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Walker, M.G., Jarman, P.J., Gill, M.R. et al. (9 more authors) (2016) A self-Assembled Metallomacrocycle Singlet Oxygen Sensitizer for Photodynamic Therapy. Chemistry - A European Journal, 22 (17). pp. 5996-6000. ISSN 0947-6539

https://doi.org/10.1002/chem.201600852

DOI: 10.1002/chem.200((will be filled in by the editorial staff))

A self-assembled metallomacrocycle singlet oxygen sensitizer for photodynamic therapy

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Dedication ((optional))

Abstract: Although metal ion directed self-assembly has been widely used to construct a vast number of macrocycles and cages, it is only recently that the biological properties of these systems have begun to be explored. However, up until now, none of these studies have involved intrinsically photo-excitable self-assembled structures. Herein we report the first metallomacrocycle that functions as an intracellular singlet oxygen sensitizer. Not only does this Ru_2Re_2 system possess potent photocytotoxicity at light fluences below those used for current medically employed systems, it offers an entirely new paradigm for the construction of sensitizers for photodynamic therapy.

Keywords: Self-assembly • singlet oxygen • ruthenium • PDT • luminescence

Introduction

Photodynamic therapy, PDT, is a non-invasive therapy regime in which light is used to selectively damage diseased tissue, usually through the *in situ* creation of reactive oxygen species (ROS).[1-5] Commonly, this is accomplished through a prodrug photosensitizer molecule that generates cytotoxic singlet oxygen *via* excited-state energy transfer. PDT has been successfully employed to treat a range of cancers[6] and other disease states.[7]

One advantage of PDT is that cell death can be targeted to specific locations through a carefully selected combination of sensitizer and light; minimising many of the undesired side-effects of conventional therapeutic regimes. The majority of photosensitizers in clinical use are tetrapyrrolic structures. [8,9] However, these structures have a number of disadvantages; for example, as extended aromatic systems they often display relatively low water solubility and they can be difficult to synthesize and purify. In recent years - due to their good water solubility, attractive optical properties, and high photostability - transition metal coordination compounds have been investigated as alternative photosensitizer leads.[10] In particular, Ru^{II} complexes have attracted much attention and one such complex is currently entering Phase I trials as a PDT sensitizer for bladder cancer.[11]

Meanwhile, over the last two decades, interest in metal directed self-assembly has exploded, leading to a huge variety of often very complex molecular architectures.[12-14] Many of these systems are hosts, and have been developed as devices such as sensors for ions and molecules or even catalysts.[15-17] Although the interaction of such architectures with biomolecules has been investigated; originally much of this work focused on helicate structures, producing several promising potential therapeutics. [18-20]

It is only more recently that the bioactivity of metallomacrocycles and cages has begun to be examined.[21,22] In this context, perhaps the most well-studied cage systems are those

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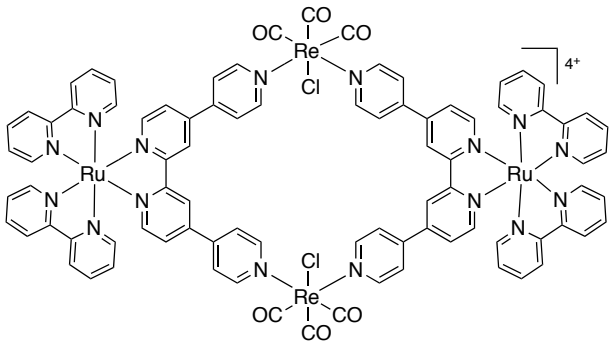
reported by the Therrien group; for example, these architectures have been successfully employed as *in cellulo* delivery vectors for a variety of "payloads" including cytotoxic therapeutics, [23,24] luminescent DNA probes,[25] and porphyrin-based sensitizers for PDT.[26] Furthermore, a number of metallocages have been identified as promising anticancer therapeutic leads in themselves, although the exact molecular basis of observed cytotoxicity in such systems is largely unidentified or poorly understood. [27,28]



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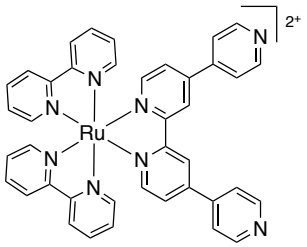

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Scheme 1 Structures discussed in this study.

In this context, we have previously reported on the synthesis[29,30] and structure[31] of self-assembled, kinetically inert, water-soluble metallomacrocycles, such as **1** (Scheme 1). These systems which – because they are based on inert d^6-metal ions - are high stable in aqueous solution possess highly structured, binding pockets composed of hydrophobic aromatic residues that bind to biomolecules with high affinity in biologically relevant conditions.[32-34] Since these macrocycles incorporate polypyridyl Ru^{II} and Re^{I} units that are related to systems being explored as PDT sensitizers, [10,11,35] we investigated the cellular internalisation properties and photocytotoxicy of **1** towards human cancer cells. Employing clinically relevant light doses, we find that metallomacrocycle **1** is successfully internalised by cells where it functions as a potent photosensitizer though ROS generation that results in plasma membrane damage.

Results and Discussion

In combination with light microscopy, the MLCT (metal to ligand charge-transfer) emissive properties of Ru(II) polypyridyl complexes provide a useful property by which to assess the cellular internalisation of a complex of interest;[36,37] a concept that may be extended to hetero bi-metallic systems.[38] As both macrocycle **1** and its "building block" mononuclear complex **2** display $Ru^{II}\rightarrow$qtpy ^{3}MLCT-based luminescence on photoexcitation at 458 nm[30,31] we initially explored cellular localisation using confocal laser scanning microscopy (CLSM) and fixed MCF7 human breast cancer cells. Surprisingly - even though **2** is emissive whether or not it is bound to biomolecules such as DNA and the barrier to transport across the plasma membrane has been removed by fixation - no intracellular emission from mononuclear complex **2** was observed. In contrast, macrocycle **1** *does* localise in fixed MCF7 cells. Although some non-specific nuclear staining is observed, the macrocycle particularly brightly stains the lipid rich regions of the cell, such as the nuclear membrane and endoplasmic reticulum (ER) – Figure 1 and SI.

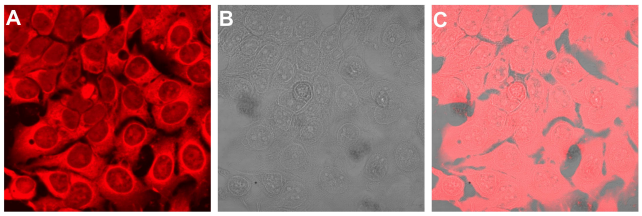

Figure 1. (**A**) CLSM images of fixed MCF7 cells stained with **1** for emission at 640-700 nm (^{3}MLCT emission of **1**). (**B**) Phase Contrast image at 485 nm (^{1}MLCT absorbance of **1**). (**C**) Merged image. [**1**] = 100 μM..

As expected from the fixed cell studies, live MCF7 cells shows no intracellular emission from **2**, indicating that live cells do not take up the complex; however addition of macrocycle **1** produced very different results – Fig 2. After 20 minutes incubation with 200 μM of the macrocycle, the media was removed. Following washing with PBS, the cells were placed in complex-free fresh media and imaged through CLSM using monochromated laser light exciting into the MLCT of **1** at 488 nm. Although initial images showed only plasma membrane staining over a period of <10 mins, cells began to display dramatic changes in both dye localisation and cellular morphology with prolonged light exposure - Fig 2A. During the first hour after exposure, **1** begins to localize within cells with evidence of nuclear accumulation and particularly intense emission being observed from nucleoli. After this period, general staining of the nuclear membrane and other membrane-enclosed structures in the cytoplasm such as endosomes, lysosomes, and the ER was increasingly observed. Gross cell swelling and the generation of growing amounts of apparently dead cells, along with complex-stained cell debris, accompanied the shifts in cellular localization of **1** - Fig 2 B.

These cellular responses, which are characteristic of oncosis/necrosis processes,[39,40] are often consequences of *in cellulo* $^1O_2$ generation.[41] Furthermore, these changes only occur after exposure of treated cells to light, clearly indicating a phototoxic response: if cells are treated with the same concentration of **1** but kept in the dark they still display normal morphology, even after two hours exposure – See SI.

It is notable that final localization in necrotic cells, where membranes have been disrupted, is very similar to that observed in fixed cells, indicating that the initial target of **1** in live cells is the plasma membrane. This hypothesis is consistent with the images in Fig 2 showing the complex accumulating at the plasma membrane at early time-points before it localizes within intracellular compartments, including the cell nucleus, as plasma membrane integrity is increasingly compromised. A similar effect has been reported to occur on cell treatment with lipophilic mononuclear Ru^{II} complexes.[42] Given these striking results, the ability of **1** and **2** to generate singlet oxygen on photo-excitation was investigated.

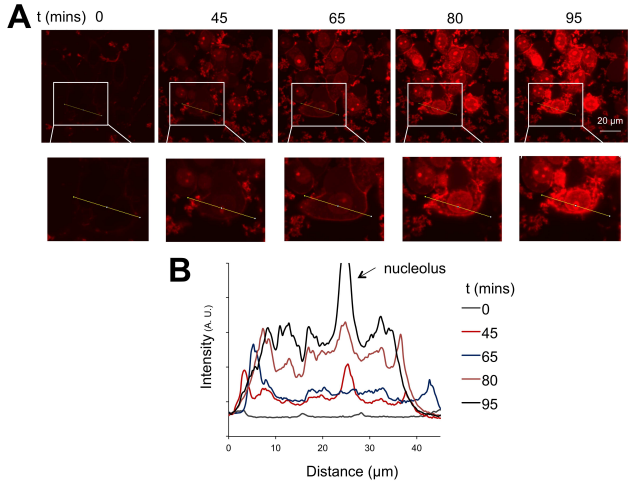

Figure 2. (**A**). Top panels: CLSM images of live MCF7 cells pre-treated with **1** ([**1**] = 200 µM, 20 mins) before exposure to 488 nm light over a 100 minute time course. MLCT emission (640-700 nm) Lower panels: detailed Image of the single cell shown in the white box in the upper panels (**B**). Time dependent emission profile of the same cell illustrating the increase in intensity and the appearance of strong nucleolus signal after initial plasma membrane staining.

Singlet oxygen quantum yields were directly measured by assessing luminescence at 1270 nm following photoexcitation of hexafluorophosphate salts of the complexes in acetonitrile. An optically matched solution of phenalenone was used as a reference sensitizer. This led to $\phi(^1O_2)$ estimates of 54 ± 5 % and 75 ± 2 % for **1** and **2** respectively. These figures probably reflect the lower energy, slightly shorter excited state lifetime of **1** in water (667 nm, 490 ns) compared to **2** (657 nm, 511 ns).

Since **1** binds to biomolecules such as DNA with high affinities $>10^6$ M^{-1} and has a $\phi(^1O_2)$ value comparable to previously reported sensitizers, it seems the biological action of **1** is through singlet oxygen sensitization. To test this hypothesis, DNA was selected as a representative biomolecule to examine the ability of **1** to induce singlet-oxygen mediated damage upon MLCT photoexcitation in cell-free experiments.

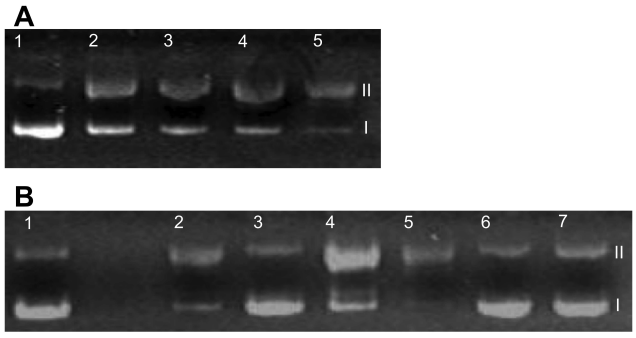

Figure 3. Photocleavage of supercoiled pBR322 DNA (3 µg) y **1** under illuminated condition (476 nm, 100 mW, 10 minutes exposure) in 50 mM Tris-HCL buffer**.** Forms **I** and **II** are supercoiled and nicked circular forms of DNA, respectively. **(A)** Lane 1, DNA control; lane 2, DNA + **1** (10 μM); lane 3, DNA + **1** (20 μM); lane 4, DNA + **1** (30 μM) ; lane 5, DNA + **1** (40 μM). (**B**) Lane 1, DNA control; lane 2, DNA + **1**; lane 3, DNA + **1** + NaN_3 (200 μM) ; lane 4, DNA + **1** + DMSO (2 μL); lane 5, DNA + **1** in D_2O; lane 6, DNA + **1** in argon; lane 7, DNA + **1** in dark..

Treatment with 25 µM **1** followed by 10 minutes illumination at 480 nm produces significant cleavage of supercoiled (form I) pBR322 plasmid DNA in buffered aqueous solutions, Fig 3A.

Complete cleavage is observed at concentrations above 40 µM. The mechanism of photocleavage was explored through further experiments involving pBR322 and a range of different inhibitors – Fig 3B. In the dark no significant cleavage is observed, confirming light is necessary for nuclease activity. Since hydroxyl radical scavengers such as DMSO do not inhibit plasmid cleavage, it is unlikely that this radical is responsible for cleavage. In contrast, photocleavage is not observed under argon indicating the involvement of dioxygen. To confirm that photoinduced cleavage involves singlet oxygen, experiments were carried out in the presence of sodium azide and D_2O. Sodium azide is a very effective $^1O_2$ quencher and - since $^1O_2$ has a longer lifetime in D_2O compared to H_2O - the deuterated solvent potentiates the activity of singlet oxygen. As shown in Fig 3B (lanes 3 and 5), the cleavage of pBR322 is inhibited by the presence of sodium azide and enhanced in D_2O, confirming that $^1O_2$ is responsible for the cleavage reaction.

Having confirmed that macrocycle **1** is phototoxic to MCF7 cells and induces damage to biomolecules by singlet oxygen generation, we quantified its photo-cytotoxicity towards A2780cis ovarian cancer cells, we chose this second cell line as it is highly resistant to the commonly employed Pt^{II}-based therapeutic cisplatin. To aid comparisons, complex **2** was investigated in parallel studies.

Cells were pre-treated with concentration gradients of **1** or **2** for 24 h before complex removal and exposure to increasing fluences of white light. The impact of each treatment upon cell viability was determined through MTT assay (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 24 h after light treatment. Data were normalized against cells not exposed to the complexes but treated with the relevant light dose, yielding the half-inhibitory IC50 values summarized in Table 1 and Figure 4.

Table 1. IC_{50} Values for **1** and **2** towards the cisplatin-resistant cell line A2780cis in the presence and absence of light.

Fluence (J cm^{-2})	IC_{50} concentration (µM) for complexes.[a]	
	1	**2**
0	61.7	> 100
8	8.5	> 100
24	1.2	28.8
48	0.3	22.9

[a] In same conditions, the cytotoxicity of cisplatin is IC_{50} = 22.9 µM.

These data show that in the dark both **1** and **2** display low cytotoxicity. Complex **2** is particularly inactive (IC50 > 100 µM) and, although **1** does demonstrates some potency, it is still approximately three-fold less toxic (IC_{50} =61.7 µM) towards a cisplatin resistant line than cisplatin itself. However, on exposure to light, **1** produces a dramatic impact upon cell viability.

As illustrated in Fig 4a, cell viabilities are sensitive to both the concentration of **1** and light dosage: even when exposed to low fluences of 8 or 24 Jcm^{-2}, **1** now demonstrates potent cytotoxicity, over an order of magnitude greater than its dark value. In fact at a higher light dose of 48 Jcm^{-2}, the IC_{50} decreases to 0.3 μM; a value two orders of magnitude more potent than cisplatin.

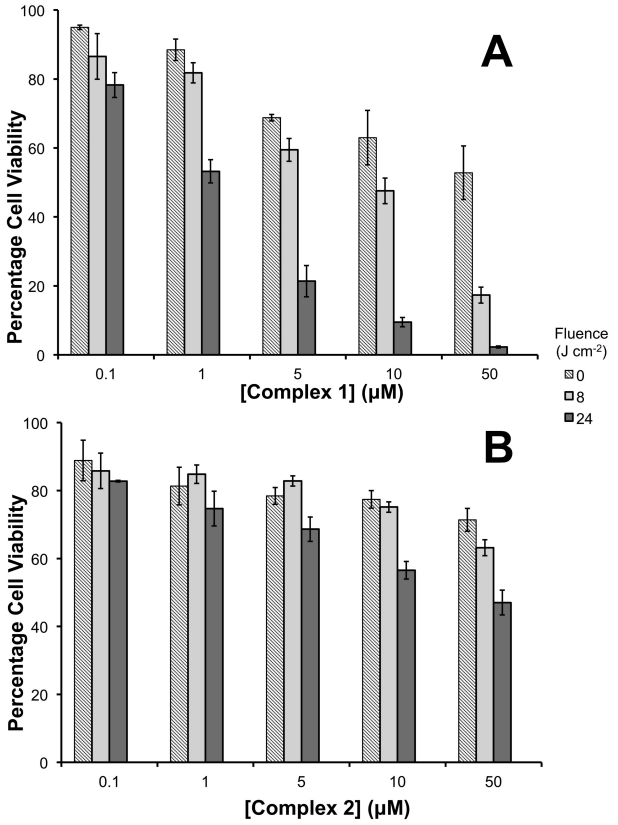

Figure 4. Light and dark cell viability figures on exposure to varying light fluences and different concentration of macrocycle **1** and mononuclear complex **2**.

In contrast, **2** produces only minimal phototoxic effects at high concentrations and light doses – Fig 4b. For example, at a fluence of 48 Jcm^{-2} (a fluence less than half of that used with the licenced PDT sensitizer photofrin) the photocytotoxicity (PI) index - where PI = IC50(light)/IC50(dark) – for the complexes showed that whilst PI(**2**) < 5, PI(**1**) = 206. Thus, on exposure to light, macrocycle **1** displays nanomolar cytotoxicity in cisplatin resistant cells at fluences that are lower than those required for a clinically approved sensitizer.

Given that **2** actually has a higher $\phi(^1O_2)$ than **1**, the dramatic differences in cytotoxicities and PI between the mononuclear complex and the macrocycle can be attributed to their contrasting cellular uptake properties; a well-established principle in the cell biology of ruthenium polypyridyl complexes.[43] To quantify this effect, relative cellular accumulation of both compounds in A2780CIS cells was determined using inductively coupled plasma mass spectrometry (ICP- MS). Cells were incubated for 12 hours with 50 μM concentrations of **1** or **2** - the highest concentration employed in the experiments summarized in Figure 4 – before intracellular Ru and Re content was determined. This analysis confirmed that ruthenium intracellular concentrations are approximately seven times higher on treatment with **1** compared to **2** – see SI. Since the macrocycle contains two Ru centres, in terms of molarity, this corresponds to a 3.5-fold preferential increase in **1** over **2**. Further analyses of the ICP-MS experiments with **1** confirm that the intracellular ratio of [Ru]:[Re] is 1:1, providing further evidence of macrocycle uptake into cells. Furthermore, the fact that low levels of **2** enter cells explains why some phototoxicity is observed for this complex at higher exposure concentrations and fluences.

Conclusion

In summary, for the first time, a metal complex PDT sensitizer has been constructed through metal directed self-assembly of photo-excitable oligonuclear metallomacrocycles. The striking bioactivity of this system can be largely attributed to its more favourable cellular uptake properties compared to its mononuclear building block. Presumably, this observation is due to a previously reported effect, the overall charge density/lipophilicity of the macrocyclic assembly is lower than that of the corresponding mononuclear complex. Since the novel macrocyclic PDT sensitizer discussed in this study has been self-assembled from mononuclear complex "modules", architecturally complex systems with new properties and functions can be readily constructed from relatively simple, previously reported, building blocks. Through this new paradigm, systems with enhanced uptake and tuneable photo-redox properties can be targeted. For example, as our previous studies indicate, [32-34] by judicious selection of ancillary and bridging ligand, cellular and subcellular binding properties of these novel PDT sensitizers can be selectively modulated to produce highly targeted PDT regimes.

Furthermore, as recently demonstrated in a number of studies, it is possible to construct Ru^{II}-based systems that can be photo-excited within the therapeutic optical window.[44-47] For example, since oligonuclear Ru^{II} complexes often display high two-photon absorption, 2PA, cross-sections,[48-50] using this modular approach, the design of macrocyclic systems for 2PA-PDT can be readily envisioned. Such studies will provide the basis of future publications.

Experimental Section

See SI for experimental details.

Acknowledgements ((optional))

PJJ and HA are grateful to the EPSRC and Malaysian Government for studentships. We also thank the Wellcome Trust, the EU MC-IIF programme, and Leverhulme Trust for Postdoctoral Fellowships (MRG, PANR, and MGW).

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Published online: ((will be filled in by the editorial staff))

Entry for the Table of Contents (Please choose one layout only)

Layout 1:

The Oxygen (Macro)cycle

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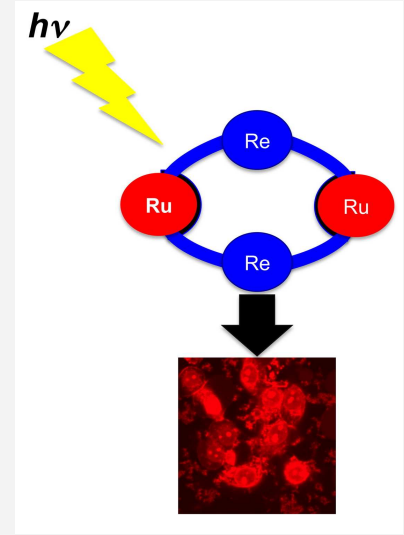
A self-assembled metallomacrocycle singlet oxygen sensitizer for photodynamic therapy



In cellulo studies show that a self-assembled Ru_2Re_2 metallomacrocycle possesses potent photocytotoxicity through singlet oxygen sensitization.

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