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1 **Photoactivatable Ru(II) complex bearing 2,9-diphenyl-1,10-phenanthroline: unusual**
2 **photochemistry and significant potency on cisplatin-resistant cell lines**

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8

9 *KEYWORDS cancer, photoactivatable chemotherapy, ruthenium polypyridyl complexes.*

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11 [sciences/people/rony-khnayzer.php](http://sas.lau.edu.lb/natural-sciences/people/rony-khnayzer.php)

12 **Abstract**

13 The current study investigates [Ru(bipy)₂(dpphen)]Cl₂ [where bipy= 2,2'-bipyridine and dpphen
14 = 2,9-diphenyl-1,10-phenanthroline] (complex **1**) for photoactivatable chemotherapy (PACT)
15 application on five cancer cell lines. [Ru(bipy)₂(phen)]Cl₂ [where phen = 1,10-phenanthroline]
16 (complex **2**) was included as an unstrained control. Upon excitation with visible light, complex **2**
17 proved to be photostable while complex **1** underwent a quantitative dissociation of the bipy ligand
18 and formation of a Ru(II) polypyridyl aqua complex in water. Complex **1** demonstrated only
19 marginal activity in the dark; its cytotoxicity increased significantly upon photoactivation with a
20 high phototoxicity index (PI = [IC₅₀ dark] / [IC₅₀ light]) ranging from 39.2-fold in A549 to over
21 100-fold in MDA-MB-231. Complex **2**, on the other hand, did not show much difference in
22 anticancer activity between dark and light conditions. Importantly, the IC₅₀ of the photoproduct of
23 complex **1** was several folds lower than that of cisplatin in all tested cell lines. Furthermore, the
24 dissociating ligand (bipy) was biologically inert in almost all cell lines investigated confirming
25 that phototoxicity was mediated primarily by the Ru aqua complex that is released upon irradiation.
26 In conclusion, the Ru-centered complex **1** could represent a potential photoactivatable
27 chemotherapeutic drug that increases selectivity to tumors and offers alternative treatment in the
28 light of increasing cisplatin resistance.

29

1 Introduction

2 Platinum-based complexes have been extensively used as chemotherapeutic agents since the
3 discovery of cisplatin, which was proved to treat a variety of cancers.¹ However, due to acquired
4 resistance and dose-limiting side effects, other metals have been investigated.² Among these
5 metals, Ru has gained interest due to the tunable physical and chemical properties of the associated
6 complexes. Furthermore, variation in ligand framework allows Ru compounds to target specific
7 organelles within cancer cells.³ Moreover, the Ru(II) state was found to be the most active form in
8 the hypoxic environment of tumors thus conferring selectivity to cancer cells.⁴ The cellular uptake
9 of some Ru complexes by transferrin receptors which are overexpressed in cancer cells further
10 increases tumor selectivity and accordingly limits systemic toxicity.⁵ Once inside the cell, Ru
11 prodrugs can undergo light-mediated activation classified as photodynamic therapy (PDT) or
12 photoactivated chemotherapy (PACT). The latter uses light to change the chemical and physical
13 properties of the Ru complex by altering its electronic state thus generating a form of the molecule
14 that mediates cytotoxicity through DNA binding, ligand ejection or caging mechanisms. Unlike
15 PDT, PACT is not dependent on the presence of oxygen and thus offers a greater advantage in
16 targeting cancer cells which are hypoxic.^{6,7} Strained Ru complexes are particularly attractive as
17 light-activated agents because they absorb in the visible range and are kinetically inert and
18 photochemically labile. Although most Ru(II) polypyridyl complexes with pseudo-octahedral
19 geometry are rather stable upon irradiation, those having a distorted octahedral geometry undergo
20 a rapid deactivation of the excited state due to the decreased ligand-field splitting. This induces
21 ligand dissociation resulting from the population of the ³d-d state which is thermally accessed from
22 the ³MLCT excited state. There are many approaches for the use of Ru complexes in PACT
23 depending on the photoactivation strategy adopted. Ru(II) polypyridyl complexes with distorted
24 octahedral geometry can undergo ligand dissociation upon irradiation and possibly bind DNA in a
25 similar fashion to cisplatin.^{6,8} Using this approach, Glazer and coworkers prepared a series of
26 Ru(II) complexes bearing methyl substituents near the coordination sphere.⁸
27 $[\text{Ru}(\text{bipy})_2(\text{dmbipy})]^{2+}$ [where dmbipy = 6,6'-dimethyl-2,2'-bipyridyl] was tested on leukemia and
28 lung cancer cell lines. As expected, photoejection of the dimethyl substituted ligands upon
29 irradiation resulted in the formation $[\text{Ru}(\text{bipy})_2(\text{H}_2\text{O})_2]^{2+}$ and the free dmbipy photoproducts in
30 aqueous solution. When added to pUC19 plasmid, the light-activated complex induced DNA
31 photobinding as evidenced by delayed migration of the supercoiled DNA on the gel and decreased

1 EtBr intercalation. DNA damage occurred only upon photoactivation which was correlated with a
2 decreased cell viability in leukemia and lung cancer cells, as compared to dark conditions.⁸
3 Another methylated derivative, $[\text{Ru}(\text{bipy})_2(\text{dmdp})]^{2+}$ [where $\text{dmdp} = 2,9'$ -dimethyl-
4 dipirido[3,2-*f*:2',3'-*h*]-quinoxaline] formed the same aquated complex as above following the
5 photoejection of the straining ligand. However, the photoactivated complex exhibited a dual effect
6 on DNA, inducing both photobinding and photocleavage.⁸ Later, the same group used a similar
7 strategy to explore $[\text{Ru}(\text{bipy})_2(\text{dmdop})]^{2+}$ [where $\text{dmdop} = 2,3$ -dihydro-1,4-dioxino
8 [2,3-*f*]-2,9-dimethyl-1,10-phenanthroline] and $[\text{Ru}(\text{dmphen})_2(\text{dop})]^{2+}$ [where $\text{dmphen} = 2,9$ -
9 dimethyl-1,10-phenanthroline].⁹ The latter exhibited photoinduced DNA damage via covalent
10 binding whereas the former induced both single-stranded breaks (SSBs) and covalent binding to
11 DNA. However, phototoxicity against leukemia cells did not linearly correlate with the degree of
12 DNA damage.⁹ Turro and coworkers investigated the photoinduced binding of *cis*-
13 $[\text{Ru}(\text{bipy})_2(\text{NH}_3)_2]^{2+}$ to DNA to mimic the mode of action of cisplatin. The resulting complex, *cis*-
14 $[\text{Ru}(\text{bipy})_2(\text{H}_2\text{O})(\text{OH})]^+$, was shown to bind covalently to 9-methyl and 9-ethyl guanine as well as
15 to single-stranded and double-stranded DNA.¹⁰ In another study, Turro and coworkers evaluated
16 four $[\text{Ru}(\text{bipy})_2(\text{L})]^{2+}$ complexes where L corresponded to bithioether (3,6-dithiaoctane or 1,2-
17 bis(phenylthio)ethane) or nitrogen bidentate (ethylenediamine or 1,2-dianilinoethane) ligands. The
18 bis-aqua complexes resulting from ligand exchange of bithioether were shown to efficiently bind
19 guanosine 5-monophosphate as well as linear double-stranded DNA.¹¹ Gasser and coworkers
20 investigated $[\text{Ru}(\text{II})(\text{bipy})_2\text{dppz}]^{2+}$ derivatives [where $\text{dppz} = \text{dipyrido}[3,2\text{-}a:2',3'\text{-}c]$ phenazine].
21 When irradiated, all complexes produced O_2 as efficiently as photosensitizers available in the
22 market and were shown to intercalate DNA. Complexes with the highest nuclear accumulation and
23 intercalative potential induced efficient DNA photocleavage which reflected in higher
24 phototoxicity in HeLa cells.¹² Other ligand frameworks were also tested and showed potential use
25 for PACT with DNA being the main biological target. MacFarland and coworkers explored three
26 strained Ru(II) metal-organic dyads bearing the crowded 6,6'-*dmb* ligand (6,6'-dimethyl-2,2'-
27 bipyridine) and imidazo[4,5-*f*] [1,10]phenanthroline (IP) appended with *n*-thiophene units (*n*T; *n*
28 = 1 to 3). The three complexes were shown to have a dual mode of action; as the number of
29 thiophene units decreased, the dissociation rate of the 6,6'-*dmb* ligand increased which correlated
30 with the formation of cisplatin-like DNA adducts, whereas the complex with the highest *n* number
31 had the highest singlet oxygen yield which translated in more DNA single-stranded breaks. All

1 three complexes exhibited a significantly higher phototoxicity than cisplatin in tested cancer cell
2 lines.¹³ Bonnet and coworkers were the first to develop Ru(II) prodrugs with a *trans* configuration
3 that allows photoactivation with green light. [Ru(biqbipy)(dmsO)Cl]Cl [where biqbipy = 6,6'-
4 bis[N-(isoquinolyl)1-amino]-2,2'-bipyridine)] and [Ru(biqbipy)(Amet)(HAmet)]PF₆ [where
5 HAmet = N-acetyl-L-methionine, Amet = deprotonated N-acetyl-L-methionine] were shown to
6 eject the *trans* ligands and become bis-aquated. The latter interacted more strongly with DNA than
7 the prodrug, however, their confirmed apoptotic activity could not be conclusively attributed to
8 this interaction.¹⁴ Glazer's group investigated Ru(II) polypyridyl complexes with one or two
9 bisquinoline ligands. Upon photoactivation with high wavelengths (700 or 800 nm), the resulting
10 bis-aqua complexes were found to bind DNA and proved to be cytotoxic against leukemia cells.¹⁵
11 While most studies focused on the role of Ru(II) aqua complexes in inducing cytotoxicity, we have
12 investigated the possible contribution of the dissociating ligand to the biological activity of
13 [Ru(bipy)₂(dmphen)]Cl₂ [where dmphen = 2,9-dimethyl-1,10-phenanthroline] on ML-2 Acute
14 Myeloid Leukemia cell line. Results of cell viability assays showed that the toxicity of dmphen
15 was significantly higher than the aqua complex formed and thus proved that the Ru(II) center can
16 act as a cage for the cytotoxic ligand. These findings uncovered the important role of the
17 coordinating ligands in the biological activity of strained Ru(II) polypyridyl complexes.¹⁶
18 Similarly, another report by Bonnet and coworkers illustrated that the photoreleased 6,6'-dimethyl-
19 2,2'-bipyridine (dmbipy) ligand is responsible for the phototoxicity of [Ru(bipy)₂(dmbipy)]²⁺,
20 whereas the Ru bis-aqua photoproduct was found to be biologically inert.¹⁷ Turro and coworkers
21 previously reported similar results on the pseudo-octahedral complex [Ru(tpy)(5CNU)₃]²⁺ (3)
22 [where tpy = 2,2':6',2''-terpyridine and 5CNU = 5-cyanouracil].¹⁸ Upon excitation with visible
23 light, the ligand 5CNU was ejected from the caging Ru(II) complex and the photoproduct exerted
24 a dual cytotoxic activity; 5CNU induced cell death through the inhibition of pyrimidine catabolism
25 whereas the Ru(II) center was capable of binding DNA¹⁸. In the present study, two Ru(II)
26 polypyridyl complexes were investigated: [Ru(bipy)₂(dpphen)]Cl₂ (complex **1**) which is sterically
27 strained by the phenyl groups on the 2,9-positions of dpphen, along with the unstrained control
28 [Ru(bipy)₂(phen)]Cl₂ (complex **2**), Figure 1.

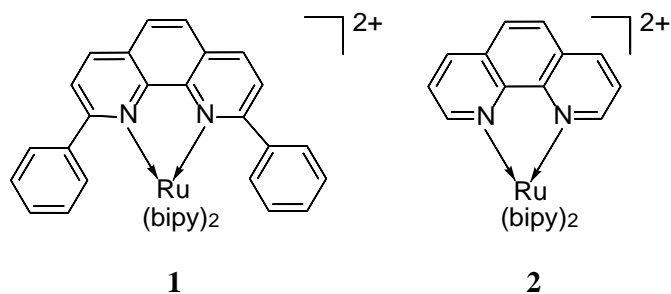
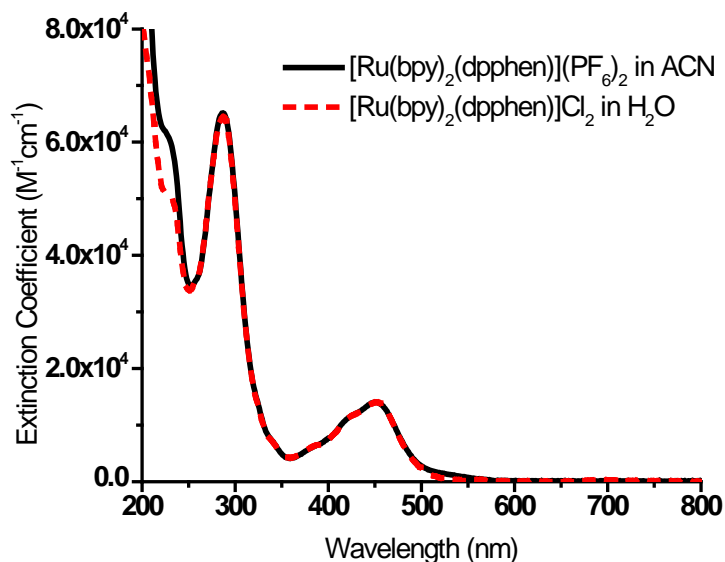


Figure 1. Structures of complexes **1** and **2** where bipy = 2,2'-bipyridine.

The photochemistry of $[\text{Ru}(\text{bipy})_2(\text{dpphen})](\text{PF}_6)_2$ was previously studied by Sauvage and coworkers who reported the quantitative ejection of bipy in acetonitrile instead of the hindering dpphen ligand thus generating the photoproduct $[\text{Ru}(\text{bipy})(\text{dpphen})(\text{CH}_3\text{CN})_2]^{2+}$.¹⁹ Unlike phenanthroline derivatives, bipy was shown to be inactive in cancer cells since it only forms electrostatic interactions with DNA and is unable to intercalate.²⁰ In the present work, the photochemistry of $[\text{Ru}(\text{bipy})_2(\text{dpphen})](\text{PF}_6)_2$ was also re-evaluated in acetonitrile for comparison with reported results and the chloride salt of complex **1** was explored as a potential photochemotherapeutic candidate. The photochemistry of the chloride complex was examined in water to determine if bipy photorelease would occur and the phototoxicity of the new product was evaluated on a panel of cancer cell lines to probe the PACT of complex **1**.

Results

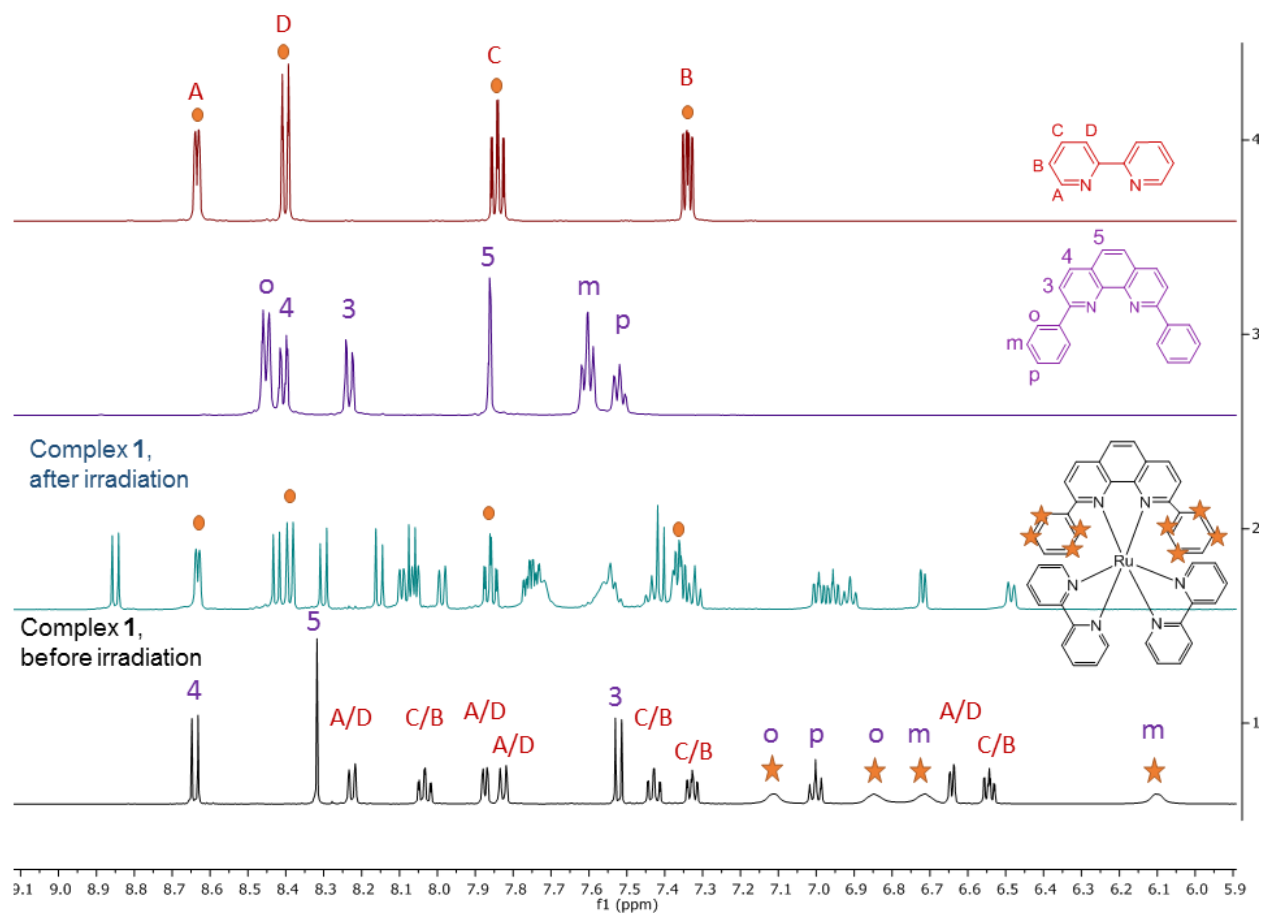
In all photochemical and photobiological experiments, the light output from a home-built blue LED setup²¹ (460 nm, $\sim 100 \text{ mW/cm}^2$) was used to provide the excitation source. This light source provides a direct metal-to-ligand charge transfer (MLCT) excitation. The extinction coefficient of the MLCT transition for complex **1** was $14,000 \pm 1,000 \text{ M}^{-1}\text{cm}^{-1}$ at 448 nm in both acetonitrile ($[\text{Ru}(\text{bipy})_2(\text{dpphen})](\text{PF}_6)_2$) and water ($[\text{Ru}(\text{bipy})_2(\text{dpphen})]\text{Cl}_2$), Figure 2. The peak at 285 nm can be assigned to intra-ligand $\pi \rightarrow \pi^*$ transition.



1
 2 **Figure 2.** UV-vis spectra of complex **1** as hexafluorophosphate and chloride salts in acetonitrile
 3 and water, respectively.

4
 5 To determine the chemical composition of the photochemical products, ¹H NMR was performed
 6 on [Ru(bipy)₂(dpphen)](PF₆)₂ (before and after photoactivation) along with the free ligands
 7 dpphen and bipy for comparison. As shown in Figure 3, the ¹H NMR spectrum in CD₃CN of the
 8 sample after irradiation showed a quantitative conversion of the [Ru(bipy)₂(dpphen)](PF₆)₂ into
 9 photoproducts. The resulting solution contained peaks assigned to the free bipy (labeled with
 10 circles) while dpphen could not be detected among the photolysis products. A broadening of the
 11 proton signals assigned to the hydrogen atoms at the ortho and meta positions of the phenyl groups
 12 of [Ru(bipy)₂(dpphen)](PF₆)₂ was observed before photoactivation and disappeared in the
 13 photoproducts.

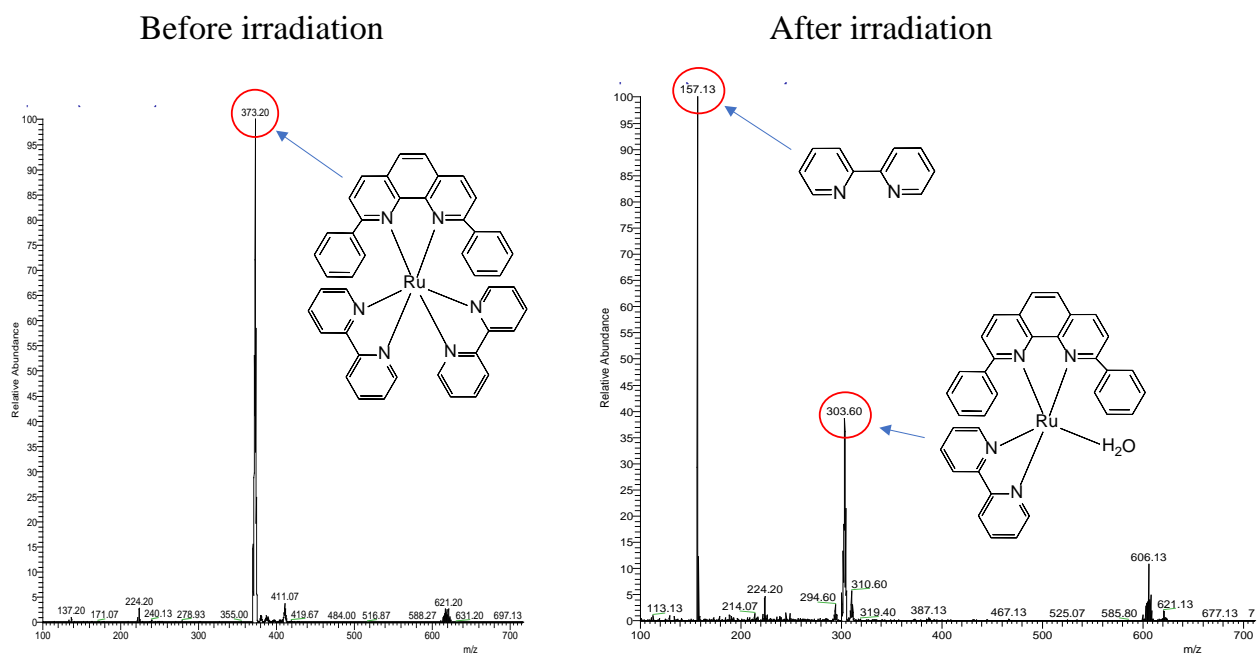
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1
 2 **Figure 3.** ^1H NMR spectra (500 MHz) in CD_3CN of complex **1** $[\text{Ru}(\text{bipy})_2(\text{dpphen})](\text{PF}_6)_2$ before
 3 photolysis (black), after 6 h of photolysis (green) as well as the free ligands 2,9-diphenyl-1,10-
 4 phenanthroline (dpphen) (purple) and 2,2'-bipyridine (bipy) (light blue). The full spectra are found
 5 in Figure S9. Peaks marked with a circle are for the bipy free ligand, the ones marked with stars
 6 are assigned to the ortho and meta hydrogens of the phenyl groups in complex **1**. The remaining
 7 assigned peaks were done based on the expected chemical shift of the bipy (red) and dpphen
 8 (violet) ligands. A or D peaks and C or B peaks could not be distinguished in complex **1**.

9
 10 The photochemistry of $[\text{Ru}(\text{bipy})_2(\text{dpphen})]\text{Cl}_2$ in water was confirmed by LC/MS. While only
 11 the peak assigned to $[\text{Ru}(\text{bipy})_2(\text{dpphen})]^{2+}$ appeared at $m/z = 373.20$ $[\text{M}-2\text{Cl}]^{2+}$ (Figure 4 left)
 12 before irradiation, bipy was identified at $m/z = 157.13$ $[\text{M}+\text{H}]^+$ after irradiation along with the
 13 aquated form of the photoproduct, $[\text{Ru}(\text{bipy})(\text{dpphen})(\text{H}_2\text{O})]^{2+}$ assigned at $m/z = 303.60$ $[\text{M}-$
 14 $2\text{Cl}]^{2+}$, Figure 4. Dpphen, which produced a signal at $m/z = 333.33$ $[\text{M}+\text{H}]^+$ (Figure S10) was
 15 absent in the photoproduct in water. LC/MS spectra of complex **2** before and after irradiation

- 1 presented a peak at $m/z = 297.07$ $[M-2Cl]^{2+}$ (Figure S11), and therefore demonstrate that complex
 2 **2** did not undergo any detectable ligand dissociation upon blue light irradiation.

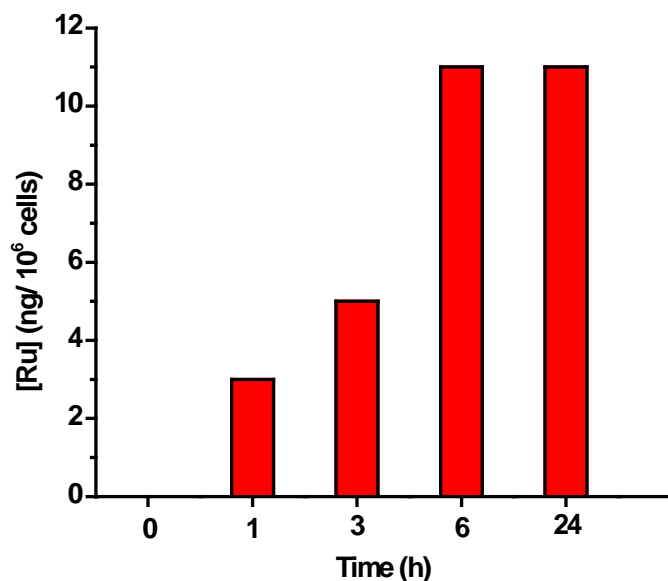


4 **Figure 4.** ESI-MS spectra of $[Ru(bipy)_2(dpphen)]Cl_2$ ($15 \mu M$) in water before (left) and after
 5 (right) photolysis with blue light for 30 min at an output of 100 mW/cm^2 . The peak at $m/z = 606.1$
 6 depicts the singly charged $[Ru(bipy)(dpphen)(OH)]^+$ and at 303.6 for $[Ru(bipy)(dpphen)(H_2O)]^{2+}$.

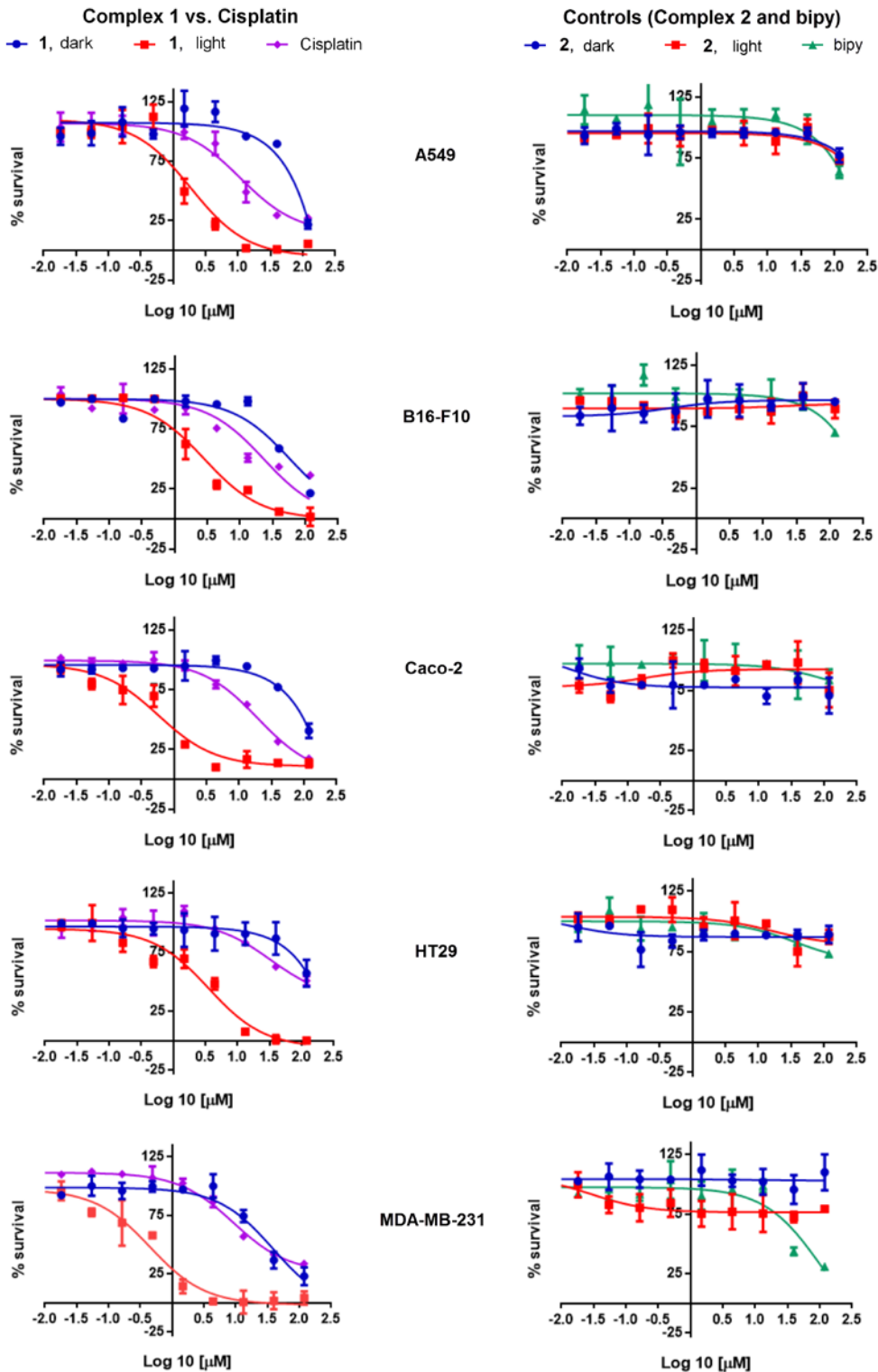
7 The UV-vis spectra of $[Ru(bipy)_2(dpphen)]Cl_2$ ($45 \mu M$) were measured as a function of irradiation
 8 time (460 nm , 100 mW/cm^2), Figure S12. The half-life ($t_{1/2}$) in water was found to be 4.3 min
 9 whereas it was 2.2 min in acetonitrile. As expected, $t_{1/2}$ in water was larger than in acetonitrile
 10 since the latter provides enhanced solvation of the photoproducts.¹⁶ However, it is important to
 11 note that the rate of photoejection is dependent on the concentration and volume of the solution as
 12 well as the incident power density. In the NMR experiment, the concentration was >100 -folds
 13 larger than that used in ESI-MS, hence the time required to complete photolysis was 6 h in Figure
 14 **3** and 30 min in Figure **4** even though photolysis under identical concentration is faster in
 15 acetonitrile than water.

16 The quantification of cellular uptake of complex **1** was performed using ICP-MS. The optimum
 17 uptake time was used to set the incubation period prior to irradiation in order to maximize cellular
 18 concentration of prodrug. While at $t = 0 \text{ h}$ no Ru could be detected ($C < 2 \text{ ng}/10^6 \text{ cells}$), the uptake
 19 started within the first hour ($2.73 \text{ ng}/10^6 \text{ cells}$) then increased until it reached a maximum between

1 6 and 24 h of approximately 11 ng/10⁶ cells, Figure 5. WST-1 kit was used to assess the anticancer
2 activity of complexes **1** and **2** and controls on five cell lines: murine melanoma B16-F10, human
3 alveolar adenocarcinoma A549, triple negative human breast adenocarcinoma MDA-MB-231,
4 human colorectal adenocarcinoma HT29 and human colon adenocarcinoma Caco-2. The cell
5 survival assay of complex **1** was performed to assess its biological activity, before and after
6 photoactivation. Cisplatin was used as a positive control and complex **2** as an unstrained control.
7 While complex **2** showed no significant activity in both dark and light conditions (IC₅₀ > 100 μM)
8 (Figure 6 and Table 1), complex **1** exhibited different cytotoxicity profile. In the dark, its IC₅₀
9 values ranged from 55.1 μM in B16-F10 to higher than 100 μM in the other cells lines. But, upon
10 irradiation, the cytotoxicity of complex **1** increased dramatically with IC₅₀ values ranging from 0.82
11 μM in B16-F10 cells to a maximum of 2.55 μM in A549 cells. Calculation of the phototoxicity
12 index (PI = [IC₅₀ dark] / [IC₅₀ light]) showed PI values ranging from 39.2-fold in A549 to greater
13 than 100-fold in MDA-MB-231 (Table 1). According to Table 1, the IC₅₀ values of cisplatin
14 ranged between 7.70 μM in B16-F10 cells and greater than 10 μM in HT-29 cells. Also, the
15 dissociating ligand bipy was found to be biologically inert on all cell lines (IC₅₀ over 100 μM)
16 except on MDA-MB-231 where an IC₅₀ of 37.5 μM was recorded (Figure 6 and Table 1).



17
18 **Figure 5.** ICP-MS analysis using the EPA 200-7/8 M method for compound **1** uptake by A549
19 cells at different time points. Results are expressed in ng/10⁶ cells.



1

2 **Figure 6.** Cytotoxic effect of complexes **1**, **2**, cisplatin and bipy on A549, B16-F10, Caco-2, HT29
 3 and MDA-MB-231. Cells were treated with 3-fold dilutions of all agents starting at 120 μM .
 4 Complexes **1** and **2** were irradiated with blue LED at a power of 100 mW/cm^2 . The effect of bipy
 5 and cisplatin was only evaluated in the dark. IC_{50} values are reported in Table 1.

1 **Table 1.** IC₅₀ values in dark/light and phototoxicity indices (PI) of complexes **1** and **2** on various
 2 cell lines. Data on the free bipy ligand and cisplatin are reported only in the dark. Numbers are
 3 averages of three separate experiments and each experiment included samples in triplicates.
 4 Numbers are expressed as mean ± SEM.

Cell Line	IC ₅₀			
	Compound 1	Compound 2	bipy free ligand	Cisplatin
A549	Dark: >100 ^a Light: 2.55 ^b ± 1.74 PI: >39.2	Dark: >100 ^a Light: >100 ^a PI: ---	>100 ^a	7.74 ^b ± 1.00
B16	Dark: 55.1 ^a ± 5.84 Light: 0.82 ^b ± 0.83 PI: >68.8	Dark: >100 ^c Light: >100 ^c PI: ---	>100 ^c	7.70 ^d ± 0.19
Caco-2	Dark: >100 ^a Light: 1.48 ^b ± 0.22 PI: >67.6	Dark: >100 ^a Light: >100 ^a PI: ---	>100 ^a	21.8 ^c ± 0.53
HT-29	Dark: >100 ^a Light: 1.98 ^b ± 2.35 PI: >50.5	Dark: >100 ^a Light: >100 ^a PI: ---	>100 ^a	>100 ^a
MDA-MB-231	Dark: >100 ^a Light: 1.00 ^b ± 0.78 PI: >100	Dark: >100 ^a Light: >100 ^a PI: ---	37.5 ^c ± 0.14	30.9 ^c ± 0.82

5 ^{a, b, c, or d} In each row, values that are not sharing the same letter are significantly different (p < 0.05).

6
 7 **Discussion**

8 The use of prodrugs in anticancer therapy has recently become a very attractive approach mainly
 9 because of its potential selectivity to tumors which could reduce systemic toxicity and acquired
 10 resistance induced by conventional chemotherapy.⁶ Ru(II) polypyridyl complexes having a
 11 distorted octahedral geometry have gained interest as prodrugs due to their chemical stability in
 12 the dark and their ability to undergo ligand dissociation upon visible light excitation.⁸ In the present
 13 study, we have exploited these features to synthesize the complex [Ru(bipy)₂(dpphen)]Cl₂
 14 (complex **1**) and explore its cytotoxic potential *in vitro* upon photoactivation. The complex is
 15 sterically strained by virtue of introducing a phenyl group on the 2,9-positions of the
 16 phenanthroline group. It is intuitive to think that the sterically hindering ligand (dpphen) is
 17 expected to dissociate from the complex upon photoactivation, however results showed that the
 18 ligand bipy was ejected when the complex was irradiated in acetonitrile thus generating the
 19 photoproduct [Ru(bipy)(dpphen)(CH₃CN)₂]²⁺. Such behavior could be related to the distorted

1 geometry of the complex, an effect that might be attributed to the twisted bipy moieties or
2 prolonged Ru-N(bipy) distances. Therefore, the ejection of bipy would be easier since the rotation
3 of the pyridine ring about the C²-C^{2'} bond becomes possible after the first Ru-N bond is broken.¹⁹
4 This is not the case with the dpphen whose plane is resistant to distortion, thus rendering the ligand
5 less labile. This unexpected ligand substitution is perhaps due to the asymmetrical distortion of the
6 complex.¹⁹ To confirm these results, we have conducted ¹H NMR and ESI-MS studies on the
7 photochemistry of the complex in deuterated acetonitrile and water respectively. The MLCT
8 absorption of complex **1** was measured by UV/vis spectrophotometry and recorded at around 448
9 nm (blue range). This wavelength was the basis for choosing blue LED (460 nm) to induce
10 photolysis for characterization and biological studies. When performed on the irradiated complex,
11 ¹H NMR suggested that only bipy was photolabilized in CD₃CN which is consistent with
12 Sauvage's results.¹⁹ At room temperature, broadening of the proton signals was observed for
13 hydrogen atoms assigned to the ortho and meta positions of the phenyl groups of
14 [Ru(bipy)₂(dpphen)](PF₆)₂ before photoactivation. This ¹H NMR broadening²² could be attributed
15 to the intramolecular π-π stacking²³ of the phenyl groups at 2,9 positions of the 1,10-
16 phenanthroline and the pyridine ring of the ancillary ligands. In this perspective, the lack of
17 dissociation of the dpphen ligand could be due to two main factors: (1) the rigidity of the 1,10-
18 phenanthroline ligand as compared to 2,2'-bipyridine which possesses a free rotation around C²-
19 C^{2'} (2) possible intramolecular π-π stacking of the phenyl groups which likely increases the
20 activation energy for dissociation of this ligand. LC-MS analysis confirmed that
21 [Ru(bipy)₂(dpphen)]Cl₂ has a similar photochemical behavior in water with a quantitative release
22 of bipy upon irradiation. Similarly to cisplatin, complex **1** is expected to undergo aquation and this
23 was also verified by LC-MS which identified another photolysis product
24 [Ru(bipy)(dpphen)(H₂O)]²⁺. However, the electrospray ionization used by the LC-MS may affect
25 the aquation process as exemplified by the detection of ligand deficient [Ru(bipy)₂]²⁺,
26 [Ru(bipy)₂(H₂O)₂]²⁺, and [Ru(bipy)₂(H₂O)(OH)]⁺ for similar photoproducts in water.^{8,10,17}
27 Therefore, the results obtained above do not necessarily translate the actual number of aqua ligands
28 bound to the Ru center in solution. Moreover, the complex was highly stable in the solid state as
29 well as in water solution under dark conditions, as verified by unchanged ¹H NMR and LC-MS
30 spectra respectively after months of storage. This constitutes a practical convenience since when
31 protected from light, the complex can be stored for a long period with no signs of degradation.

1 Unlike complex **1**, complex **2** did not undergo photolysis and was found to be photostable as
2 confirmed by ^1H NMR and LC-MS data (Figure S11) which were identical before and after
3 irradiation of the complex. Complex **2**, with distorted octahedral geometry, therefore exhibits
4 completely different photochemical properties as compared to complex **1**.

5 The main purpose of photochemotherapy is to mediate phototoxicity in cancer cells while
6 minimizing damage to healthy tissues that are not irradiated.²⁴ To be considered for
7 photochemotherapeutic applications, complex **1** should be primarily active upon photolysis and
8 thus exhibit a large difference in cytotoxicity between dark and light conditions, as measured by
9 the phototoxicity index (PI). Cellular uptake of complex **1** was maximized to about 11 ng/10⁶ cells
10 after 6-24 h of incubation, which compares well with other Ru anticancer drug uptake.²⁵ PI values
11 measured by cell survival assays were consistently high among all cell lines tested (they ranged
12 from >39-fold in A549 to >100-fold in MDA-MB-231). This was the result of high difference in
13 toxicity between dark and light conditions; while complex **1** showed little effect in the dark (with
14 a minimum IC₅₀ value of 55 μM in murine melanoma cells, B16-F10), cytotoxicity increased
15 considerably upon irradiation (IC₅₀ between 0.83 μM in B16-F10 cells to 2.6 μM in A549 cells).
16 As compared to cisplatin, the photoactivated form of complex **1** induced higher toxicity to all cell
17 lines tested and the difference was mostly noticeable in human colorectal adenocarcinoma cells
18 (HT-29) which were found to be resistant to cisplatin (IC₅₀ > 100 μM). This finding is consistent
19 with previous studies that confirmed the refractory response of colon cancer cells to cisplatin due
20 to their ability to induce DNA repair.²⁶ The free bipy ligand was included in cell survival assays
21 to test whether the dissociating ligands contribute to phototoxicity of complex **1**. Results showed
22 that the bipy ligand was not potent (IC₅₀ >100 μM) in all cell lines tested except in MDA-MB-231
23 where IC₅₀ was still high (37.5 μM). These results were comparable to the data obtained by our
24 group on ML-2 Acute Myeloid Leukemia cell line¹⁶ and in line with previous results that showed
25 moderate to no potency of bipy on K562, MDA-MB-231 and MCF-7 cells.^{27,28} This favors the
26 hypothesis that phototoxicity of complex **1** is predominantly mediated by the Ru-aqua
27 photoproduct rather than the liberated ligand. According to Lincoln and Nordén, dark toxicity can
28 be reduced by altering the structure of the Ru complex in a way to avoid DNA binding or
29 intercalation. This can be achieved by replacing phenanthroline ligands by bipyridine.²⁹ Our results
30 suggest that complex **1** and complex **2** had reduced DNA interaction *in vitro* which significantly
31 reduced their dark toxicity. However, upon irradiation, bipy was photoejected from complex **1**

1 leading to product $[\text{Ru}(\text{bipy})(\text{dpphen})(\text{H}_2\text{O})]^{2+}$ as a cytotoxic agent. Complex **2**, on the other side
2 proved to be inactive in both dark and light conditions on all cell lines tested ($\text{IC}_{50} > 100 \mu\text{M}$)
3 which is in accordance with the results found by Glazer and coworkers.⁸ pUC8 plasmid gel
4 electrophoresis experiments³⁰ using Ru(II) polypyridine complexes have previously demonstrated
5 potential DNA single and double-stranded breaks¹¹. However, these findings do not necessary
6 correlate with the mechanism of compound **1** in living cells. A thorough study is currently
7 underway to elucidate the *in vitro* mechanistic details of this compound.

8 **Conclusions**

9 Ru(II) polypyridyl complexes with distorted octahedral geometry are potential candidates for
10 photochemotherapy due to their tunable photochemical properties. $[\text{Ru}(\text{bipy})_2(\text{dpphen})]\text{Cl}_2$
11 (complex **1**) was able to mediate cytotoxicity selectively upon photoejection of the inert bipy
12 ligand and release of a highly toxic Ru(II) aqua complex. $[\text{Ru}(\text{bipy})_2(\text{phen})]\text{Cl}_2$ (complex **2**)
13 control was shown to be photostable and biologically inert in the dark and upon photoexcitation.
14 Complex **1** exhibited superior cytotoxicity and selectivity upon photoexcitation when compared
15 with cisplatin, and may be considered for potential application in PACT. Further *in vitro* and *in*
16 *vivo* investigations are underway to elucidate the biological mechanism of action.

17 **Experimental Section**

18 **Instrumentation**

19 Elemental analysis was provided by Atlantic Microlab and HR-ESI MS data were acquired at
20 Michigan State University Mass Spectrometry Core Facility. MALDI-TOF MS spectra were
21 collected on a 4800 MALDI TOF/TOF MS/MS Analyzer instrument (Applied Biosystems)
22 operated by a 4000 Series Explorer software. Briefly, aqueous solutions of Ru(II) complexes (120
23 μM) were premixed with CHCA matrix (saturated α -cyano-4-hydroxy-cinnamic acid in a mixture
24 containing 1:1 of 50% acetonitrile and 0.1% TFA. The resulting samples were then spotted on a
25 stainless-steel plate (Opti-TOF TM 384 Well Insert), dried by vacuum and inserted for data
26 acquisition. 13 calibration points were included on each plate (4700 Proteomics Analyzer
27 Calibration mixture). UV/vis absorption was measured by a Cary 60 spectrophotometer from
28 Agilent Technologies). NMR spectra were acquired on an AC500 Bruker spectrometer (^1H NMR
29 at 500 MHz). Chemical shifts were recorded in delta (δ) units and expressed as ppm values

1 relatively to the internal standard TMS. LC-MS/MS data were recorded by Ultimate 3000
2 RSLC/TSQ Endura system (ThermoScientific). Briefly, aqueous solutions of complexes **1** and **2**
3 (20 μM) were prepared and infused by direct injection into the heated ESI probe [(2 μL injection
4 at a flow rate of 100 $\mu\text{L}/\text{min}$ of LC-MS grade water containing 0.05% formic acid, sheath gas (2.71
5 L/min), auxiliary gas (5.78 L/min), sweep gas (1.5 L/min), ion transfer tube temperature (325 $^{\circ}\text{C}$),
6 vaporizer temperature (200 $^{\circ}\text{C}$), positive ion spray voltage (3700 V)]. ICP-MS was conducted at
7 the American University of Beirut (environment core lab) on Agilent 7500 ICP-MS at 0.3 seconds
8 (integration time/mass) with 3 repetitions and an uptake speed of 1 rotation per second. Blue LED
9 was obtained from LED Engin (LZ4-40B208-000) and operated at 50% of its full power (130
10 lumen). The lamp was regulated by an LED dimmer (home-built) using a Philips Xitanium LED
11 driver LEDINTA0700C210FO and light was condensed using a focusing lens with narrow beam
12 output (LLNS-1T06-H). Star LED heat sink was used to cool the system.

13 **Materials**

14 Cis-bis(2,2'-bipyridine)dichlororuthenium(II) hydrate ($\text{Ru}(\text{bipy})_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$), 1,10-
15 phenanthroline, silica gel, sephadex LH 20, Dowex 22 chloride, and all other chemicals and
16 solvents were purchased from Aldrich and used without any further purification. LC-MS grade
17 water and formic acid were from Fisher Chemical. DMEM medium, FBS and penicillin G sodium
18 salt were purchased from Aldrich and WST-1 reagent (tetrazolium salt) was obtained from
19 Roche $^{\circ}$. All cell lines used were from the American Type Culture Collection (ATCC).

20 **Synthesis of $[\text{Ru}(\text{bipy})_2(\text{dpphen})](\text{PF}_6)_2$ (Complex 1)**

21 2,9-diphenyl-1,10-phenanthroline was synthesized according to a published procedure.³¹
22 Synthesis of the complex was performed according to a modified procedure.¹⁹ $\text{Ru}(\text{bipy})_2\text{Cl}_2 \cdot 2\text{H}_2\text{O}$
23 (100 mg, 0.2 mmol) and 2,9-dpphen (69 mg, 0.19 mmol) were mixed with ethylene glycol (8 mL)
24 in a pressure vessel. The solution was degassed for 1 h under argon pressure then refluxed for six
25 h. After cooling at room temperature, filtration was carried out using micropores (0.45 μm). The
26 product was precipitated by adding a saturated solution of KPF_6 .² The precipitate was filtered then
27 purified by column chromatography (silica gel, 92% acetonitrile, 7% H_2O , 1% KNO_3). Yield:
28 153.5 mg, 72%. ^1H NMR (CD_3CN , 500 MHz): δ = 8.66 (d, J = 8 Hz, 2H), 8.34 (s, 1H), 8.24 (d, J
29 = 8 Hz, 2H), 8.06 (td, J = 7.73, 1.47 Hz, 2H), 7.89 (d, J = 6.22 Hz, 2H), 7.84 (d, J = 7.98 Hz, 2H),

1 7.54 (d, $J = 8.29$ Hz, 2H), 7.45 (td, $J = 7.71$ Hz, 1.46 Hz, 2H), 7.35 (dq, $J = 6.02$ Hz, 1.3 Hz, 2H),
2 7.14 (br. s, 2H), 7.02 (t, $J = 7.56$ Hz, 2 H), 6.87 (br. s, 2H), 6.74 (br. s, 2H), 6.66 (d, $J = 4.93$ Hz,
3 2 H), 6.56 (td, $J = 6.09$, 1.3 Hz, 2H), 6.13 (br. s, 2 H). ^{13}C NMR (CD_3CN , 500 MHz): $\delta = 168.91$,
4 158.99, 157.05, 154.02, 151.96, 149.55, 139.96, 138.65, 138.16, 137.06, 131.48, 129.96, 129.63,
5 129.02, 127.60, 127.57, 125.33, 124.14. Elemental Anal. Calcd for $\text{C}_{44}\text{H}_{32}\text{F}_{12}\text{N}_6\text{P}_2\text{Ru}\cdot\text{H}_2\text{O}$: C,
6 50.15; H, 3.25; N, 7.98. Found: C, 50.32; H, 3.17; N, 7.98. HRMS (ESI/QTOF) m/z $[\text{M}]^{2+}$ Calcd
7 for $\text{C}_{44}\text{H}_{30}\text{N}_6\text{Ru}$ 373.0872; found 373.0903. MS (MALDI/TOF) m/z , $[\text{M}]^+$ 746.15. UV/Vis
8 (ACN): $\lambda_{\text{max}}(\epsilon \text{ M}^{-1}\text{cm}^{-1})$ 448 nm (14,000). For biological testing the hexafluorophosphate salt was
9 converted to chloride salt using Dowex chloride ion exchange resins to promote the solubility of
10 the complex in water.

11 **Synthesis of $[\text{Ru}(\text{bipy})_2(\text{phen})]\text{Cl}_2$ (Complex 2)**

12 Synthesis was performed according to a modified procedure.³² $\text{Ru}(\text{bipy})_2\text{Cl}_2\cdot 2\text{H}_2\text{O}$ (100 mg, 0.20
13 mmol) and 1,10-phenanthroline (39.64 mg, 0.22 mmol) were added to a solution of 1:1
14 EthOH:H₂O (8 mL) in a round bottom flask. The mixture was stirred under argon for 1 h then
15 refluxed for three h. After cooling at room temperature, solvents were evaporated, and the residue
16 was dissolved in methanol (2 mL) and added dropwise to rapidly stirred diethyl ether (300 mL)
17 producing an orange precipitate that was collected by vacuum filtration and washed thoroughly
18 with ether. The compound was then purified on a sephadex LH-20 column with methanol as eluent
19 and finally dried under reduced pressure. Yield: 121.4 mg; 91 %. ^1H NMR (CD_3CN , 500 MHz):
20 $\delta = 8.63$ (dd, $J = 8.27$ Hz, 1.26 Hz, 2H), 8.60 (d, $J = 7.99$ Hz, 2H), 8.55 (d, $J = 8.11$ Hz, 2H), 8.26
21 (s, 2H), 8.10 (m, 4H), 7.99 (td, $J = 7.63$ Hz, 1.49 Hz, 2H), 7.85 (d, $J = 6.4$ Hz, 2H), 7.74 (dd, $J =$
22 8.26 Hz, 5.25 Hz, 2H), 7.53 (d, $J = 5.64$ Hz, 2H), 7.45 (dq, $J = 6.31$ Hz, 1.33 Hz, 2H), 7.22 (dq, J
23 = 6.31 Hz, 1.33 Hz, 2H). ^{13}C NMR (CD_3CN , 500 MHz): $\delta = 158.26$, 157.99, 153.45, 152.96, 152.81,
24 148.49, 138.79, 138.66, 137.80, 132.00, 129.05, 128.47, 128.35, 127.03, 125.30, 125.23. Elemental Anal.
25 Calcd for $\text{C}_{32}\text{H}_{24}\text{Cl}_2\text{N}_6\text{Ru}\cdot 3\text{H}_2\text{O}$: C, 53.49; H, 4.21; N, 11.7. Found: C, 53.25; H, 4.53; N, 11.42. HRMS
26 (ESI/QTOF) m/z $[\text{M}]^{2+}$ Calcd for $\text{C}_{32}\text{H}_{22}\text{N}_6\text{Ru}$ 297.0557; found 297.0568. MS (MALDI/TOF) m/z , $[\text{M}]^+$
27 594.07. UV/Vis (H₂O): $\lambda_{\text{max}}(\epsilon \text{ M}^{-1}\text{cm}^{-1})$ 448 nm (16,000).²⁰

28

29 **^1H NMR and LC-MS/MS of complexes 1 and 2 to evaluate their photochemistry**

1 The photochemistry of complex **1** (PF₆ salt) was determined by ¹H NMR in deuterated acetonitrile
2 (CD₃CN), after which the complex was converted to a chloride salt and subjected an LCMS
3 analysis in order to determine whether the complex exhibits the same photochemical
4 transformation in water. Complex **2** was evaluated by LC-MS and all measurements were
5 performed before and after irradiation. For ¹H NMR studies, samples were irradiated for 6 h as
6 opposed to 35 min for LC-MS (at an output of 100 mW/cm²) and the ligands dpphen and bipy
7 were included as controls to allow their detection in photolysis products.

8

9 **Determination of the rate of ligand dissociation of complex 1 by UV-vis spectroscopy**

10 The chloride form of complex **1**, [Ru(bipy)₂(dpphen)]Cl₂ was irradiated with blue LED light (460
11 nm, 100 mW/cm²) in water and acetonitrile for different durations. UV-vis absorption spectra were
12 then acquired to determine the half-life of the complex under identical conditions.

13

14 **Quantification of cellular uptake by ICP-MS**

15 Quantification of cellular uptake was carried out by ICP-MS following a modified procedure.³³
16 Briefly, 1.0 x 10⁶ cells (A549) were plated in 60 mm petri dishes and allowed to adhere for 12 h
17 at 37° with 5% CO₂. Complexes **1** (30 μM) were then added and the plates were incubated for 1,
18 3, 6, 12 and 24 h. Cells were then harvested by scraping, precipitated by centrifugation at 800 RCF
19 for 20 minutes at 4° and washed 3 times with PBS. The pellets were resuspended in milli-Q water,
20 transferred to a glass vial containing 65% HNO₃ and completely digested at 120 °. The product
21 obtained was then dissolved in 1 mL milli-Q water containing 2% HNO₃. Quantification of Ru
22 was performed using Agilent 7500 inductively coupled plasma mass spectrometry (ICP-MS): EPA
23 200-7/8 M method and data were analyzed using ICP-MS Mass Hunter B.01.01 software.

24 **Cell survival assay**

25 Murine melanoma cell line B16-F10, human alveolar adenocarcinoma cell line A549, triple
26 negative human breast adenocarcinoma cell line MDA-MB-231, human colorectal
27 adenocarcinoma cell line HT29 and human colon adenocarcinoma Caco-2 cell line were
28 maintained in DMEM containing 10% FBS and 1.5% pen-strep at 37° with 5% CO₂. Cells were
29 seeded in a 96 well-plate at a concentration of 10⁴ cells/well and allowed to adhere for 6 h at 37°
30 with 5% CO₂. Treatment with serial dilutions of complexes **1** and **2**, cisplatin and the ligands

1 dpphen and bipy was performed, followed by a 12 h incubation period. Cells were then irradiated
2 with blue LED purchased from LED Engin possessing 460 nm peak wavelength and operated at
3 50% of its 130 lumen full power which was controlled by a home-built LED dimmer (Light output
4 $\sim 100 \text{ mW/cm}^2$). The light was focused on the plate using a focusing lens with narrow beam output.
5 Irradiation was typically done for 35 minutes for “light conditions” or else the plates were left in
6 the dark for all other experiments. Cell proliferation was assessed after 72 h using WST-1 reagent
7 (Roche©) according to the manufacturer’s instructions and data were analyzed using GraphPad
8 Prism 6 software (GraphPad Software). Cell viability was expressed as percent survival relatively
9 to the negative control (non-treated cells). IC_{50} was calculated from 3 separate experiments using
10 a non-linear regression (curve fit) on GraphPad Prism 6 software (GraphPad Software) and
11 expressed as mean \pm SEM, calculated using Microsoft Excel (version 1705, 2016). Two-way
12 analysis of variance (ANOVA) was used to determine statistical significance (GraphPad) and
13 differences between all groups were considered significant if $p < 0.05$ (Computed by Tukey's
14 multiple comparisons test).

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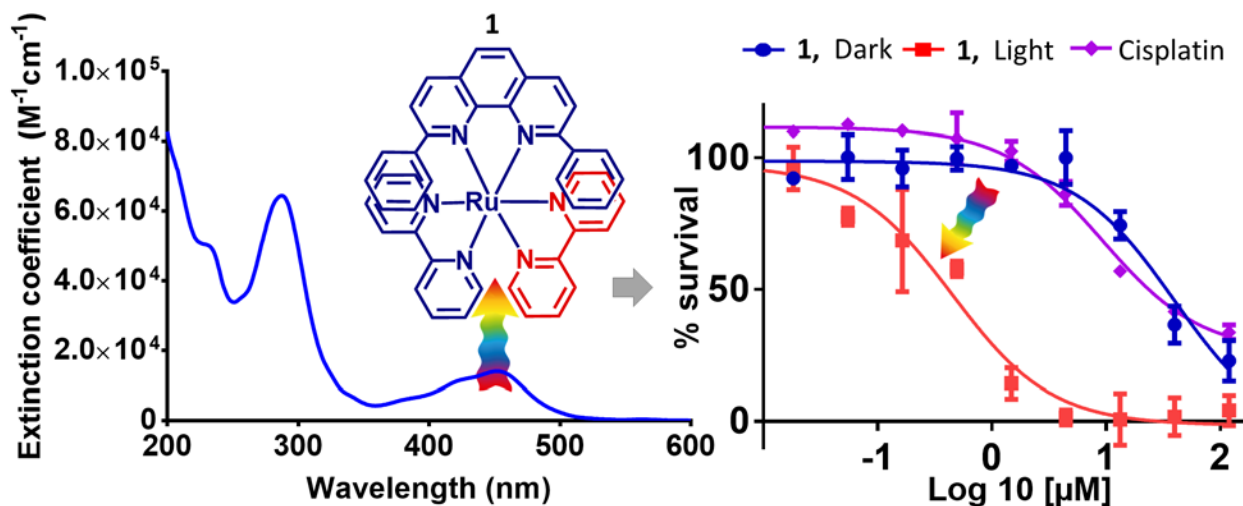
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1 **Table of Contents Graphic**



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3 **Table of Contents Text**

4 [Ru(bipy)₂(dpphen)]Cl₂ [where bipy= 2,2'-bipyridine and dpphen = 2,9-diphenyl-1,10-
5 phenanthroline] (complex 1) exhibited up to 100-fold increase in cytotoxicity upon irradiation
6 when tested on five cancer cell lines including cisplatin-resistant ones. The ejected bipy ligand
7 displayed minimal potency and therefore phototoxicity was attributed to the formation of the Ru-
8 aqua complex.

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