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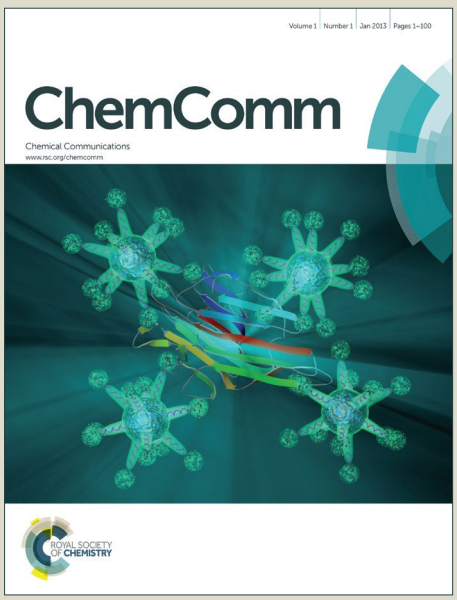

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Uncaging a Catalytic Hydrogen Peroxide Generator through the Photo-Induced Release of Nitric Oxide from a {MnNO}^{6} Complex

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The photo-initiated cytotoxicity of a newly developed manganese nitrosyl {MnNO}^{6} complex (UG1NO) to HeLa cells is described. The complex was found to be strongly cytotoxic after being exposed to light with a wavelength of 650 nm. Cell death was caused by a manganese(II) complex, UG1, generated from UG1NO through the photo-dissociation of NO, rather than by NO directly. Mechanistic studies revealed that UG1 consumes O_2 only in the presence of a reducing agent to catalytically produce H_2O_2.

The development of photo-caged generators of small molecules has been intense in the past decade because these compounds can allow the spatially and temporally controlled release of biologically relevant small molecules, such as CO, H_2O_2, H_2S, NO and peroxynitrite ($ONOO^-$), by the application of light.[1] For instance, Chang and co-workers recently developed an interesting compound, CPG1, which can be photo-activated to produce H_2O_2 on demand, and they demonstrated that CPG1 can be used to optically regulate cofilin-actin rod formation.[2] Xian and co-workers described a unique caged compound, WSP-1, which releases H_2S in cultured cells when stimulated by light.[3] CPG1 and WSP-1 both contain one or more *ortho*-nitrobenzyl groups, and this group acts as a photo-cleavable protecting group.[4] Nakagawa and co-workers have developed other unique photo-caged compounds without *ortho*-nitrobenzyl groups for releasing NO and $ONOO^-$.[5] Transition metal NO or CO complexes that release NO or CO when the ligands photo-dissociate have also been developed.[6] However, no photo-caged catalytic generators of the small molecules mentioned above have been developed. Here, we describe a manganese-nitrosyl {MnNO}^{6} complex that acts as a photo-caged catalytic generator of H_2O_2. When irradiated, the complex was found to induce changes in cell morphology and cell death efficiently. We discovered the compound as part of our development of intracellular manganese-NO complexes for the photo-induced release of NO.

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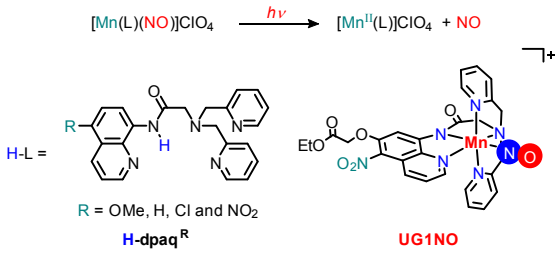
† Electronic Supplementary Information (ESI) available: Synthesis and characterization of UG1 and UG1NO. Figs. S1 to S7. Scheme S1. See DOI: 10.1039/x0xx00000x



Scheme 1 Photo-induced release of NO from {MnNO}^{6} complexes with pentadentate mono-carboxylamido ligands.

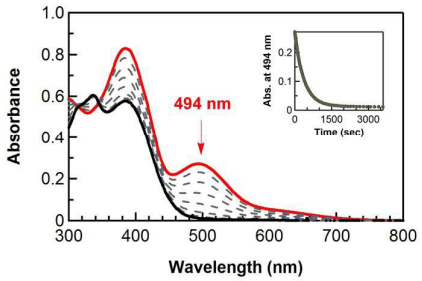


Fig. 1 Absorption spectral changes that occurred when UG1NO dissolved in a MES buffer (containing 5% DMSO and at pH 7.2) at 20 °C was irradiated with light with a wavelength of 650 nm. The arrow indicates where the band intensities decreased as the reaction proceeded. The inset shows the profile over time for absorbance at 494 nm.

We recently prepared manganese nitrosyl {MnNO}^{6} complexes with the general formula [Mn(dpaq^R)(NO)]ClO_4, where dpaq^R denotes a series of pentadentate mono-carboxylamido ligands, 2-[*N*,*N*-bis(pyridin-2-ylmethyl)]-amino-*N*'-quinolin-8-yl-acetylamido, with R = OMe, H, Cl or NO_2 at the 5-position of the quinoline moiety. We found that NO was released from this series of {MnNO}^{6} complexes when they were irradiated with light (Scheme 1).[7] The R = NO_2 derivative released NO more efficiently than the other derivatives when irradiated with red light with a wavelength of 650 nm. Encouraged by this result, we developed a strategy for achieving the light-induced release of NO from {MnNO}^{6} complexes under the conditions in which cells are cultured. We synthesized a new {MnNO}^{6} derivative with an ethyl ester moiety with the aim of increasing the intracellular retention of the complex (the ethyl ester

group being expected to become hydrolysed by esterase inside a cell, giving the corresponding acid derivative, which would not be able to permeate through the cell membrane). The newly synthesized complex $[Mn(L)(NO)]ClO_4$ (UG1NO, shown on the right in Scheme 1), in which L is the carboxylamide-deprotonated form of ethyl [{8-({[bis(pyridin-2-ylmethyl)amino]acetyl}amino)-5-nitroquinolin-6-yl}oxy]acetate, was synthesized as shown in Scheme S1. The UG1NO absorbed light in the Vis-NIR region and responded to irradiation with red light in a similar way to $[Mn(dpaq^{NO2})(NO)]ClO_4$, despite the presence of an additional electron-donating 1-(ethoxycarbonyloxy)ethyl ester at the 6-position of the quinoline. As expected, UG1NO in a buffer solution at pH 7.2 was stable in the dark but was quickly converted into the corresponding Mn(II) complex (UG1) through the dissociation of NO when it was irradiated with light with a wavelength of 650 nm (Fig. 1). The same behaviour was observed in cell culture media containing serum. The quantum yield of NO release from UG1NO by light irradiation at 650 nm was determined to be 0.74 ± 0.01, which is close to that reported for $[Mn(dpaq^{NO2})(NO)]ClO_4$.[7]

Table 1. IC_{50} values for the compounds that were tested on HeLa cells after 24 h exposure

Compound	Conditions	IC_{50} (μM)[a]
UG1NO	dark	> 100
UG1NO[b]	white light for 10 sec[f,h]	17 ± 1
UG1NO[b]	650-nm light for 1 h[g,h]	33 ± 3
NOC-7[c]	dark	> 100
SIN-1[d]	dark	> 100
UG1	dark	12.4 ± 0.2
UG1[e]	dark	> 100
cisplatin	dark	19 ± 3

[a]The errors are the standard deviations. [b]The systems were exposed to light and incubated for 24 h. [c]NOC-7 = 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine, an NO donor. [d]SIN-1 = 3-(4-morpholinyl)sydnonimine, hydrochloride, an ONOO– donor. [e]Co-incubated with esterase. [f]385 to 740 nm with 0.1 mW/cm^2. [g]5 μW/cm^2 through a band path filter (FWHW = 6 nm). [h]The light induced no cell death in the absence of the Mn complex.

The ability of UG1NO to deliver NO to living cells was then investigated. Before the experiments were performed, we assessed the anti-proliferative effects of UG1NO on HeLa cells in the dark. HeLa cells were incubated with different concentrations of UG1NO for 24 h, and the cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The IC_{50} (the concentration required to induce 50% inhibition) was determined from the dose-response curves (Fig. S1). We found that UG1NO was only weakly cytotoxic with an IC_{50} of more than 100 μM in the dark (Table 1). Therefore, HeLa was incubated with both UG1NO (10 μM) and an intracellular NO-selective fluorescent probe DAF2-DA[8] (10 μM) for 30 min to visualize the NO released by UG1NO when light was applied. Green fluorescence emitted by the product of the reaction of DAF2 with NO was observed only when light was applied (Fig. 2A). These results clearly indicate that UG1NO is a photo-activated NO-releasing molecule that can deliver NO to the insides of cells. It is likely that the resulting Mn(II)L complex should have a weakly coordinated water molecule in replace of NO in aqueous media.

We next determined whether the photo-triggered dissociation of NO from UG1NO could induce the death of HeLa cells. HeLa cells were incubated with different concentrations of UG1NO for 24 h before being exposed to white light for 10 s or to light with a wavelength of 650 nm for 1 h, after which the cell viability was determined using the MTT assay. We found that UG1NO was strongly cytotoxic to HeLa cells, the IC_{50} values being 17 ± 1 and 33 ± 3 μM after the white and 650 nm light treatments, respectively. The low IC_{50} values suggested that the release of NO was not the main cause of the cells dying, because NO is rather weakly cytotoxic (Okuno and co-workers reported that 500 μM NO was required to induce cell death in HeLa cells, when *S*-nitroso-*N*-acetyl-DL-penicillamine was used as a thermally induced generator of NO).[9] Even 500 μM of a thermally induced NO generator, NOC-7, did not induce cell death in HeLa cells in the tests we have performed. $ONOO^-$ is another potential toxic agent, as it can be formed quickly by the reaction of NO with a superoxide anion radical ($O_2^{\bullet-}$). However, the IC_{50} of SIN-1, a thermally induced $ONOO^-$ donor, was above 100 μM. Taking these results together we concluded that NO and $ONOO^-$ were not the main causes of the photo-induced cell death that was observed even though NO was released from UG1NO exposed to light.

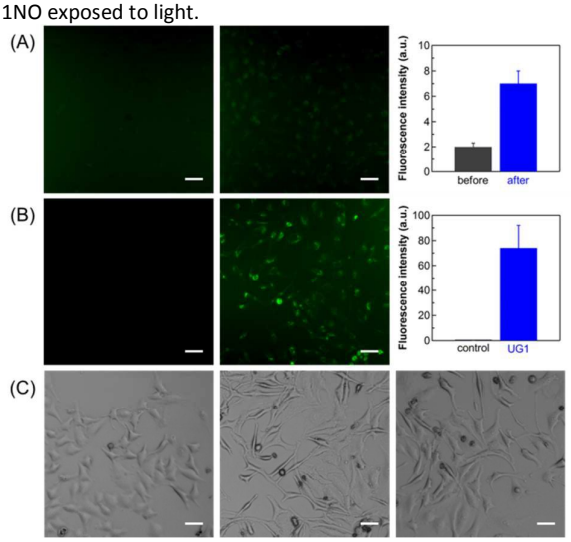


Fig. 2 (A) Fluorescence images of cells incubated with DAF-2DA and UG1NO before (left) and after (middle) being exposed to light with a wavelength of 650 nm for 30 min. Each scale bar indicates 50 μm. The relative fluorescence intensities are shown on the right. (B) Fluorescence images of cells exposed to PF1 (left) and to PF1 and UG1 (middle) after being exposed to light. Each scale bar indicates 50 μm. The relative fluorescence intensities are shown on the right. (C) Bright field images of cells incubated with 10 μM UG1 (middle) and 100 μM H_2O_2 (right). The control is shown in the left.

We later found that the Mn(II)L complex UG1, which lacks an NO ligand, had a strong anti-proliferative effect on HeLa cells (the IC_{50} was 12.4 ± 0.2 μM after 24 h incubation). The IC_{50} was comparable to that found for UG1NO after white light was applied for 10 sec or after light with a wavelength of 650 nm was applied for 1 h (Table 1). Notably, UG1 was found to be even more cytotoxic than cisplatin, which is an anti-cancer drug (IC_{50} = 19 ± 3 μM).[10] It should be noted that the ester functionality of UG1 was important to the ability of UG1 to cause cell death. The acid form of UG1 was found to be not cytotoxic (IC_{50} > 100 μM), and the structurally similar manganese(II) complexes without ester functionality (i.e., $[Mn^{II}(dpaq^R)]ClO_4$, R = OMe, H, Cl or NO_2) shown in Scheme 1 were

also found to be only moderately cytotoxic (with IC_{50} values between 30 and 70 μM, data not shown). We speculated that UG1 may cause cell death via oxidative stress. Therefore, we next visualized the production of reactive oxygen species using a H_2O_2-specific fluorescent probe, PF1,[11] and a cell-membrane permeable fluorescent reactive-oxygen-species probe, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). We observed green fluorescence inside cells incubated with UG1 and PF1 or DCFH-DA (Figs. 2B and S2). These results suggest that UG1 can produce H_2O_2 inside a cell.

Interestingly, HeLa cells became elongated when incubated with 10 μM UG1, as shown in Fig. 2C (middle). A similar morphological change was observed when HeLa cells were treated with 100 μM H_2O_2 (Fig. 2C, right). HeLa cells have been found to become elongated after exposure to isophthalate derivatives[12] and as a consequence of knock-down by prohibitins.[13] Isophthalate derivatives affect the reorganization of the cytoskeleton by binding to the C1 domain of protein kinase C. However, prohibitin plays an important role in mitochondrial function, and its overexpression protects against oxidative stress.[14] Bokoch and co-workers found that exogenously generated H_2O_2 can modulate cytoskeleton reorganization via the activation of the actin depolymerization factor cofilin.[15] The similarities in the morphological changes caused by UG1 and H_2O_2 suggested that UG1 can produce H_2O_2 inside a cell. We speculated that H_2O_2 may be catalytically produced through the reductive dioxygen activation by UG1, resulting in the consumption of biological reducing agents inside the cell.

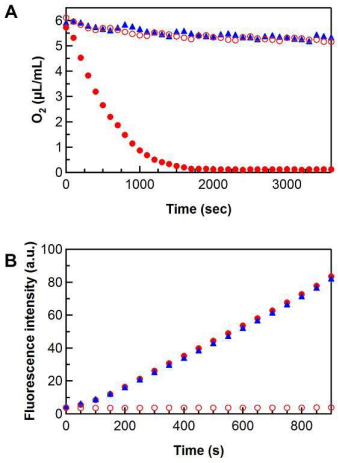


Fig. 3 (A) Oxygen consumption by UG1 (100 μM) in an air-saturated 20 mM phosphate buffer (pH 7.5) at 37 °C. The open and filled red circles are data collected in the absence and presence of 1 mM ascorbate, respectively. The blue filled triangles are data for the control (without UG1 present). (B) The generation of H_2O_2 by UG1 (25 μM) in an air-saturated 20 mM phosphate buffer (pH 7.5) at 37 °C. The open and filled red circles are data collected in the absence and presence of 1 mM ascorbate, respectively. The H_2O_2 was detected using PF1 (10 μM). The blue filled triangles are data obtained in the presence of superoxide dismutase (100 unit/mL).

It has been found by several research groups that redox-active metal complexes, including water-soluble cationic manganese(III) porphyrins[16] and iron(III) porphyrins,[17] can cause enough oxidative stress to induce cell death through the formation of $O_2^{\bullet-}$ and H_2O_2. Batinic-Haberle and co-workers found that a series of water-soluble cationic manganese(III) porphyrin complexes (MnPs) showed anti-cancer activities in several cancer models, and to achieve this the complexes acted as pro-oxidants (even though the complexes were originally developed for use as superoxide dismutase (SOD) mimics). Batinic-Haberle and co-workers showed that MnPs can promote the aerobic oxidation of ascorbic acid, producing $O_2^{\bullet-}$ and H_2O_2, and that H_2O_2 is a major cytotoxic species, formed through the disproportionation of $O_2^{\bullet-}$ and through the reaction between $O_2^{\bullet-}$ and Mn(II) porphyrins. The latter reaction is part of the SOD cycle catalysed by MnPs. The SOD activity of SOD mimics strongly depends on the redox potential of SOD mimics, as the SOD cycle is made up of the reduction and oxidation reactions of $O_2^{\bullet-}$ (Scheme 2A). SOD mimics based on MnPs have redox potentials in the range −50 to 350 mV vs. NHE, which are between the reduction potential of $O_2^{\bullet-}$ (891 mV vs. NHE; $O_2^{\bullet-}/H_2O_2$) and the oxidation potential of $O_2^{\bullet-}$ (−160 mV vs. NHE; $O_2/O_2^{\bullet-}$).[18] We therefore measured the cyclic voltammogram of UG1 in 5% DMSO in H_2O (Fig. S3). UG1 was found to have a Mn(II)/Mn(III) redox potential of 732 mV vs. NHE. As suggested from the redox potential, UG1 showed SOD activity in vitro, although the SOD activity of UG1 was rather lower than the SOD activities that were found for the MnPs mentioned above (log k_2 = 6.2 ± 0.2 M^{-1} s^{-1}; Fig. S4). This SOD activity of UG1 suggests that, in the Mn(II) state, it can produce H_2O_2 by reacting with $O_2^{\bullet-}$ (eq 2 in Scheme 2). However, the positive reduction potential of UG1 suggested that the Mn(II) centre of UG1 cannot reductively activate dioxygen to produce $O_2^{\bullet-}$. As expected, UG1 stably retained the Mn(II) state in aqueous solution and did not consume dioxygen (Fig. 3A). Jackson and co-workers found that the Mn(II) centre of a structurally related manganese(II) complex, Mn(dpaq^H), was aerobically oxidized to the corresponding Mn(III)OH species in acetonitrile.[19] UG1 has an additional nitro-group at the 5-position of the quinoline moiety, which would make its redox potential more negative than the redox potential of Mn(dpaq^H), and therefore UG1 cannot react with dioxygen. However, we observed that UG1 catalytically consumed dioxygen in the presence of ascorbic acid (Fig. 3A), as Batinic-Haberle and co-workers found for SOD mimics based on MnPs.[16g]

We also found that UG1 facilitated the oxidation of glutathione (GSH) to give GSSG under aerobic conditions, and this was monitored using the glutathione reductase/NADPH coupled method (Fig. S5). We confirmed that UG1 could produce H_2O_2 only in the presence of the biological reducing agents GSH (−0.26 V vs. NHE)[20] and ascorbic acid (0.06 V vs. NHE)[21] (Figs. S6 and 3B) using a H_2O_2-specific fluorescent probe, PF1.[11] These results indicate that UG1 can reductively activate dioxygen at the manganese centre, with an assistance from biological reducing agents, to produce H_2O_2.

A) superoxide dismutase activity

$$[Mn^{III}(L)]^{2+} + O_2^{\bullet-} \longrightarrow [Mn^{II}(L)]^{+} + O_2 \quad (1)$$

$$[Mn^{II}(L)]^{+} + O_2^{\bullet-} \xrightarrow{2H^+} [Mn^{III}(L)]^{2+} + H_2O_2 \quad (2)$$

B) catalytic H_2O_2 generation

$$[Mn^{II}(L)]^{+} \underset{}{\overset{O_2}{\rightleftharpoons}} [Mn(O_2)(L)]^{+} \quad (3)$$

$$[Mn(O_2)(L)]^{+} + AH \longrightarrow [Mn^{III}(OOH)(L)]^{+} + A\cdot \quad (4)$$

$$[Mn^{III}(OOH)(L)]^{+} + H^{+} \longrightarrow [Mn^{III}(L)]^{2+} + H_2O_2 \quad (5)$$

$$[Mn^{III}(L)]^{2+} \xrightarrow{\text{reductant}} [Mn^{II}(L)]^{+} \quad (6)$$

Scheme 2 (A) Plausible reactions during superoxide disproportionation catalysed by UG1. (B) Proposed reaction mechanism for the generation of H_2O_2 catalysed by UG1. AH denotes a reductant.

The formation of H_2O_2, catalysed by UG1 in the presence of ascorbic acid, was not altered by adding SOD (Fig. 3B), excluding the possibility that some free $O_2^{\bullet-}$ may be produced by the reaction of UG1 (in the Mn(II) state) with dioxygen. It is more likely that the dioxygen adduct of the UG1 in the Mn(II) state will oxidize a biological reductant to yield the corresponding Mn(III)OOH, which would afford H_2O_2 and the Mn(III) complex (eqs 3-5 in Scheme 2). The dioxygen adduct, manganese(III)-superoxo complex, would have enough reactivity toward hydrogen atom abstraction from a biological reductant.[22] A similar reaction has been reported in which an air-stable mononuclear nonheme Fe(II) complex produced the corresponding Fe(III)OOH species in the presence of an NADH analogue.[23] These in vitro results suggest that H_2O_2 could be catalytically produced by UG1 inside a cell, although small amounts of H_2O_2 may be produced by the reaction between UG1 and $O_2^{\bullet-}$ generated endogenously through cellular processes, such as the mitochondrial respiratory chain (eq 2 in Scheme 2).

In summary, we have found that a newly developed manganese nitrosyl complex releases NO when treated with light, and is also a photo-caged catalytic generator of H_2O_2 when a reducing agent is present. Many photo-caged molecules based on photo-labile protection groups, such as the *ortho*-nitrobenzyl group, have been prepared.[24] Our results demonstrate that ligand photo-dissociation is a useful way of achieving the photo-activation of the catalytic activity of metal complexes. We are now investigating the mechanism involved in the activation of dioxygen to produce H_2O_2 by UG1 in detail.

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