A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

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Accepted Article

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To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201814519
Angew. Chem. 10.1002/ange.201814519

Link to VoR: http://dx.doi.org/10.1002/anie.201814519
http://dx.doi.org/10.1002/ange.201814519

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10.1002/anie.201814519

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COMMUNICATION

Universal Anticancer $Cu(DTC)_2$ Discriminates Between Thiols and Zinc(II) Thiolates Oxidatively

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Abstract: Aerobic organisms must rely on abundant intracellular thiols to reductively protect various vital functional units, especially ubiquitous zinc(II)-thiolate sites of proteins, from deleterious oxidations due to oxidizing environments. Here we disclose the first well-defined model study for reactions between zinc(II)-thiolate complexes and copper(II) complexes. Among all studied ligands of copper(II), diethyldithiocarbamate (DTC) displays a unique redox-tuning ability that enables copper(II) to resist the reduction by thiols while retaining its ability to oxidize zinc(II) thiolates to form disulfides. This work proves for the first time that it's possible to develop oxidants to discriminate between thiols and zinc(II) thiolates, alluding to a new chemical principle for how oxidants, especially universal anticancer $Cu(DTC)_2$, might circumvent the intracellular reductive defense around certain zinc(II)-thiolate sites of proteins to kill malignant cells.

Living organisms cannot survive and thrive without the vital functions of various oxidatively unstable functional units, especially ubiquitous zinc(II)-thiolate sites of proteins.[1] However, aerobic organisms also rely on oxygenated living environments. Thus, a well-maintained reductive intracellular media is necessary to protect those vital functional units from deleterious oxidations. Such a reductive protection is chemically achieved by abundant intracellular thiols that serve as the primary reductant to quench aberrant oxidants. Highly reactive species generated from the combination of copper and dioxygen can lead to severe oxidative damages *in vivo*.[2] Thus copper, especially oxidizing copper(II), is usually regulated tightly in its protein-bound state in living organisms. It has been estimated that each cell has <1 free copper ion under normal physiological conditions.[3] On the other hand, cancer therapy using small-molecule copper complexes has been long pursued due to the abnormal ability of tumors to accumulate copper specifically.[4] A recent epidemiological analysis shows that a clinical combination of copper(II) and disulfiram, a classical alcohol-abuse drug, can reduce the death risk of almost all cancers.[5] Biological evidence indicates for the first time that copper(II) diethyldithiocarbamate ($Cu(DTC)_2$), a metabolite from copper(II) and disulfiram, can target a zinc(II)-binding thiolate site of NPL4 protein to kill cancer cells.[5] A molecular-level chemical mechanism for how such a copper(II) complex interacts with the reductive intracellular environment to target the zinc-thiolate site remains unclear.

Zinc is one of the essential micro-elements in living organisms. Its bivalent state, zinc(II), is the most abundant redox-inactive transition metal ion *in vivo* under typical physiological conditions. As a borderline Lewis acid in hard-soft-acid-base theory, zinc(II) can coordinate with either hard bases (e.g. O or N-based donors) or soft bases such as thiolates.[6] These features make zinc(II) a natural choice to coordinate with sp^2-hybridized nitrogenous donors of histidine residues and thiolate donors of cysteine residues in various proteins, especially zinc finger proteins[7] and zinc metallothioneins,[8] to form tetrahedral sites, which are usually indispensable for proteins' structural integrity and functional control. Actually, it was found that coding sequences of zinc finger proteins constitute *ca.* 1% of all mammalian genes[9] (> 10% for zinc proteins),[10] an unusually high proportion. In addition, zinc(II)-thiolate sites have been found in many important enzymes, such as methionine synthase,[11] bacteriophage T7 lysozyme,[12] alcohol dehydrogenase,[13] and 5-aminolevulinate dehydratase.[14] Given the ubiquity of the zinc(II) thiolates *in vivo* and their essential physiological functions,[10] molecular-level insights into how $Cu(DTC)_2$ targets intracellular zinc(II)-thiolate sites for cancer therapy may provide an important new chemical strategy to kill malignant cells.

Considering the structural complexity of zinc(II)-thiolate enzymes/proteins and the lack of characteristic spectroscopic features to monitor chemical changes of the zinc(II) sites,[15] probing clean bioinorganic model reactions of synthetic zinc(II) complexes with well-defined structures mimicking those in living organisms is an effective way to obtain molecular-level insights into their core mechanisms. Although fundamental curiosities about zinc enzymes have led intense research attention to zinc(II) model complexes,[16] the bioinorganic model reactivity between copper(II) and zinc(II)-thiolate complexes remains surprisingly unexplored. Here we show the first well-defined bioinorganic model study for reactions between copper(II) and zinc(II)-thiolate

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10.1002/anie.201814519

complexes. In contrast to the previously speculated non-redox binding between copper(II) and the zinc(II)-thiolate site[5], oxidations of zinc(II) thiolates by various copper(II) complexes to form disulfides were observed. Among all tested ligands, DTC exhibits a unique ability to tune the oxidation power of copper(II) center specifically into the fine reduction window between zinc(II) thiolates and several other reductants, including thiols and nicotinamide adenine dinucleotide (NADH). The redox selectivity provides an unprecedented chemical mechanism that may potentially allow copper(II) to resist intracellular reductions while retaining its ability to oxidize zinc(II)-thiolate sites.

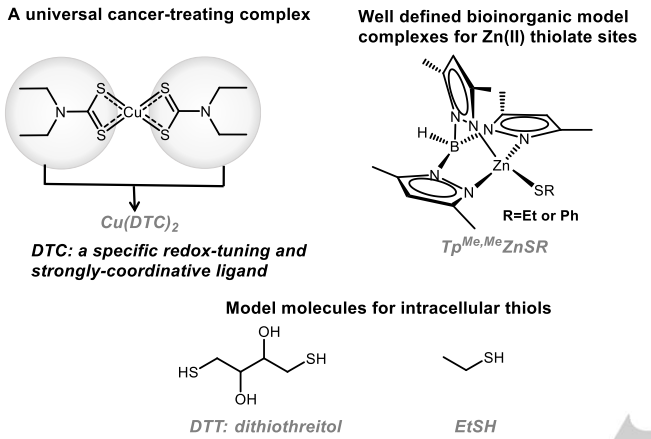

Figure 1. Molecular structures of zinc(II)-thiolate model complexes, $Cu(DTC)_2$ and model thiols.

Two zinc-thiolate complexes ($Tp^{Me,Me}ZnSR$, R= Et or Ph, Figure 1) were chosen to furnish a hybrid N/S coordination sphere mimicking zinc(II)-binding histidine/cysteine sites *in vivo*. There are five key design features for the model complexes: (1) The strong facially-tricoordinative mode of $Tp^{Me,Me}$ imposes a well-defined structure around zinc(II) with three sp^2-hybridized nitrogenous donors during reactions; (2) The methyl substituents of $Tp^{Me,Me}$ provides characteristic NMR signals that are sensitive to chemical changes in the coordination sphere, allowing for unambiguous characterizations of zinc speciation during the reactions; (3) The Zn-SEt ($Et=CH_2CH_3$) moiety serves as a close structural model for the 'zinc(II)-S-CH_2-CH' fragment in proteins; (4) Replacement of Et by Ph ($Ph=C_6H_5$) on the sulfur introduces a useful chemical probe for reaction mechanisms; and (5) The excellent solubility of these model complexes in organic solvents is also preferred for the desired reactivity study since $Cu(DTC)_2$ is virtually insoluble in water (*e.g.* $Cu(DTC)_2$ is barely detected even for its saturated aqueous solution by UV-Vis spectroscopy, as shown in Figure S23).

To unambiguously elucidate the reactivity between copper(II) and the zinc(II) model complexes, we first identified reaction conditions for clean conversions with two simple copper salts, CuX_2 (X=Cl or OAc). The color of copper(II) disappeared and precipitates formed immediately upon mixing CuX_2 (X=Cl or OAc) with 2 equivalents of $Tp^{Me,Me}ZnSR$ in organic solvents with various polarities (chloroform, acetonitrile or tetrahydrofuran). Surprisingly, the starting materials were cleanly converted to 4:1 mixtures of $Tp^{Me,Me}ZnX$ (X=Cl or OAc) and RSSR (R=Et or Ph) (Figure 1, Figure S7, S8), indicating that 50% of the starting RS^- ended up in the precipitates and the zinc(II) thiolates formally act as a 0.5-electron reductant. These results were consistently observed regardless of X, R and solvent. In contrast, oxidations of thiolates by copper(II) to disulfides, in which thiolates always formally act as 1-electron reductants, were observed in the absence of zinc(II) previously.[17]

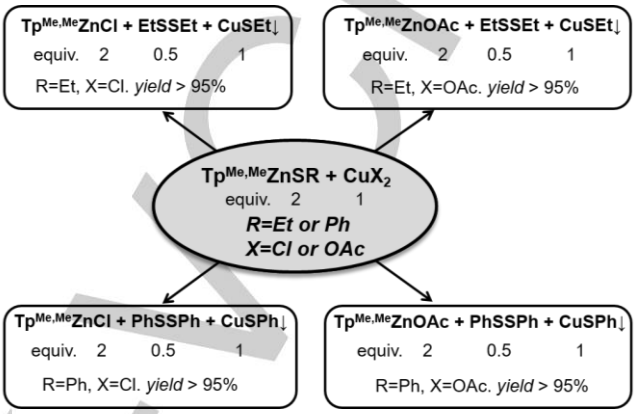

Figure 2. Clean redox reactions between well-defined zinc(II)-thiolate model complexes and simple copper(II) complexes: **a,** oxidations of $Tp^{Me,Me}ZnSR$ (R=Et or Ph) by CuX_2 (X=Cl or OAc).

The precipitates appear brownish-red for R=Et and yellow for R=Ph, respectively (Figure S9). No visible color difference was observed for the precipitates from different X's, consistent with the virtually quantitative formation of $Tp^{Me,Me}ZnX$. Mass/redox-balance analyses suggest that complete reductions of copper(II) to copper(I) should occur and the composition of the precipitates should be $Cu^{I}SR$, which is supported by elemental analysis (Table S6). Attempts to crystalize the precipitates were not successful due to their poor solubility. Thus, X-ray absorption spectroscopy (XAS) was employed to characterize the structures of the precipitates. The Cu K-edge X-ray absorption near-edge structures (XANES) are showed in Figure 3a. The position of adsorption edge reflects the oxidation state of the copper ion. Similar positions were observed for both precipitates and two reference samples, Cu_2O and Cu_2S. The absence of the pre-edge feature from 8977 to 8978 eV, characteristic of copper(II) due to a formally dipole-forbidden 1s to unfilled 3d electronic transition, further supports the assignment that the copper ions in both precipitates are copper(I). Fourier-transformed (FT) k^3-weighted EXAFS data (Figure 3b) show the appearance of the peak for Cu−S shell in *R* space in both precipitates, which was further confirmed by comparisons with the data from three control samples, including copper foil, Cu_2O and Cu_2S. Fitting of the data (Figure S10, Table S7) shows that the bond length between Cu and S is 2.26 Å and 2.25 Å for $Cu^{I}SEt$ and $Cu^{I}SPh$, respectively. The coordination number is *ca.* 3.0 for both samples. These results revealed that RS^- act as bridging ligands and the copper sites prefer a trigonal-planar coordination geometry, consistent with previous results of related copper thiolates.[18]

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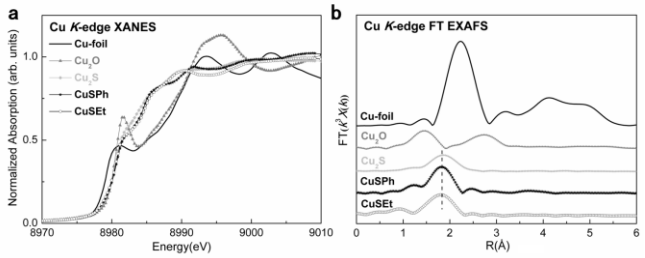

Figure 3. a, Cu K-edge XANES spectra of Cu-containing products and reference samples; **b**, Fourier-transform (FT) plots of the Cu K-edge EXAFS (FT range: 2.9-10.6 $Å^{-1}$).

The clean model reactions with simple CuX_2 clearly point to a two-step mechanism (steps 1 and 2, Figure 4). $Tp^{Me,Me}ZnSR$ is first oxidized by 1 equivalent of CuX_2 to afford a 0.5:1:1 ratio of RSSR, $Tp^{Me,Me}ZnX$, and $Cu^{I}X$. Subsequent metathesis between $Cu^{I}X$ and the second equivalent of $Tp^{Me,Me}ZnSR$ leads to $Cu^{I}SR$ and another equivalent of $Tp^{Me,Me}ZnX$. As observed previously in the literature, copper(II) thiolate can dimerize and undergo further redox tautomerizations to form copper(I) and disulfides.[17a],[17b] A similar process might also be involved in step 1. The yield relative to $Tp^{Me,Me}ZnSR$ (50%) is constant for the disulfides under all circumstances suggests that the second step is much faster than the first step; otherwise, higher yields are expected for the disulfides and $Tp^{Me,Me}ZnSR$ would formally act more similar to a typical 1-electron reductant. In fact, the 50% yield remained unchanged even upon increasing the starting copper(II)/zinc(II) ratio from 0.5:1 to 1:1 (**Figure S11**), further supporting the notion that the first step is rate-determining and the abstraction of thiolate from the zinc(II) center by copper(I) is much faster than the redox step.

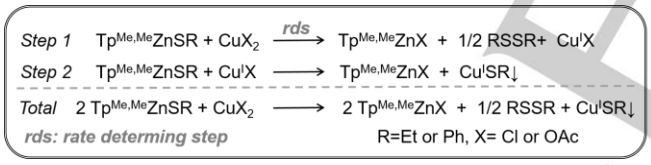

Figure 4. a proposed reaction mechanism between $Tp^{Me,Me}ZnSR$ and CuX_2.

Competitive oxidations of both $Tp^{Me,Me}ZnSR$ complexes were performed to further probe the mechanism (Figure S13, Table S8). In addition to EtSSEt and PhSSPh, the cross coupling product EtSSPh was also formed. Since PhSSPh doesn't react with $Tp^{Me,Me}ZnSEt$ (Figure S14), the formation of EtSSPh should arise from cross coupling reaction of relevant intermediates in step 1 (Figure 4). As observed previously in the literature, copper(II) thiolate can dimerize and undergo further redox tautomerizations to form copper(I) and disulfides[17]. A similar process might also be involved in step 1 (Figure S15), which is consistent with statistical analyses of disulfide product distributions in the competition reactions upon a dimeric mechanism (Table S8, S9).

We next studied the impact of DTC ligation onto the reactivity between copper(II) and the zinc(II) model complexes. A 2:1 mixture of $Tp^{Me,Me}ZnSR$ and $Cu(DTC)_2$ shows a redox reactivity similar to those with simple CuX_2, albeit not as clean. Broad ^1H NMR signals typical of paramagnetic species were observed (Figure S12), indicating the incomplete conversion of $Cu(DTC)_2$. Thus, UV-Vis spectroscopy was used to probe the reactions by monitoring the characteristic absorption band in the visible region of copper(II) (Figure S17)**.** $Cu(DTC)_2$ shows much slower reaction speeds relative to $CuCl_2$ (Figure 5a, Figure S17, S19). Additionally, the reaction with R=Et (*ca.* 64% conversion) appears several times faster than that with R=Ph (*ca.* 25% conversion) in the initial 10 minutes, consistent with the results of the competition experiments. After 3 hours, the conversion of $Cu(DTC)_2$ is *ca.* 78% for $Tp^{Me,Me}ZnSEt$ and *ca.* 48% for $Tp^{Me,Me}ZnSPh$, respectively. PhSSPh is nonvolatile and can thus be conveniently isolated by thin-layer chromatography for yield analysis. A molar amount equal to a half of the consumed $Cu(DTC)_2$ was observed (Figure S18), confirming the same redox stoichiometry as simple CuX_2 (Figure 2). Further kinetic studies indicate that the reaction is 2.57 ± 0.33 order in $Cu(DTC)_2$ and 1.62 ± 0.03 order in $Tp^{Me,Me}ZnSEt$ (Figure S24), which seems to be consistent with a dimeric mechanism in step 1 (*vide supra*). The consistent oxidation of zinc(II) thiolates by various copper(II) complexes to form disulfides suggests that the intracellular clustering of NPL4 proteins induced by $Cu(DTC)_2$[5] might potentially involve a similar chemical process. In contrast to the hard Cl/O donors in CuX_2, DTC has two soft sulfur donors conjugated with one lone-pair-donating nitrogen. As such, the coordination bonds in $Cu(DTC)_2$ are more covalent and its copper center is less electron-deficient than those in CuX_2, which presumably makes $Cu(DTC)_2$ less oxidizing relative to CuX_2. The minor negative shift of the redox potential for $Cu(DTC)_2$ (0.02V *vs* Fc^+/Fc) (Figure S16) relative to $CuCl_2$ (0.09V *vs* Fc^+/Fc) implies that the former should play a more important role.

The most decisive difference between $Cu(DTC)_2$ and other copper(II) complexes was observed from their reactions with two thiols—EtSH and dithiothreitol—mimicking intracellular thiols such as glutathione and cysteinyl thiols of proteins. Similar to $Tp^{Me,Me}ZnSEt$, both thiols reduced three simple copper(II) complexes—$Cu(OAc)_2$, $CuCl_2$, and $CuBr_2$—virtually completely within 2 minutes under the same conditions (Figure S20), which is consistent with the role of intracellular thiols in quenching aberrant oxidants. In stark contrast, < 5% reduction of $Cu(DTC)_2$ by both thiols was observed even after 3 hours (Figure 5b, S20), indicating that thiols are much less reactive than zinc(II) thiolates in their reactions with $Cu(DTC)_2$. Such extraordinary redox specificity is unprecedented, clearly showcasing the unique ability of DTC in altering the common reactivity of copper(II) via tuning its oxidation power to discriminate between thiols and zinc(II) thiolates. Addition of base was found to promote the oxidation of EtSH by $Cu(DTC)_2$ (*ca.* 20% conversion, Figure 5b, S21), suggesting that the formation of proton acid due to the oxidation of EtSH should account for the reactivity difference between $Tp^{Me,Me}ZnSEt$ and thiols. This interesting discovery suggests that acidic cancerous environments might enhance the resistance of thiol oxidation by $Cu(DTC)_2$. A further experiment showed that $Cu(DTC)_2$ is similarly inert in the presence of NADH (Figure S22, a close analogue of nicotinamide adenine dinucleotide phosphate (NADPH)). At this point, whether intracellular zinc(II)-binding histidine/cysteine sites are susceptible to similar oxidations by

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$Cu(DTC)_2$ remains an important open question that deserves extensive future investigations.

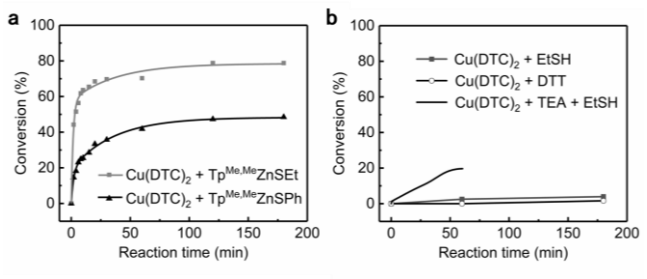

Figure 5. Redox specificity of $Cu(DTC)_2$ and ultra-strong binding of DTC to Cu(II) underlying anticancer activities of Cu(II)/alcohol-abuse drug disulfiram: **a,** slow consumption of $Cu(DTC)_2$ by $Tp^{Me,Me}ZnSR$ over 3 hours; **b,** < 5% consumptions of $Cu(DTC)_2$ by EtSH or dithiothreitol (DTT) over 3 hours; *ca.* 20% consumptions of $Cu(DTC)_2$ by EtSH in the presense of triethylamine (TEA).

In summary, we have provided the first clear chemical evidence proving that oxidants, especially universal anticancer agent $Cu(DTC)_2$, may avert the quenching of primary intracellular reductants, including both thiols and NADPH, and retain the ability to oxidize zinc(II) thiolates that structurally mimics vital physiological functional sites. This work further alludes to a new anticancer chemical principle—that strongly-coordinating small ligands possessing such redox-tuning specificity may enable extracellular copper(II) pre-accumulated at cancerous tumors to oxidize certain intracellular zinc(II)-thiolate sites and kill cancer cells despite the reducing intracellular environments.

Acknowledgements

We thank Professor Min Zhuang and Professor Haiming Liu of ShanghaiTech University for helpful discussions. The research is supported by the National Natural Science Foundation of China (No. U1532125).

Keywords: Anticancer chemical mechanism • Redox chemistry • Zinc thiolates • Copper • EXAFS spectroscopy

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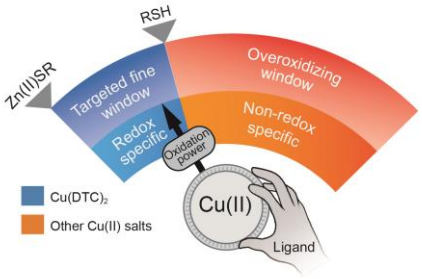

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