or $Cu^{II}$(atsm) (500 nM). Total intracellular copper was measured by ICP-MS. (C) $Cu^{II}$(gtsm) and not $Cu^{II}$(atsm) increases bioavailable intracellular copper. PC3 cells transfected with a metal-responsive element (MRE)-luciferase reporter constructs were treated for 3 h with either 20 $\mu$M $CuCl_2$, $Cu^{II}$(gtsm) or $Cu^{II}$(atsm). Ionophore concentrations are shown. Firefly and *Renilla* luciferase activities were measured, normalized, and expressed as fold-induction relative to no-treatment control. (D) $Cu^{II}$(gtsm) and not $Cu^{II}$(atsm) can exploit extracellular copper to increase bioavailable intracellular copper. PC3 cells transfected with a MRE-luciferase reporter were treated for 3 h with either $Cu^{II}$(gtsm) or $Cu^{II}$(atsm) alone or in combination with 20 $\mu$M $CuCl_2$. Ionophore concentrations are shown. Firefly and *Renilla* luciferase activities were measured, normalized, and expressed as fold-induction relative to no-treatment control. (E) $Cu^{II}$(gtsm) redistributes intracellular copper, making it bioavailable. PC3 cells transfected with a MRE-luciferase reporter were pretreated for 1 h with either basal medium (control) or $Cu^{II}$(gtsm) (500 nM) and then were further cultured for 2 h with basal medium or medium containing 20 $\mu$M $CuCl_2$. As further controls, cells were treated with either $Cu^{II}$(gtsm) (500 nM, or 200 $\mu$M $CuCl_2$ for the full 3 h. Firefly and *Renilla* luciferase activities were measured, normalized, and expressed as fold-induction relative to no-treatment control.

NCT01118741). Clioquinol and disulfiram form neutral membrane-permeable metal complexes, and their anticancer activities are dependent upon their metal-ionophoric properties.[11,13−15] Both clioquinol and disulfiram transport copper into mammalian cells and consequently cause the death of cancerous cells by inhibiting the proteasomal system and inducing apoptosis.[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 glyoxalbis(thiosemicarbazone) ($H_2$gts) inhibited sarcoma 180 tumor growth in Swiss mice.[17] Subsequently, other bis(thiosemicarbazone) analogues were found to similarly possess anticancer activities, reliant on either coordinated copper or zinc.[18−20] However, preclinical pharmacological studies demonstrated hepatic toxicity and weight loss in animals treated with these compounds.[21] Renewed interest in bis(thiosemicarbazone) copper complexes has focused on developing less toxic analogues and better understanding of their mechanisms of action.[22,23] Bis(thiosemicarbazone) ligands doubly deprotonate upon coordination with copper(II) to form neutral complexes, with the copper in a square planar $N_2S_2$ coordination geometry. Changes to the backbone of the ligand can dramatically alter the biological activity of copper complexes.[17,19] This subtle control of biological activity is exemplified by the differing cellular metabolism of glyoxalbis[*N*4-methylthiosemicarbazonato]$Cu^{II}$ [$Cu^{II}$(gtsm)] and diacetylbis[*N*4-methylthiosemicarbazonato]$Cu^{II}$ [$Cu^{II}$(atsm)] (Figure 1A). Unlike $Cu^{II}$(atsm), $Cu^{II}$(gtsm) releases its coordinated copper intracellularly under normoxic conditions,[24] and importantly,

both analogues have been administered to mice at appreciable doses (10–30 mg/kg/day for >6 weeks) without reported side effects.[25,26] $Cu^{II}$(gtsm) is being investigated as a therapeutic for Alzheimer's disease,[25] while $Cu^{II}$(atsm) has also shown potential as a therapy for Parkinson's disease in animal models.[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 *in vitro* and *in vivo*, directly comparing their distinct chemistry and cell metabolism with their anticancer efficacy.

## ■ RESULTS AND DISCUSSION

**$Cu^{II}$(gtsm) and $Cu^{II}$(atsm) Selectively Kill Human Prostate Cancer in Vitro.** In comparison to the $Cu^{II}$(gtsm) complex, the methyl group substituents on $Cu^{II}$(atsm) do not alter ionophoric capacity but lower the potential for coordinated divalent copper(II) to be reduced (Figure 1A) ($E_m$ = 0.44 mV for $Cu^{II}$(gtsm) compared to $E_m$ = 0.60 mV for $Cu^{II}$(atsm), versus SCE where $Fc/Fc^+$ = 0.54 V).[24] This difference in reduction potential results in $Cu^{II}$(atsm) retaining coordinated copper better under the reductive intracellular environment, whereas copper dissociates readily from $Cu^{II}$(gtsm), increasing intracellular bioavailable copper.[24] Despite this difference, both $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) proved to be efficient at killing human prostate carcinoma PC3 cells ( approx $LD_{50}$ of 1.5 and 7 $\mu$M, respectively) (Figure 1B); however, $Cu^{II}$(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 $\mu$M $CuCl_2$) enhanced their capacity to kill PC3 cells, especially for $Cu^{II}$(gtsm) (approx $LD_{50}$ of 150 nM) (Figure 1B). Note: standard growth medium contains <1 $\mu$M copper. When compared to clioquinol, a copper-ionophore recently evaluated in patients with advanced hematological malignancies (phase 1), $Cu^{II}$(gtsm) is more than 100-fold more efficient at killing PC3 cells in the presence of 20 $\mu$M copper (Figure 1B). Importantly, the metal-free ligands $H_2$gtsm and $H_2$atsm are not toxic unless combined with a physiological concentration of copper (20 $\mu$M $CuCl_2$) (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 and 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-methylthiosemicarbazonato]$Zn^{II}$ [$Zn^{II}$(gtsm)] complex likewise does not elicit toxicity toward PC3 cells even at high concentrations (32 $\mu$M) (Supplementary Figure 1C and D). Both $Cu^{II}$(gtsm) and $Cu^{II}$(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 $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) treatment (Figure 1E). However, cell cycle analysis indicated that a high concentration of $Cu^{II}$(gtsm) (>64 $\mu$M), 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 $Cu^{II}$(gtsm), with the upper range for drug dosage being at least 16 $\mu$M (Figure 1F). $Cu^{II}$(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 $\mu$M) (Figure 1F). Together, these results demonstrate that $Cu^{II}$(gtsm) and $Cu^{II}$(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 $Cu^{II}$(gtsm) is more potent.

**$Cu^{II}$(gtsm) Redistributes Copper, Making It Bioavailable.** The role of extracellular (medium) copper in enhancing the toxicities of $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) toward cancerous prostate cells (PC3) (Figure 1B) was further investigated through a competition assay using the copper-chelator tetrathiomolybdate (TTM) (Figure 2A). $Cu^{II}$(gtsm) when applied to cells alone (4 $\mu$M) ( approx $LD_{80}$, Figure 1C) was to a small extent dependent on available extracellular copper (medium contains <1 $\mu$M copper) for toxicity (Figure 2A). $Cu^{II}$(gtsm) provided at a lower concentration (250 nM) in combination with a physiological concentration of copper (20 $\mu$M $CuCl_2$) (approx $LD_{80}$, Figure 1C) was completely reliant on extracellular copper for toxicity (Figure 2A). Therefore, $Cu^{II}$(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 $Cu^{II}$(atsm) toward cancerous prostate cells (Figure 2A).

Both $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) increased intracellular copper when applied to cells (Figure 2B), but distinctively, $Cu^{II}$(gtsm) undergoes intracellular reduction, causing the dissociation of its coordinated copper and thereby increasing bioavailable copper.[24] We therefore investigated whether $Cu^{II}$(gtsm) can utilize extracellular copper to further increase the level of intracellular bioavailable copper. Intracellular dissociation of copper from $Cu^{II}$(gtsm) was examined using PC3 cells transfected with a metal-responsive element (MRE)-luciferase reporter, as previously described (Figure 2C).[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 promotor region.[27] When PC3 cells transfected with the MRE-luciferase reporter were treated with $Cu^{II}$(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 $Cu^{II}$(atsm) was used at the same concentration (500 nM) or a physiological level of copper (20 $\mu$M $CuCl_2$) was added, there was no reportable increase in bioavailable intracellular copper. Consistent with dose dependence, treatment with a lower concentration of $Cu^{II}$(gtsm) (125 nM) produced a more modest increase in bioavailable intracellular copper (only ~2-fold induction of reporter) (Figure 2D). However, when the same low concentration of $Cu^{II}$(gtsm) (125 nM) was combined with a physiological level of copper (20 $\mu$M $CuCl_2$), 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 $Cu^{II}$(atsm) to dissociate copper intracellularly and activate the reporter (Figure 2D). Taken together, these results indicate that $Cu^{II}$(gtsm), but not $Cu^{II}$(atsm), can exploit extracellular copper to increase bioavailable intracellular copper. We next pretreated PC3 cells with $Cu^{II}$(gtsm) (500 nM) for 1 h, removed the treatment, and further cultured the cells with either fresh basal medium or medium containing a physiological level of copper (20 $\mu$M $CuCl_2$), for 2 h (Figure 2E). Thus, following pretreatment extracellular $Cu^{II}$(gtsm) was removed, leaving reduced $H_2$gtsm ligands inside the cells. The $Cu^{II}$(gtsm) pretreatment in

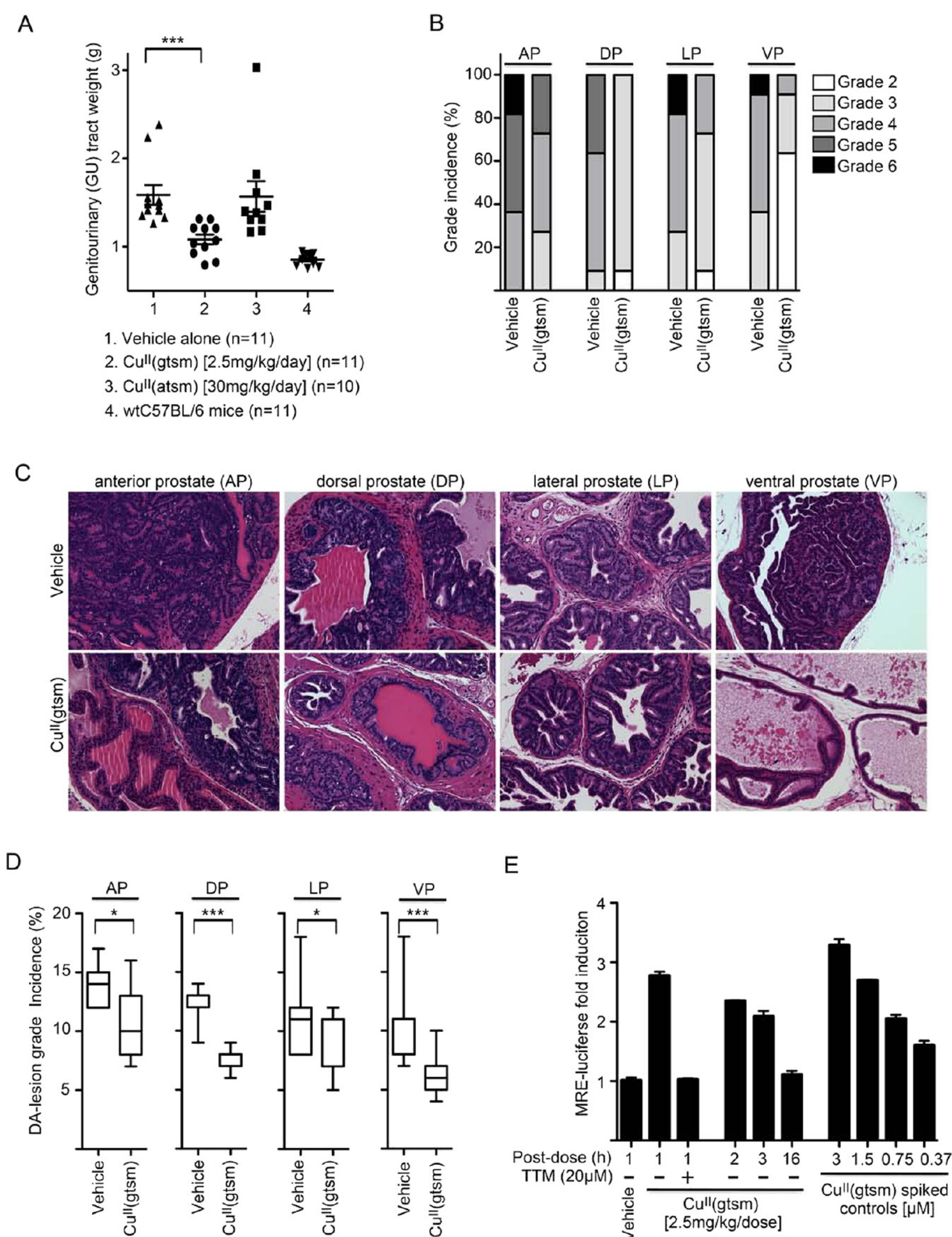


**Figure 3.** $Cu^{II}$(gtsm) reduces prostate cancer burden and grade in TRAMP mice. (A) $Cu^{II}$(gtsm) significantly reduced the weight (burden) of prostate cancer in TRAMP mice. Twenty-week-old heterozygous TRAMP mice were gavaged with either vehicle, $Cu^{II}$(gtsm), or $Cu^{II}$(atsm) daily for 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) $Cu^{II}$(gtsm) significantly reduced prostate cancer severity in the TRAMP mice. Prostate disease in the above vehicle and $Cu^{II}$(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 $Cu^{II}$(gtsm)-treated mice. Images are at 20× magnification. (D) $Cu^{II}$(gtsm) significantly reduced the distribution-adjusted (DA) lesion grade in each prostate lobe in TRAMP mice. Grade data presented in panel 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 $Cu^{II}$(gtsm) evaluation in mice orally gavaged with 2.5 mg/kg. Subsequent to 20-week-old male wtC57BL/6 mice being administered 2.5 mg/kg of $Cu^{II}$(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 $Cu^{II}$(gtsm) (1 h time point only). To estimate the serum $Cu^{II}$(gtsm) concentration, normal mouse serum was spiked with $Cu^{II}$(gtsm) at the concentrations shown. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

combination with the physiological level of copper (20 μM $CuCl_2$) profoundly raised bioavailable intracellular copper, as measured by a ∼4-fold increase in MRE-luciferase reporter activity (Figure 2E). These results indicate that intracellular

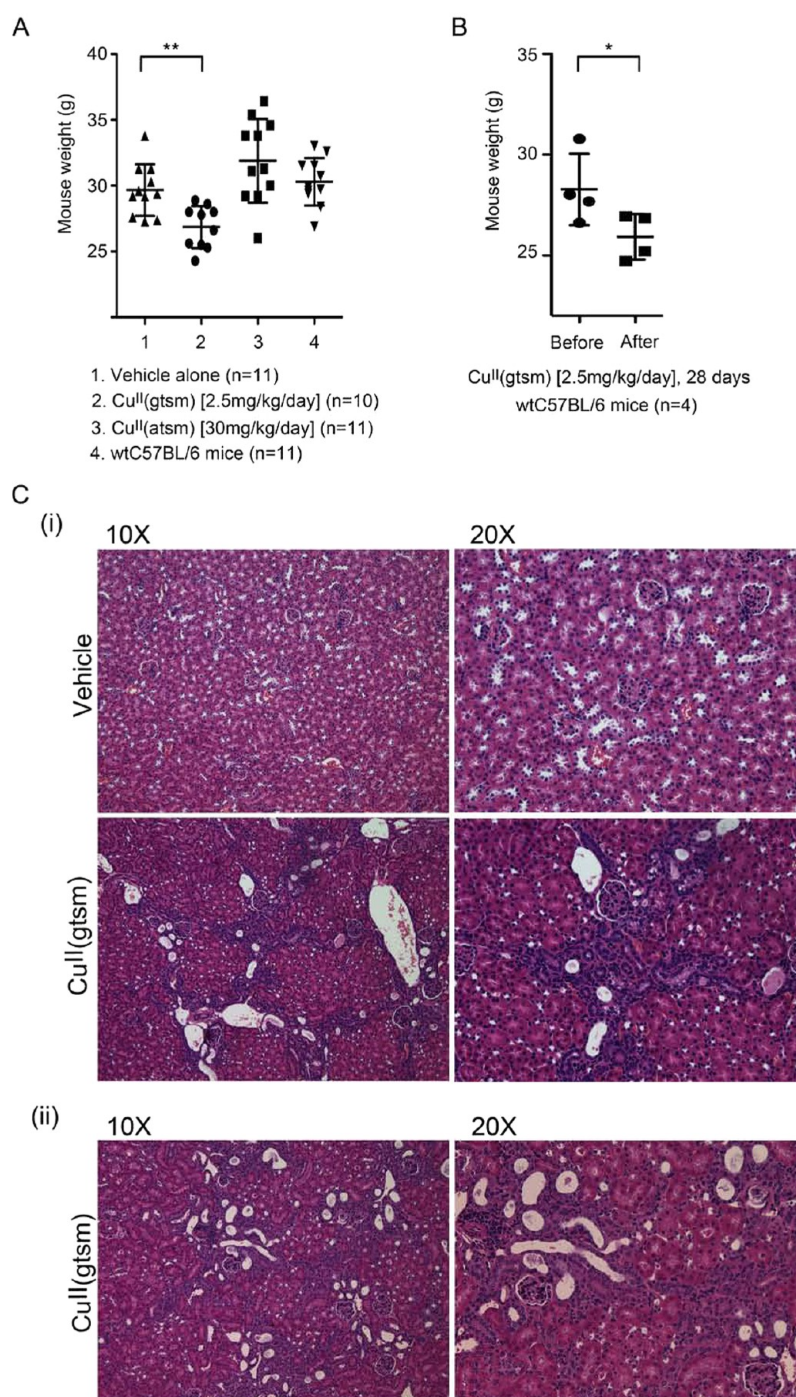


**Figure 4.** Cu$^{II}$(gtsm) causes weight loss and renal toxicity in C57BL/6 mice. (A) Cu$^{II}$(gtsm) significantly reduced the weight of TRAMP mice. Twenty-week-old heterozygous TRAMP mice were gavaged with either vehicle, Cu$^{II}$(gtsm), or Cu$^{II}$(atsm) daily for 28 days. The weights of the mice were compared after 28 days. (B) Cu$^{II}$(gtsm) significantly reduced the weight of wtC57BL/6 mice. Twenty-week-old wtC57BL/6 mice were gavaged with Cu$^{II}$(gtsm) for 28 days. The weights of the mice were compared before and after treatment. (C) Cu$^{II}$(gtsm) causes kidney toxicity. (i) Representative H&E-stained sections of kidneys from TRAMP mice orally gavaged with either vehicle or Cu$^{II}$(gtsm) for 28 days. Magnifications are shown. (ii) Representative H&E-stained sections of kidney from wtC57BL/6 mice orally gavaged with Cu$^{II}$(gtsm) for 28 days. Magnifications are shown. (*$p$ < 0.05; **$p$ < 0.01; ***$p$ < 0.001)

$H_2$gtsm [reduced Cu$^{II}$(gtsm)] can continue to redistribute copper from conventional homeostasis pathways and redistribute this copper into a bioavailable pool.

**Cu$^{II}$(gtsm) Reduces Prostate Cancer Burden and Grade in TRAMP Mice.** The therapeutic utility of Cu$^{II}$(gtsm) and Cu$^{II}$(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

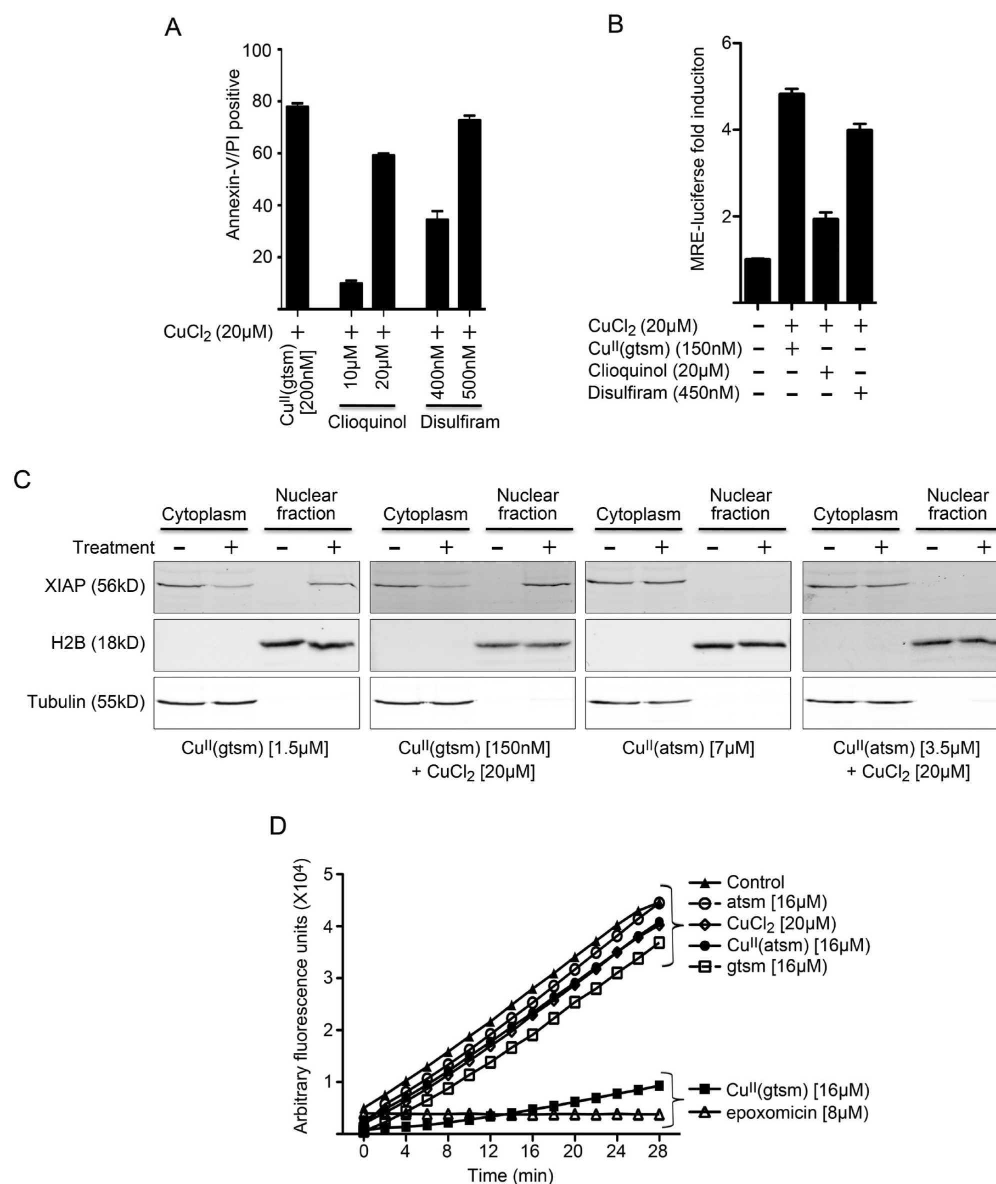


**Figure 5.** Bioavailable intracellular copper inhibits proteasomal activity. (A) $Cu^{II}$(gtsm), clioquinol, and disulfiram induce apoptotic cell death. PC3 cells treated for 16 h with either $Cu^{II}$(gtsm), clioquinol, or disulfiram in combination with 20 $\mu$M $CuCl_2$. Ionophore concentrations 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 MRE-luciferase reporter construct were treated for 3 h with either with $Cu^{II}$(gtsm), clioquinol, or disulfiram in combination with 20 $\mu$M $CuCl_2$. Ionophore concentrations are shown. Firefly and *Renilla* luciferase activities were measured, normalized, and expressed as fold-induction relative to no-treatment control. (C) $Cu^{II}$(gtsm) induces the cytoplasmic clearance of XIAP, indicative of proteasome inhibition. PC3 cells were treated for 5 h with either $Cu^{II}$(gtsm) or $Cu^{II}$(atsm) alone or in combination with 20 $\mu$M $CuCl_2$. Ionophore concentrations are shown. Cytoplasmic and nuclear enriched fractions were analyzed by Western blotting. Endogenous XIAP, Histone H2B (nuclear fraction control), and anti-$\beta$-tubulin III (cytoplasmic fraction control) were all immunolabeled as described in the Methods section. (D) $Cu^{II}$(gtsm) inhibits intracellular proteasomal chymotrypsin-like activity. PC3 cells were treated for 1.5 h with either $Cu^{II}$(gtsm), $Cu^{II}$(atsm), $H_2$gtsm, or $H_2$atsm, alone or in combination with 20 $\mu$M $CuCl_2$. The proteasome inhibitor epoxomicin and 20 $\mu$M $CuCl_2$ 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.

mice 20 weeks of age were treated (oral gavage) daily with vehicle, $Cu^{II}$(gtsm) (2.5 mg/kg/day) or $Cu^{II}$(atsm) (30 mg/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.[28] TRAMP mice treated with $Cu^{II}$(gtsm) displayed a significant reduction (~70%) in the weight of their GU tracts, while $Cu^{II}$(atsm) had no effect at the given dose (Figure 3A). Note: GU tract weights were normalized against the respective mouse weights. We next evaluated the effect of $Cu^{II}$(gtsm) and $Cu^{II}$(atsm) treatment on the grade of prostate disease (Figure 3B–D). We employed the scoring system developed by Suttie and colleagues (2003)[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


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each lobe signified the grade of disease (Figure 3B). In TRAMP mice treated with $Cu^{II}$(gtsm) there was a significant reduction in the severity of prostate lesions in every lobe (Figure 3B and C). A representative image of the mean histological lesion seen in each lobe for both vehicle- and $Cu^{II}$(gtsm)-treated mice is shown (Figure 3C). Consistent with $Cu^{II}$(atsm) treatment having no effect on tumor burden (Figure 3A), it likewise did not decrease 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)[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 $Cu^{II}$(gtsm) (Figure 3D), but not in mice treated with $Cu^{II}$(atsm) (Supplementary Figure 3). We estimated the serum levels of $Cu^{II}$(gtsm) in mice dosed with 2.5 mg/kg using the MRE-luciferase bioavailable copper assay in PC3 cells (Figure 3E). The concentration of $Cu^{II}$(gtsm) exceeded 1.5 $\mu$M 1 h after administration and was mostly cleared from serum after 16 h. Copper-chelation by TTM abrogated the capacity of $Cu^{II}$(gtsm)-containing serum to activate the MRE-luciferase reporter assay (Figure 3E), suggesting that indeed $Cu^{II}$(gtsm) continues to coordinate copper and no other divalent metal (e.g., zinc) *in vivo*. Taken together, these results demonstrate that increasing bioavailable copper through administration of $Cu^{II}$(gtsm) significantly reduces prostate cancer burden and severity in the TRAMP mouse model.

**$Cu^{II}$(gtsm) Treatment Causes Microrenal Toxicity.** TRAMP mice treated with $Cu^{II}$(gtsm) for the 28 days displayed on average a ~2.5 g (~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) (2.5 mg/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 nonsuppurative interstitial nephritis of moderate severity with mild luminal distention and occasional tubular epithelial degeneration/necrosis (Figure 4Ci and 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 toward cancerous prostate cells (PC3) (Figure 5). Two copper-ionophores, clioquinol and disulfiram, previously were shown to be selectively toxic toward 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: 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 h with $Cu^{II}$(gtsm) (1.5 $\mu$M) alone or at a low concentration (150 nM) in combination with a physiological level of copper (20 $\mu$M $CuCl_2$), 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 $Cu^{II}$(gtsm) (16 $\mu$M) treatment for 1.5 h was sufficient to inhibit intracellular proteasomal chymotrypsin-like activity (Figure 5D). This effect was specific to the $Cu^{II}$(gtsm) complex as intracellular proteasomal activity was not inhibited by $Cu^{II}$(atsm) (16 $\mu$M), $H_2$gtsm, or $H_2$atsm free ligands (16 $\mu$M) or with a physiological level of copper (20 $\mu$M $CuCl_2$) (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-ethoxybutyraldehydebis[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 toward prostate cancerous cells that contain predisposing elevated intracellular copper concentrations (Figure 1B and 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(thiosemicarbazone) 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 that of clioquinol (>100-fold) (Figures 1B and 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 $\mu$M) (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 and D).[35,36] However, the anticancer activity of clioquinol did not correlate well with its ability to increase bioavailable copper (Figure 5A and 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 $H_2$gtsm, which has relatively weak affinities for these metals. The anticancer activities of both $Cu^{II}$(gtsm) and disulfiram paralleled their ability to increase bioavailable copper ($LD_{50}$ = 4- to 5-fold increase) (Figure 5A and 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 hematological malignancies, where copper levels are significantly elevated.[1] Using copper-ionophores may also offer enhanced selectivity toward 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 10 mg/kg/day for 6 weeks without reported side effects.[25] In C57BL/6 background mice (wt and TRAMP) treated with only 2.5 mg/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 while 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)-$NH_2$ (BBN) have recently been developed.[23] The receptors for BBN are overexpressed 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 toward 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.[15] 5-Chloro-7-iodo-8-quinolinol (Clioquinol), tetrathiomolybdate (TTM), and carboxymethylcellulose were purchased from Sigma Aldrich. Diacetylbis[*N*4-methylthiosemicarbazonato]$Cu^{II}$ [$Cu^{II}$(atsm)], glyoxalbis[*N*(4)-methylthiosemicarbazonato]$Cu^{II}$ [$Cu^{II}$(gtsm)], glyoxalbis[*N*(4)-methylthiosemicarbazonato]$Zn^{II}$ [$Zn^{II}$(gtsm)], and respective metal-free ligands were synthesized following published procedures.[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 ($3 \times 10^5$ cells) separately into 24-well plates and cultured for 6−8 h to allow adhesion. Human primary prostate epithelial cells (PrEC) were seeded at $1.5 \times 10^5$ into 24-well plates and cultured for 6−8 h. Cells were treated as outlined in the Results and Discusion section. Cell viability was measured by propidium iodide (Merck) dye exclusion assay and flow cytometry analysis (BD FACSCantoII), as previously described.[15] To determine annexinV/PI double positive cells, cells were prepared as outlined above, and then 100,000 cells were incubated in 50 $\mu$L of binding buffer [10 mM Hepes (pH 7.4), 140 mM NaCl, 5 mM $CaCl_2$)] containing 0.5 $\mu$g/mL (0.74 $\mu$M) propidium iodide and AnnexinV-APC conjugated antibody (diluted 1/100) (BD Pharmigen 550475). Cells were incubated at RT for 15 min, and then 150 $\mu$L 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 FACSCantoII).

**Western Blot Analysis.** Prostate carcinoma PC3 cells seeded ($2 \times 10^5$ 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.[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,[28] using the forward primer 5′-CCGGTCGACCGGAAGCTTCCACAAGTGCATTTA-3′ and reverse primer 5′-CTCCTTTCAAGACCTAGAAGGTCCA-3′. Primers against $\beta$-casein were used as an internal PCR control (forward: 5′-GATGTGCTCCAGGCTAAAGTT-3′ and reverse: 5′-AGAAACGGAATGTTGTGGAGT-3′). Twenty-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), $Cu^{II}$(gtsm) (2.5 mg/kg/day) or $Cu^{II}$(atsm) (30 mg/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 being 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,[29] and results are 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 (± SD) were used for comparisons.

The following methods are provided in the Supporting Information: cell cycle analysis; proteasome activity assay; copper analysis; and MRE-luciferase bioavailable copper assay.

## ■ ASSOCIATED CONTENT

### Supporting Information

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

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### Notes

The authors declare no competing financial interest.

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