

# Evaluation of the Potential of Cobalamin Derivatives Bearing Ru(II) Polypyridyl Complexes as Photosensitizers for Photodynamic Therapy

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The current photosensitizers (PSs) for photodynamic therapy (PDT) lack selectivity for cancer cells. To tackle this drawback, in view of selective cancer delivery, we envisioned conjugating two ruthenium polypyridyl complexes to vitamin B<sub>12</sub> (Cobalamin, Cbl) to take advantage of the solubility and active uptake of the latter. Ultimately, our results showed that the transcobalamin pathway is unlikely involved for the delivery of these ruthenium-based PDT PSs, emphasizing the difficulty in successfully delivering metal complexes to cancer cells.

**Keywords:** bioinorganic chemistry, cobalamines, medicinal inorganic chemistry, photodynamic therapy, ruthenium polypyridyl complexes.

## Introduction

Photodynamic therapy (PDT) is an approved medical technique that relies on the use of a photosensitizer (PS) to ultimately generate reactive oxygen species (ROS) or radicals that can trigger cell death.<sup>[1]</sup> The interest of this method is its spatio-temporal control. The PS is activated only when and where the physician applies light. In brief, upon irradiation at a specific, defined wavelength, an electron of the ground state of the PS reaches a singlet excited state (<sup>1</sup>PS\*), which then reaches a triplet state (<sup>3</sup>PS\*) through an intersystem crossing (ISC) event.<sup>[2]</sup> The PDT process can then rely on two types of mechanism: 1) in Type I, an electron or proton transfer from the species <sup>3</sup>PS\* to a biological substrate that generates radicals which can further react with molecular oxygen and form superoxides, hydroxyl radicals, or peroxides, or 2) in Type II, an energy transfer from <sup>3</sup>PS\* to molecular oxygen in

its ground triplet state (<sup>3</sup>O<sub>2</sub>) to generate the highly toxic singlet oxygen (<sup>1</sup>O<sub>2</sub>).<sup>[3]</sup>

The currently used PSs in the clinic are mainly based on cyclic tetrapyrrolic scaffolds (chlorins, phthalocyanines, and porphyrins<sup>[4]</sup>). Their main drawbacks are a lack of selectivity towards cancer cells, low water solubility, an important photobleaching and, sometimes, serious problems of photosensitivity for the treated patients.<sup>[5]</sup> Ru(II) polypyridyl complexes were found to be an interesting alternative to the current PDT PSs. Although the use of such compounds as PDT PS against cancer is relatively recent, the results are spectacular with one of such compounds, TLD-1433, having recently completed phase I clinical trial against bladder cancer.<sup>[2,6-10]</sup> We note that to reach the therapeutic window for PDT treatment (ca. 600 to 800 nm), some Ru(II) polypyridyl complexes were found to be good PSs for two-photon PDT,<sup>[11-13]</sup> further illustrating the versatility of ruthenium in medicinal chemistry. To further improve the properties of the Ru(II)-based PDT PSs, it is also possible to conjugate them with targeting moieties, or to associate them in non-covalent manner with serum or

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membrane proteins.<sup>[14–16]</sup> Another possible strategy envisioned by our group and by others is the encapsulation of the Ru(II)-based PDT agents in polymers or their functionalization to nanoparticles.<sup>[17–19]</sup>

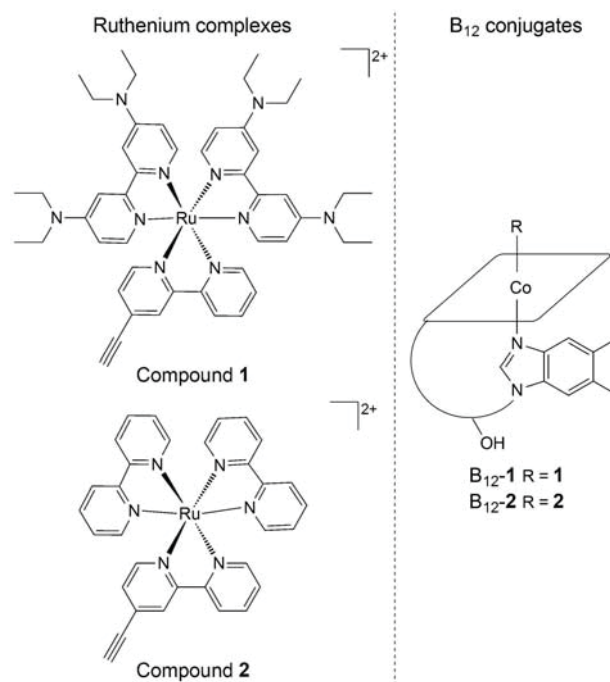
Vitamin B<sub>12</sub> is a vital nutrient that is characterized by a low bioavailability. Because it is playing an essential role in cell proliferation, it is crucial for fast growing cells.<sup>[20]</sup> This interesting characteristic was already used in several studies<sup>[21]</sup> in which cobalamin was employed as a targeting moiety for metal complexes to direct them towards fast dividing malignant cells.<sup>[22–24]</sup> With this in mind, in this work, we aimed at developing a system for improving the solubility and uptake of Ru(II)-based PSs into cancer cells. Our hope was that the resulting conjugates would have a good water solubility and an active cellular uptake.<sup>[25,26]</sup> Indeed, in the systemic circulation, Cbl is brought to the cells by a carrier protein named transcobalamin and ultimately taken up following a receptor-mediated endocytosis.<sup>[13,27]</sup> Therefore, two trisbipyridyl ruthenium(II) complexes were conjugated to vitamin B<sub>12</sub> (Cobalamin, Cbl). After characterization of the new conjugate B<sub>12</sub>-**2**, both molecules were tested *in vitro* to evaluate their efficiency in PDT as well as their cellular uptake. This data were compared with the Ru(II) complexes themselves.

## Results and Discussion

### Compounds Design and Chemistry

As a cofactor, inside cells, cob(III)alamin is ultimately reduced to cob(I)alamin. During this process, the  $\beta$ -upper ligand of cobalamin becomes labile.<sup>[28]</sup> This feature has been explored in the past to attach drugs/drug candidates at this position.<sup>[29]</sup> However, chemical modifications at the  $\beta$ -position were for a long time restrained by synthetic constraints as well as the instability of the resulting derivatives.<sup>[30]</sup> Recent advances in organometallic chemistry of cobalamin have allowed to generate stable derivatives and to rethink this prodrug approach.<sup>[31,32]</sup> As a prerequisite, the chosen compounds should bear an accessible alkyne group which can be directly attached to the cobalt center of Cbl following a copper-mediated reaction as described by Gryko and coworkers.<sup>[33]</sup> Two bispyridyl ruthenium(II) complexes were chosen and adapted to the need of this coupling reaction: a cytotoxic compound, which was previously reported to accumulate at the plasma membranes of ovarian carcinoma cell line A2780, [Ru(NN bpy)<sub>3</sub>]<sup>2+</sup> (where NNbpy = diethylamino-2,2'-bipyridine) and the standard [Ru

(bpy)<sub>3</sub>]<sup>2+</sup> (bpy: 2,2'-bipyridine).<sup>[34]</sup> These two compounds were synthesized asymmetrically in order to substitute one of the original bipyridyl ligands by a 4-ethynyl-2,2'-bipyridine ligand (C≡Cbpy), as previously reported to give [Ru(NNbpy)<sub>2</sub>(C≡Cbpy)]<sup>2+</sup> (**1**) and [Ru(bpy)<sub>2</sub>(C≡Cbpy)]<sup>2+</sup> (**2**) as shown in Figure 1.<sup>[35,36]</sup>



**Figure 1.** Ruthenium complexes and B<sub>12</sub> conjugates used in this study.

The complexes **1** and **2** were then coupled to cobalamin in good yield by adapting Gryko's procedure<sup>[31]</sup> to give two B<sub>12</sub> derivatives: B<sub>12</sub>-**1** and B<sub>12</sub>-**2** (see Figure 1). The compounds were unambiguously characterized by <sup>1</sup>H-NMR and HR-ESI-MS and their purity verified by HPLC (see Supporting Information). Very importantly, all compounds were found stable in water for at least seven days as well as light stable over the same time period.

### Photophysical Properties

With both compounds in hand, we investigated their photophysical properties to evaluate their potential as PDT PSs (Tables 1 and 2). As a first experiment, the absorption of the compounds was measured in MeOH and compared with their B<sub>12</sub>-conjugates (Figure 1, right side). Since the necessary <sup>3</sup>MLCT band centered at 450 nm did not significantly change, we assume that the photophysical properties of the conjugate should

**Table 1.** Photophysical properties of **1** and **2** in MeCN.  $\lambda_{em}$  = emission maximum,  $\Phi_{em}$  = luminescence quantum yield,  $\tau$  = luminescence lifetime, n.d. = not detectable.

Compound	$\lambda_{em}$ [nm]	$\Phi_{em}$	$\tau$ [ns]	
			air	degassed
<b>1</b>	695	> 0.001	n.d.	n.d.
<b>2</b>	635	0.021	226	679

**Table 2.** Singlet oxygen quantum yields in MeCN and aqueous solution determined at 450 nm. Average of three independent measurements, n.d. = not detectable.

Compound	direct	direct	indirect	indirect
	450 nm MeCN	450 nm D <sub>2</sub> O	450 nm MeCN	450 nm PBS
<b>1</b>	n.d.	n.d.	8%	3%
<b>2</b>	n.d.	n.d.	19%	7%

not be influenced through the conjugation. As a second experiment, the emission of the compounds was investigated upon excitation at 450 nm in MeCN. Compound **2** has an emission maximum at 635 nm and a luminescence quantum yield of 0.02. These values are in the same range as other Ru(II) polypyridine complexes.<sup>[37,38]</sup> However, the emission of **1** was barely measurable with the apparatus in our laboratory. As a third experiment, the luminescence lifetimes were determined and their influence on the presence of air investigated. Due to the very low emission of complex **1**, its lifetime was not detected. This contrasts with the lifetime of compound **2** which was found to be in the same range than other Ru(II) polypyridyl complexes.<sup>[37,38]</sup> Importantly, the excited state lifetime changed drastically upon the presence of oxygen indicating that <sup>3</sup>O<sub>2</sub> is able to interact with the excited state of **2**.

After showing that our compounds are able to interact with oxygen, we investigated quantitatively the production of singlet oxygen (<sup>1</sup>O<sub>2</sub>) upon light exposure. This is a crucial factor for a PS since <sup>1</sup>O<sub>2</sub> is known to be the major active species for most applied

PSs in the clinics. For this purpose, two different methods have been used: 1) direct by measurement of the phosphorescence of <sup>1</sup>O<sub>2</sub>, 2) indirect by measurement of the change in absorbance of a reporter molecule.<sup>[39]</sup> Worthy of note, only singlet oxygen quantum yields over 20% can be detected via the direct method with our apparatus. The results shown in Table 2 demonstrate that compounds **1** and **2** are producing <sup>1</sup>O<sub>2</sub> only poorly. This could be explained by the weak population of the excited state indicated by the poor luminescence properties of the complexes (Table 1) which is a necessary requirement for the production of <sup>1</sup>O<sub>2</sub>.

### Evaluation of PDT Activity

Dark and light cytotoxicity of the complexes was investigated in the cervical cancer cell line (HeLa) and non-cancerous retina pigmented epithelium (RPE-1) cell lines (Table 3). It was expected that the B<sub>12</sub> derivatives would be more toxic to both cell lines due to the presence of B<sub>12</sub> that should increase their uptake. Surprisingly, compound **2** and its derivative B<sub>12</sub>-**2** showed no cytotoxicity either in the dark or upon light irradiation. On the contrary, complex **1** was found to be cytotoxic in the dark (*IC*<sub>50</sub>: 9.33 ± 1.43 μM and 6.08 ± 0.085 μM in HeLa and RPE-1 cell lines, respectively). Irradiation at 480 nm (10 min; 3.21 J cm<sup>-2</sup>) did not significantly increase its toxicity. Photoindex (*PI*) values (*IC*<sub>50</sub> dark/*IC*<sub>50</sub> light) of 1.3 and 1.1 for HeLa and RPE-1 cell lines, respectively, were determined. To our surprise, the B<sub>12</sub>-**1** complex was found to be not toxic in the dark. Light irradiation of cells treated with B<sub>12</sub>-**1** did not cause toxicity in the RPE-1 or in the HeLa cell lines (see results in Table 1). Overall, these studies did not show any correlation between the presence of vitamin B<sub>12</sub> and (photo-) toxicity, clearly emphasizing that the coupling of Cbl was not helping in the delivery of our Ru(II) complexes. An obvious reason could be the bulkiness of the Ru(II) complexes. In a more general context, these disap-

**Table 3.** *IC*<sub>50</sub> values of complexes incubated with RPE-1 or HeLa cell line in the dark and upon light irradiation (in μM).

Compound	Cell line		<i>PI</i> value	HeLa		<i>PI</i> value
	RPE-1 Dark	Light		Dark	Light	
<b>1</b>	6.08 ± 0.085	5.43 ± 0.060	1.1	9.33 ± 1.43	7.14 ± 0.13	1.3
B <sub>12</sub> - <b>1</b>	> 100	> 100	–	> 100	> 100	–
<b>2</b>	> 100	> 100	–	> 100	> 100	–
B <sub>12</sub> - <b>2</b>	> 50	> 50	–	> 50	> 50	–

pointing results highlight the difficulty in specifically delivering metal complexes to cancer cells.

Ru(II) polypyridyl complexes are usually known to be highly luminescent.<sup>[40]</sup> We have therefore used this characteristic to further investigate the cellular biodistribution of the complexes in cells, and confocal microscopy studies were performed. Disappointingly, these two Ru(II) complexes as well as their B<sub>12</sub> derivatives showed very weak or no luminescent signal in treated HeLa cells (see *Figure S13*). For this reason, cellular localization could not be precisely determined, although localization in the cytoplasm could be faintly observed.

## Conclusions

In this article, we have presented the evaluation of trisbipyridyl Ru(II) complexes **1** and **2** conjugated with vitamin B<sub>12</sub> as potential photosensitizers for PDT. The conjugation with cobalamin increased the water solubility of the compounds, especially for complex **1** which was found to be extremely poorly soluble in this solvent. Unfortunately, our ruthenium-containing conjugates were found to not have any significant phototoxic activity to the cell lines studied in this work. In addition, we could not precisely determine the cellular localization of the complexes by confocal microscopy due to either the lack of luminescence of the Ru(II) complexes or due to the very poor uptake of the compounds. Overall, this study suggests that the transcobalamin pathway is unlikely involved for the uptake of our Ru(II) conjugates. It would be interesting to assess if this is true with other Ru(II) polypyridyl complexes.

## Experimental Section

### General Experimental Details

All chemicals were purchased from *Sigma-Aldrich* (St. Louis, MO) and used without further purification. The ligand 4-ethynyl-2,2'-bipyridine was synthesized according to a published procedure as well as the Ru complexes **1** and **2** and the B<sub>12</sub> derivative B<sub>12</sub>-**1**.<sup>[15,16]</sup> HPLC Analyses were performed on a *Merck-Hitachi L7000*. The analytical separations were conducted on a *Macherey-Nagel Nucleodur PolarTec* column (5 µm particle size, 110 Å pore size, 250×3 mm). The preparative separations were conducted on a *Macherey-Nagel Nucleodur C18 HTec* column (5 µm particle size, 110 Å pore size, 250×21 mm). HPLC solvents were water (A)

and methanol (B). The compounds were separated using the following gradient: 0–5 min (75% solvent A), 5–35 (75% solvent A→0% solvent A), 35–45 min (100% solvent B). The flow rate was set to 0.5 ml min<sup>-1</sup> for analytical separations and 5 ml min<sup>-1</sup> for the preparative ones. The eluting bands were detected at 320 nm. High resolution ESI-MS was performed on a *Bruker FTMS 4.7-T Apex II* (positive mode) and the UV/Vis spectra recorded on a *Jasco V-730*. nmR Analyses were recorded on a *Bruker Avance III 500* MHz. The corresponding <sup>1</sup>H and <sup>13</sup>C chemical shifts are reported relative to residual solvent protons and carbons.

### Synthesis and Characterization of the Derivative B<sub>12</sub>-**2**

The following procedure was adapted from the literature to achieve the synthesis of the B<sub>12</sub> derivatives.<sup>[13]</sup> A mixture of cyanocobalamin (20 mg, 0.013 mmol, 1 equiv.), CuOAc (2.3 mg, 0.0013 mmol, 0.1 equiv.) and the alkyne **2** (0.07 mmol, 5 equiv.) in DMA (3.5 ml) was stirred until dissolution. DBU (0.01 ml, 0.7 mmol, 5 equiv.) was added and the solution was allowed to react at room temperature for 4 h. The respective crudes were precipitated by dropwise addition to a stirred solution of diethyl ether/CH<sub>2</sub>Cl<sub>2</sub> (50 ml, 1:1). The residue was dissolved in a mixture of MeOH and water (2 ml, 1:1), filtered again, and purified by preparative HPLC. The eluting band containing the desired product was isolated and lyophilized.

*Data for B<sub>12</sub>-**2***: Isolated as a brownish powder, yield 19.8 mg (70%). HPLC: t<sub>R</sub> = 14.5 min. UV/Vis (MeOH): 330, 363, 460, 519, 552. <sup>1</sup>H-NMR (500 MHz, (D<sub>4</sub>)methanol): 8.53 (t, J = 9.5, 4 H); 8.25 (t, J = 9.37, 1 H); 8.09–7.99 (m, 5 H); 7.85–7.69 (m, 6 H); 7.54 (dd, J = 6.0, 2.37, 1 H); 7.40–7.32 (m, 5 H); 7.29 (s, 1 H); 7.14 (s, 1 H); 6.82–6.76 (m, 1 H); 6.50 (s, 1 H); 6.37 (d, J = 3.2, 1 H); 6.05 (d, J = 3.8, 1 H); 4.38–4.23 (m, 2 H); 4.13–4.07 (m, 1 H); 3.95 (dd, J = 13.0, 2.45, 1 H); 3.78 (dd, J = 13.0, 4.0, 2 H); 3.62 (d, J = 14.3, 1 H); 3.43–3.35 (m, 2 H); 3.32–3.25 (m, 2 H); 2.99 (dd, J = 9.0, 5.3, 2 H); 2.79–2.32 (m, 16 H); 2.27 (s, 6 H); 2.14 (t, J = 12.0, 1 H); 2.09–1.93 (m, 7 H); 1.89–1.77 (m, 5 H); 1.45 (d, J = 4.2, 3 H); 1.40 (d, J = 3.4, 3 H); 1.34 (s, 3 H); 1.27 (d, J = 6.3, 3 H); 1.18 (s, 1 H); 1.15 (s, 3 H); 1.13–1.02 (m, 2 H); 0.52 (s, 3 H). HR-ESI-MS (pos.): 960.8315 (M<sup>2+</sup>, C<sub>94</sub>H<sub>111</sub>Co<sub>11</sub>N<sub>19</sub>O<sub>14</sub>P<sub>1</sub>Ru<sub>1</sub><sup>2+</sup>; calc. 960.8342).

### Spectroscopic Measurements

The emission was measured by irradiation of the sample in fluorescence quartz cuvettes (width 1 cm)

using a *NT342B Nd-YAG* pumped optical parametric oscillator (*Ekspla*) at 450 nm. Luminescence was focused and collected at right angle to the excitation pathway and directed to a *Princeton Instruments Acton SP-2300i* monochromator. As a detector a *XPI-Max 4 CCD* camera (*Princeton Instruments*) has been used.

#### Luminescence Quantum Yield Measurements

For the determination of the luminescence quantum yield, the samples were prepared in a MeCN solution with an absorbance of 0.2 at 450 nm. This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a *NT342B Nd-YAG* pumped optical parametric oscillator (*Ekspla*) at 450 nm. The emission signal was focused and collected at right angle to the excitation pathway and directed to a *Princeton Instruments Acton SP-2300i* monochromator. As a detector a *XPI-Max 4 CCD* camera (*Princeton Instruments*) has been used. The luminescence quantum yields were determined by comparison with the reference  $[\text{Ru}(\text{bipy})_3]\text{Cl}_2$  in MeCN ( $\Phi_{\text{em}} = 0.059$ )<sup>[41]</sup> applying the following formula:

$$\Phi_{\text{em},S} = \Phi_{\text{em},R} * (F_R/F_S) * (I_S/I_R) * (n_S/n_R)^2$$

$$F = 1 - 10^{-A}$$

$\Phi_{\text{em}}$  = luminescence quantum yield, F = fraction of light absorbed, I = integrated emission intensities, n = refractive index, A = absorbance of the sample at irradiation wavelength, R = reference, S = sample.

#### Lifetime Measurements

For the determination of the lifetimes, the samples were prepared in an air saturated and in a degassed MeCN solution with an absorbance of 0.2 at 450 nm. This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a *NT342B Nd-YAG* pumped optical parametric oscillator (*Ekspla*) at 450 nm. The emission signal was focused and collected at right angle to the excitation pathway and directed to a *Princeton Instruments Acton SP-2300i* monochromator. As a detector a *R928* photomultiplier tube (*Hamamatsu*) has been used.

#### Singlet Oxygen Measurements

##### Direct Evaluation

The samples were prepared in an air saturated MeCN or D<sub>2</sub>O solution with an absorbance of 0.2 at 450 nm.

This solution was irradiated in fluorescence quartz cuvettes (width 1 cm) using a mounted *M450LP1 LED* (*Thorlabs*) whose irradiation, centered at 450 nm, has been focused with aspheric condenser lenses. The intensity of the irradiation has been varied using a *T-Cube LED Driver* (*Thorlabs*) and measured with an optical power and energy meter. The emission signal was focused and collected at right angle to the excitation pathway and directed to a *Princeton Instruments Acton SP-2300i* monochromator. A longpass glass filter was placed in front of the monochromator entrance slit to cut off light at wavelengths shorter than 850 nm. As a detector an *EO-817L* IR-sensitive liquid nitrogen cooled germanium diode detector (*North Coast Scientific Corp.*) has been used. The singlet oxygen luminescence at 1270 nm was measured by recording spectra from 1100 to 1400 nm. For the data analysis, the singlet oxygen luminescence peaks at different irradiation intensities were integrated. The resulting areas were plotted against the percentage of the irradiation intensity and the slope of the linear regression calculated. The absorbance of the sample was corrected with an absorbance correction factor. As reference for the measurement in an MeCN solution phenaleneone ( $\Phi_{\text{phenaleneone}} = 95\%$ )<sup>[42]</sup> and for the measurement in a D<sub>2</sub>O solution  $[\text{Ru}(\text{bipy})_3]\text{Cl}_2$  ( $\Phi_{\text{Ru}(\text{bipy})_3\text{Cl}_2} = 22\%$ )<sup>[43]</sup> was used and the singlet oxygen quantum yields were calculated using the following formula:

$$\Phi_S = \Phi_R * (S_S/S_R) * (I_R/I_S)$$

$$I = I_0 * (1 - 10^{-A})$$

$\Phi$  = singlet oxygen quantum yield, S = slope of the linear regression of the plot of the areas of the singlet oxygen luminescence peaks against the irradiation intensity, I = absorbance correction factor, I<sub>0</sub> = light intensity of the irradiation source, A = absorbance of the sample at irradiation wavelength, R = reference, S = sample.

##### Indirect Evaluation

For the measurement in MeCN: The samples were prepared in an air-saturated MeCN solution containing the complex with an absorbance of 0.2 at the irradiation wavelength, *N,N*-dimethyl-4-nitrosoaniline (RNO, 24  $\mu\text{M}$ ) and imidazole (12 mM). For the measurement in PBS buffer: The samples were prepared in an air-saturated PBS solution containing the complex with an absorbance of 0.1 at the irradiation wavelength, *N,N*-dimethyl-4-nitrosoaniline (RNO, 20  $\mu\text{M}$ ) and

histidine (10 mM). The samples were irradiated on 96 well plates with an *Atlas Photonics LUMOS BIO* irradiator for different times. The absorbance of the samples was measured during these time intervals with a *SpectraMax M2 Microplate Reader (Molecular Devices)*. The difference in absorbance ( $A_0 - A$ ) at 420 nm for the MeCN solution or at 440 nm a PBS buffer solution was calculated and plotted against the irradiation times. From the plot the slope of the linear regression was calculated as well as the absorbance correction factor determined. The singlet oxygen quantum yields were calculated using the same formulas as used for the direct evaluation.

#### Cell Culture

HeLa cell line was cultured in DMEM (*Gibco, Life Technologies, USA*) supplemented with 10% of fetal calf serum (*Gibco*). RPE-1 cells were cultured in DMEM/F-12 (*Gibco*) supplemented with 10% of fetal calf serum. Cell lines were complemented with 100 U/ml penicillin-streptomycin mixture (*Gibco*), and maintained in humidified atmosphere at 37 °C and 5% of CO<sub>2</sub>.

#### Cytotoxicity Studies

Dark and light cytotoxicity of the Ru(II) complexes and Ru(II) conjugates was assessed by fluorometric cell viability assay using resazurin (*ACROS Organics*). For light and dark cytotoxicity, HeLa and RPE-1 cells were seeded in triplicates in 96 well plates at a density of 4000 cells per well in 100  $\mu$ l, 24 h prior to treatment. Cells were then treated with increasing concentration of compounds for 48 h. After that time, medium was replaced by fresh complete medium. For light cytotoxicity experiments HeLa and RPE-1 cells were exposed to 480 nm light for 10 min in a 96-well plate using a *LUMOS-BIO* photoreactor (*Atlas Photonics*). Each well was individually illuminated with a 5 lm LED at constant current (light dose 3.21 J cm<sup>-2</sup>). After 44 h in the incubator medium was replaced by fresh complete medium containing resazurin (0.2 mg ml<sup>-1</sup> final concentration). After 4 h incubation at 37 °C, fluorescence signal of resorufin product was read by *SpectraMax M5* microplate reader (ex: 540 nm; em: 590 nm). IC<sub>50</sub> values were calculated using GraphPad Prism software.

#### Localization Studies

Cellular localization of the Ru(II) compounds was assessed by fluorescent microscopy. HeLa cells were

grown on the 12 mm *Menzel-Gläser* coverslips in 2 ml of complete medium at a density of 1.3 × 10<sup>5</sup> cells per ml. Cells were then treated with the compounds (IC<sub>50</sub> concentration in the dark) for 2 h, with *NucBlue* (two drops per 1 ml of media) for the last 25 min and with 100 nm *Mitotracker Green FM* for the last 15 min. HeLa cells were then fixed with paraformaldehyde solution in PBS (4%) and mounted on glass slides using *Prolong Glass Antifade Mountant. Leica SP8* confocal microscope was used to analyze the samples. Ru compounds were excited at 488 nm and emission above 650 nm was recorded. Images were recorded in Cellular and Molecular Imaging Technical Platform, INSERM UMS 025-CNRS UMS 3612, Faculty of Pharmacy of Paris, Paris Descartes University, Paris, France.

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#### Author Contribution Statement

*M. J., J. R., J. K., and J. D.* performed the experiments, analyzed the data, and wrote the paper. *B. G., F. Z., and G. G.* conceived and designed the experiments.

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