

Cellular Consequences of Copper Complexes Used To Catalyze Bioorthogonal Click Reactions

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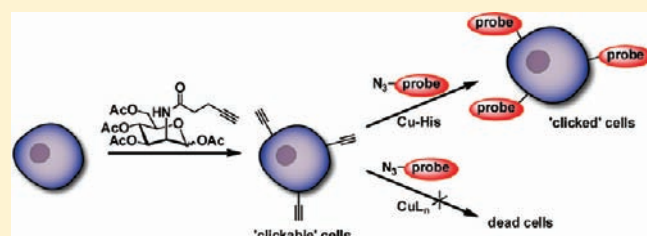
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S Supporting Information

ABSTRACT: Copper toxicity is a critical issue in the development of copper-based catalysts for copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reactions for applications in living systems. The effects and related toxicity of copper on mammalian cells are dependent on the ligand environment. Copper complexes can be highly toxic, can induce changes in cellular metabolism, and can be rapidly taken up by cells, all of which can affect their ability to function as catalysts for CuAAC in living systems. Herein, we have evaluated the effects of a number of copper complexes that are typically used to catalyze CuAAC reactions on four human cell lines by measuring mitochondrial activity based on the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to study toxicity, inductively coupled plasma mass spectrometry to study cellular uptake, and coherent anti-Stokes Raman scattering (CARS) microscopy to study effects on lipid metabolism. We find that ligand environment around copper influences all three parameters. Interestingly, for the Cu(II)–bis-*L*-histidine complex (Cu(his)₂), cellular uptake and metabolic changes are observed with no toxicity after 72 h at micromolar concentrations. Furthermore, we show that under conditions where other copper complexes kill human hepatoma cells, Cu(I)–*L*-histidine is an effective catalyst for CuAAC labeling of live cells following metabolic incorporation of an alkyne-labeled sugar (Ac₄ManNAL) into glycosylated proteins expressed on the cell surface. This result suggests that Cu(his)₂ or derivatives thereof have potential for in vivo applications where toxicity as well as catalytic activity are critical factors for successful bioconjugation reactions.



INTRODUCTION

Chemical reactions that proceed efficiently and do not cross-react with biological functionality have far reaching applications that span chemistry, biology, and materials science.^{1–3} The concept of ‘click’ chemistry pioneered by Sharpless, Finn, and Kolb in 2001⁴ describes a set of highly specific, high yielding reactions for rapid generation of molecules with desired structure and useful properties. Bioorthogonal click chemistry has emerged as a general strategy for the study of biomolecule dynamics and function in cells and living organisms.^{2,3,5–7} To improve on this strategy and to extend it to living organisms, there is increased interest to develop rapid bioorthogonal click reactions that proceed with exceptionally fast kinetics and do not compromise the function and metabolic processing of biomolecules.

The classic click reaction, copper-catalyzed azide–alkyne cycloaddition (CuAAC),^{8–12} has been applied in diverse fields spanning bioconjugation in vitro^{13–15} and in cell lysates,^{16–19} polymer ligation,^{20–25} dendrimer synthesis,^{26,27} surface science,^{28–32} and combinatorial organic synthesis.^{33,34} Only recently has CuAAC

been utilized for live-cell labeling of metabolically incorporated azide-containing^{35–37} or alkyne-containing³⁸ glycoconjugates at cell surfaces.^{35–40} Effective labeling of biomolecules via CuAAC requires a copper complex that enhances the reaction rate. This usually involves a ligand that is coordinated to copper that stabilizes the Cu(I) oxidation state, and ideally prevents the formation of undesired byproducts. The ligand can also function to sequester Cu(I) ions to prevent biomolecule damage and facilitate their removal.^{39,40} Prototypical ligands include bathophenanthroline disulfonate disodium salt (BPS),^{14,41} tris((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine (TBTA),^{42,43} and tris-(hydroxypropyltriazolyl)-methylamine (THPTA)^{42,44} (Figure 1). Other ligands such as tris(2-benzimidazolyl-methyl)amines^{45–47} have also been shown to accelerate CuAAC reactions in vitro. Modifications of TBTA to enhance the water solubility of resulting Cu(I) complexes have led to ligands such as 2-[4-{(bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)-methyl}-1H-1,2,3-triazol-1-yl]ethyl

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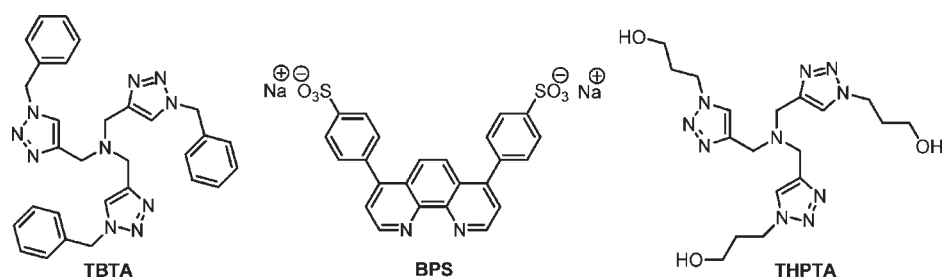


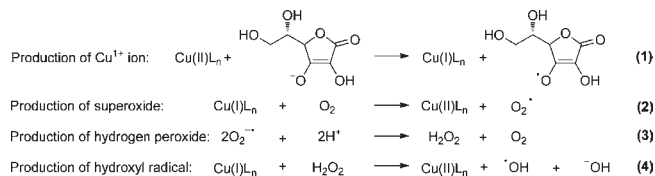
Figure 1. Commonly used Cu(I)-chelating ligands used in CuAAC reactions.

hydrogen sulfate (BTTEs)^{37,40} and 2-[4-{bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl]-1H-1,2,3-triazol-1-yl]-acetic acid (BTAA)³⁹ that require shorter labeling times and have thus exhibited fewer toxic effects when administered to cells. Still, a lack of copper catalysts effective for in vivo labeling strategies, where prolonged catalyst exposures cannot be avoided, hamper application of CuAAC reactions in more complex living systems.

With regards to bioconjugate labeling, CuAAC has primarily been effective for in vitro biological studies at the cell surface, due to its reliance on delivery of the copper catalyst to the reaction site and the toxicity of the copper complexes at concentrations employed for labeling.^{19,48} Attempts to move this versatile chemistry from cell culture to more complex physiological settings have proven challenging. To overcome this obstacle, strain-promoted azide–alkyne cycloaddition (SPAAC)^{3,49–56} reactions have been exploited as copper-free alternatives that have been applied to live cells^{57–59} and even organisms^{60,61} with minimal toxic effects. However, the synthesis of cyclooctynes used for SPAAC reactions are often quite laborious and these reagents are not as easily adapted for all in vivo applications as compared to the use of smaller and more stable terminal alkyne reporter groups used in CuAAC reactions. Therefore, the development of a nontoxic copper catalyst would be highly beneficial, but first a better understanding of the mechanism and factors that contribute to copper catalyst toxicity is needed.

Copper is an essential metal required by all cells and is typically found in serum as Cu(II). The molecular biology of copper-binding proteins has been studied and reviewed extensively.^{62–68} The toxicity of copper complexes is typically ascribed to oxidative damage from reactive oxygen species (ROS),⁶⁹ primarily hydroxyl radicals (and alkoxyl radicals) formed according to eqs 1–4 involving the Fenton reaction.^{70–73} The toxicity of copper complexes is highly dependent on both the number of ligands coordinated (this controls the ability of oxygen to bind to copper) and the reduction potential of the copper complex that is required for reduction of oxygen. Fenton chemistry requires the Cu(II)/Cu(I) reduction potential fall within –160 to 460 mV range in order to generate the reactive oxygen species that give rise to toxicity.⁷⁴ It is interesting to note that reducing agents such as ascorbate are used in both CuAAC and Fenton reactions,^{71–73} but it is the redox potential of the copper complex that controls the degree of ROS generation, whereas the stereoelectronics of the ligand bound to copper appears to be more important for the catalysis of CuAAC reactions. In fact, depending on the redox potential and ligand environment, copper can induce or protect against oxidative damage. Protective behavior is observed particularly when copper is bound in the active site of porphyrins⁷⁵ or CuZn-superoxide dismutase, for example.

For bioconjugation via CuAAC, ideal catalysts for in vivo applications should therefore preferentially ligate the alkyne and catalyze the CuAAC reaction, rather than participate in eqs 2–4.



Depending on the application of CuAAC, cellular copper uptake may or may not be desirable. Therefore, it would be useful to directly compare how well the different catalysts are taken up by cells. The mechanisms by which copper ions enter cells are not well understood and reduction of Cu(II) to Cu(I) is a critical first step in copper transport.⁶⁴ It is believed that in mammalian circulation Cu(II) is bound to ligands such as albumin and other serum proteins as well as amino acids and that these chelate complexes, specifically those that form with smaller molecules such as amino acids, may facilitate cellular uptake of copper.⁷⁶ Cu(II) may be reduced at the cell surface to Cu(I) by the action of duodenal metalloredutase dCytb^{77,78} prior to being taken up by the human copper transporter protein 1 (hCtr1).^{62,64,79} The hCtr1 transport protein has also been hypothesized to work in concert with other metalloredutases^{80,81} for Cu(I) movement across cell membranes.⁶⁴ Cu(I) complexes are the active catalytic species in CuAAC reactions. Both the structure and oxidation state of the metal complexes may influence its bioavailability, ligand transfer, cellular uptake, and toxicity. Herein, we describe a general strategy for studying the behavior of CuAAC catalysts in living systems toward understanding their potential for in vivo applications in complex multicellular organisms.

RESULTS AND DISCUSSION

We have investigated the effects of commonly employed CuAAC catalysts, Cu-BPS, Cu-TBTA, Cu-THPTA, and Cu-L-histidine on cell viability, cellular uptake, and lipid metabolism. To establish a general measure of toxicity across several cell types, cell viability was measured using an MTT colorimetric assay (see Experimental Section) for the following cell lines: human hepatoma cells (Huh7.5), cervical cancer cells (HeLa), human embryonic kidney cells (Hek 293T), and breast cancer cells (MDA-MB-468) exposed to each copper complex (Table 1, Figure 2). Toxicity was measured in the four cell lines using Cu(II) complexes because ascorbate is both a reducing agent and an antioxidant and therefore would lead to countervailing effects on toxicity. Since any in vivo application of copper complexes would require them to become reduced at the site of action

Table 1. Toxicity of Copper Complexes in Four Different Human Cell Lines

ligand	Cu(II/I) reduction potential (mV) vs NHE ^a	IC ₅₀ values for MTT toxicity assays in human cells			
		Huh7.5 cells	MDA-MB-468 cells	Hek293T cells	Hela cells
SO ₄ ²⁻	0	73.9 μM	124 μM	107 μM	50.5 μM
L-histidine	-120	>1000 μM	>1000 μM	>1000 μM	>1000 μM
EDTA	N/A ^b	118 μM	192 μM	111 μM	116 μM
TBTA	260 ^c	16.3 μM	29.6 μM	13.5 μM	12.2 μM
BPS	140	0.74 μM	8.65 μM	33.0 μM	45.6 μM
THPTA	300	93.3 μM	22.4 μM	117 μM	183 μM

^a Values are from literature but in aqueous buffers of different compositions.^{43,82–84} ^b EDTA destabilizes Cu(I) and no potential is available for this complex. ^c Value is given for a derivative of TBTA that is water-soluble.

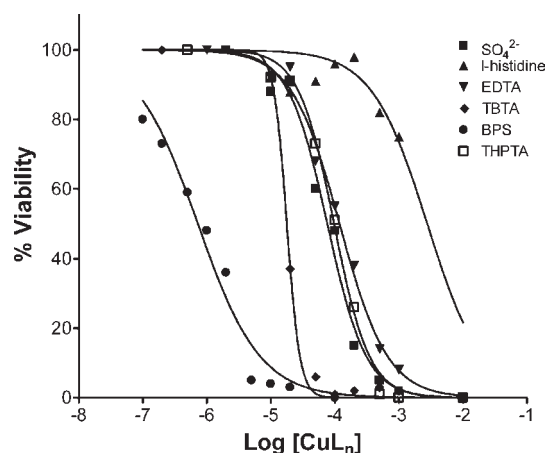


Figure 2. Dose–response curves for MTT toxicity assays for the different copper complexes in the Huh7.5 human hepatoma cell line. Curve fitting for the TBTA ligand employed a variable slope, whereas all other curves were calculated using a nonlinear least-squares fitting of a standard sigmoidal dose–response equation.

within the given organism, circulating catalytic species would most likely begin as Cu(II) complexes. The IC₅₀ value for (+)-sodium-L-ascorbate in the absence of Cu(II) was found to be greater than the concentrations used ($\gg 1$ mM) against all four cell lines and therefore not a contributing factor to the measurement of copper complex toxicity. IC₅₀ values were also determined for L-histidine, EDTA, TBTA, BPS, and THPTA ligands. In each case, the IC₅₀ value was significantly greater than for the corresponding copper-complex, suggesting that the presence of free ligand in solution is also not a determining factor for toxicity.

While absolute toxicities varied from cell line to cell line for each complex, the general trends within a given cell line were similar. Both Cu–TBTA and Cu–BPS were found to be more toxic than the other copper complexes tested against all four cell lines (Table 1), though most acutely toxic to hepatic cells. The previously measured redox potentials for the different ligands are also presented in Table 1.^{43,82–84} The toxicity of Cu(II)–THPTA was found to be comparable to that of Cu(II) in the absence of added ligand with appreciable toxicity at 50 μM (approximately 75% cell viability), the concentration typically used in labeling studies.^{38,85} It should be noted that Cu(II)–TBTA was solubilized in DMSO/H₂O (1:1) and precipitation of the complex upon addition to media was observed at concentrations well above the IC₅₀ value for the complex, though it may have acted as a suspension at lower concentrations as well.

Table 2. Metal Uptake of Copper(II) Complexes in Four Different Cell Lines

cell line	metal uptake after 24 h (ng/10 ⁶ cells \pm 10%)						
	mock	no ligand	L-histidine	EDTA	TBTA	BPS	THPTA
Huh7.5	8	120	195	185	170	190	195
MDA-MB-468	6	11	10	12	12	10	10
Hek293T	12	75	95	98	102	0	15
HeLa	5	45	45	42	39	6	40

Cu(II)–TBTA and Cu(II)–BPS were also found to be significantly more toxic than unligated Cu(II). In contrast to the other complexes studied, the IC₅₀ value for Cu(his)₂ is much higher than for unligated copper. The different toxicities of the copper complexes, which are themselves clearly more toxic than the free ligands, suggests that the cellular toxicity is mediated via interactions with intact or only partially substituted complexes. Intact complexes may result in different rates of oxidative damage either inside or outside of the cell and/or differential recognition and uptake of the complexes resulting in different final concentrations. To probe this latter hypothesis further, the uptake of copper was measured for each complex to determine if there was a link between cellular uptake and toxicity.

Cells were analyzed for copper content after 24-h treatments with 10 μM of each of the Cu(II) complexes (Table 2) using inductively coupled plasma mass spectrometry (ICP-MS). Huh 7.5 cells were found to uptake more Cu(II) on a per cell basis after 24 h than the other cell lines, likely because the liver is the primary site in the body for copper homeostasis. For this cell line, ligated forms of Cu(II) were taken up more readily than unligated Cu(II); however, there was no correlation between uptake and toxicity. MDA-MB-468 cells took up only negligible Cu(II) and there was no apparent preference for the different Cu(II)–ligand complexes tested. The difference in uptake between these cell lines is striking considering that the IC₅₀ values do not vary nearly as much. Hek293T cells exhibited about half the copper uptake as Huh 7.5 cells; however, for complexes of THPTA and BPS, copper uptake was essentially zero. HeLa cells exhibited uptake levels approximately 20% that of Huh 7.5 cells, except in the case of complexes of BPS, where almost no copper was taken up into the cells compared to mock treated cells. This behavior of cells treated with the Cu(II)–BPS complex suggests that this complex may act as an inhibitor of copper uptake. Our results show no apparent correlation between uptake and toxicity. In fact, with a few exceptions, there is little

difference in uptake of the complexes when compared within one cell line, but toxicity varies significantly.

We then sought to determine whether Cu(II)–TBTA, Cu(II)–BPS, and Cu(II)–THPTA complexes also contributed to changes in metabolism as we had previously observed for Cu(his)₂ and Cu–EDTA in hepatocytes.^{86,87} Huh 7.5 cells were treated with 10 μM of each of the copper complexes to determine the effects on lipid metabolism using coherent anti-Stokes Raman scattering (CARS) microscopy, a label-free technique for visualizing and quantifying lipid content in cells (Figure 3, Table 3).^{86,88,89} Cells were incubated with Cu–ligand complexes in the presence and absence of an excess of (+)-sodium-L-ascorbate for 24 h. Changes in lipid metabolism were measured using a previously described image analysis method called voxel analysis, which in this case determines the volume of lipids in a given stack of images.⁸⁶ We observed minimal effects on lipid storage between samples of Cu(II) (Figure 3 and Table 3) versus Cu(I) (Figure S1, see Supporting Information) with the same ligand at 24 h. For both Cu(II)–TBTA and Cu(II)–THPTA over 24 h, the lipid content increased from 2 to 20% and 15%, respectively. This increase in lipid content is consistent with what was previously observed for Cu(his)₂ and Cu–EDTA under the same conditions.⁸⁶ The increase in lipid content observed from

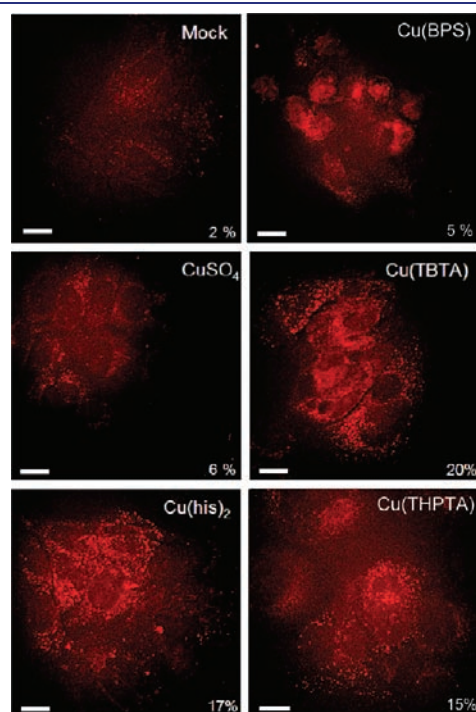


Figure 3. CARS microscopy was used to determine increases in lipid metabolism from copper treatments of cells. (Top left) Mock treated cells where no copper was added. All measurements are on cells fixed after a 24-h treatment with 10 μM copper. Scale bar is 20 μm. Percentages are for average lipid content by volume per cell determined by voxel analysis of ten or more cells. Complexes between CuSO₄ and ligands were preformed before adding to media. CuSO₄ · 5H₂O was the copper source for the formation of all of these complexes.

Table 3. Effects of Copper(II) Complexes on Lipid Storage in Huh7.5 Cells As Determined by CARS Microscopy

Huh 7.5 cells	mock	none	L-histidine	EDTA	TBTA	BPS	THPTA
% Lipids at 24 h	2 ± 1%	6 ± 1%	17 ± 3%	19 ± 3%	20 ± 3%	5 ± 2%	15 ± 3%

treatment with Cu–BPS was considerably less in all replicate experiments. At 10 μM Cu(II)–BPS, the density of cells after 24 h was ~10% that of the mock cells and the morphology of the cells suggested that they were under considerable stress and appeared to be either in the process of dying or had already died. The lipid content of the viable cells was ~5% (Figure 3 and Table 3) which is similar to that of cells treated with unligated copper. The experiment was repeated using 1 μM Cu(II)–BPS, and at this concentration, slightly more than 50% of the cells were still viable after 24 h and the increase in lipid content was similar to that observed at 10 μM. Results for CuSO₄ treated cells are similar to those previously observed when CuCl₂ is used as a copper source; only a small increase from 2 to 6% in lipid content was observed after 24 h.³⁰ These results suggest that the effects on lipid metabolism and storage by Cu–BPS, CuCl₂, and CuSO₄ differ from those of Cu(his)₂, Cu–EDTA, Cu–TBTA, and Cu–THPTA complexes. Altered lipid metabolism as a result of copper complex treatment does seem to correlate with copper uptake (Table 2 and Table 3), although toxicity and effects on lipid metabolism appear to be independent processes. Although oxidation of lipids can result from ROS and thus affect lipid metabolism, we see changes to lipid metabolism by copper complexes that do not have favorable redox potentials for ROS formation (i.e., Cu(his)₂, Table 1). This suggests that changes to intracellular lipid content are likely to occur through an ROS-independent mechanism that involves cellular copper uptake, although it is also possible that changes in copper coordination in the cellular milieu could alter the redox potential to promote ROS generation.

Having observed that L-histidine was considerably less toxic than TBTA, BPS, and THPTA, yet readily absorbed by cells compared to other copper catalysts, we sought to determine the catalytic utility of L-histidine as a ligand in a CuAAC reaction between benzyl azide and phenyl acetylene for 24 h (Table 4). It is noteworthy that the copper complex of L-histidine has previously been reported as an additive in similar cycloaddition reactions^{46,90} but has not been used in catalytic amounts. Using a set of standard conditions,⁸² the addition of L-histidine as a ligand

Table 4. Comparison of Ligand Efficiency in CuAAC^a

ligand	yield % ^b
none	23
histidine	90
EDTA	18
TBTA	93
BPS	88
THPTA	89

^a Reactions were performed in scintillation vials with no effort to exclude oxygen other than capping with screw cap. ^b Yield % was determined by gas chromatography.

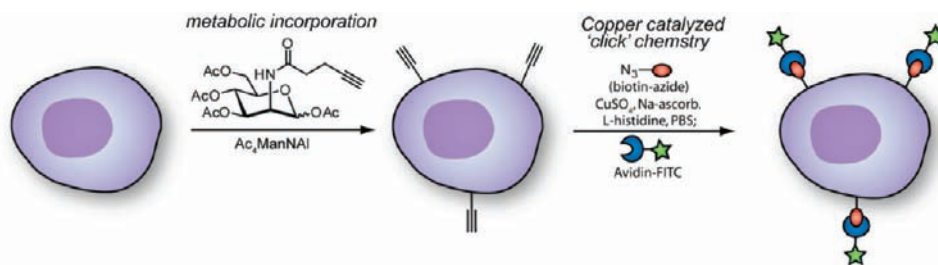


Figure 4. Metabolic incorporation of $Ac_4ManNAL$ onto Huh7.5 human hepatoma cell surfaces for fluorescence detection via CuAAC.

was as effective as the more commonly employed THPTA, TBTA, and BPS ligands after 24 h of reaction. $Cu(EDTA)$ was unable to catalyze the reaction likely due to the destabilizing effect of EDTA on the $Cu(I)$ oxidation state (Table 1).

One major drawback in the development of copper catalysts for in vivo CuAAC labeling has been the concern that relatively high doses of copper will result in significant oxidative damage and resulting cytotoxicity. The relatively nontoxic nature of the $Cu(his)_2$ complex suggests that L-histidine or modified histidine-like ligands have the potential to be applied in CuAAC labeling studies in complex organisms. To test the utility of $Cu(I)$ –L-histidine complex to catalyze CuAAC reactions on live cells, we adapted a procedure for metabolic incorporation of an alkyne tagged mannosamine derivative ($Ac_4ManNAL$)¹² (Figure 4) onto the surface of 4 cell lines: Huh7.5, HeLa, Hek 293T, and MDA-MB-468, and used L-histidine as a ligand in the subsequent CuAAC labeling step (Figure 4). Following metabolic incorporation of $Ac_4ManNAL$, the alkyne tagged glycans were treated with biotin-azide⁸⁵ ($50 \mu M$), $CuSO_4 \cdot 5H_2O$ ($50 \mu M$), (+)-sodium-L-ascorbate ($500 \mu M$), and L-histidine ($50 \mu M$). Secondary labeling was accomplished using a streptavidin–FITC conjugate for fluorescence imaging. Under identical labeling conditions, $Cu(I)$ –L-histidine served as an effective CuAAC catalyst for labeling cell surface glycans for all four cell lines (Figure 5) in a manner comparable to the other three commonly used catalysts and much more efficiently than unligated copper. These results demonstrate the utility of histidine as a biocompatible ligand for the CuAAC reaction.

To evaluate the competing reaction pathways of ROS formation and CuAAC reactions for the $Cu(I)$ –L-histidine complex and other $Cu(I)$ –ligand complexes, we examined their toxicities over time while labeling cell surface glycans via CuAAC. We found that 20 min exposures to any of the complexes at catalytic concentrations did not result in any significant changes to cellular viability following a removal of the copper species. Toxicity only arises on a longer time scale or at much higher doses of copper for short times. $Cu(I)$ –L-histidine appears to avoid the toxic effects at longer exposure times perhaps because it is a worse Fenton reducing agent due to the more negative $Cu(II)/Cu(I)$ reduction potential in comparison to $Cu(I)$ –THPTA, $Cu(I)$ –TBTA, and $Cu(I)$ –BPS.^{78,79} It is important to examine the effects of prolonged exposure as this is essential to developing a labeling strategy that could be implemented in vivo. For this purpose, we treated cells with $CuSO_4$, TBTA, or L-histidine, (+)-sodium-L-ascorbate, and biotin azide for 24 h. After the 24-h treatment, virtually all the cells treated with TBTA were dead (Figure 6). Cells treated with $Cu(his)_2$ in the presence of (+)-sodium-L-ascorbate were still healthy. Secondary labeling with streptavidin–FITC resulted in fluorescent labeling, although now considerable labeling was also observed inside the cell.

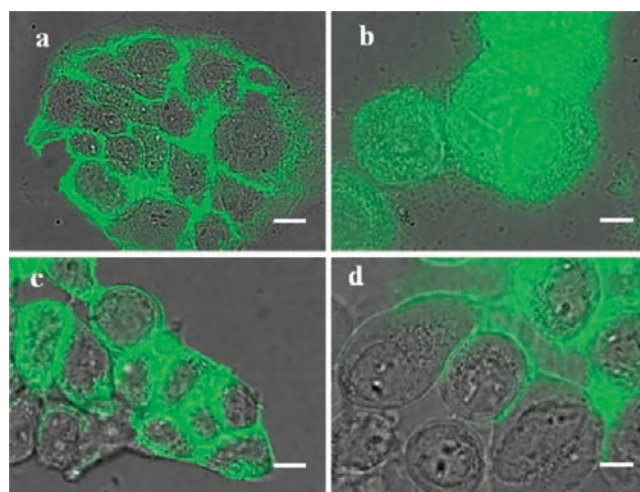


Figure 5. Overlaid bright field and fluorescence images of four cell lines: (a) Huh7.5 cells, (b) MDA-MB-468 cells, (c) Hek 293T cells, and (d) HeLa cells following metabolic incorporation of $Ac_4ManNAL$ onto cell surface glycans (3 days), CuAAC labeling ($CuSO_4 \cdot 5H_2O$ ($50 \mu M$), (+)-sodium-L-ascorbate ($500 \mu M$), L-histidine ($50 \mu M$), and biotin azide ($50 \mu M$)) for 10 min and secondary labeling with streptavidin–FITC ($5 \mu g/mL$ in PBS) for 30 min. No fluorescence was observed for control experiments lacking $Ac_4ManNAL$, L-histidine, biotin azide, or streptavidin–FITC. Scale bars = $20 \mu m$ for (a) and $10 \mu m$ for (b–d).

Considerable effort has been devoted toward the development of copper-free variants of CuAAC as biocompatible alternatives,³ however, the assumption that all copper catalysts are toxic in a uniform manner is not the case. Indeed some copper complexes, particularly those that employ TBTA or BPS as ligands, are toxic upon prolonged exposure. However, others that employ L-histidine are not. Here, we have shown that $Cu(his)_2$ in the presence of (+)-sodium-L-ascorbate can be tolerated at millimolar concentrations for at least 3 days, illustrating the point that the toxicity of copper species is ligand-dependent and can span at least 3 orders of magnitude.

While it is true that many copper-complexes generate ROS in cells by reducing small molecules such as hydrogen peroxide, it is also true that specific ligation of copper can not only prevent the formation of ROS, but also exhibit antioxidant properties to protect the cell.⁶⁷ Typically, bioavailable copper is ligated by one or more L-histidine ligands depending on pH.⁹¹ This bioavailable form of copper represents a pool of copper that could potentially be used to catalyze in vivo labeling without the addition of exogenous ligands. Exposure to higher concentrations of $Cu(his)_2$ in the presence of (+)-sodium-L-ascorbate results in changes in lipid metabolism and storage in hepatocytes, but does not lead

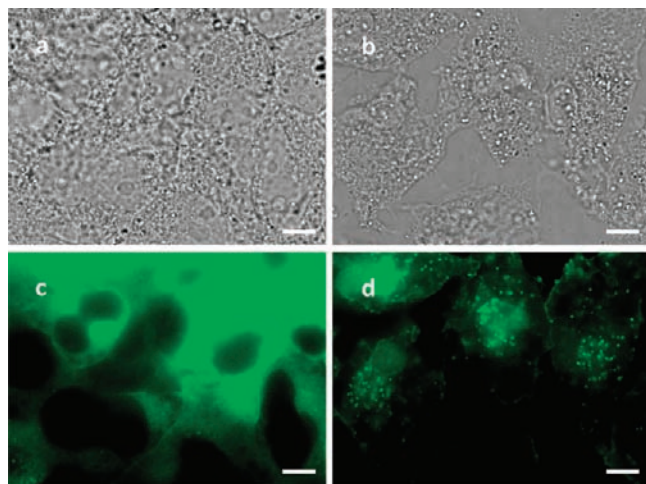


Figure 6. Brightfield (a and b) and fluorescence (c and d) images of Huh7.5 cells following metabolic incorporation of $Ac_4ManNAc$ onto cell surface glycans (3 d), a 24-h treatment with $CuSO_4 \cdot 5H_2O$ ($50 \mu M$), (+)-sodium-L-ascorbate ($500 \mu M$), (a and c) L-histidine ($50 \mu M$) or (b and d) TBTA ($50 \mu M$), and biotin azide ($50 \mu M$) and secondary labeling with streptavidin-FITC. The pattern of fluorescence in panel c is consistent with cell surface labeling and some cytosolic labeling presumably due to internalized biotinylated glycoproteins. Cells treated with $Cu(I)$ -TBTA in panel d were predominantly dead or dying and labeling efficiency was low compared to cells treated with L-histidine giving lower fluorescence intensity and sparse labeling. Scale bars = $10 \mu m$.

to toxicity over several days in the four cell lines we tested. The IC_{50} values for these copper complexes in Huh 7.5 cells do not correlate directly with changes in lipid metabolism, nor do they correlate with total copper content of the cell. Toxicity and cellular absorption are independent properties that depend on the nature of the ligand environment surrounding copper. Specifically, the competition between ROS generation and the CuAAC reaction is dependent on the ligand environment. Our results show that it is possible for efficient CuAAC catalysis to occur without significant toxicity due to ROS generation. These findings are important both for designing catalysts that will be better suited for in vivo labeling, but also for providing insights toward the process of cellular copper uptake, which appears to be accelerated by ligated forms of copper in Huh 7.5 cells and suggests that copper complexes may also utilize different entry pathways for different cell types.

CONCLUSIONS

In summary, we have investigated the effects of copper complexes used in the CuAAC reaction on cellular uptake, toxicity, and metabolism. Our results suggest unique biochemical properties for each copper-complex and emphasize the importance of studying these properties on a per copper-complex basis. Copper toxicity generally follows the trend of redox potential for the complexes; however, it is interesting that these do not correlate with copper uptake and with effects on lipid metabolism. We have shown under our catalytic conditions that L-histidine serves as an efficient ligand for catalyzing the CuAAC reaction as compared to the TBTA, THPTA, and BPS ligands. We have also tested the effect of $Cu(I)$ -L-histidine toward the CuAAC reaction at live cell surfaces.¹² Successful labeling of four different human cell lines was accomplished using the Cu -L-histidine

catalyst. This suggests that the design of similar less toxic catalysts could prove valuable for developing CuAAC to be adaptable to in vivo labeling where prolonged exposure is unavoidable and residence time is dictated by how efficiently the compounds are cleared. Furthermore, our data suggests that complexes that are poor initiators of the formation of reactive oxygen species can still function as efficient CuAAC catalysts. Complexes with a similar ligand environment to that of $Cu(his)_2$ that demonstrate similar toxicity profiles but have enhanced catalytic efficiency should have even greater potential for in vivo applications.

EXPERIMENTAL SECTION

Cell Culture. Cells were cultured as previously described.⁸⁶ Specifically, Huh7.5, Hek293T, and HeLa cells were maintained in Falcon T-75 flasks in an incubator at $37^\circ C$ with 5% CO_2 . Cultures were maintained in Dulbecco Minimal Essential Media (DMEM) with 10% fetal bovine serum (FBS) and nonessential amino acids, penicillin, and streptomycin. MDA-MB-468 cells were maintained in Falcon T-75 flasks with Liebowitz's media with 10% FBS, nonessential amino acids, penicillin, and streptomycin in an incubator at $37^\circ C$. Cells were passaged twice per week.

Coherent Anti-Stokes Raman Scattering (CARS) Microscopy. The CARS microscopy procedure has been previously described.^{86,89} Briefly, the CARS microscopy system uses a single femtosecond Ti:sapphire oscillator (Spectra Physics Tsunami operating at 80 MHz) as the excitation source. The frequency difference between two input lasers, Stokes and pump beam, is equal to that of the Raman resonance of interest. The second longer wavelength (Stokes beam) is generated through use of a photonic crystal fiber (PCF), which produces power in the wavelength range of 1035 nm with negligible amplitude fluctuations. When overlapped with the 800 nm (pump beam) from the Ti:sapphire laser, this corresponds to the 2850 cm^{-1} Raman resonance of the C-H stretch. A modified Olympus Fluoview 300 laser scanning system and IX71 inverted microscope was used to carry out all CARS and two-photon imaging. A 40X 1.15 NA UAPO water immersion lens with a coverslip correction collar was used as the objective and the 0.55 NA long working distance condenser lens for collection in the forward direction. Light was directed to photomultiplier tubes (PMT) with enhanced red sensitivity (Hamamatsu R3896) and operated at a gain of about 530 V. Imaging was completed when the combined average powers reached approximately 120 mW for the pump and the Stokes. Fixed cell samples were imaged in 4.2 cm^2 Lab-Tek Chambers Slide System (NUNC, Rochester, NY). Optical sectioning of lipid droplets were imaged at $1 \mu m$ z-slices for a total z-stack analysis ranging from 7 to 12 μm depending on thickness of cell sample. Mock images were obtained to set the normal lipid content and droplet distribution for comparison. Images presented in the manuscript represent z-projections of 7–12 slices compiled together in one image and were produced using ImageJ software. Voxel analysis was used to determine changes in lipid droplet storage in the CARS images using ImageJ software. Simultaneous two-photon fluorescent images were collected and used to define the border outlines of cells in the z-projected stacks. A voxel counting routine was used to determine the number of voxels in a defined region that meet a set threshold intensity that was typically set at 50, but was optimized for each individual image. The regions of interest (ROIs) were hand drawn to outline the cells as observed using the fluorescence images, and then the % volume of lipid was determined by counting the percentage of voxels exceeding the threshold value in each defined cell. For each sample, 20 cells were counted, 5 cells per image, from four distinct and randomly selected regions of each sample.

MTT Assays. A general procedure used for the MTT assay has been previously reported.^{69,81,82} Specifically, $100 \mu L$ aliquots of a suspension

of 1×10^5 cells in media were added to each well. After 24 h, the control cells were treated with 100 μL of PBS while experimental wells were treated with dilutions of the copper complexes dissolved in PBS buffer and diluted accordingly to span a range from 2 mM to 1 μM . In the case of the Cu–BPS complex, the range was shifted to span from 20 μM to 100 nM. Cells were treated for 72 h at which time 50 μL of a solution of 2.5 mg/mL MTT in PBS was added to each well. The cells were incubated with MTT for 3 h. After 3 h, the media was aspirated from each well and the remaining formazan crystals were solubilized in 150 μL of DMSO. Absorbance of the wells was measured on a Spectra Max M2 (Molecular Devices) and the data recorded using Softmax Pro 4.7 software with a preshake time of 10 s. The average absorbance of 6 replicates, each repeated 3 times, was normalized to untreated cells and plotted versus concentration to determine IC_{50} values. MTT assay data were analyzed using the software package Graphpad Prism 4.0 using nonlinear least-squares fitting using the sigmoidal dose–response curve for fitting. In some cases, variable slope sigmoidal dose–response curve fitting was employed to accurately fit the data. Standard errors are provided for LogIC_{50} values. Some differences were observed in the total shape of the toxicity profile. Standard errors were determined based on LogIC_{50} values as reported in Table 1.

Metal Uptake Studies. The metal uptake assay has been previously described.⁸⁶ Suspensions (10 mL) of cells (10^5 cells/mL) were plated into 5 cm Petri dishes. These samples were incubated for 24 h, at which time the media was removed and the cells were rinsed twice with PBS. Trypsin–EDTA (2 mL) was then added to detach the cells from the plate surface, and an additional 5 mL of media was added to resuspend the cells. These suspensions were transferred to 15 mL conical Falcon tubes and centrifuged for 5 min at 1000 rpm. The supernatant was discarded and the cells were resuspended and rinsed twice with PBS in this manner. Cell pellets were then resuspended in 2 mL of PBS and counted on a microscope using a hemacytometer. Cell suspensions ranged between 0.2 and 2×10^6 cells per sample, those with SBO being the lowest in cell count, only 10% of mock. After determining the number of cell in each sample, the cells were again pelleted, and dried overnight. To each dried pellet, 100 μL of concentrated HNO_3 was added and the sample was left for 24 h to be digested. A total of 250 μL of H_2O was then added to dilute the acidic samples and the samples were centrifuged to remove any insoluble cell debris (this debris was later checked for Cu content and found to be below the detection limit of the ICP-MS). The prepared samples were then submitted for ICP-MS analysis to determine the copper content and the results normalized to the number of cells in each sample.

CuAAC Model Reactions. Phenyl acetylene (125 μL , 1.14 mmol) and benzyl azide (147 μL , 1.14 mmol) were added to a stirred solution of (+)-sodium-L-ascorbate (22 mg, 0.114 mmol), ligand (0.0114 mmol), and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (2.6 mg, 0.0114 mmol) in DMSO/ H_2O (2:1, 3 mL). The reaction mixture was stirred at room temperature for 24 h. Upon completion, the resultant suspension was cooled to 0 $^\circ\text{C}$, diluted with water (10 mL), and the precipitate was filtered and dried. Yields were determined by gas chromatography of the crude reaction mixture.

Fluorescence Microscopy^{35,56,83}. Cells were seeded at 4×10^4 cells/well in borosilicate Lab-Tek chambers (VWR, Mississauga, ON) in 2 mL of DMEM supplemented with 10% FBS (CANSERA, Rexdale, ON), 100 nM nonessential amino acids, 50 $\mu\text{g}/\text{mL}$ penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. After 24 h, appropriate samples were treated with Ac_4ManNAI (50 μM) and incubated for 3 days. The cells were washed with PBS (3 \times) and treated with the following reagents for 10 min at 37 $^\circ\text{C}$ in 2 mL of PBS: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (50 μM), (+)-sodium-L-ascorbate (500 μM), L-histidine (50 μM), and biotin azide (50 μM). Following CuAAC labeling, cells were washed with PBS (3 \times), blocked with 1% BSA (in PBS) for 20 min at room temperature, and then stained with streptavidin–FITC (5 $\mu\text{g}/\text{mL}$ in PBS) for 30 min at room temperature; cells were then washed with PBS (2 \times), media (1 \times), and then imaged

live-cell in 1 mL of phenol-red free DMEM. Imaging was done with an Olympus 1 \times 81 spinning-disk confocal microscope equipped with a FITC filter (Semrock, Excitation: 465–499 nm, Emission: 516–556 nm) and a Photometrics (Coolsnap ES) camera using either 60 \times or 100 \times magnification. Images were taken of samples and controls (absence of Ac_4ManNAI) using both bright-field and the FITC channel (10 s exposure). Image processing was done using ImageJ software, using the Color Merge plugin to apply pseudocolour to FITC channel images and merging with bright-field images. The same pixel-intensity ranges were applied and displayed for samples and controls. For 24-h exposure experiments, cells were treated for 24 h with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (50 μM), (+)-sodium-L-ascorbate (500 μM), L-histidine (50 μM), or TBTA (50 μM) and biotin azide (50 μM). Post 24-h treatment, the cells were washed with PBS (3 \times), blocked with 1% BSA (in PBS) for 20 min at room temperature, and then stained with streptavidin–FITC (5 $\mu\text{g}/\text{mL}$ in PBS) for 30 min at room temperature. Cells were then washed with PBS (2 \times), media (1 \times), and then imaged live-cell in 1 mL of phenol-red free DMEM.

■ ASSOCIATED CONTENT

Supporting Information. Synthetic details and characterization data for compounds are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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