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Green light-induced apoptosis in cancer cells by a tetrapyrridyl ruthenium prodrug offering two *trans* coordination sites

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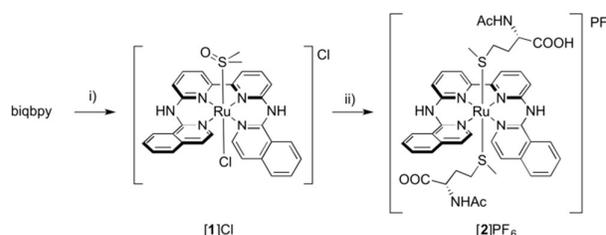
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In this work, two new photopharmacological ruthenium prodrugs are described that can be activated by green light. They are based on the tetrapyrridyl biqbpby ligand (6,6'-bis[N-(isoquinoly)]-1-amino]-2,2'-bipyridine), which coordinates to the basal plane of the metal centre and leaves two *trans* coordination sites for the binding of monodentate sulphur ligands. Due to the distortion of the coordination sphere these *trans* ligands are photosubstituted by water upon green light irradiation. *In vitro* cytotoxicity data on A431 and A549 cancer cell lines shows an up to 22-fold increase in cytotoxicity after green light irradiation (520 nm, 75 J.cm⁻²), compared to the dark control. Optical microscopy cell imaging and flow cytometry indicate that the cancer cells die *via* apoptosis. Meanwhile, very low singlet oxygen quantum yields (~1-2%) and cell-free DNA binding studies conclude that light-induced cell death is not caused by a photodynamic effect but instead by the changes induced in the coordination sphere of the metal by light, which modifies how the metal complexes bind to biomolecules.

Introduction

In classical chemotherapy side effects are a burden for patients, limit treatment doses, and lower prognosis. Light-activated anti-cancer prodrugs have appeared as an alternate strategy to increase the selectivity of chemotherapeutic agents.¹ Ideally, their inactive form should minimally interact with biological molecules to limit the toxicity of the prodrug to non-irradiated tissues. Upon *in vivo* light irradiation these prodrugs are locally activated to selectively kill tumour cells. Among light-activated compounds those based on ruthenium(II) have been extensively studied due to their superior light absorption properties and rich photoreactivity. The majority of light-activated ruthenium-based anticancer compounds described to date belong to the class of photodynamic therapeutic agents (PDT agents) that generate singlet oxygen (¹O₂) as a means to locally kill cancer cells.² For example, clinical trials recently started with ruthenium-oligothiophene dyads TLD1411 and TLD1433, which are red-light activated, water-soluble, and resistant to photobleaching.^{2c} A less common family of ruthenium compounds consist in photoactivated chemotherapy agent (PACT agents), where visible light excitation (350 – 800 nm) leads to the cleavage of a protecting group. This irreversible photoreaction releases a toxic ligand,³ modifies part of it,⁴ or

generates open coordination sites on the metal centre, which enables biological ligands to bind.⁵ In PACT a light-induced modification of the interaction between the metal compound and biological molecules triggers cell death.^{3b, 4a, 5a, 6} The major advantage of this mode of activation, compared to PDT, is that it does not depend on the presence of molecular oxygen, and hence may be applied to treat hypoxic tumours, a type of tumour characterised by low response to standard chemotherapy and faster cancer progression.⁷



Scheme 1. Synthesis of [1]Cl and [2]PF₆. Conditions: i) 1.1 eq. [Ru(DMSO)₂Cl₂], 80 °C, in EtOH under argon, 16 h, yield 43%; ii) 20 eq. HAmet, 80 °C, in water under argon, 16 h, yield, 43 %.

Many ruthenium PACT agents known to date contain two bidentate ligands based on the 2,2'-bipyridine scaffold.^{5a, 8} After irradiation, bis-aqua photoproducts are formed with a *cis* configuration that mimic the binding pattern of cisplatin to DNA.⁹ Transplatin, on the other hand, is not active *in vivo* and less cytotoxic than cisplatin *in vitro*, so that anticancer metallodrugs with a *trans* geometry, usually based on platinum(II), have not been considered until recently.¹⁰ New *trans* platinum(IV) compounds have also been prepared as PACT agents that can be activated with UV A (320-400 nm) or high-energy visible light (400-450 nm).^{5b, 11} This type of light is,

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however, harmful to cells¹² and penetrates biological tissues sub-optimally.¹³ We embarked hence on developing ruthenium-based PACT agents with a *trans* geometry that can be activated at higher wavelengths, *i.e.*, closer to the photodynamic window.¹⁴

Here we report on two *trans* ruthenium-based PACT compounds that can be activated using green light. The two ruthenium complexes, [Ru(biqbpy)(DMSO)Cl]Cl (**[1]Cl**, biqbpy = 6,6'-bis[N-(isoquinolyl)1-amino]-2,2'-bipyridine) and [Ru(biqbpy)(Amet)(HAMet)]PF₆ (**[2]PF₆**, HAMet = N-acetyl-L-methionine, Amet⁻ = deprotonated N-acetyl-L-methionine, see Scheme 1), are based on a tetrapyridyl ligand (biqbpy) specifically developed to coordinate in the basal plane of octahedral metal complexes and to leave two *trans* positions for the coordination of monodentate ligands.¹⁵ In order to minimize interactions of the metal centre with biomolecules in the dark, sulphur-based monodentate ligands were selected, *i.e.*, one dmsol in **[1]Cl**, and two Amet⁻ ligands in **[2]PF₆**, that can be removed by visible light irradiation.¹⁶ The synthesis, photochemistry, and biological properties of these compounds is reported, which demonstrates that they can trigger apoptosis in human cancer cell lines upon green light irradiation.

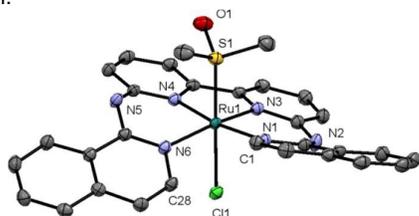


Figure 1. Displacement ellipsoid of cationic *M*-**[1]**⁺ (50% probability level) as observed in the crystal structure of (**[1]Cl**·CH₃OH)₂. chloride counter-anions, H atoms, lattice CH₃OH, and disorder, have been omitted for clarity.

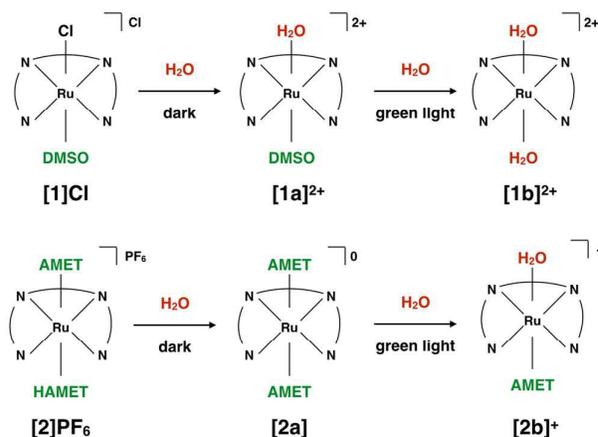
Results and Discussion

Synthesis and characterization. Complex **[1]Cl** was synthesized by reacting biqbpy with 1.1 equivalents of [Ru(DMSO)₄Cl₂] in ethanol overnight at 80 °C (Scheme 1). After filtration **[1]Cl** was obtained as a red brown powder. Slow vapour diffusion of a methanol solution containing **[1]Cl** into ethyl acetate gave ruby-coloured crystals suited for X-ray diffraction (Figure 1). In the structure of (**[1]Cl**·CH₃OH) the ligand biqbpy is coordinated to ruthenium(II) in a highly distorted fashion with an N1-N3-N4-N6 torsion angle of 12.78°. The difference between the bond angle N1-Ru1-N6 = 97.80° at the open-ended site of the complex and the angle N4-Ru1-N3 = 80.78° at the bpy site highlights the distortion of the coordination octahedron. Strain is caused by the repulsion between the hydrogen atoms borne by C1 and C28, and forces **[1]**⁺ to assume a helical, thus chiral configuration. The crystal structure of (**[1]Cl**·MeOH) is a racemate containing both the right-handed (*P*) and left-handed (*M*) helices.

Reacting **[1]Cl** with 20 equivalents of HAMet in water overnight at 80 °C was required to substitute both *trans* ligands by the monodentate thioethers (Scheme 1). Anion exchange to the PF₆ salt increased the lipophilicity of **[2]**⁺ allowing extraction of

the compound using ethyl acetate. Purification using size exclusion chromatography resulted in analytically pure **[2]PF₆**. Coordination of two N-acetylmethionine (Amet) ligands was confirmed using high-resolution mass spectrometry (HRMS), NMR, and elemental analysis (see ESI).

Dark Stability. Testing the dark stability of anticancer metallodrugs in conditions relevant for biological testing is critical for interpreting uptake and cytotoxicity data. Stability assays were thus performed in the dark in aqueous and DMSO solutions. Like for cisplatin the dark stability of **[1]Cl** in aqueous solution depends on chloride concentration. According to ¹H NMR (Figure S3) and mass spectrometry upon dissolution in deionized water or D₂O the chloride ligand of **[1]**⁺ was immediately hydrolysed to afford [Ru(biqbpy)(dmsol)(H₂O)]²⁺ (**[1a]**²⁺, see Scheme 2). Upon adding chlorides the concentration of **[1]**⁺ increased, to reach a ratio **[1]**⁺:**[1a]**²⁺ in solution of 1:3 at 0.15 M of NaCl. By contrast, a DMSO solution of compound **[1]Cl** was stable in the dark at -20 °C for at least six months (Figure S5), which allowed storage in stock solutions for all biological studies. Dissolving **[1]Cl** in DMSO first, and adding in a second step a physiological relevant NaCl aqueous solution (0.11 M), led to a 2:3 mixture of **[1]**⁺:**[1a]**²⁺ (Figure S7). Whether **[1]Cl** was in aqueous or DMSO solutions, the Ru-S bond with the dmsol ligand remained stable in the dark at 298 K. The dark behaviour of **[2]PF₆** was quite different. Although the protonation of one HAMet ligand in the solid state is corroborated by elemental analysis, in aqueous solution at neutral pH the complex is deprotonated into the neutral species **[2a]** (Scheme 2). In D₂O, this species remained stable in the dark for 6 weeks (Figure S4). In pure DMSO, however, **[2a]** degraded over 16 h, also at -20 °C (Figure S6). Thus, DMSO-containing stock solutions of **[2]PF₆** need to be freshly prepared, or, the compound being soluble in water, DMSO should simply be avoided. Overall, in aqueous solution **[2]PF₆** appears as a “protected” version of **[1]Cl**, as the hydrolysable Ru-Cl bond of **[1]Cl** has been replaced by thermally stable Ru-S bonds.



Scheme 2. Ligand exchange processes upon dilution of **[1]Cl** and **[2]PF₆** in aqueous solutions, and upon green light irradiation.

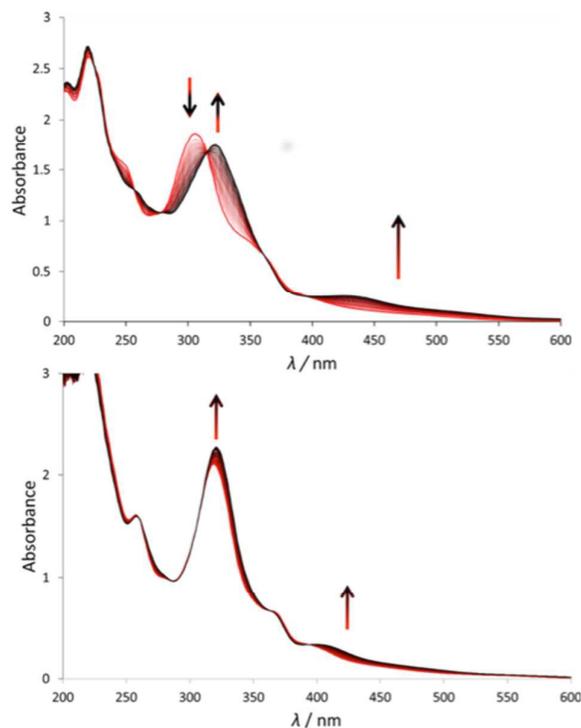


Figure 2. Evolution of the electronic absorption spectra of a solution of [1]Cl (a) and [2](PF₆) (b) in demi-water upon green light irradiation under argon ($\lambda = 530$ nm, $\Delta\lambda_{1/2} = 25$ nm, 3.02 mW, 2.1×10^{-8} Einstein.s⁻¹). Time: 0 min (red curve) to 120 min (black curve, a) or 160 min (black curve, b). Conditions [Ru]₀ = 7.5×10^{-5} (a), 7.8×10^{-5} (b), irradiated volume was 3.0 mL at 298 K.

Photoreactivity of [1]Cl and [2]PF₆. Under green light irradiation ($\lambda_{\text{irr}} = 520$ nm) and under argon a solution of [1]Cl in water, which mostly contains [1a]²⁺, resulted in a shift of the absorption maximum from 305 nm to 320 nm, and a slight increase of the absorbance in the visible region (Figure 2a). Mass spectrometry after light irradiation showed new peaks at $m/z = 288.7$ corresponding to [Ru(biqbpy)(H₂O)₂]²⁺ ([1b]²⁺ in Scheme 2, calc. $m/z = 288.8$). Thus, the dmsoligand was photosubstituted by water (Scheme 2). This reactivity is typical of geometrically distorted ruthenium(II) compounds that possess low-lying triplet metal-centred (³MC) excited states with a strongly dissociative character.¹⁷ ¹H NMR confirmed this analysis, as a new resonance at 2.72 ppm, characteristic of free dmsoligand, appeared after green light irradiation, but not in the dark (Figure S16). Similar evolutions were observed under blue light irradiation (450 nm, Figure S10-S11), which also allowed measuring a photosubstitution quantum yield (Φ_{PS}) of 0.3% (see ESI). Overall, cleavage of the Ru-S bond of [1]⁺ is a photochemical process, and compound [1]Cl can be seen as a semi-protected light-activated prodrug. One of the two *trans* ligands is thermally labile in water, while the other is only labile under visible light irradiation.

For [2]PF₆, green light irradiation in aqueous solution under argon (Figure 2b) was accompanied by increased intensity of the metal-to-ligand charge transfer (MLCT) absorption band near 400 nm and of the transition near 325 nm, and several isosbestic points. Mass spectrometry gave a clearer indication

about the photoreaction occurring in such conditions. The initial peak at $m/z = 923.4$ characteristic for [2]⁺ (calc. $m/z = 923.2$) was gradually replaced by a signal at $m/z = 732.4$ characteristic for [Ru(biqbpy)(Amet)]⁺ (calc. $m/z = 732.1$), which showed the formation of [Ru(biqbpy)(Amet)(OH₂)]⁺, [2b]⁺. A signal at $m/z = 605.1$ for [Ru(biqbpy)(MeOH)(OMe)]⁺ (calc. 605.1) or $m/z = 386.6$ for [Ru(biqbpy)(CH₃CN)₂]²⁺ (calc. 387.1) could only be obtained under extensive blue light irradiation (450 nm, see Figure S9, S12, and S13; MeOH and CH₃CN were solvents used for MS and HPLC, respectively). Irradiation with high-energy visible light was hence necessary to form the bis-aqua complex [1b]²⁺ from [2b]⁺ (Scheme 2). In our conditions the formation of [1b]²⁺ under green light irradiation was too slow to be observed. This result was confirmed by ¹H-NMR (Figure S17), as only one ligand was photosubstituted by water under green light irradiation at a dose of 75 J.cm⁻². In conclusion, complex [2]PF₆ is a water-soluble, fully protected complex: both *trans* N-acetyl-L-methionine ligands remain coordinated to the metal in the dark, while one of them is cleaved off by green light irradiation, and the second one is removed by high doses of blue light.

(Photo)cytotoxicity Studies. The cytotoxicity of compounds [1]Cl and [2]PF₆ was investigated against three cell lines, *i.e.*, A549 (human adenocarcinoma human alveolar basal epithelial cells), A431 (human epidermoid carcinoma cells), and MRC-5 (noncancerous human foetal lung fibroblasts). The effective concentrations (EC₅₀), defined as the compound concentration that reduces cell viability by 50% compared to untreated wells, were measured, in the dark and after light activation, following a protocol described in details in Hopkins *et al.*^{12a} These studies aimed at establishing whether the photosubstitution reactions observed in a chemical environment may translate into *in vitro* light activation. Although both blue and green light resulted in photosubstitution, green light (520 nm) was chosen for the photocytotoxicity tests because it is much less toxic to human cell lines than blue light^{12a} and it penetrates further into biological tissues. Preliminary studies in a 96-well plate (Figure S14 and S15) demonstrated that in the conditions of our cell-irradiation setup (21 mW.cm⁻²) a 60 min irradiation time, corresponding to a dose of 75 J.cm⁻², was necessary to activate 0.8-1.6 nmol of the compounds (the maximum amount present in each well for concentrations of 40-80 μ M). The EC₅₀ of complexes [1]Cl, [2]PF₆, and cisplatin, against A431, A549, and MRC-5 cell lines, measured in the dark and after green light irradiation, are reported in Table 1.

In the dark, the EC₅₀ values of ~ 10 and ~ 35 μ M were observed for [1]Cl and [2]PF₆, respectively in A431 cells (Table 1). For the A549 cell line similar trends were observed with EC₅₀ values of 6-9 μ M for [1]Cl and 11-20 μ M for [2]PF₆. Thus, [1]Cl has similar cytotoxicity in the dark as cisplatin (Tables S1), whereas the two thioether ligands in [2]PF₆ decreased the cytotoxicity by a factor of two to four compared to [1]Cl. This result suggested that coordination of the sulphur ligands may slow down or diminish the cellular response to these ruthenium compounds.

Although identical doses of green light did not induce phototoxicity by themselves (Figure S23), nor modify the cytotoxicity of cisplatin (Figure S24, S25, Table 1), a dramatically decreased cell population was observed when the cells were incubated with compound [1]Cl or [2]PF₆ for 6 h or 24 h, and then irradiated with 75 J.cm⁻² of green light (Table 1,

Table 1. Cytotoxicity (EC₅₀ with confidence interval (95%) in μM) of [1]Cl, [2]PF₆ and cisplatin on skin (A431) and lung (A549) cancer cell lines given with photo index (PI).^{[a],[b]} In addition, the complexes were tested against a non-cancerous lung cell line (MRC-5) for comparison.

cell line	t _{incubation} (h)	light dose (J.cm ⁻²)	[1]Cl			[2]PF ₆			Cisplatin		
			EC ₅₀ (μM)	± CI (95%)	PI	EC ₅₀ (μM)	± CI (95%)	PI	EC ₅₀ (μM)	± CI (95%)	PI
A431	6	0	13.0	1.30	22	38.0	8.8	4.9	4.3	1.5	0.9
		75	0.60	0.05		7.80	1.0		4.6	1.5	
	24	0	10.0	0.59	11	30.0	4.3	2.1	4.8	1.6	1.0
		75	0.88	0.24		14.0	1.1		4.9	1.6	
A549	6	0	9.30	2.30	16	20.0	6.1	5.6	3.3	0.55	1.0
		75	0.58	0.08		3.60	1.0		3.3	0.54	
	24	0	6.20	0.86	9.5	11.0	1.0	2.2	3.1	0.55	0.8
		75	0.65	0.0		5.00	0.4		3.6	0.77	
MRC-5	6	0	13.0	1.3	8.1	8.50	3.5	> 8.5	3.8	1.5	n.d
		75	1.60	2.4		< 1	n.d.				
	24	0	8.30	1.0	4.9	18.3	1.4	> 18	6.9	1.2	n.d
		75	1.70	2.3		< 1	n.d.				

[a] "light"=green light irradiation (520 nm, 60 min, 75 J.cm⁻²). [b] Incubation time is the time the Ru complex is incubated (37 °C, 7% CO₂) with cells in the dark before light irradiation. [c] n.d. stands for not determined.

Figure 4 and S26-28). For complex [1]Cl, EC₅₀ values close to 1 μM or lower were observed for all cell lines independently of when irradiation was performed. For A549 cells treated with complex [2]PF₆, the EC₅₀ decreased from 20 μM to 3.6 μM when irradiation occurred 6 h after treatment, and from 11 μM to 5 μM when it was done 24 h after treatment. Similar trends were observed for A431 cells. After green light irradiation, complex [2]PF₆ showed cytotoxicity comparable to the dark toxicity of [1]Cl, although compound [2]PF₆ was less toxic in the dark than [1]Cl. For both compounds, the photo index (PI) increased when irradiation occurred 6 h after treatment, compared to 24 h. This effect was mostly a consequence of lower EC₅₀ values in the dark after 24 h incubation, which suggested a higher degree of thermal activation with longer dark incubation times. Overall, these results suggest that the sulphur ligands of [1]Cl (dmsO) and of [2]PF₆ (Amet⁻) partially inhibit the cytotoxicity of the ruthenium centre in the dark, and that ligand photosubstitution is accompanied by an increase of the cytotoxicity of the compound. In other words, selectivity will be obtained by light irradiation.

Singlet Oxygen Production. Due to the long-lived triplet excited states of many photostable ruthenium polypyridyl complexes, singlet oxygen (¹O₂) generation is often a dominant pathway upon light irradiation.^{1e, 18} In fact, promising photodynamic therapeutic agents also include ruthenium-based sensitizers.^{2c, 19} However, photosubstitution reactions observed with distorted ruthenium(II) complexes often lead to quenching of their long-lived ³MLCT states by nearby ³MC excited states, which lowers the quantum yields of phosphorescence and ¹O₂ generation. These trends represent

a unique opportunity for PACT, as the hypoxic conditions in many tumour tissues, requires new oxygen-independent photoactivation strategies. In order to test whether compounds [1]Cl and [2]PF₆ would qualify better as PDT or as PACT agents their quantum yields of ¹O₂ generation (Φ₁₀₂) were measured under 450 nm excitation by direct detection of the 1274 nm infrared emission of ¹O₂ in CD₃OD. The prototypical [Ru(bpy)₃]Cl₂ complex was used as a reference (Φ^{ref} = 73%).²⁰ Φ₁₀₂ values of 1.3% and 2.3% were found for [1]Cl and [2]PF₆, respectively (Table S1 and Figure S19). According to these results, both [1]Cl and [2]PF₆ are extremely poor ¹O₂ generators, and the photoactivation observed *in vitro* is not a PDT effect.

Light-induced Apoptosis. To investigate which type of cell death occurred, the morphology of A549 cells was inspected in the dark and after green light irradiation using bright field microscopy (Figure 3 and Figure S29-30). Directly after irradiation (520 nm, 75 J.cm⁻²), cells treated with [1]Cl (1.5 μM) displayed cell shrinkage, loss of cell-cell contact, and membrane blebbing as depicted in Figure 3b. An enhanced effect was detected when the cells were incubated for an additional 24 h after light irradiation (Figure 3d). The changes in cell morphology are characteristic for apoptotic cell death.²¹

To confirm that a majority of the A549 cells treated with [1]Cl or [2]PF₆ and irradiated with green light died by apoptosis, their fate was investigated using the Annexin V-propidium iodide assay and analysed using flow cytometry (FC).²² Figure 5 shows representative density plots of non-irradiated A549 cells treated with [1]Cl (1.5 μM, Figure 4a) or [2]PF₆ (10 μM, Figure 4c). The majority of the cells are in the lower left quadrant, *i.e.*, alive (see also Figure S31). However,

upon green light irradiation (1 h, 75 J.cm⁻²) a clear shift of the cell population to the bottom right quadrant indicates, for both [1]Cl and [2]PF₆, Annexin V binding, thus apoptotic cells.

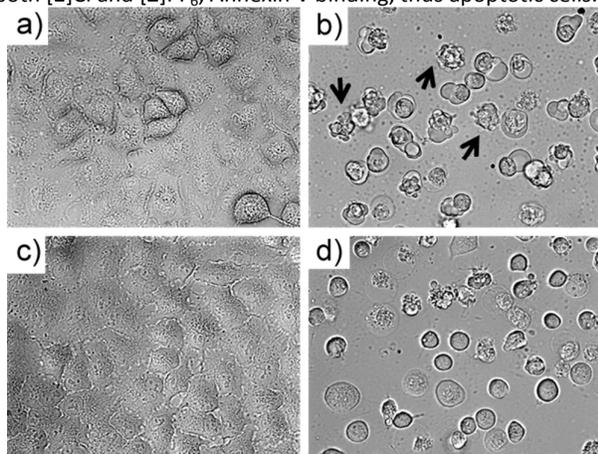
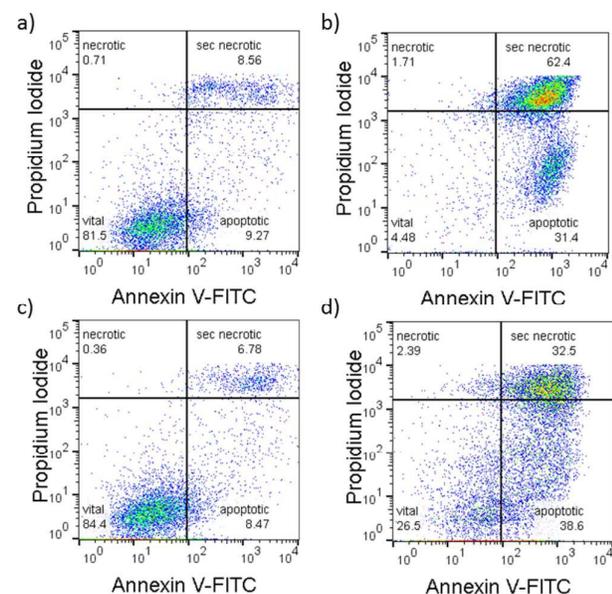


Figure 3. Bright field microscopy images (40× magnification) of A549 cells treated with [1]Cl (1.5 μM) for 6+1 h in the dark (a) and 6 h in the dark followed by 1 h green light irradiation (b, 520 nm, 75 J.cm⁻²). Images (c) and (d) show sample a) and b) after an additional 24 h incubation in the dark. Arrows in (b) show examples of membrane-blebbing, which is characteristic for early apoptosis.

The lack of cells in the top left quadrant indicates the absence of purely necrotic cells. Cells in the top right are commonly referred to as “secondary necrotic”, and are a known artefact in *in vitro* assays.

According to the flow cytometry data, the photocytotoxicity of [1]Cl and [2]PF₆ occurs *via* apoptosis without any sign of necrosis. In addition, confocal microscopy of A549 cells stained with tetramethylrhodamine ethyl ester (mitochondria) and DRAQ5 (nuclear DNA) showed that light irradiation diminished the mitochondrial membrane potential and induced chromosomal condensation, especially for [1]Cl (Figure S31). All of the tested cellular responses clearly demonstrate that compounds [1]Cl and [2]PF₆ belong to a rare sub-family of



metallodrugs that can trigger apoptosis with green light.²³

Figure 4. Representative flow cytometry density plots (Annexin V-FITC (525 nm)/Propidium iodide (670 nm)) of A549 cells incubated with [1]Cl (1.5 μM) in the dark for 6+1+24 h (a), or in the dark for 6 h, followed by irradiation with green light for 1 h, followed by 24 h incubation (b) or treated with [2]PF₆ (10 μM) and left in the dark for 31 h (c) or irradiated 1 h with green light 6 h after treatment and further incubated for 24 h in the dark (d). Irradiation conditions: 520 nm, 60 min, 75 J.cm⁻². Quantification: see Figure S20.

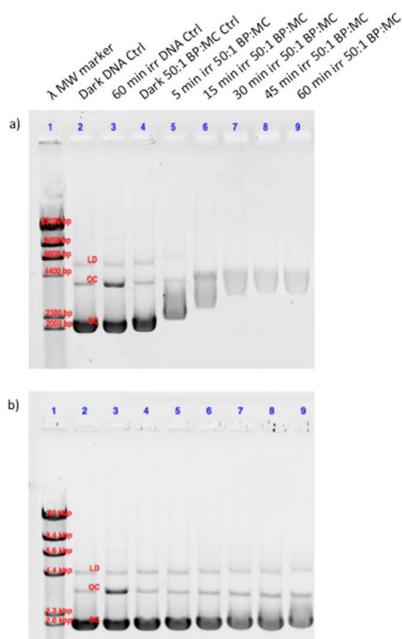
Intracellular Distribution and Uptake. In order to gather information on the intracellular localisation of [1]Cl and [2]PF₆, and to investigate whether the difference in cytotoxicity between [1]Cl and [2]PF₆ in the dark was due to differences in cell-uptake and/or of intracellular distribution, cell fractionation was performed. For this experiment, A549 cells were incubated with [1]Cl or [2]PF₆ for 6 h in the dark at concentrations corresponding to the EC₅₀ value. The cells were then harvested, the cytosol, membrane, nuclei, and cytoskeleton fractions were separated (see ESI), and the ruthenium concentration in each fraction was measured by ICP-MS (Figure S32). The observed total uptake of [2]PF₆ (7.5 ng/10⁶) was significantly lower compared to that of [1]Cl (16 ng/10⁶ cells). As the effect of both treatments was identical (*i.e.*, reducing the cell population by 50%), [2]PF₆ seems to be more potent than [1]Cl, although larger EC₅₀ values were found for [2]PF₆. This result suggests that the dark cytotoxicity of [2]PF₆ might be limited by a lower uptake. In terms of intracellular distribution both complexes were found in all fractions, with a slight ([2]PF₆) to strong ([1]Cl) preference for the membrane fraction, and to a lesser extent in the nuclear fractions. The membrane fraction does not only contain the cell membrane but also mitochondria, endosomes, lysosomes, etc. These results are in agreement with contemporary literature suggesting an endocytosis-dependent uptake mechanism for polypyridyl metal complexes (thus high Ru content in endosomes and lysosomes), and accumulation of lipophilic cationic species in the mitochondrial membranes.²⁴

Cell-free DNA Binding Studies. Thermal and photoinduced DNA binding studies were performed to establish whether the photolabile sulphur-based ligands in [1]Cl and [2]PF₆ were protecting the compounds from interaction with biomolecules. The pUC19 plasmid used for this study (2686 bp) exists in three forms: supercoiled (SC, most condensed form, migrates the fastest), single-nicked open circular (OC, relaxed form of the SC, migrates in between the SC and LD) and linear dimer (LD, largest form at 5372 bp, migrates the slowest). For both the thermal and photoinduced DNA binding studies, chloride-free phosphate buffer was used to model a pseudo intracellular environment. For the dark thermal binding experiments, [1]Cl and [2]PF₆ were incubated at varied DNA base pair (BP) to metal complex (MC) ratios for 24 h (Figure S33). Both [1]Cl and [2]PF₆ showed negligible binding (minimal change in migration of the OC or SC forms), even at the largest concentration of metal complex (5:1 BP:MC ratio). Cisplatin was included as a positive control (5:1 BP:MC ratio) and displayed typical DNA binding results as those observed in literature (Figure S34).²⁵ In the dark, [1]Cl and [2]PF₆ have a low affinity and negligible association with any of the forms of the plasmid DNA.

In a second experiment, the ruthenium complexes and cisplatin were photolysed ($\lambda_{irr} = 520$ nm) for different amounts of time (0-60 min) in the presence of pUC19 plasmid (Figure 5).

For these experiments, a 50:1 BP:MC ratio was used, which displayed insignificant dark thermal binding. However, following green light irradiation complex [1]Cl showed significant retardation of the SC form (Figure 5a, lanes 5-9) compared to [2]PF₆ (Figure 5b, lanes 5-9), which itself showed slight changes in the OC and SC forms compared to the control. Additionally, a change in the intensity of the staining indicates that increased photoinduced binding of the metal complexes interferes with the intercalation of ethidium bromide. These studies clearly show that after light activation, [1]Cl interacts strongly with the pUC19 plasmid, whereas [2]PF₆ interacts less but still significantly more than in the dark. Clearly, ¹O₂-based DNA cleavage was not observed under irradiation in presence of either ruthenium compound. Although these simple results neither allow to specify in details the binding mode of [1]Cl and [2]PF₆ to DNA, nor to say whether this interaction is relevant for cell death, they clearly demonstrate that the photosubstitution reactions occurring under green light irradiation critically changes the way these two ruthenium compounds interact with biomolecules.²⁶

Figure 5. Photoinduced binding of [1a]²⁺ (a) and [2]PF₆ (b) to pUC19 plasmid DNA. The lanes correspond to (1) lambda DNA MW marker, (2) dark DNA only



control, (3) irradiated DNA only control, (4) dark 50:1 BP:MC control, (5-9) 5, 15, 30, 45, and 60 min irradiated 50:1 BP:MC samples, respectively. The bands of the lambda MW marker correlate to 23, 9.4, 6.6, 4.4, 2.3, and 2.0 kpb. The dark DNA control bands are labelled according to the form, linear dimer (LD), open circular (OC), and supercoiled (SC).

Conclusions

Complexes [1]Cl and [2]PF₆ are the first light-activated *trans* ruthenium-based anticancer prodrugs. In the dark these water-soluble complexes are well taken up and display mild cytotoxic to A431 and A549 cancer cells. However, upon green light irradiation, [1]Cl and [2]PF₆ are activated resulting in highly cytotoxic therapeutics, with EC₅₀ values below 1 μM and photo-indices of up to 22. Clearly the combination of these

compounds and green light irradiation induces apoptosis, and the low singlet oxygen generation efficiency and the absence of DNA photocleavage conclude that cell death is not due to a photodynamic effect. The dose of light necessary to activate [1]Cl and [2]PF₆ *in vitro* (75 J.cm⁻²) is somewhat higher compared to values published for other photoactivated ruthenium or *trans*-platinum complexes (typically 10 J.cm⁻²). However, the green light used in this work (520 nm) is much less harmful to cells than the shorter wavelength (UV or blue light) reported previously,^{4a, 5b, 12a, 12c, 19a, 27} so that high doses do not represent *per se* a problem. Green light also penetrates deeper into the skin,²⁸ which makes it more relevant for phototherapy.

Overall, the data presented in this article suggests that the activation mechanism for this new type of *trans* ruthenium polypyridyl complexes relies on ligand photosubstitution reactions. The ruthenium species [2a] bound to two sulphur reacting ligands is the least cytotoxic, followed by the two mono-protected species [1a]²⁺ and [2b]²⁺ bound to a single sulphur ligand, while the bis-aqua, fully deprotected species [1b]²⁺ shows the highest cytotoxicity. Although cell-free DNA studies showed clear photoinduced DNA-binding by [1]Cl and, to a lesser extent, by [2]PF₆, DNA only represents one of the possible biological target(s) of these compounds, as they distribute in the whole cell. It will be necessary to follow for example chemical biological methods described by Hartinger et al,²⁹ to determine which interaction with which biomolecule is actually responsible for the green light-induced apoptosis observed with [1]Cl and [2]PF₆.

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References

- (1) a) N. J. Farrer, L. Salassa and P. J. Sadler, *Dalton Trans.*, 2009, DOI: 10.1039/b917753a, 10690-10701; b) J. D. Knoll and C. Turro, *Coord. Chem. Rev.*, 2015, **282-283**, 110-126; c) S. Yano, S. Hirohara, M. Obata, Y. Hagiya, S.-i. Ogura, A. Ikeda, H. Kataoka, M. Tanaka and T. Joh, *J. Photochem. Photobiol. C*, 2011, **12**, 46-67; d) D. Crespy, K. Landfester, U. S. Schubert and A. Schiller, *Chem. Commun.*, 2010, **46**, 6651-6662; e) U. Schatzschneider, *Eur. J. Inorg. Chem.*, 2010, **2010**, 1451-1467; f) W. A. Velema, W. Szymański and B. L. Feringa, *J. Am. Chem. Soc.*, 2014, **136**, 2178-2191.
- (2) a) H.-J. Yu, S.-M. Huang, L.-Y. Li, H.-N. Jia, H. Chao, Z.-W. Mao, J.-Z. Liu and L.-N. Ji, *J. Inorg. Biochem.*, 2009, **103**, 881-890; b) H. Huang, P. Zhang, B. Yu, C. Jin, L. Ji and H. Chao,

- Dalton Trans.*, 2015, **44**, 17335-17345; c) G. Shi, S. Monro, R. Hennigar, J. Colpitts, J. Fong, K. Kasimova, H. Yin, R. DeCoste, C. Spencer, L. Chamberlain, A. Mandel, L. Lilge and S. A. McFarland, *Coord. Chem. Rev.*, 2015, **282–283**, 127-138; d) J. Fong, K. Kasimova, Y. Arenas, P. Kaspler, S. Lazic, A. Mandel and L. Lilge, *Photochem. Photobiol. Sci.*, 2015, **14**, 2014-2023; e) S. P. Foxon, M. A. H. Alamiry, M. G. Walker, A. J. H. M. Meijer, I. V. Sazanovich, J. A. Weinstein and J. A. Thomas, *J. Phys. Chem. A*, 2009, **113**, 12754-12762; f) H. Huang, B. Yu, P. Zhang, J. Huang, Y. Chen, G. Gasser, L. Ji and H. Chao, *Angew. Chem. Int. Ed.*, 2015, **54**, 14049-14052; g) M. R. Gill and J. A. Thomas, *Chem. Soc. Rev.*, 2012, **41**, 3179-3192; h) P. Agostinis, K. Berg, K. A. Cengel, T. H. Foster, A. W. Girotti, S. O. Gollnick, S. M. Hahn, M. R. Hamblin, A. Juzeniene, D. Kessel, M. Korbelik, J. Moan, P. Mroz, D. Nowis, J. Piette, B. C. Wilson and J. Golab, *CA Cancer J. Clin.*, 2011, **61**, 250-281; i) C. Mari, V. Pierroz, R. Rubbiani, M. Patra, J. Hess, B. Spingler, L. Oehninger, J. Schur, I. Ott, L. Salassa, S. Ferrari and G. Gasser, *Chem. Eur. J.*, 2014, **20**, 14421-14436.
- (3) a) R. Sharma, J. D. Knoll, P. D. Martin, I. Podgorski, C. Turro and J. J. Kodanko, *Inorg. Chem.*, 2014, DOI: 10.1021/ic500299s; b) M. A. Sgambellone, A. David, R. N. Garner, K. R. Dunbar and C. Turro, *J. Am. Chem. Soc.*, 2013, **135**, 11274-11282.
- (4) a) T. Joshi, V. Pierroz, C. Mari, L. Gemperle, S. Ferrari and G. Gasser, *Angew. Chem. Int. Ed.*, 2014, **53**, 2960-2963; b) A. Presa, R. F. Brissos, A. B. Caballero, I. Borilovic, L. Korrodi-Gregório, R. Pérez-Tomás, O. Roubeau and P. Gamez, *Angew. Chem. Int. Ed.*, 2015, DOI: 10.1002/anie.201412157, n/a-n/a.
- (5) a) B. S. Howerton, D. K. Heidary and E. C. Glazer, *J. Am. Chem. Soc.*, 2012, **134**, 8324-8327; b) N. J. Farrer, J. A. Woods, L. Salassa, Y. Zhao, K. S. Robinson, G. Clarkson, F. S. Mackay and P. J. Sadler, *Angew. Chem. Int. Ed.*, 2010, **49**, 8905-8908.
- (6) a) S. Bonnet, B. Limburg, J. D. Meeldijk, R. J. M. K. Gebbink and J. A. Killian, *J. Am. Chem. Soc.*, 2010, **133**, 252-261; b) A.-C. Laemmel, J.-P. Collin and J.-P. Sauvage, *Eur. J. Inorg. Chem.*, 1999, **1999**, 383-386; c) G. Ragazzon, I. Bratsos, E. Alessio, L. Salassa, A. Habtemariam, R. J. McQuitty, G. J. Clarkson and P. J. Sadler, *Inorg. Chim. Acta*, 2012, **393**, 230-238; d) F. Barragan, P. Lopez-Senin, L. Salassa, S. Betanzos-Lara, A. Habtemariam, V. Moreno, P. J. Sadler and V. Marchan, *J. Am. Chem. Soc.*, 2011, **133**, 14098-14108.
- (7) J. P. Cosse and C. Michiels, *Anti-Cancer Agents Med. Chem.*, 2008, **8**, 790-797.
- (8) a) A. N. Hidayatullah, E. Wachter, D. K. Heidary, S. Parkin and E. C. Glazer, *Inorg. Chem.*, 2014, **53**, 10030-10032; b) R. N. Garner, J. C. Gallucci, K. R. Dunbar and C. Turro, *Inorg. Chem.*, 2011, **50**, 9213-9215; c) O. Filevich and R. Etchenique, *Photochem. Photobiol. Sci.*, 2013, **12**, 1565-1570; d) L. Salassa, C. Garino, G. Salassa, R. Gobetto and C. Nervi, *J. Am. Chem. Soc.*, 2008, **130**, 9590-9597.
- (9) a) F. Arnesano, M. Losacco and G. Natile, *Eur. J. Inorg. Chem.*, 2013, **2013**, 2701-2711; b) P. M. Takahara, A. C. Rosenzweig, C. A. Frederick and S. J. Lippard, *Nature*, 1995, **377**, 649-652.
- (10) a) U. Kalinowska-Lis, J. Ochocki and K. Matlawska-Wasowska, *Coord. Chem. Rev.*, 2008, **252**, 1328-1345; b) A. G. Quiroga, *J. Inorg. Biochem.*, 2012, **114**, 106-112; c) S. M. Aris and N. P. Farrell, *Eur. J. Inorg. Chem.*, 2009, **2009**, 1293-1302; d) Y. Zhao, J. A. Woods, N. J. Farrer, K. S. Robinson, J. Pracharova, J. Kasparkova, O. Novakova, H. Li, L. Salassa, A. M. Pizarro, G. J. Clarkson, L. Song, V. Brabec and P. J. Sadler, *Chem. Eur. J.*, 2013, **19**, 9578-9591.
- (11) F. S. Mackay, J. A. Woods, P. Heringová, J. Kašpárková, A. M. Pizarro, S. A. Moggach, S. Parsons, V. Brabec and P. J. Sadler, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 20743-20748.
- (12) a) S. L. Hopkins, B. Siewert, S. H. C. Askes, P. Veldhuizen, R. Zwier, M. Heger and S. Bonnet, *Photochem. Photobiol. Sci.*, 2016, 10.1039/C1035PP00424A; b) T. J. McMillan, E. Leatherman, A. Ridley, J. Shorrocks, S. E. Tobi and J. R. Whiteside, *J. Pharm. Pharmacol.*, 2008, **60**, 969-976; c) S. Wäldchen, J. Lehmann, T. Klein, S. van de Linde and M. Sauer, *Scientific Reports*, 2015, **5**, 15348.
- (13) S. L. Jacques, *Phys. Med. Biol.*, 2013, **58**, 5007-5008.
- (14) S. Bonnet, *Comments Inorg. Chem.*, 2015, **35**, 179-213.
- (15) Z. Arcis-Castillo, S. Zheng, M. A. Siegler, O. Roubeau, S. Bedoui and S. Bonnet, *Chem. Eur. J.*, 2011, **17**, 14826-14836.
- (16) a) A. Bahreman, B. Limburg, M. A. Siegler, E. Bouwman and S. Bonnet, *Inorg. Chem.*, 2013, **52**, 9456-9469; b) R. E. Goldbach, I. Rodriguez-Garcia, J. H. van Lenthe, M. A. Siegler and S. Bonnet, *Chem. Eur. J.*, 2011, **17**, 9924-9929.
- (17) P. C. Ford, *Coord. Chem. Rev.*, 1982, **44**, 61-82.
- (18) a) O. J. Stacey and S. J. A. Pope, *RSC Advances*, 2013, **3**, 25550-25564; b) A. Ruggi, F. W. B. van Leeuwen and A. H. Velders, *Coord. Chem. Rev.*, 2011, **255**, 2542-2554.
- (19) a) C. Mari, V. Pierroz, S. Ferrari and G. Gasser, *Chem. Sci.*, 2015, **6**, 2660-2686; b) R. Lincoln, L. Kohler, S. Monro, H. Yin, M. Stephenson, R. Zong, A. Chouai, C. Dorsey, R. Hennigar, R. P. Thummel and S. A. McFarland, *J. Am. Chem. Soc.*, 2013, DOI: 10.1021/ja408426z.
- (20) M. C. DeRosa and R. J. Crutchley, *Coord. Chem. Rev.*, 2002, **233–234**, 351-371.
- (21) a) Z. Darzynkiewicz, G. Juan, X. Li, W. Gorczyca, T. Murakami and F. Traganos, *Cytometry*, 1997, **27**, 1-20; b) G. Melino and D. Vaux, *Cell Death*, Wiley, 2010.
- (22) M. van Engeland, L. J. W. Nieland, F. C. S. Ramaekers, B. Schutte and C. P. M. Reutelingsperger, *Cytometry*, 1998, **31**, 1-9.
- (23) a) M. Dickerson, Y. Sun, B. Howerton and E. C. Glazer, *Inorg. Chem.*, 2014, **53**, 10370-10377; b) L. He, Y. Huang, H. Zhu, G. Pang, W. Zheng, Y.-S. Wong and T. Chen, *Adv. Funct. Mater.*, 2014, **24**, 2754-2763.
- (24) a) M. Groessl, O. Zava and P. J. Dyson, *Metallomics*, 2011, **3**, 591-599; b) S. M. Zeman and D. M. Crothers, in *Drug-Nucleic Acid Interactions*, Elsevier, 2001, vol. 340, pp. 51-68.
- (25) M. V. Babak, S. M. Meier, K. V. M. Huber, J. Reynisson, A. A. Legin, M. A. Jakupc, A. Roller, A. Stukalov, M. Gridling, K. L. Bennett, J. Colinge, W. Berger, P. J. Dyson, G. Superti-Furga, B. K. Keppler and C. G. Hartinger, *Chem. Sci.*, 2015, **6**, 2449-2456.
- (26) B. Zhang, S. Seki, K. Akiyama, K. Tsutsui, T. Li and K. Nagao, *Acta Med Okayama*, 1992, **46**, 427-434.

- (27) a) G. M. Findlay, *The Lancet*, 1928, **212**, 1070-1073; b) C. Kielbassa, L. Roza and B. Epe, *Carcinogenesis*, 1997, **18**, 811-816; c) B. H. Mahmoud, C. L. Hexsel, I. H. Hamzavi and H. W. Lim, *Photochem. Photobiol.*, 2008, **84**, 450-462; d) C. Opländer, S. Hidding, F. B. Werners, M. Born, N. Pallua and C. V. Suschek, *J. Photochem. Photobiol. B*, 2011, **103**, 118-125; e) M. Frasconi, Z. Liu, J. Lei, Y. Wu, E. Strelakova, D. Malin, M. W. Ambrogio, X. Chen, Y. Y. Botros, V. L. Cryns, J.-P. Sauvage and J. F. Stoddart, *J. Am. Chem. Soc.*, 2013, **135**, 11603-11613.
- (28) L. J. Steven, *Phys. Med. Biol.*, 2013, **58**, R37.
- (29) M. V. Babak, S. M. Meier, K. V. M. Huber, J. Reynisson, A. A. Legin, M. A. Jakupec, A. Roller, A. Stukalov, M. Gridling, K. L. Bennett, J. Colinge, W. Berger, P. J. Dyson, G. Superti-Furga, B. K. Keppler and C. G. Hartinger, *Chem. Sci.*, 2015, **6**, 2449-2456.