Responsive and mitochondria-specific ruthenium(II) complex for dual *in vitro* applications: two-photon (near-infrared) induced imaging and regioselective cell killing†

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A mitochondria-permeable ruthenium(II) complex has been designed as a responsive probe which may be used to sensitize the formation of singlet oxygen (Φ_Δ = 0.93) and cause local damage *in cellulo* when exposed to UV and near-infrared laser excitation.

Photodynamic therapy (PDT) has received considerable attention as a promising modality for cancer treatment.[1] This therapy requires a photosensitizer, visible light, and molecular oxygen. Upon irradiation with a specific wavelength, the photosensitizer undergoes a transition from its ground state to an excited singlet state. Subsequently, the excited singlet state may decay back to its ground state with emission of fluorescence or may undergo intersystem crossing to its excited triplet state. The excited triplet state can react with molecular oxygen to generate reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) and other radical species, which ultimately cause cellular and tissue damage. $^1O_2$ is known to be the major cytotoxic agent responsible for photobiological activity.[2]

Porphyrin-based photosensitizers, such as haematoporphyrin derivatives (HpD), have been studied extensively[3] and used in clinical work during the past two decades.[4] However, their non-specific localization and toxic UV excitation have still severely limited their achievable efficiency. In fact, requests have been made for the development of target specific-vectors for particular signal pathways and organelles, the mitochondria in particular.[5] By targeting the mitochondria, the power supply of the cell, the rate of cell death can be increased. The treatment efficiency of porphyrin-based sensitizers is also limited by their small extinction coefficient excitation in the body's therapeutic window (650–900 nm).[6] However, this can be overcome by a new generation of photosensitizers that absorb strongly at wavelengths greater than 650 nm through two-photon absorption (TPA).[7] Lower energy, longer wavelength photons readily penetrate the surface and if absorbed simultaneously, their combined energy may be sufficient to cross the energy gap between the excited-state energy levels and the ground-state energy levels of the photosensitizer.[8]

Herein, we describe the synthesis of a new mitochondria-specific PDT agent, **PZn–Ru**, an amphiphilic conjugate in which a porphyrin moiety and a polypyridyl Ru(II) moiety are covalently linked together *via* an ethynyl bridge. **PZn–Ru** exhibits a strong two-photon absorption cross-section and is capable of generating $^1O_2$ through linear and two-photon excitation. It is also cell permeable and can be excited selectively with high spatial control *in cellulo* using a laser, giving rise to high local concentrations of singlet oxygen, which can cause sufficient damage to trigger apoptosis. The insensitive **PZn-Phen** complex was used as negative control for the PDT therapy of **PZn–Ru** *in vitro*.

The precursor complex **PZn-Phen** can be readily synthesised *via* the Sonogashira cross-coupling reaction between 5-ethynyl-1,10-phenanthroline and 5-bromo-10,15,20-tri(3′,4′,5′-trimethoxyphenyl)porphyrinato zinc(II).[9] It is then reacted with *cis*-Ru(bpy)$_2$Cl$_2$ in THF–ethanol at 80 °C to give the title product **PZn–Ru** in 72% yield (see the ESI† for further experimental details).

The linear and two-photon induced photophysical properties of **PZn–Ru** in solution were measured at room temperature. **PZn–Ru** and showed marked peak-broadening and bathochromic shifts of 32 nm in its Soret bands compared to its intermediate (Fig. S2†). This suggests that there is rather strong electronic interaction between the porphyrin and the phenanthroline moieties. In $CHCl_3$, **PZn–Ru** displays identical normalized linear and two-photon induced visible emission spectra with maxima at 646 nm (Fig. 1).

The two-photon absorption cross section σ_2 value was measured at 800 nm using the open-aperture *Z*-scan method.[10] The inset of Fig. 1 shows the *Z*-scan traces of **PZn–Ru**, from which the absolute σ_2 value can be determined. **PZn–Ru** exhibits a large σ_2 value of 1104 GM (GM = 10^{-50} cm s^4 photon^{-1} molecule^{-1}), which is 40 times larger than that of tetraphenylporphyrin (H_2TPP) (σ_2 = 28 GM),[11] and also much higher than the recently reported two-photon bio-available organometallic molecular probes.[12] The large enhancement is mainly due to the expansion of π-conjugation and the molecular polarization induced by the asymmetrical (D–π–A) structure between the porphyrin and Ru(II)-polypyridyl moieties linked with an ethynyl bridge.

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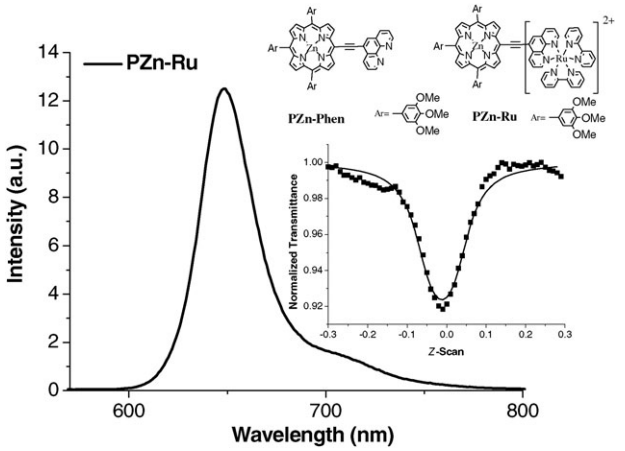

Fig. 1 The normalized linear and two-photon induced emission spectra of **PZn–Ru**; Inset: Open-aperture *Z*-scan traces of **PZn–Ru** (250 μM DMSO) excited at 800 nm. The average power of the laser beam used was 0.20 mW, the two-photon absorption cross-section of **PZn–Ru** is 1104 GM, λ_{ex} = 800 nm.

In order to evaluate the photosensitizing efficiency of **PZn–Ru**, the $^1O_2$ quantum yields (Φ_Δ) were determined by measuring the near-infrared (NIR) phosphorescence intensity of $^1O_2$ (at 1.27 μm) produced from these compounds upon irradiation at 424 nm, using H_2TPP as a reference (Φ_Δ = 0.55), in $CHCl_3$. The $^1O_2$ quantum yield of **PZn–Ru** (Φ_Δ = 0.93) is found to be significantly higher than that of H_2TPP. The subcellular localization of **PZn–Ru** in the HeLa (Fig. 2a and b), and HK-1 cells (Fig. 2c–f) was studied using both two-photon and linear fluorescence microscopy. After 40 min incubation, upon excitation at 850 nm, strongly solid red emission can be observed apparently in the mitochondria of the HeLa cells (Fig. 2a and b). To confirm the observed mitochondria localization, co-localization of **PZn–Ru** (red emission) with a commercial mitochondria tracker (green emission) was investigated and Fig. 2c–f shows the confocal images of cells co-stained with **PZn–Ru** and this mitochondria tracker.

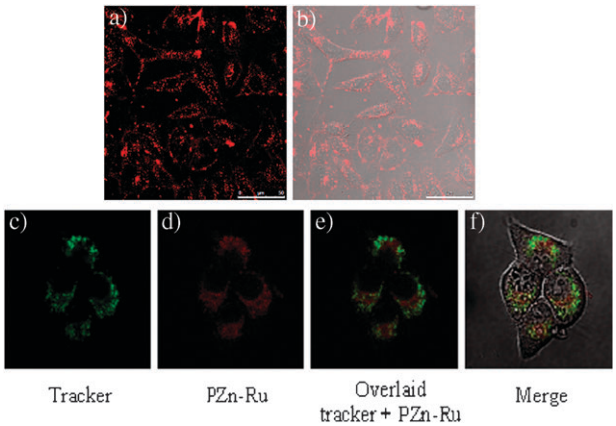

Fig. 2 (a and b) Two-photon induced *in vitro* mitochondria staining microscopy with **PZn–Ru** for 1 h (HeLa cells, dose conc. = 50 μM, λ_{ex} = 850 nm); (c, d, e and f) co-localization experiment with intracellular fluorescence of HK-1 cells treated with **PZn–Ru** (50 μM, 24 h) and Mito tracker (λ_{ex} = 450 nm).

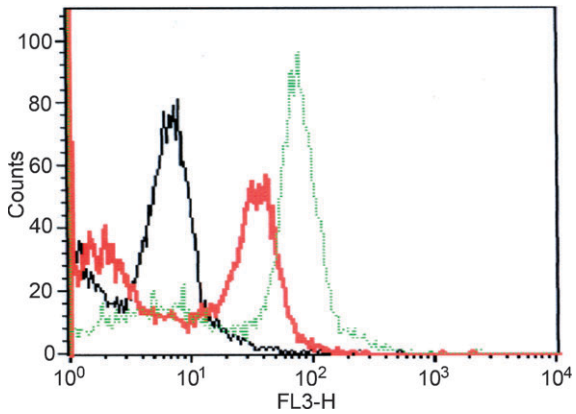

Fig. 3 Flow cytometric analysis of the cellular uptake of **PZn–Ru** in the HK-1 cells. The cells were incubated with 1 μM of **PZn–Ru** for 0 h (control, black line), 6 h (red line) or 24 h (green line) in dark. The cells were trypsinized and washed twice with PBS. The fluorescence profiles of the treated cells were analyzed using FACSCalibur (Becton Dickinson). The 488 nm laser line was used for the excitation of **PZn–Ru** and the fluorescence signal was collected using the FL-3 channels equipped with long pass filter (> 650 nm). At least 10 000 events were counted.

The uptake of a photosensitizer by tumour cells is a critical determinant of treatment efficacy. The cellular uptake of **PZn–Ru** by the human nasopharyngeal carcinoma HK-1 cells was studied by flow cytometry (Fig. 3). The fluorescence intensity of the HK-1 cells after incubation with **PZn–Ru** for 6 and 24 h was about 7 and 10 times, respectively, higher than that of the untreated cells. The results clearly indicated that **PZn–Ru** was taken up rapidly by the HK-1 cells. This is due to the amphiphilic structure of **PZn–Ru**, which has an appropriate hydrophobic/hydrophilic balance to enhance its cellular uptake.

We designed two experiments to evaluate the therapeutic efficacy of **PZn–Ru**. Firstly, its photocytotoxicity towards HK-1 cells was measured by a MTT-reduction assay (Fig. 4). It can be seen that **PZn–Ru** was essentially non-cytotoxic in the absence of light, but exhibited very high

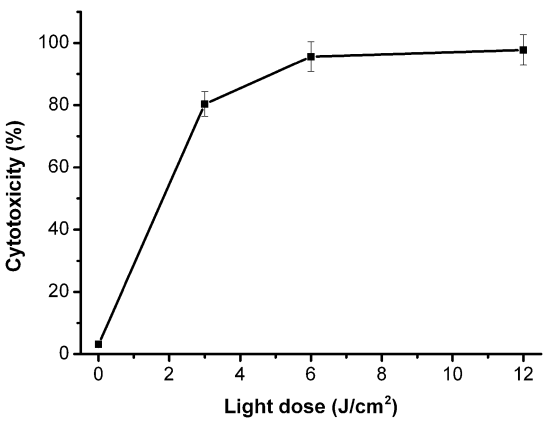

Fig. 4 Photocytotoxicity of **PZn–Ru** on HK-1 cells. HK-1 cells were treated with **PZn–Ru** (1 μM) for 24 h, then washed with fresh medium, and irradiated with various doses of light. The MTT-reduction assay was carried out 24 h after PDT. The results were expressed as the mean ± S.D. of three separate trials.

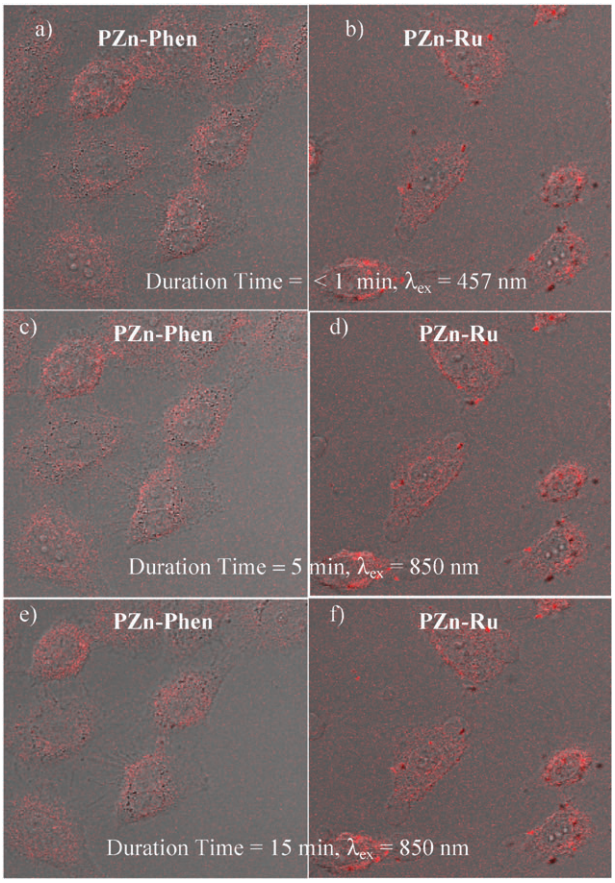

Fig. 5 Confocal microscopy and bright-field images of HeLa cells loaded with **PZn-Phen** (a, c, e) and **PZn–Ru** (b, d, f). (a and b) Images were observed with the incubation of **PZn–Ru** and **PZn-Phen** for 1 h before snap flash with a laser at 457 nm; (c and d) confocal microscopy images observed after flash with a laser at 850 nm for 5 min; (e and f) after further flash for 15 min with the laser line at 850 nm.

photocytotoxicity. At the concentration of 1 μM and a light dose of 3 J cm^{-2}, 80% of HK-1 cells were killed. The light dose required to kill 50% of HK-1 cells (LC_{50}) was *ca.* 1.8 J cm^{-2}. This remarkably high photocytotoxicity may be attributed to the high cellular uptake, high $^1O_2$ quantum yield and proper subcellular localization of **PZn–Ru**.

Andrasik *et al.* showed that the quantum yield of two-photon induced $^1O_2$ generation is around 30–40% even though the linear process and the mechanism of two-photon and linear $^1O_2$ generation are the same.[13] Thus, to further show the potential of its therapeutic efficiency, two-photon induced $^1O_2$ generation was also performed *in vitro* with **PZn–Ru**. 5 μM **PZn–Ru** or **PZn-Phen** (negative control, incapable of generating $^1O_2$ in the NIR region) was incubated in cervical carcinoma HeLa cells for 1 h and the uptake was visualized using a confocal microscope equipped with laser excitation sources at 457 nm (linear) or 850 nm (two-photon excitation). Cells in the field of view were then excited at 457 nm for a short period of time (to avoid $^1O_2$ generation, Fig. 5a and b). We observed that both **PZn–Ru** and **PZn-Phen** were taken up by the cells and localized in mitochondria and cytoplasm, respectively. There was no significant cell death seen in either case. After 5 min flash excitation by 850 nm laser on the cells loaded with **PZn–Ru**, 90% of the HeLa cells were deformed or had lost their integrity. However, the same was not observed for **PZn-Phen**. No significant cell death occurred and there was no observable change in the bright-field images of the HeLa cells (Fig. 5c and d). The cells dosed with **PZn–Ru** and **PZn-Phen** were further flashed with an NIR laser line for 15 min, after which all the cells incubated with **PZn-Phen** were seen to be alive (Fig. 5e).

In summary, a novel ethyne-linked amphiphilic porphyrin–polypyridyl Ru(II) conjugate with relatively high two-photon absorption cross-section has been synthesized for mitochrondra localization. The compound shows a substantial $^1O_2$ quantum yield under visible and NIR excitation. Significant cell death occurred upon suitable photoexcitation in the presence of **PZn–Ru**, suggesting that **PZn–Ru** may be a promising candidate for TPA photodynamic therapy.

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