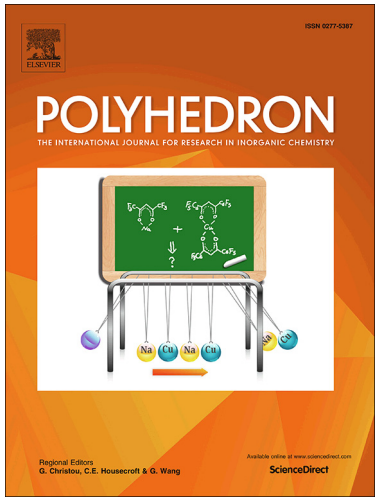

Investigation of photo-activation on Ruthenium(II)-arene complexes for the discovery of potential selective cytotoxic agents

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Investigation of photo-activation on Ruthenium(II)-arene complexes for the discovery of potential selective cytotoxic agents

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Abstract: Rational design of ruthenium complexes with optimized ligands is a promising approach to modulate their stability and antiproliferative effects in cancerous cells. The release of ligands from the coordination sphere of the ruthenium complexes can lead to the formation of biologically active Ru species that are able to exert cytotoxic effects. Herein, we have studied this approach on four ruthenium(II) complexes bearing (3,5-cycloheptadienyl)diphenylphosphine or (cycloheptyl)diphenylphosphine and ethylbenzoate or p-cymene and an unusual bimetallic analogue. The stability of the complexes was investigated in DMF as well as DMEM/FBS using ^{1}H and $^{31}P\{^{1}H\}$ NMR spectroscopy. We studied the photocytotoxicities of the complexes in two cell lines – HeLa and RPE-1 – to understand their behavior and cytotoxicity upon ligand dissociation. All complexes showed moderate to high cytotoxicity in the two cell lines upon light irradiation.

1. Introduction

Despite the success of cisplatin, its heavy off target side-effects and eventual appearance of resistance prompted numerous chemists to explore other metal complexes, especially organometallic complexes, for their anticancer properties.[1–8] Among them, ruthenium complexes appear to be promising alternatives.[9] NAMI-A, (N)KP-1339 and their derivatives are to date the most well-known representative complexes since they have reached phase II clinical tests (**Figure 1**).[10,11] The exploration of the potential of ruthenium complexes is not limited to its +III oxidation state; numerous Ru(II) complexes such as those of the RAPTA (Ru(η^6-*p*-cymene)Cl_2(pta)) family have also been investigated (**Figure 1**). Concerning these neutral Ru(II)-based-arene-phosphine complexes, they usually displayed poor cytotoxicity *in vitro*.

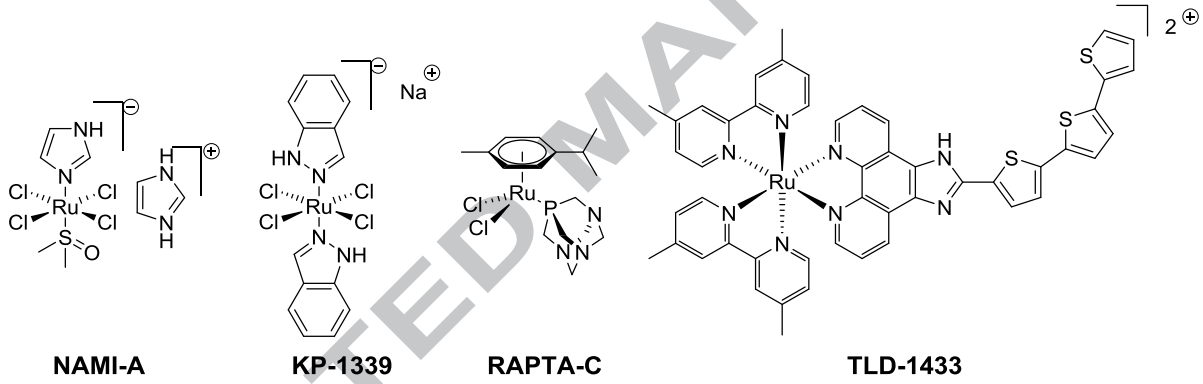


Figure 1. Chemical structures of some ruthenium-based drug candidates and of the photodynamic therapy photosensitizer TLD-1433.

Organometallic ruthenium(II)–arene complexes have promising potential for cancer therapy because the coordination ligands in such complexes can be modulated for their pharmacological properties such as cellular accumulations and kinetic reactivities. Naphthalimide tagged Ru(II)-arene complexes, which showed moderate to high toxicity and selectivity towards cancerous cells, were reported by Dyson *et al.*[12] and Sadler and coworkers showed that half-sandwich ruthenium(II)-arene complexes with chelated diamine ligands can undergo reversible oxidation to diimine complexes and this was accompanied by strong changes in their cytotoxic behavior in ovarian cancer A2780 cells.[13] A very recent work by Xu *et al*. reported a few of ruthenium(II)−arene complexes containing a hypoxia inducible factor-1α inhibitor, which allowed the organometallic complexes to target hypoxia in cancer cells, enhancing their anticancer activities.[14] We note that the ruthenium(II) polypyridyl

complex of McFarland, namely TLD-1433, has completed phase I clinical trial as a photodynamic therapy (PDT) photosensitizer (PS).[15]

Very recently, some of us published a study highlighting the cytotoxicity profile of some Ru(II) arene complexes (Figure 2). Six Ru(II) complexes were designed and two of them (**IV** and **V**) displayed low micromolar IC_{50} in CT26, 4T1, and LLC1 cancer cell lines (**Figure 1**).[16] More interestingly, we were able to link directly the toxicity of each complex of the family to its ability to be taken up by cells (correlation between IC_{50} and ruthenium uptake determined by ICP-MS analysis).

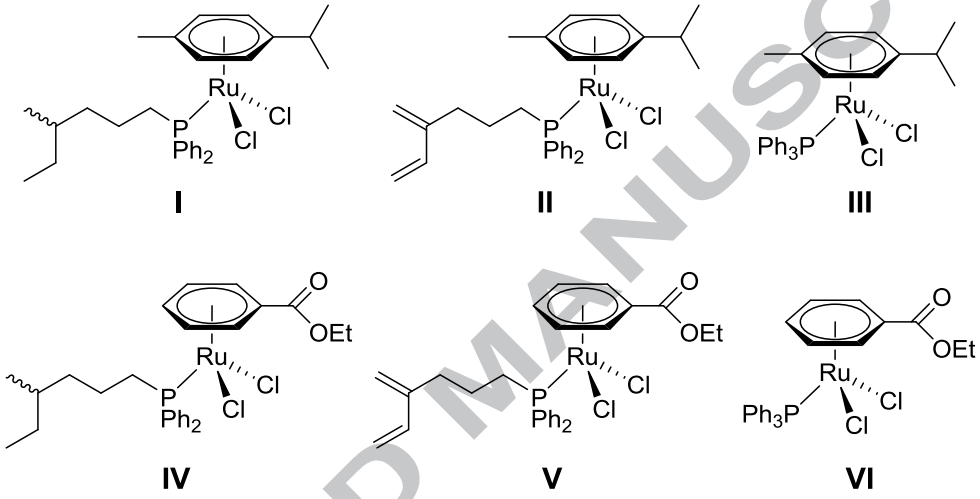


Figure 2: Structures of ruthenium(II) previously prepared by some of us for their anticancer activity.

More specifically, the complexes bearing an ethylbenzoate as an arene ligand displayed an uptake up to twenty times higher than the one containing a *p*-cymene ligand. These results imply that the ruthenium(II) cation itself is the biologically active species and that the ligands are used to promote the cellular uptake of the complexes. For this reason, we looked for a manner of forcing the release of the ligands, especially of the arene. Being able to do so will either confirm that the role of the ligands is just for the transport or to give access to activatable ligands (removing the arene will lead to very active ruthenium species).

With this idea in mind, we capitalized on the work that some of us developed for catalysis. We have recently described the ruthenium complexes **1-5** bearing either (3,5-cycloheptadienyl)diphenylphosphine or (cycloheptyl)diphenylphosphine groups and studied their catalytic activity in ATRA (Atom transfer Radical Addition) reactions (**Figure 3**).[17] We showed that upon light irradiation, complexes **1**-**4** lose their arene

ligand. It is worth noting that under the same conditions, the ligand ethylbenzoate in compounds **3** and **4** is released in 15 minutes, while it took more than two hours for the p-cymene derivatives (compounds **1** and **2**). Consequently, we decided to investigate the photo-activation of these Ru(II)-based complexes *in vitro*. Moreover, we have investigated compound **5** in this study as an important control. As shown previously, this cationic dinuclear compound is generated upon light treatment of the (cycloheptadienyl)phosphine-containing complexes **1** and **3** in organic solvents (dichloromethane, chloroform *etc.*).

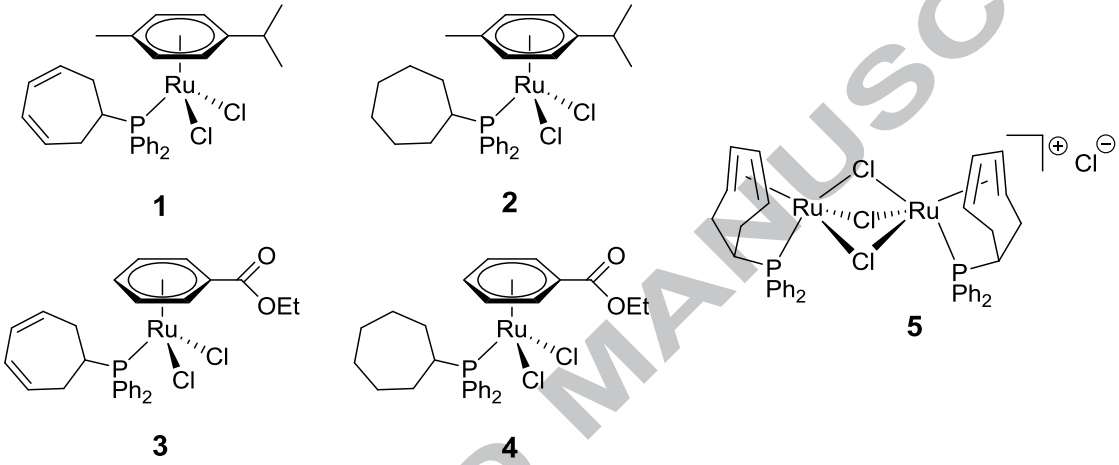


Figure 3: Ruthenium complexes described by some of us for their catalytic activity in ATRA reactions.

In this article, we describe the study of the photocytotoxicity of these ruthenium complexes on two cell lines, namely a cancerous one (HeLa) and a model of non-cancerous one (RPE-1), as well their stability in DMEM-10% FBS solution**.**

2. Experimental

*2.1. Photocytotoxicity of Ruthenium Complexes **1-5***

2.1.1. Materials:

Dulbecco's Modified Eagles Medium (DMEM, catalogue number 11500416), Dulbecco's Modified Eagles Medium supplemented with nutrient mixture F-12 (DMEM/ F-12, catalogue number 11580546), fetal bovine serum (FBS, catalogue number 11573397), Gibco™ Penicillin-Streptomycin-Glutamine (penstrep, catalogue number 12090216) Dulbecco's Phosphate-Buffered Saline (DPBS, catalogue number 12559069), Trypsin-EDTA (catalogue number 11560626) and resazurin (catalogue

number 10751244 were purchased from ThermoFisher Scientific. N, N-Dimethyl formamide (DNAse, RNAse and protease free, molecular biology grade, catalogue number 327175000) was purchased from ACROS Organics. The pooled human plasma was obtained from Biowest. Complexes **1-5** were prepared as previously reported.[17] Analytical data matched that previously reported.

2.1.2. Cell culture

HeLa and RPE-1 cells were cultured in DMEM and DMEM/ F-12, respectively reconstituted with 10 % FBS and 1 % penstrep. Cells were grown in a humidified cell culture incubator at 37 °C and with 5 % CO_2 and passaged three times before being used for the experiments.

2.1.3. Photocytotoxicity Studies

The experiments were performed using a similar procedure previously used in our laboratories.[10,18,19] More specifically, HeLa and RPE-1 cells were seeded at a density of 4000 cells/ well in 100 µL media and grown overnight in a humidified cell culture incubator at 37 °C and with 5 % CO_2. All experiments were performed in triplicates. The media was removed and replenished with varied concentrations of the ruthenium complexes diluted in appropriate volume of the culture media in a total volume of 200 µL. The stock solutions of the Ru complexes were made in DMF and the final concentration of DMF in culture media was 1% or less. They were incubated for 4 h and replaced with 200 µL of fresh culture media. Three of the plates were irradiated at 450 nm (10 mins for HeLa cells, dose: 10 J cm^{-2} and 2 mins for RPE-1 cells, dose: 2 J cm^{-2}). Irradiation was performed in the 96-well culture plates using a LUMOS-BIO photoreactor (Atlas Photonics, Switzerland). Each well was individually illuminated with a 50 mW LED at constant current and the temperature of the plates was maintained constant at 37 °C with a cooling system. The cells were then allowed to grow in the incubator for another 44 h. From the three other plates for the dark cytotoxicity control, the media was removed after 4 h, replaced with 200 µL of fresh culture media and incubated for another 44 h. After 48 h of the initial treatment, the media was exchanged for resazurin solution (0.2 mg/ mL) made in the respective culture media and sterile filtered. After 4 h incubation, the fluorescence of the product resorufin was measured at 590 nm with excitation at 540 nm using a Spectramax M5

UV-visible spectrophotometer. The data was analyzed and plot using Graph Pad Prism 8.

2.2. Stability studies

2.2.1. Stability in DMF

In NMR tubes, 0.01 mmol (5.8-6 mg) of complexes **1-4** or 0.005 mmol (4.5 mg) of the dimer **5** were dissolved in 0.5 mL of DMF-H7 (protected from air) to give clear solutions. NMR experiments were conducted on each compound under light protection at 10 min, 1.5 h, 2.5 h, 3 h, 4.5 h, 21 h and 45 h (0.01 mmol/0.5 ml = 0.02 mmol/1 ml = 20 mM)

2.2.2. Stability in DMF + DMEM-10% FBS

On the samples at 48 h, 0.1 mL solution (DMEM-10%FBS) was added on each compound directly in the NMR tube (under air). The samples were light protected. At the beginning, the solutions were clear and no obvious change was observed. 2 h after DMEM addition the samples **1**-**4** presented a precipitate (~ suspension at the bottom) while sample **5** remain clear. After 5 days, the samples were let to evolve for 24 h under natural light.

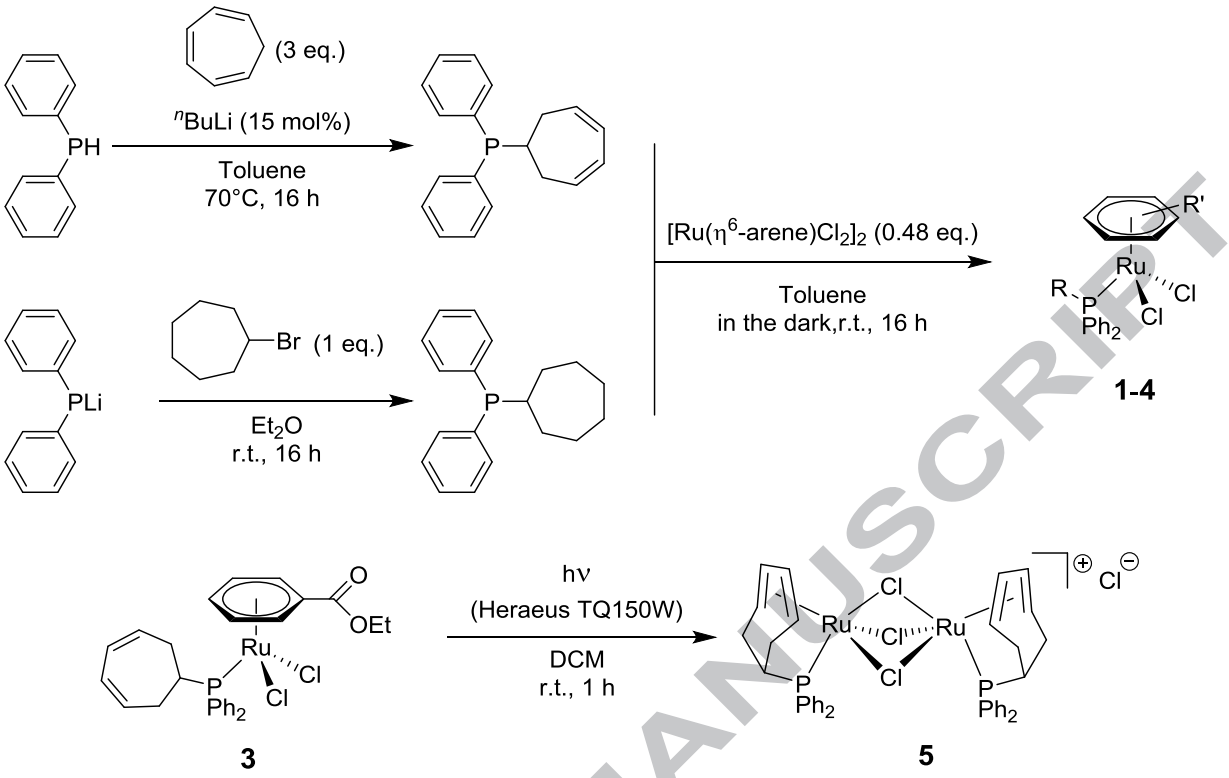
2.3. NMR Studies

The acquisition of $^{31}P\{^{1}H\}$ NMR spectra DMF-H7 was recorded at 300K using a no-lock sequence on a Bruker Avance III HD 600 MHz spectrometer (equipped with double resonance broad band probes). Monitoring the reaction by ^{1}H NMR was possible as the diene signals (4-6 ppm) do not overlap with the DMF-H7 signals.

3. Results and discussion

3.1. Synthesis of the complexes

The five complexes **1**-**5** were synthesized in good yield following procedures previously reported by some of us (**Scheme 1**).[17] All the products are air-stable. However, they need to be protected from light, especially when they are in solution.



Scheme 1: Synthetic pathways of the ruthenium complexes reported by some of us earlier.[17]

3.2. Preliminary stability investigation

Since the stability of a compound in a biological environment is a crucial property in view of its use as a PDT PS or in photo-activated chemotherapy (PACT),[20] this parameter has been thoroughly investigated in this study. When the biological studies have been performed, the complexes were first dissolved in DMF since this solvent was shown to be suitable for biological applications due to its low propensity for coordination compared to DMSO.[21] These stock solutions were then dissolved in culture media (DMEM-10% FBS). Thus, the stability of ruthenium complexes **1**-**5** was further evaluated by ^{1}H and $^{31}P\{^{1}H\}$ NMR analysis in pure DMF, then after dilution with DMEM-10% FBS.

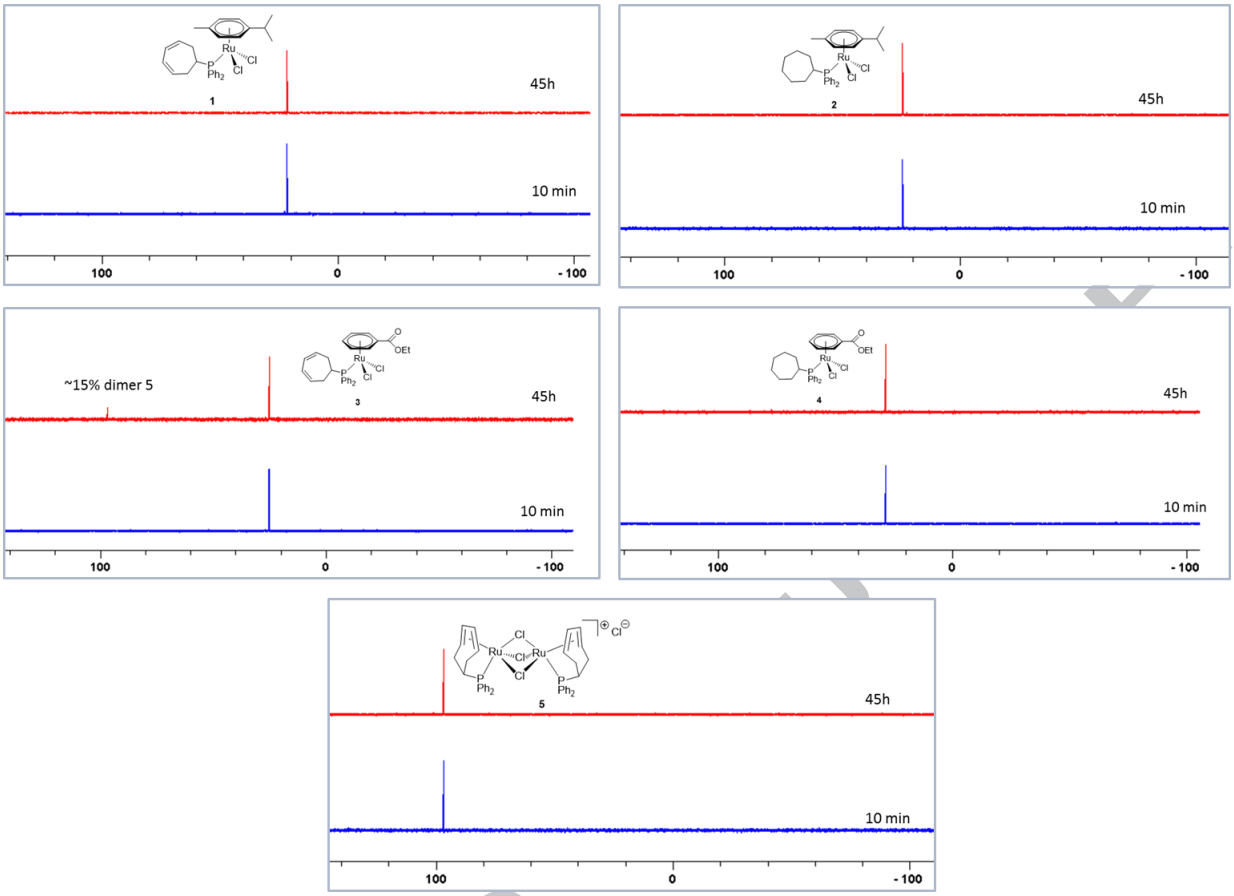


Figure 4: $^{31}P\{^{1}H\}$ NMR spectra of ruthenium complexes **1-5** after 10 min and 45 h in DMF-H7.

For this purpose, solutions of complexes **1**-**5** were prepared in DMF under argon at a concentration of 20 mM of ruthenium. Clear solutions were obtained, which were analysed by NMR after being kept in the dark from 10 min to 45 h. The ^{1}H and $^{31}P\{^{1}H\}$ NMR analyses showed that complexes **1**, **2**, **4**, **5** are stable over time, no other signal being detected after 45 h (**Figure 4**). Only compound **3** is transforming very slowly into the dimer **5** (after 21 h, 5 % of **5** were detected; after 45 h, 15 % of **5** were detected by $^{31}P\{^{1}H\}$ NMR). This result agrees with our previous observation regarding complex **3**, which had a higher sensitivity than complex **1**.[17] This stability in DMF is interesting since a recent investigation of the stability of *N*-heterocyclic-[Ru(η^{6}-arene)Cl_2] complexes in DMSO, a much more coordinating solvent, concludes to their instability (the heterocyclic ligand was replaced with DMSO).[22]

We were further interested in the behavior of these complexes in the presence of DMEM-10% FBS in the dark. For this, in the previous NMR samples, 0.1 mL solution (DMEM-10%FBS) was added on each compound under an air atmosphere. This

rapidly allowed the formation of a suspension in the bottom of samples **1** to **4** (the high concentration required for NMR investigation are not compatible with the limited solubility of the complexes in culture medium). Only compound **5** remained as a clear orange solution. However, we were able to evaluate the evolution of these samples in the dark by ^{1}H and $^{31}P\{^{1}H\}$ NMR analysis (**Figure 5**).

After 5 days under these conditions, both complexes **1** and **2** were present as the major compound in solution, even if two new signals slowly appeared on $^{31}P\{^{1}H\}$ NMR spectra (in the case of complex **2** one of the signals corresponds to free diphenylcycloheptylphosphine). Compared to those two, complex **3** displays lower stability. After 5 days, very low signals were detected by $^{31}P\{^{1}H\}$ NMN. We noticed that the dimer **5** present in 15 % before adding DMEM-10% FBS disappeared also very quickly. The only complex, which presents higher stability than complex **2**, is complex **4**, which shows lower degradation after 5 days. Concerning dimer **5**, the $^{31}P\{^{1}H\}$ NMR signal disappeared as soon as DMEM-10% FBS was added. This suggests that this complex reacts immediately with the biological media.

After 5 days in dark, all solutions were exposed to natural light for 24 h and analyzed again by NMR. The obtained spectra lead to three conclusions, namely 1) the signals of the complexes **1**, **2** and **4**, which were still present as the major compound in solution, totally disappears. This confirms the light-induced arene released; 2) the signals of the “degradation products” – at ≈ 70 ppm for complex **1** and at ≈ 40 ppm for complexes **2** and **4** – are still present after light exposure. This suggests that these complexes do not any longer bear an arene on their ruthenium; 3) it highlights the necessity to protect these complexes from light during the studies.

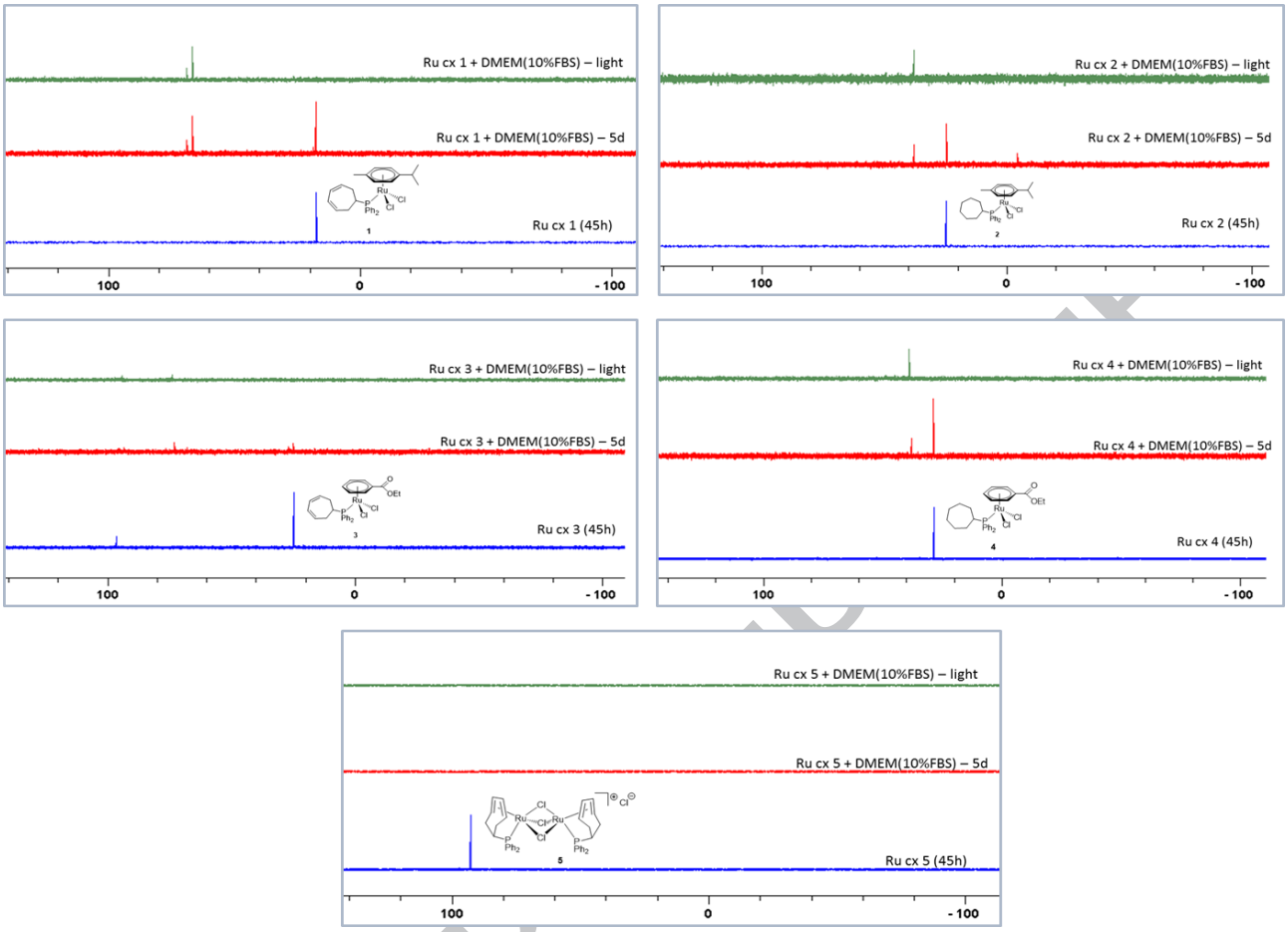
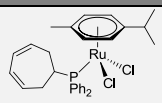
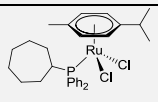
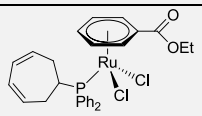
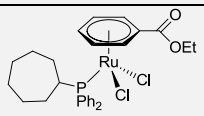
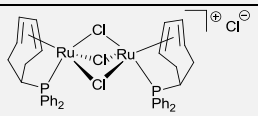


Figure 5: $^{31}P\{^{1}H\}$ NMR spectra of ruthenium complexes **1**-**5** with DMEM 10% FBS (blue line: 45 h in DMF; red line: 5 days under dark; and green line: after additional 1 day of light exposure).

*3.3. Photo-cytotoxicity of Ruthenium Complexes **1**-**5***

The photo-cytotoxicity of the ruthenium complexes **1-5** was evaluated in two cell lines, namely HeLa (human cervical cancer cells) and RPE-1 (human Retinal Pigmented Epithelial cells). After 4 h incubation in both cell lines with the ruthenium complexes **1**-**5**, the cells were washed and half of the samples were irradiated at 450 nm (10 mins for HeLa cells, dose: 10 J cm^{-2} and 2 mins for RPE-1 cells, dose: 2 J cm^{-2}) and then allowed to grow in the incubator for another 44 h. Longer irradiation times are not feasible in RPE-1 cells because these cells are not robust enough and prolonged irradiation leads to profound cell death. Similar experiments were also conducted without light irradiation to serve as controls. The antiproliferative properties of the ruthenium complexes on both samples – with and without irradiation – were determined by the resazurin assay where the fluorescence of resorufin was measured and the results are presented in Table 1.

Table 1: IC_{50} values of complexes **1**-**5** in different cell lines with and without light irradiation.

Complex	Structure	HeLa - IC_{50} (µM)			RPE-1 - IC_{50} (µM)		
		(450 nm, ~ 10 J.cm^{-2})	In the dark	PI	(450 nm, ~ 2 J.cm^{-2})	In the dark	PI
1		11.3 ± 0.9	20.3 ± 2.2	1.8	76.3 ± 2.7	75.7 ± 3.0	1.0
2		7.6 ± 0.5	11.0 ± 1.6	1.4	15.4 ± 1.7	19.9 ± 2.1	1.3
3		22.9 ± 0.7	29.9 ± 2.6	1.3	83.6 ± 4.0	79.2 ± 2.6	0.9
4		14.0 ± 0.4	17.9 ± 1.4	1.3	8.1 ± 0.8	8.9 ± 1.5	1.1
5		12.3 ± 1.8	30.4 ± 4.6	2.5	27.7 ± 2.7	28.6 ± 0.4	1.0

From these data, we can observe several trends concerning the photo-activation. First, for RPE-1 cell line, there is no significant difference between irradiated and non-irradiated cells, which suggests two different possibilities: either the dose used of 2 J.cm^{-2} is not enough to induce the photo-release of the arene or the arene ligand is already gone before light irradiation. When the corresponding results with HeLa is analyzed – this time with a five-time higher dose –, a small difference is observed when the arene ligand is an ethylbenzoate, but a more significant one appeared for the complexes bearing a *p*-cymene ligand. The number of compounds is too low to draw definitive conclusions. However, it suggests that most of ethylbenzoate is released before light irradiation and that *p*-cymene-complexes need more than 2 J.cm^{-2} to be photo-activated. The case of the complex **5** is even more interesting. It is clear that it requires at least 10 J.cm^{-2} to be activated, but this time, the photo-activation must involve another mechanism.

IC_{50} values themselves indicate that the complexes display interesting properties against HeLa cells. However, the main point of this study is that cycloheptadienyl complexes **1** and **3** are more efficient on the cancer cell line than on the healthy one (3.7 and 2.6 times more efficient, respectively and even 6.7 and 3.7 if we compare the value after irradiation). These results are very promising for future applications because they suggest that these products may display an *in vivo* selectivity for tumor *vs*. healthy tissues. Concerning the compound **5**, its IC_{50} needs to be taken with caution due to the fact that compound **5** contains two ruthenium ions. Thus, it can be concluded that compounds **1** and **3** display very high antiproliferative activities on the HeLa cancer cell line along with moderate to low antiproliferative properties on the non-cancerous RPE-1 cell line. Ruthenium-arene complexes bearing a *p*-cymene ligand can be photo-activated *in vitro*, but require more energy and the unusual cationic bimetallic complex display interesting antiproliferative properties, especially when photo-activated. The mechanism of action of the latter is still under investigation.

4. Conclusions

In this study, we investigated the biological activity of four ruthenium(II) complexes bearing (3,5-cycloheptadienyl) diphenylphosphine or (cycloheptyl)diphenylphosphine and ethylbenzoate or *p*-cymene and an unusual bis-ruthenium complex. All the complexes were found to be stable in DMF and three of them display good stability in culture medium in the dark. On the contrary, they rapidly degrade under light exposure. All complexes display significant antiproliferative properties against the HeLa cancer cell line. Interestingly, ruthenium-arene complexes bearing (3,5-cycloheptadienyl) diphenylphosphine present a limited toxicity against the healthy model cell line RPE-1. We could also demonstrate the possibility to further activate the different complexes by light irradiation (450 nm), which results in an increase of the antiproliferative properties by a factor up to 2.5. This photo-activation results in the formation of an activated ruthenium complex produced by the released of the arene ligand for complexes **1**-**4**. Concerning the bis-ruthenium, the photoactivation mechanism is still under investigation.

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Four Ru(II)-arene complexes which are stable in DMF were found to display significant anti-proliferative properties against HeLa cancer cell line. We could also demonstrate the possibility to further activate the different complexes by light irradiation, which results in an increase of the anti-proliferative properties to a factor up to 2.5.