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Light uncages a copper complex to induce nonapoptotic cell death†

Cite this: *Chem. Commun.*, 2013, **49**, 2460

Received 13th December 2012,
Accepted 4th February 2013

DOI: 10.1039/c3cc38927h

www.rsc.org/chemcomm

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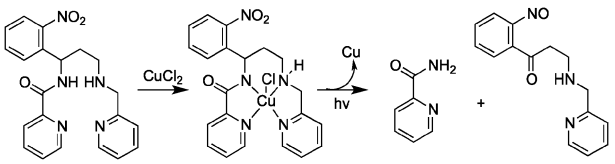
Cu3G is a Cu(II) complex of a photoactive tetradentate ligand that is cleaved upon UV irradiation to release Cu. Here we show that the cytotoxicity of Cu3G increases in response to brief UV stimulation to result in extensive cytoplasmic vacuolization that is indicative of nonapoptotic cell death.

Medicinal uses of copper and copper complexes have been documented as far back as the ancient Egyptians and continue to be a subject of intense research.[1–6] The bioactivity of copper stems in part from its requirement as a cofactor in numerous metalloenzymes,[7] its redox activity that can induce cytotoxicity under some conditions,[2] and its predilection to displace other essential metals.[8] This paradox between beneficial and toxic roles requires cells to have elaborate systems for copper regulation.[9,10] This same paradox also provides a rich opportunity to use small molecules to override or intervene in copper regulation pathways as a therapeutic strategy, for example to overcome a localized copper imbalance in neurodegenerative disease,[6,11] reduce a copper overload in Wilson's disease,[12] or hijack endogenous copper to induce oxidative stress in cancer cells.[13–15] In these various applications, the coordination environment around copper plays a pivotal role in determining the cellular uptake, localization, and ultimately the biological activity of copper.[16–18] Ideally, the biological activity would be targeted to the disease site without affecting normal cells.

We are interested in manipulating the coordination environment of copper complexes by using external stimuli to trigger a targeted biological activity as a consequence of altered coordination chemistry. Toward these goals, our laboratory and others have developed a series of photocaging ligands that incorporate a photolabile nitrophenyl group in the backbone of multidentate copper chelators.[19–22] Stimulation with UV light cleaves the ligand backbone to uncage its Cu content, as shown in Scheme 1 for our 3rd generation chelator, 3Gcage. With an effective dissociation constant for Cu(II) of 0.18 fM at pH 7.4, 3Gcage is our best cage to date, and preliminary *in vitro* investigations showed that it inhibits Cu-induced hydroxyl radical formation in the dark, whereas it promotes $OH^{\bullet}$ production by 300% after UV photolysis.[20] In principle, such a triggered alteration in coordination could be used to unleash the toxic reactivity of an otherwise nonreactive copper complex. The current study tested this concept in three human cancer cell lines to find that brief UV exposure increases the cytotoxicity of Cu3Gcage. Furthermore, treated cells showed extensive cytoplasmic vacuolization, an indication of a nonapoptotic cell death pathway.



Scheme 1

The ligand 3Gcage was synthesized by following a revised synthetic route to improve yield (ESI†). The copper complex was prepared by refluxing ethanolic solutions of 3Gcage with $CuCl_2$ and purifying the blue product by column chromatography on alumina followed by crystallization from ethanol. The crystal structure in Fig. 1 shows Cu(II) in a distorted square pyramidal environment provided by 3Gcage with a coordinated Cl in the apical position.‡ The formulation of the complex is therefore [CuCl(3Gcage)], which is abbreviated **Cu3G** hereafter.

A critical challenge in the general strategy of using an external trigger to release Cu is to ensure that Cu is retained in its complex prior to activation. Although the thermodynamic affinity of 3GCage for Cu(II) is very strong, the reducing environment within a cell could provide a pathway for release of Cu(I) even in the

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† Electronic supplementary information (ESI) available: Including experimental details, spectra of Cu3G challenge experiments, additional cell viability experiments, and X-ray crystallography data in cif format. CCDC 914779. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c3cc38927h

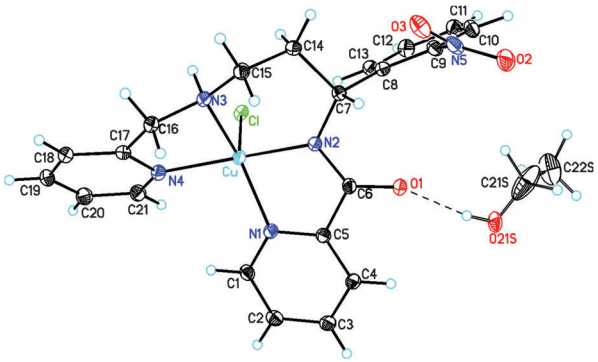

Fig. 1 ORTEP diagram of [CuCl(3Gcage)] (**Cu3G**) along with EtOH solvent molecule, showing 50% thermal ellipsoids. Selected bond distances: Cu–N2 1.961(2), •Cu–N3 2.025(2), •Cu–N1 2.045(2), •Cu–N4 2.053(2), Cu–Cl 2.5433(7) Å. Selected bond angles: N2–Cu–N3 94.20(9), •N2–Cu–N1 81.81(9), •N3–Cu–N1 153.48(9), •N2–Cu–N4 169.91(9), •N3–Cu–N4 79.59(9), •N1–Cu–N4 100.17(9), N2–Cu–Cl 100.90(7), •N3–Cu–Cl 103.65(7), •N1–Cu–Cl 102.85(6), •N4–Cu–Cl 88.36(7).

absence of light uncaging. In order to assess this possibility, we measured its reduction potential by cyclic voltammetry. While no oxidation–reduction peaks were observed between +1.0 and −1.4 V in pH 7.4 PBS buffer, a metal-based redox feature was observed in acetonitrile with a quasireversible peak-to-peak separation of 110 mV and a midpoint reduction potential $(E'_{1/2})$ of −0.60 V *vs.* Ag/AgCl (−0.40 V *vs.* NHE) (Fig. 2).§

This reduction potential of **Cu3G** matches the reported value of −0.60 V *vs.* Ag/AgCl recorded in DMSO for the bisthiosemicarbazone complex Cu(Atsm).[23] Its resistance to reduction makes Cu(Atsm) an attractive ^{64}Cu PET imaging agent of hypoxia, since the intact Cu^{II}(Atsm) complex is stable and washes out of normal cells, but can be reduced and retained under hypoxic conditions. The similar reduction potential of **Cu3G** to Cu(Atsm) suggests that **Cu3G** may remain intact under normal cellular conditions.

To further probe its stability, we challenged a solution of **Cu3G** in PBS buffer at pH 7.4 with various reducing agents or binding agents, including ascorbate, glutathione, cysteine, histidine, and the strong Cu(I) chelator BCS. We found that none of these agents on their own strip Cu from **Cu3G**, as evidenced by the persistence of its characteristic d–d band at 584 nm (ESI†). However, the combination of ascorbate and BCS did cause reductive transfer of Cu(II) from **Cu3G** to Cu(I) in $[Cu(BCS)_2]^{3-}$ (see ESI†). In contrast, Cu(Atsm) was reported to retain Cu under similar conditions.[23] These combined results

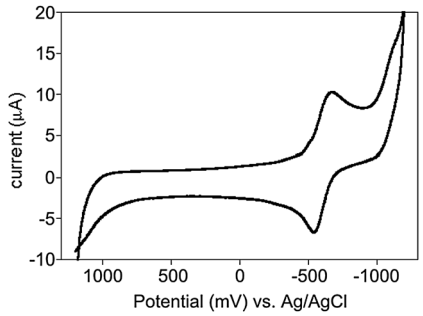

Fig. 2 Cyclic voltammogram of a 1 mM solution of **Cu3G** in CH_3CN with 0.1 M Et_4NClO_4 and a scan rate of 100 mV s^{-1}.

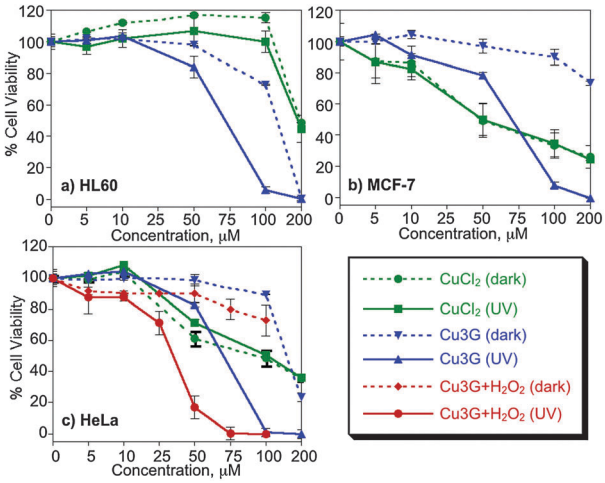

Fig. 3 Effect of $CuCl_2$ or Cu3G on cell viability of (a) HL60, (b) MCF-7, and (c) HeLa cancer cell lines. Dashed lines indicate that cells were incubated with the test compound in the dark, while solid lines indicate that cells were treated with the test compound for 1 h, irradiated with 350 nm UV light for 90 s, then further incubated overnight in the dark. Where indicated in (c), cells were treated with 50 μM H_2O_2 added with the test compound. Cell viability was assayed by CellTitre Blue after 24 h. Error bars represent standard deviation from experiments done in triplicate.

suggest that while 3Gcage does provide a robust coordination environment for retaining Cu(II) even under reducing conditions, the combination of reducing conditions and the presence of strong Cu(I) sinks could provide a pathway for Cu removal.

The cytotoxicity of **Cu3G** was evaluated in MCF-7, HeLa and HL-60 cancer cells by using CellTitre-Blue, a fluorometric assay that measures cell viability by their efficiency to metabolize resazurin to fluorescent resorufin. As shown in the dashed blue lines in Fig. 3, **Cu3G** has little effect on cell viability after 24 h incubation in the dark at concentrations below 100 μM, although the complex is cytotoxic at higher concentrations in all 3 cell lines. This trend differs from that of $CuCl_2$ (dashed green lines Fig. 3), which was toxic at most doses to MCF-7 and HeLa cells, but actually increased proliferation in HL60 cells. Such a response to added copper has been observed previously.[16] The difference in cell viability between $CuCl_2$ and **Cu3G** is consistent with the hypothesis that 3Gcage retains Cu in complexed form in cell culture.

The effect of UV light on **Cu3G**-treated cells was tested by exposing treated cells to UV light centered at 350 nm in a photoreactor for 90 s. The short exposure time was chosen because it is sufficient to cleave **Cu3G** cleanly *in vitro* in accord with Scheme 1.[20] As shown by the solid blue lines in Fig. 3, cell viability 24 h after photoirradiation was diminished in comparison to samples held in the dark. In contrast, UV light did not attenuate the viability of the $CuCl_2$-treated cells (solid green lines, Fig. 3). Furthermore, neither the 3Gcage ligand alone, nor the ligand after photoirradiation, reduced cell viability at concentrations up to 200 μM (ESI†).

When viewed under the microscope, photoirradiated cells treated with **Cu3G** looked rounded and swollen with extensive cytoplasmic vacuolization (Fig. 4e), whereas cell morphology was unchanged for untreated or 3Gcage-treated cells exposed to UV and for cells treated with **Cu3G** and kept in the dark (Fig. 4a–d). Combined, these results suggest that the cytotoxicity observed in samples treated with **Cu3G** and UV light is a consequence of

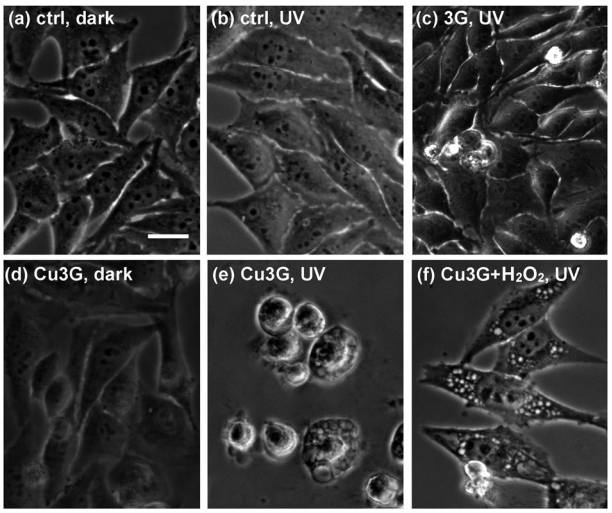

Fig. 4 Representative phase contrast images at 40× magnification of HeLa cells incubated for 24 h with treatments as indicated, where UV specifies photoirradiation prior to incubation; (a) control, no treatment, (b) control, 90 s UV irradiation, (c) 200 μM 3GCage ligand (no Cu) with 60 s UV irradiation, (d) 100 μM Cu3G, (e) same as d, but with 90 s UV irradiation, (f) 50 μM Cu3G + 50 μM H_2O_2 + 90 s UV irradiation prior to 18 h incubation. Images for treatment f were taken at a shorter timepoint to show the vacuolar structures evident prior to complete cell rounding as observed in e. Scale bar represents 10 μM.

UV-triggered changes to the **Cu3G** complex, and not the free ligand, extracellular copper, or UV irradiation alone.

While the cytotoxicity studies described above are promising, the shift to increased cytotoxicity upon photoirradiation is not dramatic, moving from an $IC_{50} \sim 150$ μM for **Cu3G** alone to ~75 μM upon UV exposure. Based on previous *in vitro* results showing that photoirradiated **Cu3G** increases the Fenton-like production of $OH^{\bullet}$ in the presence of ascorbate and H_2O_2, we were interested to see if low dose H_2O_2 could synergistically increase cytotoxicity of photoirradiated **Cu3G**. We therefore incubated HeLa cells in the presence of a non-toxic dose of 50 μM H_2O_2 with **Cu3G** with and without photoirradiation. As shown by the dashed red line in Fig. 3c, cells treated with a combination of **Cu3G** and H_2O_2 in the dark for 24 h remained greater than 80% viable up to a 100 μM dose. Cells that received the same treatment but also a 90 s exposure to UV light were much more susceptible to cell death, with an IC_{50} value ~30 μM (solid red line Fig. 3c). Interestingly, when the cells were imaged by bright field microscopy after 18 h, no change in the morphology was observed for cells treated with **Cu3G** and H_2O_2 (ESI†); however, extensive vacuole formation was observed in the cytoplasm of cells that received the combination of **Cu3G**, H_2O_2, and UV exposure (Fig. 4f and ESI†).

The cytoplasmic vacuolization observed in Fig. 4f is highly reminiscent of a hallmark of cytotoxic copper delivery agents that induce paraptotic cell death.[24–26] Most anticancer compounds induce apoptosis in cancer cells, and impairment of these pathways is associated with drug resistance.[27] The combined UV light and H_2O_2 stimulation of **Cu3G** to induce non-apoptotic cell death suggests that selective delivery of Cu to cancer cells might be of particular interest for apoptosis-resistant cell lines. The current work represents a promising step in that direction and points to several areas for improvement. Notably, ligands capable of better retaining Cu against reductive transfer would likely improve the differential between the stimulus-activated response *vs.* control. Furthermore, compounds that respond to stimuli other than UV light for metal release are desirable.

This work was supported in part by the U.S. National Science Foundation (CHE-1152054). A.A.K. acknowledges the Department of Science and Technology, Government of India, for a BOYSCAST Fellowship (SR/BY/C-10/09) and Duke University for providing facilities.

Notes and references

‡ Crystal data for [CuCl(3Gcage)]·2(EtOH): $C_{25}H_{32}ClCuN_5O_5$, M_r = 581.55 g mol^{-1}; triclinic, $P\bar{1}$; a = 10.6787(10) Å; b = 11.3255(9) Å, c = 12.1898(12) Å; α = 113.135(9)°; β = 99.670(8)°; γ = 98.548(7)°; V = 1298.4(2) Å^3; Z = 2; T = 123(2) K; density (calcd) = 1.487 Mg m^{-3}; 8680 reflections measured; 5179 independent reflections (R_{int} = 0.0256); R_1(obs) = 0.0611, wR_2(obs) = 0.1822; R_1(all) = 0.0651, wR_2(all) = 0.1876. § Abbreviations: PBS (phosphate buffered saline), BCS (bathocuproine disulfonate), Cu3G [CuCl(3Gcage)], ROS (reactive oxygen species).

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