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Study of the biological activity of photoactive bipyridyl-Ru(II) complexes containing 1,3,5-triaza-7-phosphaadamantane (PTA)

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ABSTRACT

The water-soluble ruthenium complex *cis*-[Ru(dcbpyH)$_2$(PTAH)$_2$]Cl$_2$·3H$_2$O (**1**) (dcbpy = 4,4′-dicarboxy-2,2′-bipyridine; PTA = 1,3,5-triaza-7-phosphaadamantane) has been synthesized and characterised by NMR, IR spectroscopy, elemental analysis, and single-crystal X-ray diffraction. The optical properties of **1** were studied, including photoactivation under visible light, as well as its biological properties, together with those of the previously published Ru complexes *cis*-[Ru(bpy)$_2$(PTA)$_2$]Cl$_2$ (**2**), *trans*-[Ru(bpy)$_2$(PTA)$_2$](CF$_3$SO$_3$)$_2$ (**3**) and *cis*-[Ru(bpy)$_2$(H$_2$O)(PTA)](CF$_3$SO$_3$)$_2$ (**4**) (bpy = 2,2′-bipyridine). Anticancer activities of the complexes against human lung (A549), cervical (HeLa) and prostate (PC3) carcinoma cells were evaluated under dark conditions and upon photoactivation with visible light. None of the complexes exhibited cytotoxic activity in the absence of light irradiation (IC_{50} > 100 μM). However, after photoactivation, the cytotoxicity of complexes **1**, **2** and **3** against the three cell lines markedly increased, resulting in IC_{50} values between 25.3 μM and 9.3 μM. Notably, these complexes did not show toxicity against red blood cells. These findings show the potential of complexes **1**, **2** and, particularly, **3** for selective and controlled cancer photochemotherapy. The reactivity of the Ru complexes against DNA under UV–Vis irradiation was studied by analysing plasmid mobility. Experimental data shows that **4** unfolds supercoiled DNA (SC DNA) both in the dark and under visible irradiation, while **1** and **3** are only active under light, being **2** inactive in either case. The unfolding activities of complexes **3** and **4** were dependent on the air present in the reaction. The measured intracellular levels of reactive oxygen species (ROS) upon irradiation with complexes **1**, **2** and **3** suggest that their mechanism of action is related to oxidative stress.

1. Introduction

Metal complexes containing platinum, such as cisplatin and derivates, have been extensively employed as chemotherapeutic agents against cancer [1–3]. However, the use of these Pt compounds has some limitations due to their high toxicity, poor selectivity and specificity and development of chemoresistance [4–6]. Nevertheless, the development of novel platinum-based compounds remains an active area of scientific research [7–10]. Furthermore, there has been a growing interest in designing alternative metal-based anticancer agents that overcome these drawbacks [11–14]. Ruthenium complexes have proved to be promising drug candidates because of their physicochemical properties such as their acceptance rate of ligand exchange, their accessible range of oxidation states and their ability to mimic iron in the binding to biological molecules [15–23]. Of particular interest are compounds that can be photoactivated when irradiated with light of a specific wavelength [24–29]. Using this strategy, the activation can be done in a controlled way at the desired location, increasing its selectivity and minimizing the impact on healthy tissues [30–34]. Padeliporfin, a palladium-based compound that employs this approach, has recently been approved by the European Medicines Agency for the treatment of patients with low-risk prostate cancer [35].

So far, for what concern photoactivable Ru^{II}-polypyridyl complexes, many interesting examples of potential candidates for photodynamic therapy (PDT) and photoactivated chemotherapy (PACT) have been published [36–57], and the first transition-metal based complex to enter in human clinical trial for PDT was the ruthenium complex TDL1433, created by McFarland in 2017 [58], which started the phase-II clinical

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study in 2019 [59].

The photoactivation of Ru^{II}-polypyridyl compounds containing monodentate ligands generally proceeds trough a photolytic process [60–67] that occurs following a two-steps pathway: i) a metal-to-ligand charge transfer takes place upon the absorption of the adequate wavelength, generating a triplet excited state (^{3}MLCT) and ii) the ^{3}MLCT can undergo interconversion into a low-lying triplet metal-centred state (^{3}MC) with dissociative character that gives rise to the release of a ligand [68–70]. When this phenomenon occurs, either the resulting complex or the released ligand may be the cytotoxic species, and the overall activity is determined by the kinetics of the process: the faster the photolysis, the higher the cytotoxicity [41,71–74]. After photolysis in physiological media, usually the respective aqua-derivative of the starting complex forms, which is a common responsible of the complex related cytotoxicity [75]. Within the Ru^{II}-polypyridyls, the $[Ru(bpy)_2LL']^{2+}$ family (bpy = 2,2′-bipyridyne) (L and L' = monodentate ligands) has properties that can be widely tuned through ligand modification or functionalization, changing their photophysics, molecular recognition and transport behaviour [76]. For example, common strategies employed to modify the properties of the $[Ru(bpy)_2]^{2+}$ scaffold, are directed to red-shift the active absorption band or to change the redox properties of the complex. In the first case, lower frequency wavelengths are preferable to the higher ones because they can penetrate deeper in tissues and are also less dangerous to normal cells. Recently, this was achieved by using ligands such as thioethers with phosphonate group and Schiff bases [56,57]. On the other hand, changing the redox properties of the final complex can be useful to obtain compounds which are more efficient to produce superoxide reactive oxygen, even under hypoxic conditions [55]. Finally, also Ru-(Schiff-base) nanoparticles were found photocytotoxic enough to inhibit tumour growth [57].

Among the possible functionalization of the bipyridine rings, the addition of carboxylate groups has been used to attach Ru complexes to proteins [77]. In addition, carboxylate groups are susceptible to protonation, which would allow its tunability according to pH.

In our experience, the coordination of the 1,3,5-triaza-7-phosphaadamantane (PTA) ligand and its derivatives such as the *N*-methylated mPTA and dmPTA, usually confers high antiproliferative activities to the resulting complexes, together with a very high solubility in water-rich media [78–81]. Therefore, by introducing PTA or its derivates into the coordination sphere of a Ru-bpy complex might combine strong water solubility, new optical properties, and anticancer activity [82–85]. In this work, complex **1** has been synthesized and its photochemistry and anticancer photoactivated properties, as well as its ability to interact with supercoiled DNA have been studied and compared with complexes **2**, **3** and **4** (Scheme 1), which were previously reported [86].

2. Results and discussion

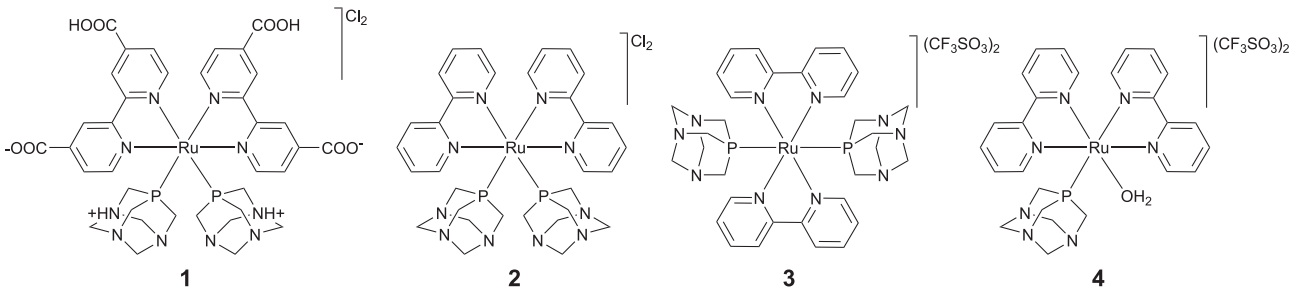
2.1. Synthesis of 1

The complex *cis*-$[Ru(dcbpyH)_2(PTAH)_2]Cl_2{\cdot}3H_2O$ (**1**) was synthesized by reaction of *cis*-$[Ru(dcbpyH_2)_2Cl_2]{\cdot}2H_2O$ with PTA (10 equivalents) during 24 h at 80 °C. The excess of PTA was removed by using a mixture of ethanol and chloroform resulting in 96% yield (Scheme 2).

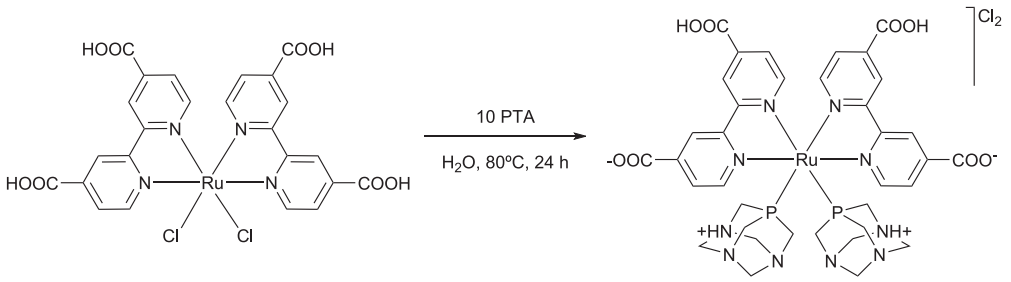
Complex **1** was characterised in water solution by ^{1}H, $^{13}C\{^1H\}$ and $^{31}P\{^1H\}$ NMR. The ^{1}H NMR spectrum in D_2O shows the signals corresponding to one PTA ligand (from $\delta = 3.82$ to 4.51 ppm) and two dcbpy ligands (from $\delta = 7.38$ to 8.92 ppm). The resonances found in the $^{13}C\{^1H\}$ NMR spectrum from 124.11 to 169.74 ppm, corresponding to dcbpy carbon atoms, confirm the *cis* geometry. In addition, the $^{31}P\{^1H\}$ NMR spectrum displays one singlet at $\delta = -37.45$ ppm, which is close to that of the previously characterised complex **2** [86]. Considering these data, we can assume a structure which consists of a ruthenium atom coordinated to two dcbpy ligands *cis* to each other and two PTA ligands bonded by the P atom. The presence of N—H bands in the IR spectrum (Fig. S1) suggested that PTA ligands are monoprotonated on one N atom.

A large number of efforts were devoted to obtaining single-crystal to fully characterise complex **1** but all the attempts were unsuccessful. Nevertheless, light orange single crystals of $[Ru(dcbpyH_2)_2(PTAH)_2]Cl_4$ (**1HCl**), which was synthesized from **1** by full protonation of the dcbpy ligands, were obtained by diffusion of HCl into a solution of **1** in pure water (Crystal data and structure refinement for **1** are provided in Table S1). The asymmetric unit is constituted by one $[Ru(dcbpyH_2)_2(PTAH)_2]^{4+}$ cation, four Cl^- anions and ten disordered molecules of water (see Supporting Information for more details). The ruthenium atom exhibits an octahedral distorted geometry, being coordinated by two dcbpy ligands *cis* to each other and to the phosphorus atoms of two PTA (Fig. 1). In Table 1, selected bond lengths and angles are summarised. The lengths of ruthenium-nitrogen bonds trans to the phosphorus atoms are 2.096(3) Å and 2.086(3) Å, while the Ru—N bond lengths trans to nitrogen atoms are 2.105(3) Å and 2.116(3) Å for N_{dcbpy} *trans* to P_{PTA}. The Ru—P bond lengths are 2.3131(10) Å and 2.3095(12) Å. All the interatomic distances between ruthenium and the atoms to which it is coordinated are consistent with those reported for complex **2** and with previously published Ru(II) complexes containing dcbpy and PTA [87]. However, the angle between PTA ligands is 100.31(4)° (P1-Ru1-P2), which is larger than that observed for **2**, indicating that the octahedral distortion is greater in **1**.

The UV–Vis spectrum of **1** in water at pH = 0 (Fig. 2) show absorption maxima at 200, 250, 308, 340 and 390 nm. The molar extinction coefficients suggest that the bands at 200 nm (39,001 $dm^3\ mol^{-1}\ cm^{-1}$) and 308 nm (19,524 $dm^3\ mol^{-1}\ cm^{-1}$) may correspond to a LC $\pi \rightarrow \pi^*$ transitions, the shoulder at 250 nm (20,203 $dm^3\ mol^{-1}\ cm^{-1}$) and the band at 390 nm (5482 $dm^3\ mol^{-1}\ cm^{-1}$) to MLCT $d \rightarrow \pi^*$ transitions, while the remaining peak at 340 nm (8750 $dm^3\ mol^{-1}\ cm^{-1}$) might correspond to a MC transition [64]. Basification of the solution to pH = 10 shifts the wavelength of the absorbance peaks: the MLCT band is red-shifted from 390 to 405 nm but for the rest of the peaks, a slightly blue shift is observed. The comparison with the absorption spectra of **1** and **2** [79] shows that bands over 400 nm are red-shifted in **1** compared to **2,** which is probably due to the lower energy of the dcbpy π^* orbitals compared to those of the bpy [59]. Water solutions of **1** displayed fluorescence under air at room temperature upon irradiation of the



Scheme 1. Molecular structure of Ru-complexes studied.



Scheme 2. Synthesis of **1**

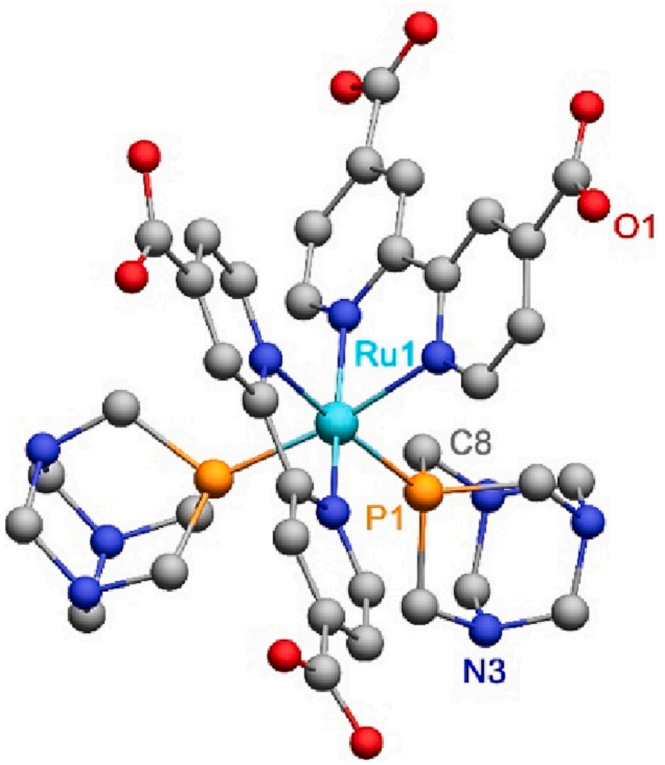


Fig. 1. Single-crystal X-ray structure of the cationic complex unit of **1HCl** including the atomic labelling scheme. Anions and hydrogen atoms are not included for clarity.

Table 1
Selected bond lengths and angles for **1**.

Atoms	Bond Length (Å)	Atoms	Angle (°)
Ru1-P1	2.3095(12) Å	P1-Ru1-N10	87.54(9) °
Ru1-P2	2.3131(10) Å	P1-Ru1-N9	90.20(10) °
Ru1-N10	2.086(3) Å	P1-Ru1-N8	171.54(9) °
Ru1-N9	2.116(3) Å	P1-Ru1-P2	100.31(4) °

sample at 450 nm (Fig. 3), emitting at 620 nm. The fluorescence quantum yield was calculated using the relative method designed by Williams et al. ($\Phi_F = 7.84{\cdot}10^{-4}$) [88]. Upon changing the pH from 1 to 10, the emission band show a decrease in intensity from pH = 0 to pH = 3, while, as the pH rises above this value, the intensity increases again up to pH = 10.

The study of the influence of pH on potential anticancer compounds is fundamental to understanding its behaviour under physiological conditions. The pK_a values of **1** were determined by both procedures: UV–Vis measurements and titration. The change of the absorbance versus the pH at a given wavelength (fig. S9) allowed obtaining values of

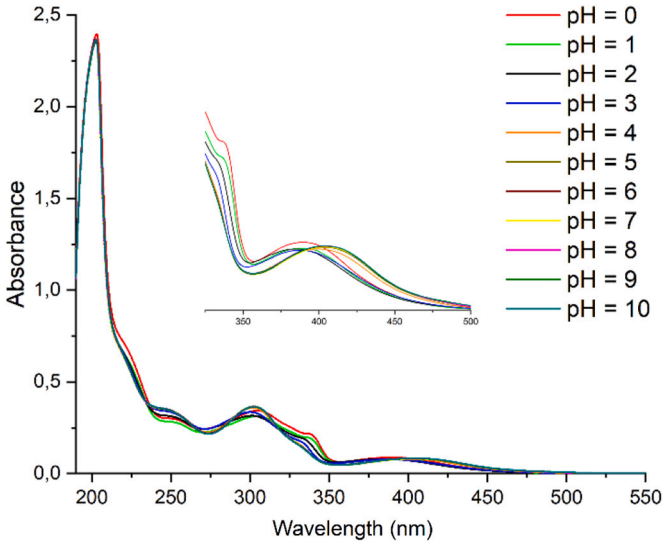


Fig. 2. UV/Vis spectra of **1** in water ($1.435{\cdot}10^{-5}$ M) at different pH.

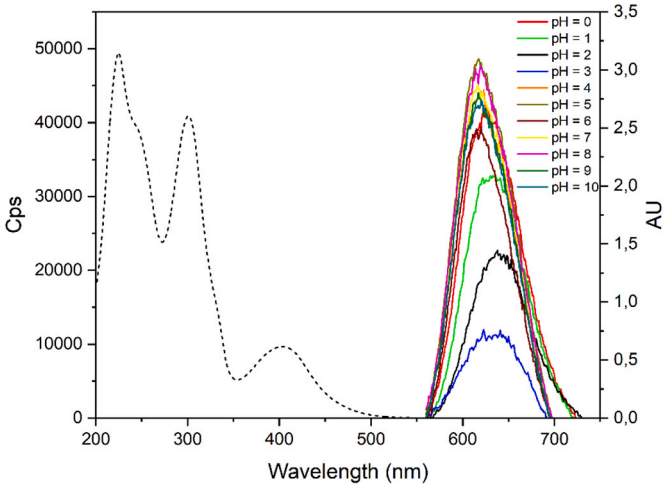


Fig. 3. Emission (solid line) of **1** vs. pH and absorption spectra (pH = 7, dotted line).

pK_{a1} = 1.5 and pK_{a2} = 3.27 for the first and second deprotonation process of the dcbpy ligand, which agrees with those of previously reported ruthenium complexes containing dcbpy [89,90]. Nevertheless, this method did not evidence the equivalence point related to the protonation of the PTA ligand. For this reason, a potentiometric titration experiment (fig. S10) was also performed. With this method, the pK_a values obtained were a pK_{a2} = 3.47, which only differs by 0.2 units from that obtained from UV–Vis experiments, and a pK_{a3} = 6.38,

corresponding to the protonation of a tertiary amine of the PTA ligand [91]. These results also support the different protonation degrees observed in this complex.

Solutions of **1** were studied under continuous visible light (λ > 320 nm) to determine whether this complex could be a good candidate to be used as a phototherapeutic agent. When water was the solvent, the $^{31}P\{^{1}H\}$ NMR showed (Fig. S11) that after 20 h of irradiation at room temperature new peaks formed, which remained stable for an additional 48 h. Unfortunately, it was not possible to isolate the species that gave rise to these new peaks. Nevertheless, comparison with irradiation effect on the analogous compound *cis*-$[Ru(bpy)_2(PTA)_2]^{2+}$ suggests that the species with a chemical shift of −26.03 ppm (26%) and − 47.39 ppm (20%) may be respectively the complexes *cis*-$[Ru(dcbpyH)_2(H_2O)(PTAH)]^{2+}$ and *trans*-$[Ru(dcbpyH)_2(PTAH)_2]^{2+}$ (Scheme 3) [86]. When the irradiation was performed on the solution of **1** in water with 1 M triflic acid (Figs. S12 and S13, respectively), the $^{31}P\{^{1}H\}$ NMR showed that the starting complex almost disappeared after 20 h and a main peak observed at −21.9 ppm (85%), which was characterised as *cis*-$[Ru(dcbpyH_2)_2(H_2O)(PTAH)]^{4+}$ [86]. It is important to stress that no evidence of the formation of the *trans*-isomer was found. A further experiment using aqueous acetic buffer as solvent (pH = 5) (Fig. S14) showed that complex **1** was not transformed after 24 h of irradiation. Evolution of solutions of **1** in D_2O and a DMSO-d_6/D_2O (1:1), which are used usually in biological assays, were studied also by $^{31}P\{^{1}H\}$ NMR at room temperature and 40 °C (Figs. S15-S18). In both experiments, the signal corresponding to the starting complex was uniquely observed after 24 h.

The stability in water and water/DMSO of complexes **2** and **3** was previously determined [86], but not in cell culture medium and under visible light irradiation. For this reason, solutions of **1**, **2**, **3** and **4** (10 mg) into 1 mL of cell culture media were irradiated with white light (λ > 320 nm) for 2.5 h. As expected, the $^{31}P\{^{1}H\}$ NMR spectrum showed that complexes **3** and **4** do not decompose under the abovementioned conditions. In contrast, complexes **1** and **2** were slightly transformed in a very small amount (< 4%) into the respective *trans* isomer. The resulting solutions were evaporated to dryness and the antiproliferative activity of the obtained solid was evaluated under the same conditions employed for the pure compounds.

2.2. Electrochemistry of *1*

The redox properties of **1** were studied by cyclic voltammetry in water and the obtained voltammogram is shown in Fig. 4. The observed events can be attributed to a one-electron process. The Ru^{II}/Ru^{III} oxidation peak was found at 1.49 V, and the corresponding reduction at 0.35 V, being the overall process irreversible (ΔE_p = 1.14 V). Only a redox waves at a lower potential than those of the Ru atom was possible to be distinguilled that only can be ascribable to the redox process of the dcbpy, which is also irreversible (E_{ox} = 1.11 V, E_{red} = 0,39 V, ΔE_p = 0,72 V). The Ru^{II}/Ru^{III} redox waves of **1** are similar to those of the *cis*-analogous compound **2**, but the dcbpy redox peaks are at somewhat higher potentials than those of bpy ligand [86]. (See Fig. 4.)

2.3. Photocytotoxic activity in cancer cells

The cytotoxicity of the complexes was evaluated against human lung

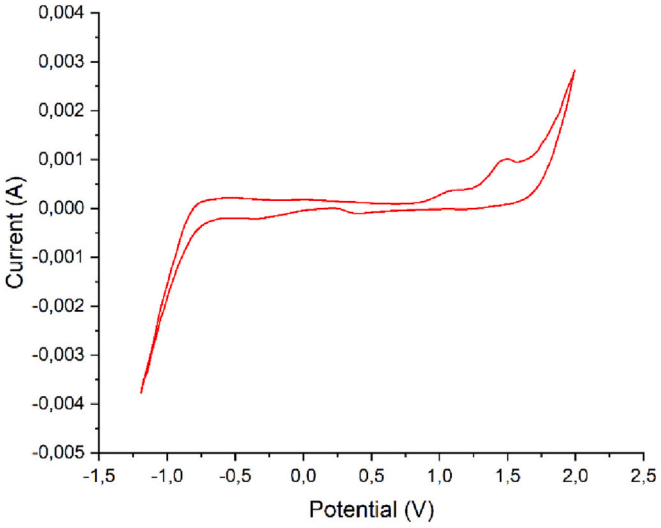
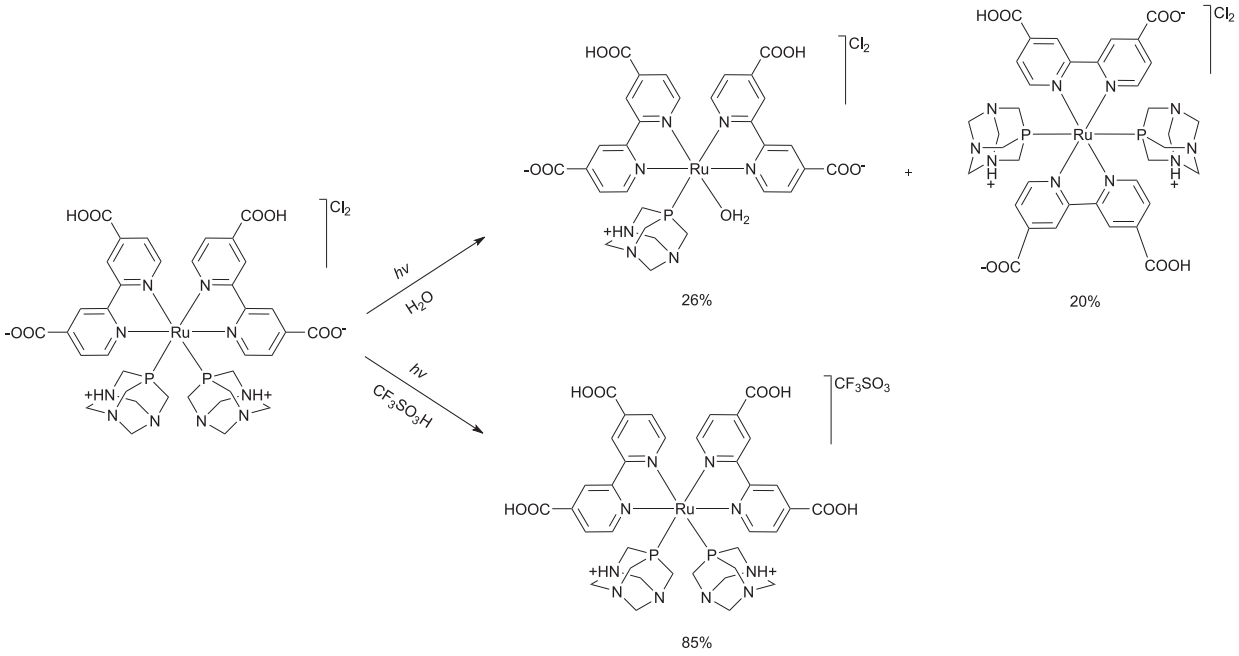


Fig. 4. Cyclic voltammogram (Ag/AgCl; 0.1 V/s) of **1** in water.



Scheme 3. Proposed products of the photolysis of **1** in water and in triflic acid.

(A549), cervical (HeLa), and prostate (PC3) cancer cells, both in the dark and after photoactivation. Cells were treated with the pure compounds as well as with the products resulting from their activation with continuous visible light (as described in the previous section). Optimally, for their application as a photosensitizer for cancer PDT, they should not be active against cells in the absence of light but should exert potent cytotoxic activity upon photoactivation. Compounds were tested at different concentrations ranging from 0 to 100 μM and the concentration that inhibits cell viability by 50% (IC_{50}) was determined. Under dark conditions, all the complexes exhibited IC_{50} values higher than 100 μM against cancer cells and were considered inactive. However, when the complexes were photoactivated a marked increase in the cytotoxicity of **1**, **2** and **3** was detected (Tables 2 and S4). In these experiments, cells were exposed to the complexes for 4 h to allow their cellular uptake and then, they were irradiated with a light of a wavelength of 460 nm (blue) since lower wavelengths have a limited tissue penetration and would therefore be ineffective in activating the complexes within tumours [92,93]. In all cases, the best results were achieved when compounds were preactivated with visible light, resulting in IC_{50} values ranging between 9.3 ± 4.0 μM for **1** in A549 cells and 25.3 ± 4.4 μM for **3** in PC3 cells (Table 2). These results also revealed that, in contrast with our first expectations, complex **1**, containing 4,4′-dicarboxy-2,2′-bipyridine, exhibits lower phototoxicity than complex **2,** except in PC3 cells. More interestingly, the IC_{50} value of **3** in A549 cells was similar to that obtained for the chemotherapeutic drug Cisplatin in this cell line (8.9 ± 2.6 μM). This finding demonstrates that, following photoactivation, complex **3** can exert an efficient anticancer activity against lung cancer cells. As shown previously [86], this complex evolves under visible light (400 nm) but it is not possible to ascertain the transformation undergone by the complex into the cell, and further in-depth studies are needed to determine the real nature of the resulting complex. Complex **4** remained non-cytotoxic after photoactivation, which contrasts with the activity observed in experiments against DNA, which will be presented below. These results show that under these conditions, complexes **2** and **3** display a notable photocytotoxic effect, reaching phototoxicity indexes higher than 6.8 and 10.7 in A549 cells, respectively, which are in the range observed for other Ru(II)-based photosensitizers [94].

In the case of pure compounds without previous exposure to visible light, in all cases higher IC_{50} values (ranging from 17.1 ± 6.7 to 49.1 ± 13.9 μM) were obtained after blue light irradiation compared to preactivated complexes (Table S3). Moreover, the compounds exhibited an irregular response to the photoactivation, resulting in less reproducible results. As previously observed, no cytotoxic effect was detected in the case of complex **4.** These results show that irradiation with blue light alone is less effective in activating the compounds, which is in line with their absorption spectra. However, prior activation of the compounds with continuous visible light renders them more sensitive to their subsequent photoactivation at the cellular level with blue light.

In addition, the toxicity of the complexes at the $IC_{50,light}$ against red blood cells was evaluated by a haemolysis assay. This is an important experiment to determine if complexes can be safely distributed to cancer cells through the bloodstream. Red blood cells were exposed to the complexes in the dark and with blue light activation, and haemoglobin release was quantified as a measure of cell lysis. None of the compounds displayed haemolytic activity (% of haemolysis <5%) (Table S4), indicating that at their cytotoxic concentration against cancer cells, they have good compatibility with red blood cells. Furthermore, these results indicate that the activity of the compounds does not damage the cell membrane, and is more likely directed against cellular organelles, such as mitochondria, or against DNA, which are not present in red blood cells.

2.4. Intracellular ROS generation

Photodynamic therapy (PDT) is based on the interaction of light-activated photosensitising agents with oxygen to generate ROS capable of damaging or destroying cells [95]. We investigated whether, in the case of complexes **1**, **2** and **3**, the generation of ROS in cells is responsible for their photo-cytotoxic effect. For this purpose, A549 cells were treated with the photoactivated complexes, and the elevation of ROS levels was determined with the 2′,7′-dichlorodihydrofluorescein diacetate probe (H_2DCFDA) that is intracellularly oxidised by a wide range of ROS, generating a highly fluorescent product that can be detected by flow cytometry. As shown in Fig. 5, ROS levels increased 1.84 + 0.17, 1.75 + 0.03 and 2.00 + 0.17-fold after treatment with photoactivated complexes **1**, **2** and **3**, respectively. The highest increase in ROS was obtained with complex **3**, which showed the highest photo-

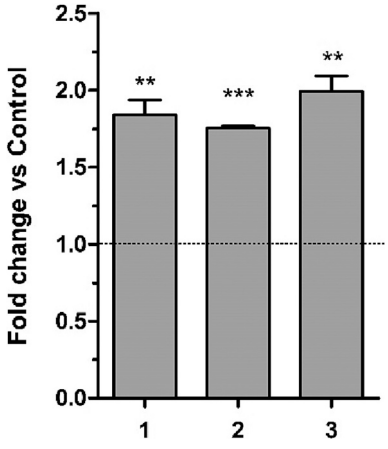


Fig. 5. Intracellular ROS generation. A549 cells were incubated with compounds **1**, **2** and **3** previously activated with visible light at the corresponding IC_{50}, light for 4 h and then irradiated with blue light for 1 h. The elevation of intracellular ROS levels was determined by flow cytometry using the H_2DCFDA probe. The bar graph shows the fold increase relative to untreated cells (Control) and represent the mean ± standard deviation from three independent experiments. $^{**}p < 0.05$ and $^{***}p < 0.01$ vs control cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Cytotoxic activity of pre-activated complexes **1**, **2**, **3** and **4** against different cancer cells.

Cells:	A549			HeLa			PC3		
	IC_{50} (μM)		IP[c]	IC_{50} (μM)		IP[c]	IC_{50} (μM)		IP[c]
	Dark[a]	Light[b]		Dark[a]	Light[b]		Dark[a]	Light[b]	
1	> 100	25.3 ± 10.1	**> 3.9**	> 100	24.4 ± 5.6	**> 4.1**	> 100	24.2 ± 6.88	**> 4.1**
2	> 100	14.6 ± 3.5	**> 6.8**	> 100	15.1 ± 2.3	**> 6.6**	> 100	23.1 ± 3.7	**> 4.3**
3	> 100	9.3 ± 4.0	**> 10.7**	> 100	17.8 ± 2.9	**> 5.6**	> 100	25.3 ± 4.2	**> 3.9**
4	> 100	> 100	–	> 100	> 100	–	> 100	> 100	–

Human lung (A549), cervical (HeLa), and prostate (PC3) cancer cells were treated with the complexes, previously irradiated for 2.5 h with continuous visible light, (a) in the dark or (b) with light photoactivation (460 nm, 24.1 J cm^{-2}) for 72 h. IC_{50} values were determined by MTT assays. Data represent the mean ± SD of three independent experiments, each performed in quadruplicates. (c) PI: phototoxicity index = $IC_{50,dark}/IC_{50,light}$.

cytotoxic activity. Overall, this marked increase in ROS levels shows that the irradiation of the compounds triggers a photodynamic effect capable of generating oxidative stress in the cells, which is probably the cause of their death.

2.5. Photoinduced DNA binding

We have investigated the interaction of the water-soluble ruthenium complexes with DNA by using the mobility shift assay to obtain some information on the action mechanism of these complexes against the cancer cells. Studies on transition metal complexes have suggested that modification of the electrophoretic mobility of plasmid DNA on agarose gels could be taken as verification of the existence of an interaction between DNA and the metal [66,96]. The alteration of the DNA structure leads to a retardation in the migration of supercoiled DNA (SC DNA), without significant mobility shift of the open circular DNA (OC DNA), to a point where both forms co-migrate (coalescence point, CP). This was performed by mixing a fixed quantity of plasmid DNA (pBluescript KS-II) with different complex amounts to achieve increasing metal-to-base (Ri) molar ratios as indicated in figure captions. Initial experiments were conducted using a standard assay commonly employed for cisplatin, with incubations performed for 14 h at 37 °C in the absence of light, in a phosphate-buffered medium at pH 7. Under these conditions, none of the assayed complexes (**1**, **2** and **3**) showed detectable activity even at Ri values over four times the coalescence point for cisplatin (Ri = 0.13) (Fig. S19).

In a further set of experiments, complexes were assayed at pH = 5, 6, and 7, and incubations were performed with or without previous irradiation of the reaction mixture with the light of a wavelength of 400 nm for 2 h and 30 min at 37 °C, followed by O/N incubation (14 h) at the same temperature. Complex **1** showed activity against SC DNA only after irradiation and mainly at pH = 5 (Fig. 6), which is not the usual pH in the cell. In this case, direct conversion of SC DNA to the OC form was observed, rather than a gradual shift. This indicates that complex **1** interaction promotes single DNA strand breaking of DNA at this pH, leading to the loss of supercoiled DNA structure, rather than a modification of supercoiled structure itself.

No activity was recorded under our conditions for complex **2** at any pH, regardless of being irradiated or not (results not shown), whereas complex **3** does not exhibit activity without irradiation at any pH up to Ri values of 3.8 (Fig. 7, lower panels). However, irradiation of the mixture at 400 nm promoted a clear DNA mobility shift of the SC form at pH = 5, reaching coalescence at Ri = 2.8 (Fig. 7, upper left panel). A much lower effect, but observed, was produced at pH = 6 (not shown) or 7 (Fig. 7, upper right panel), where coalescence was never reached up to Ri values of 3.8. The changes observed in DNA mobility at different Ri ratios (Ru/base molar ratio) are typical of DNA-binding compounds affecting plasmid supercoiling [97,98]. Thus, a progressive reduction in plasmid mobility is apparent for complex **3** at low Ri values, likely due to a reduction in the negative supercoiling of the circular DNA molecule to achieve a similar level to that of the relaxed open circular (OC) form. An increase in DNA mobility is obtained at high Ri ratios, probably due to further twisting resulting in positive supercoiling of the plasmid.

Experiments performed with complex **4** revealed a clear activity on DNA structure when the reaction mixtures were irradiated, not only at pH = 5 but also at pH = 6 and 7 (Fig. 8, left panels), with similar coalesce points at Ri around 2.1, which contrast with its lack of anticancer activity. Contrary to complex **3**, moderate activity is also recorded without irradiation (Fig. 8, right panels), although coalescence was never reached, even at a high amount of metal-to-DNA ratios (Ri = 4.3).

To determine if light activation of complex **3** is a permanent process, persisting after irradiation, an experiment was designed where the complex solution was first irradiated for 30 min at 400 nm and then incubated with DNA in the dark for 2 h 30 min at 37 °C (Fig. 9). This incubation time was previously determined to be enough to get the clear activity of the complex on DNA (the coalescence point is reached) (Fig. 9 B). As it is shown in Fig. 9 A, no activity of **3** was obtained when irradiation is performed before incubation with DNA. This indicates that interaction between DNA and the complex requires continuous irradiation of the reaction mixture. The same experiments were also performed for complex **4**, with similar results (not shown). These results indicate that light irradiation is required for the reaction itself between Ru and DNA.

To determine the influence of oxygen on the interaction between the most active Ru complexes **3** and **4** and the DNA, two parallel experiments were carried out: one of them in the presence of air oxygen and the other under an argon atmosphere. As it is shown in Fig. 10 incubations under an Ar atmosphere (lower panels) suppress the effect of both Ru complexes on DNA structure, thus indicating that the presence of oxygen is necessary for Ru interaction with DNA. In light of these results, we also checked the effect of introducing a ROS quencher such as sodium azide in the reaction mixture. However, this compound did not have any effect on the structural changes produced by complex **3**, thus indicating that changes in DNA structure promoted by the Ru complex

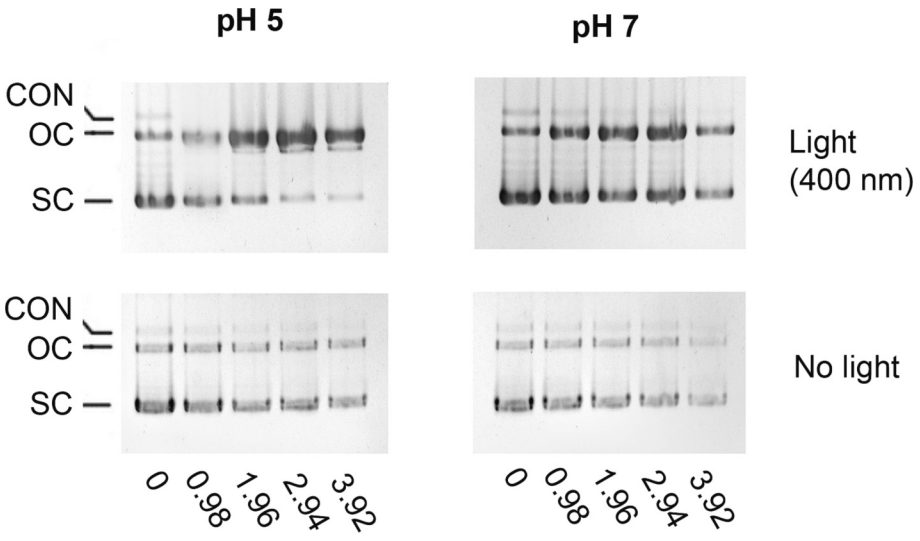


Fig. 6. DNA mobility shift assay of Ru complex **1**. Reactions were performed at different pH, either in the dark or after irradiation of the DNA plus complex mixture with 400 nm light for 2 h and 30 min at 37 °C, followed by O/N incubation (14 h) at the same temperature. The Ri (Ru/DNA base molar ratio) values are shown below for each assay. SC: supercoiled DNA. OC: open circular DNA. CON: plasmid concatemers.

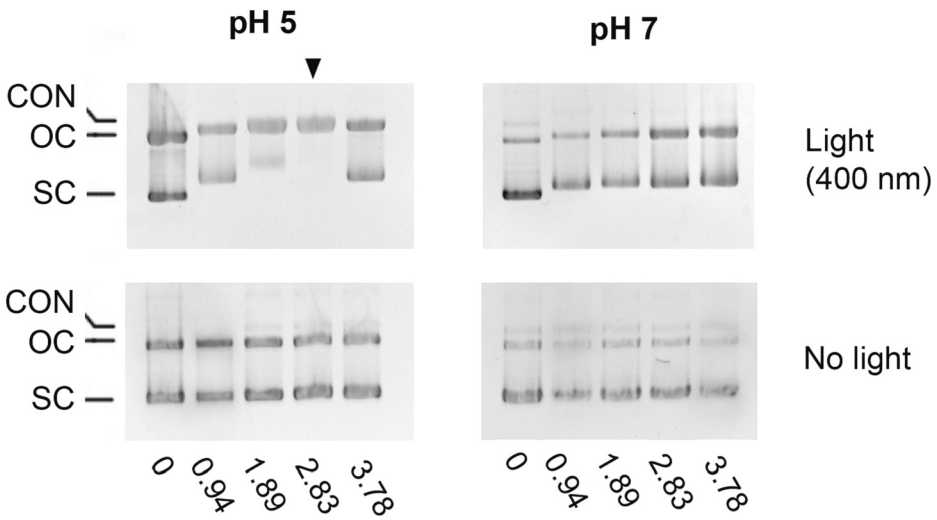


Fig. 7. DNA mobility shift assay of Ru complex **3**. Reactions were performed at different pH, either in the dark or after irradiation of the DNA plus complex mixture with 400 nm light for 2 h and 30 min at 37 °C, followed by O/N incubation (14 h) at the same temperature. The Ri (Ru/DNA base molar ratio) values are shown below for each assay. SC: supercoiled DNA. OC: open circular DNA. CON: plasmid concatemers. The Ri value corresponding to the coalescent point is indicated by an arrow.

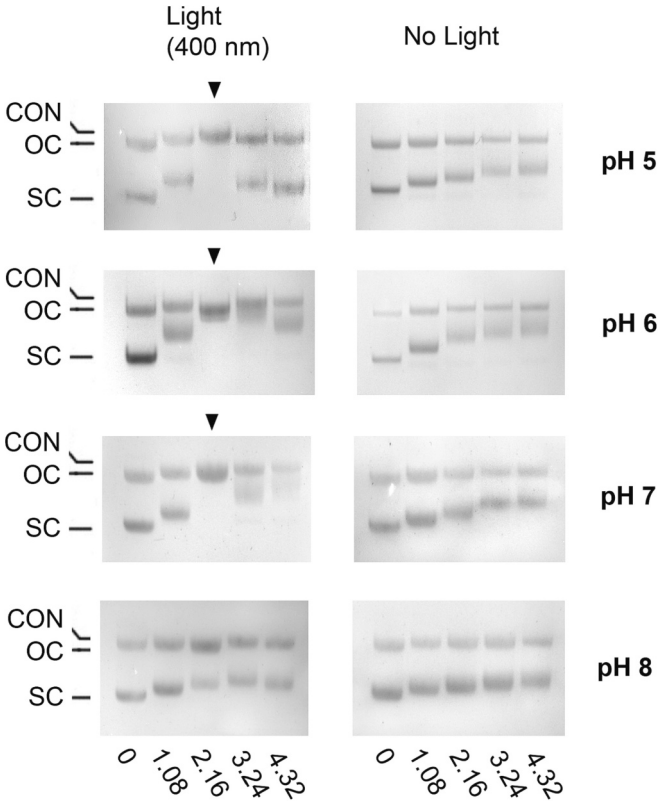


Fig. 8. DNA mobility shift assay of Ru complex **4**. Reactions were performed at different pH, either in the dark or after irradiation of the DNA plus complex mixture with 400 nm light for 2 h and 30 min at 37 °C, followed by O/N incubation (14 h) at the same temperature. The Ri (Ru/DNA base molar ratio) values are shown below for each assay. SC: supercoiled DNA. OC: open circular DNA. CON: plasmid concatemers. Ri values corresponding to the coalescent point are indicated by arrows.

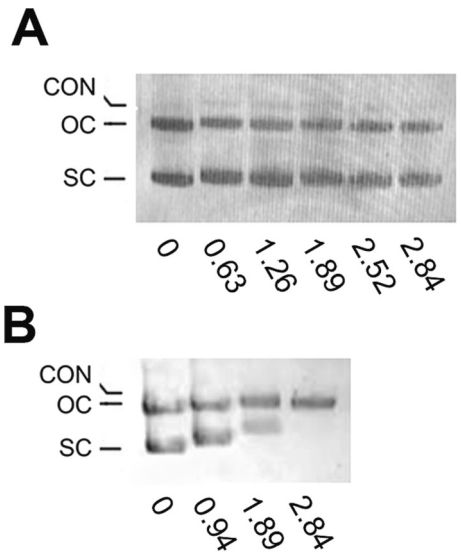


Fig. 9. Light pre-activation assay of Ru complex **3**. A complex 3 solution at pH 5 was irradiated for 30 min using 400 nm light and then incubated with DNA in the dark for 2 h 30 min at 37 °C (panel A). As a control, equivalent reaction mixtures at pH 5 were irradiated with light for 2 h 30 min before being analysed by DNA mobility shift assay (panel B). The reactions were performed at different Ri (Ru/DNA base molar ratio) values which are indicated below. SC: supercoiled DNA. OC: open circular DNA. CON: plasmid concatemers.

were due to direct interaction rather than to some oxidative damage on DNA triggered by the complex.

3. Discussion

It is important to stress that the evaluated complexes are not cytotoxic in the dark but are significantly active against cancer cells under one first step of irradiation with continuous visible light and later with a blue light when added to the cells. Complexes **2** and **3** display activity in the range of other Ru(II)-based photosensitizers and, in the case of **3** its IC_{50} value is practically similar to that for cisplatin. Nevertheless, complex **4** does not show anticancer activity both in the dark and under irradiation, despite it being one of the generated species when complex **2** is irradiated with visible light. The ability of complexes **1**, **2** and **3** to increase cellular ROS levels up to twofold after photoactivation suggests that their cytotoxic action is related to their ability to generate oxidative damage at the cellular level. Experiments against SC DNA confirmed that complexes **1**, **2** and **3** are not active in the dark under experimental conditions, showing **4** a moderate activity although coalescence was never reached even at a high amount of metal. In contrast, complexes **1**, **3** and **4** were active when experiments were performed under continuous irradiation with visible light. In this experiment, complex **1** produces single DNA strand breaking producing the loss of supercoiled DNA

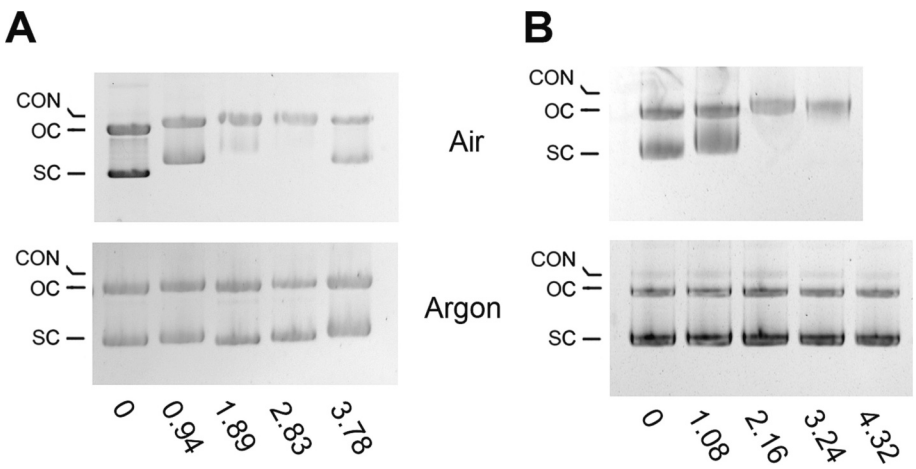


Fig. 10. Effect of O_2 on the reactivity of Ru complexes **3** (panel A) and **4** (panel B). Reactions at pH 5 (complex **3**) or pH 7 (complex **4**) were performed either under air (upper panels) or argon (lower panels) atmospheres. Incubations were performed for 2 h and 30 min at 37 °C under irradiation with 400 nm light. The Ri (Ru/DNA base molar ratio) values are shown below for each assay. SC: supercoiled DNA. OC: open circular DNA. CON: plasmid concatemers.

structure at pH = 5, which is not the cell condition, but complex **4** showed a clear activity under irradiation also at pH = 7. Therefore, the most active complexes against cancer cells are not the most reactive with SC DNA. This apparent contradiction supports several consequences: a) complex **1** is active against DNA only under an oxygen atmosphere, which confirms that oxygen and ROS generation has an important role in the action mechanism of the complex; b) complex **4** contains an easily replaceable ligand, the water molecule, and therefore it could also easily react with the plethora of biomolecules present in the cell. Therefore, probably this complex is not able to reach the cell nuclei; c) This assumption may be supported by the fact that the *cis*-complexes **1** and **2** both show anticancer activity but lower than complex **3**, nevertheless **2** is completely inactive against DNA under the reaction conditions, despite their redox properties are similar to those for **1**; d) results support that the DNA is not the only possible target that can induce the cell death, so probably the action mechanism is different from that for cisplatin. Nevertheless, an additional experiment should be done to ensure this affirmation.

In line with this, complex **3** is transformed under light irradiation into **4** notably more slowly than **2** [86]. This fact could suggest that **3** can access the cell nuclei without significant modification and there being transformed in complex **4** interacting with DNA and/or other biomolecules in the cell nuclei or stimulates reactions that finally induce the cell death. It is important to point out that activity against DNA only happens under an oxygen atmosphere, thus supporting that oxygen has an important role in the action mechanism of these Ru complexes. These pieces of evidence suggest that without discarding that interaction with DNA can also happen in the cell, the main anticancer mechanism is probably the generation of ROS species that can also directly react and damage other biomolecules of the cells such as lipids and proteins. Nevertheless, more experiments need to be made to elucidate important mechanistic aspects such as how complexes interact with DNA (intercalation, electrostatically…..) if they can interact with other biomolecules in the cell and what effect can produce, also if parent complexes containing different PTA derivatives display similar behaviour, etc.

4. Conclusions

The new water-soluble ruthenium complex *cis*-[Ru(dcbpyH)$_2$(PTAH)$_2$]Cl$_2$·3H$_2$O (**1**) (dcbpyH$_2$ = 4,4′-dicarboxy-2,2′-bipyridine; PTA = 1,3,5-triaza-7-phosphaadamantane), *cis*-[Ru(bpy)$_2$(PTA)$_2$]Cl$_2$ (**2**), *trans*-[Ru(bpy)$_2$(PTA)$_2$](CF$_3$SO$_3$)$_2$ (**3**) and *cis*-[Ru(bpy)$_2$(H$_2$O)(PTA)](CF$_3$SO$_3$)$_2$ (**4**) showed no cytotoxic activity in absence of light irradiation (IC$_{50}$ > 100 μM), but after photoactivation cytotoxicity of complexes **1**, **2** and **3** markedly increased (IC$_{50}$ = 25.3 μM (**1**), 14.6 μM (**2**) and 9.3 μM (**3**)). Complexes **2** and **3** display a similar anticancer activity that is in the range of other Ru(II)-based photosensitizers, while complex **3** showed an IC$_{50}$ similar to that of cisplatin. However, **4** does not show anticancer activity either in the dark or under irradiation, despite it being one of the generated species when **1** is irradiated with visible light. These complexes did not show toxicity against red blood cells and therefore they can be used as chemotherapeutic compounds. It is important to point out that pre-activation with visible light increased the anticancer activity when irradiated with blue light, making in these irradiation conditions complex **3** able to display an activity like the cisplatin. In addition, the reactivity of the Ru complexes against plasmid DNA under UV–Vis irradiation was studied by analysing plasmid mobility. Experimental data shows that **4** unfolds SC DNA (supercoiled DNA) both in the dark and under visible irradiation, while **1** and **3** are only active under visible light irradiation. Complex **2** was found to be inactive in either case. The unfolding activity of complexes **3** and **4** was dependent on the oxygen present in the reaction. Also, it is important to stress that, the photoactivation of compounds triggered the generation of ROS species in the cells, providing an alternative mechanism for cancer treatment. Despite the interesting results suggesting that this family of compounds are promising photoactive anticancer active agents, additional experiments are needed to determine important mechanistic aspects such as how complexes interact with DNA (intercalation, electrostatically…..) and corroborate all the indicated suspicions, but also the synthesis of new complexes should also be carried out to obtain more information about action mechanism and better and more efficient photoactive compounds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2023.112291.

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