Effects of the Bidentate Ligand on the Photophysical Properties, Cellular Uptake, and (Photo)cytotoxicity of Glycoconjugates Based on the [Ru(tpy)(NN)(L)]^{2+} Scaffold

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Abstract: Ruthenium polypyridyl complexes have received widespread attention as potential chemotherapeutics in photodynamic therapy (PDT) and in photochemistry (PACT). Here, we investigate a series of sixteen ruthenium polypyridyl complexes with general formula [Ru(tpy)(N–N(L))^{1/2±}][(tpy = 2,2’-6,2”-terpyridine, N–N = bpy (2,2-bipyridine), phen (1,10-phenanthroline), dpq (pyrazino[2,3-f][1,10]phenanthroline), dpzp (dipyrido[3,2-a:2’,3’-c]phenazine), dppn (benzo[8]dipyrido[3,2-a:2’,3’-c]phenazine), pmip (2-(4-methylphenyl)-1H-imidazo[4,5-f][1,10]phenanthroline), pym (1-(E)-N-phenyl-1-(pyridin-2-yl) methanimine), or azpy (2-(pyrrolazo)pyridine), L = Cl or 2-(2-(2-(methylthio)ethoxy)ethyl)-β-d-glucopyranoside) and their chloride analogues [Ru(tpy)(N–N’)(Cl)]^{2+} with N–N’ = bpy (2,2’-bipyridine), phen (1,10-phenanthroline), dpq (pyrazino[2,3-f][1,10]phenanthroline), dpzp (dipyrido[3,2-a:2’,3’-c]phenazine), dppn (benzo[8]dipyrido[3,2-a:2’,3’-c]phenazine), pmip (2-(4-methylphenyl)-1H-imidazo[4,5-f][1,10]phenanthroline), pym (1-(E)-N-phenyl-1-(pyridin-2-yl)methanimine), or azpy (2-(phenylazo)pyridine), and of their water-soluble derivatives [Ru(tpy)(N–N’)(L)]^{2+} upon visible light irradiation. Many other examples of ruthenium complexes used as photosensitive agents releasing anticancer molecules have been developed by the group of Turro,[12] Gasser,[13] Glazer,[14] Kodanko,[15] and Bonnet.[16] Following up on our initial work using thioether monodentate ligands to cage cytotoxic aqua ruthenium complexes,[17] we report here a series of related chloride complexes [1a][Cl]^{2+}–[8a][Cl]^{2+} having the general formula [Ru(tpy)(N–N(Cl))][Cl] with N–N = bpy (2,2’-bipyridine), phen (1,10-phenanthroline), dpq (pyrazino[2,3-f][1,10]phenanthroline), dpzp (dipyrido[3,2-a:2’,3’-c]phenazine), dppn (benzo[8]dipyrido[3,2-a:2’,3’-c]phenazine), pmip (2-(4-methylphenyl)-1H-imidazo[4,5-f][1,10]phenanthroline), pym (1-(E)-N-phenyl-1-(pyridin-2-yl)methanimine), or azpy (2-(phenylazo)pyridine), and of their water-soluble derivatives [Ru(tpy)(N–N(Cl))][PF_6]^{2+} (1b)[PF_6]^{2+}–(8b)[PF_6]^{2+}, in which R = 2-(2-(2-(methylthio)ethoxy)ethoxy)ethyl-β-d-glucopyranoside is a thioether-glucose conjugate (Figure 1).

On the one hand, [Ru(tpy)(bpy)(Cl)][Cl] is known to be poorly cytotoxic to cancer cells.[18] On the other hand, we recently demonstrated that [Ru(tpy)(dpzp)(R)][PF_6]^{2+} (1b)[PF_6]^{2+}–(5b)[PF_6]^{2+}, (Figure 1) has unique phototoxic properties based on a dual mode-of-action involving both photosubstitution of the thioether ligand and singlet oxygen generation. In this paper, we compare the photophysical properties of all conjugates [1b][PF_6]^{2+}–(8b)[PF_6]^{2+} and of their chloride analogues [1a][Cl]^{2+}–[8a][Cl]^{2+}. The proof-of-concept for ruthenium-based PACT[19] was first demonstrated that although increased lipophilicity is generally related to increased uptake of these complexes, it does not necessarily lead to increased (photo) cytotoxicity. However, the non-toxic complexes are excellent candidates as PACT carriers.

Introduction

Ruthenium based anti-cancer compounds have been investigated for several decades[20] as potential alternatives to the clinically approved cisplatin. Cisplatin is associated with serious side effects such as renal toxicity, neurotoxicity, and hearing loss.[21] The most thoroughly investigated ruthenium-based anti-cancer agents, NAMI-A and KP1019, both reached phase II clinical trials before being abandoned.[22] More recently, the tunable photophysical properties of ruthenium(II) polypyridyl complexes have been used to develop compounds combating bacterial resistance to antibiotics,[23] or new photosensitizers for photodynamic therapy as an alternative to, for example, Photofrin.[24] Recently, the group of McFarland have made a great step forward in this field, by entering phase I clinical trials with a Ru^{2+}-thiophene-polypyridyl-based photosensitizer, TLD1433.[25] Simultaneously, a great interest has been shown in the development of sterically strained ruthenium(II) complexes for the light-induced delivery of cytotoxic cargo.[26] This last approach is often referred to as photo-activated chemotherapy (PACT).[27] The proof-of-concept for ruthenium-based PACT was first demonstrated that Etchenique’s group, who demonstrated the photorelease of the potassium channel blocker 4-aminoypyridine (4AP) from [Ru(bpy)_2(4AP)]^{2+} upon visible light irradiation.[28] Many other examples of ruthenium complexes used as photosensitive agents releasing anticancer molecules have been developed by the group of Turro,[12] Gasser,[13] Glazer,[14] Kodanko,[15] and Bonnet.[16] Following up on our initial work using thioether monodentate ligands to cage cytotoxic aqua ruthenium complexes,[17] we report here a series of related chloride complexes [1a][Cl]^{2+}–[8a][Cl]^{2+} having the general formula [Ru(tpy)(N–N(Cl))][Cl] with N–N = bpy (2,2’-bipyridine), phen (1,10-phenanthroline), dpq (pyrazino[2,3-f][1,10]phenanthroline), dpzp (dipyrido[3,2-a:2’,3’-c]phenazine), dppn (benzo[8]dipyrido[3,2-a:2’,3’-c]phenazine), pmip (2-(4-methylphenyl)-1H-imidazo[4,5-f][1,10]phenanthroline), pym (1-(E)-N-phenyl-1-(pyridin-2-yl)methanimine), or azpy (2-(phenylazo)pyridine), and of their water-soluble derivatives [Ru(tpy)(N–N(Cl))][PF_6]^{2+} (1b)[PF_6]^{2+}–(8b)[PF_6]^{2+}, in which R = 2-(2-(2-(methylthio)ethoxy)ethoxy)ethyl-β-d-glucopyranoside is a thioether-glucose conjugate (Figure 1).
Chemical structure of the complexes used in this study. General formula \([\text{Ru(tpy)}(\text{N})\text{Cl}^\text{n+}]\), \(\text{N}\) = bpy, phen, dpq, dppz, dppn, pmip, pymi or azpy. \(L = \text{Cl}^–\) or \(L = \text{R} = (2-(2-(\text{methylthio})\text{ethoxy})\text{ethoxy})\text{ethyl}-\text{d-glucopyranoside}).

Figure 1. Chemical structure of the complexes used in this study. General formula \([\text{Ru(tpy)}(\text{N})\text{Cl}^\text{n+}]\), \(\text{N}\) = bpy, phen, dpq, dppz, dppn, pmip, pymi or azpy. \(L = \text{Cl}^–\) or \(L = \text{R} = (2-(2-(\text{methylthio})\text{ethoxy})\text{ethoxy})\text{ethyl}-\text{d-glucopyranoside}).

[8a]Cl in water, and correlate them to the uptake and cytotoxicity in cancer cells. Critically, the glucose-containing ligand L ensures that all thioether-ruthenium complexes are soluble in water, allowing their photochemistry to be studied independently from the lipophilicity of the N–N spectator bidentate ligand.

Results

Synthesis

Chloride complexes \([1\text{a}]\text{Cl},[16] [2\text{a}]\text{Cl},[17] [4\text{a}]\text{Cl},[18] [5\text{a}]\text{Cl},[14b] [7\text{a}]\text{Cl},[19] [8\text{a}]\text{Cl}[20]\) and the ligand \(2-(2-(\text{methylthio})\text{ethoxy})\text{ethoxy})\text{ethyl}-\text{d-glucopyranoside} (\text{R})[46]\) were synthesized as reported previously. Complexes \([3\text{a}]\text{Cl}\) and \([6\text{a}]\text{Cl}\) were synthesized by reacting \([\text{Ru(tpy)Cl}_2]\) with the bidentate ligand dpq or pmip in the presence of triethylamine as a reducing agent. The ruthenium complexes \([1\text{a}]\text{Cl}–8\text{a}]\text{Cl}\) were then reacted with an excess of the thioether ligand \(\text{R}\) in the dark in water. Silica column purification of the crude complexes, followed by size exclusion chromatography, afforded the thioether-glucose ruthenium conjugates \([1\text{b}]\text{PF}_6, [2\text{b}]\text{PF}_6, [4\text{b}]\text{PF}_6\) as orange to red solids and \([8\text{b}]\text{PF}_6\) as a purple solid. To ease purification of the pmip complex \([6\text{b}]\text{PF}_6\), the synthesis was carried out similarly to the previously reported synthesis of \([5\text{b}]\text{PF}_6,[14b]\) by first converting the chloride precursor \([5\text{a}]\text{Cl}\) to the aqua species \([\text{Ru(tpy)pmip(H}_2\text{O})]_2\text{PF}_6\) using \(\text{AgNO}_3\) and \(\text{NH}_4\text{PF}_6\) followed by reaction of the thioether ligand with the aqua complex. Similarly, the syntheses of \([3\text{b}]\text{PF}_6\) and \([7\text{b}]\text{PF}_6\) were carried out in the presence of \(\text{AgPF}_6\) to ensure in situ conversion of the chlorido precursor into the aqua species before coordination of the thioether ligand. All chloride complexes except \([4\text{a}]\text{Cl}, [5\text{a}]\text{Cl}\) and \([6\text{a}]\text{Cl}\) and all thioether complexes are soluble in water. As reported for the complex \([\text{Ru(tpy)(bpy)(Hmte)}]\text{PF}_6,[21]\) all thioether complexes showed an upfield shift of the methylsulfide group to about 1.5 ppm in the \(\text{H}^1\text{NMR spectra, confirming coordination of the thioether donor atom to the ruthenium center. All new compounds were characterized using NMR spectroscopy, thin layer chromatography, electronic absorption spectroscopy, high-resolution mass spectrometry, and elemental analysis.}

Crystal structures

Attempts to crystallize the glycoconjugates \([1\text{b}]\text{PF}_6–[8\text{b}]\text{PF}_6\) were unsuccessful and usually led to the formation of oils or colloidal suspensions. However, single crystals suitable for X-ray diffraction analyses were obtained for \([5\text{a}]\text{Cl}\) and for \([3\text{a}]\text{PF}_6\) and \([4\text{a}]\text{PF}_6\) after salt metathesis of \([3\text{a}]\text{Cl}\) and \([4\text{a}]\text{Cl}\) using aqueous \(\text{NH}_3\text{PF}_6\), followed by vapor diffusion of diethyl ether in a solution of \([3\text{a}]\text{PF}_6\) in acetone or acetone in a solution of \([4\text{a}]\text{PF}_6\) in ethyl acetate (Figure 2). The three crystal structures showed the expected distorted octahedral geometry, with a reduced (>180°) N-Ru-N angle for the coordinated terpyridine ligand (N1-Ru1-N3, 159.11–159.40°, Table 1). The bidentate ligands dpq, dppz and dppn are all bound perpendicular to tpy, with a N4-Ru1-N5 bite angle of 79.26–80.2° (Table 1). The Ru1–Cl bond lengths were found to be similar with values ranging from 2.4015 to 2.4165 Å which are very close to reported values for related complexes.[22] Selected bond lengths and angles are given in Table 1.

Photophysical properties of the \([\text{Ru(tpy)(NN)(L)}]\text{PF}_6^{2+}\) complexes

The photophysical properties of chloride complexes \([1\text{a}]\text{Cl}–[8\text{a}]\text{Cl}\) were first investigated in acetonitrile, in which the complex...
Lowest-energy absorption maxima [A] and bond angles [°] for complexes [3a][PF6], [4a][PF6], and [5a][Cl].

| Complex   |  \( \lambda_{\text{max}} \) [nm] |  \( \epsilon_{\text{max}} \) [M\(^{-1}\) cm\(^{-1}\)] |  \( \phi_{\text{MLCT}} \) |  \( \varphi \) |  \( \phi \) |
|-----------|----------------------------------|----------------------------------|----------------------------|----------------|----------------|
| [3a][Cl]  | 504 (9.1 \times 10^4)            | 4.6 \times 10^4                  | –                          | –              | 0.055          | < 1 \times 10^-5 |
| [2a][Cl]  | 501 (9.1 \times 10^4)            | 6.5 \times 10^4                  | –                          | –              | 0.048          | 8.5 \times 10^-4 |
| [3a][Cl]  | 504 (9.1 \times 10^4)            | 6.6 \times 10^4                  | –                          | –              | 0.055          | < 1 \times 10^-5 |
| [4a][Cl]  | 511 (9.6 \times 10^4)            | 5.6 \times 10^4                  | –                          | –              | 0.005          | < 1 \times 10^-5 |
| [5a][Cl]  | 498 (12.0 \times 10^4)           | 8.5 \times 10^4                  | –                          | –              | 0.023          | 4.3 \times 10^-4 |
| [6a][Cl]  | 501 (11.2 \times 10^4)           | 6.8 \times 10^4                  | –                          | –              | 0.082          | 3.2 \times 10^-3 |
| [7a][Cl]  | 523 (13.0 \times 10^4)           | 3.4 \times 10^4                  | –                          | –              | 0.012          | 1.4 \times 10^-3 |
| [8a][Cl]  | 508 (12.2 \times 10^4)           | 3.9 \times 10^4                  | –                          | < 0.001        | 1.8 \times 10^-3 |
| [1b][PF6] | 450 (7.0 \times 10^4)            | 7.0 \times 10^4                  | 0.0084                     | 59             | 0.020 (0.020)  | > 1 \times 10^-5 |
| [2b][PF6] | 448 (6.2 \times 10^4)            | 6.2 \times 10^4                  | 0.0065                     | 40             | 0.050 (0.080)  | 1.2 \times 10^-4 |
| [3b][PF6] | 448 (8.9 \times 10^4)            | 8.9 \times 10^4                  | 0.0067                     | 60             | 0.030 (0.010)  | > 1 \times 10^-5 |
| [4b][PF6] | 458 (13.1 \times 10^4)           | 12.8 \times 10^4                 | 0.020                      | 256            | 0.0010 (0.0030)| < 1 \times 10^-5 |
| [5b][PF6] | 458 (11.6 \times 10^4)           | 11.4 \times 10^4                 | 0.0095                     | 11             | 0.71(0.41)     | < 1 \times 10^-5 |
| [6b][PF6] | 460 (11.0 \times 10^4)           | 10.4 \times 10^4                 | 0.0070                     | 73             | 0.0020         | < 1 \times 10^-5 |
| [7b][PF6] | 472 (11.7 \times 10^4)           | 11.7 \times 10^4                 | 0.0053                     | 62             | 0.11 (0.14)    | 2.5 \times 10^-3 |
| [8b][PF6] | 505 (7.2 \times 10^4)            | 2.7 \times 10^4                  | –                          | –              | 0.0070 (--)    | < 1 \times 10^-5 |

[a] In MeCN for [1a][Cl] and in MilliQ H\(_2\)O for [1b][PF6] and [8b][PF6]. 
[b] In H\(_2\)O. 
[c] In CD\(_2\)OD.

The hydrophilicity of the thioether analogues [1b][PF6] and [8b][PF6] allowed for studying photosubstitution quantum yields in MilliQ water using electronic absorption spectroscopy. Monochromatic blue light (450 or 470 nm) was used to irradiate the complexes in their MLCT absorption band. Although all thioether complexes are thermally stable at room temperature, seven of the eight complexes, that is, [1b][PF6] and [7b][PF6], showed light-induced exchange of their thioether ligand for H\(_2\)O.

The ligand photosubstitution was characterized by clear isosbestic points in the UV/Vis spectra (450 to 476 nm depending on the compound), as shown in Figure 3. For each of these reactions a bathochromic shift of the MLCT band was observed,
which is consistent with earlier reports on the formation of monoaqua-ruthenium complexes in aqueous solution.\textsuperscript{[15a]} Most complexes have a photosubstitution quantum yield (\(\Phi_{\text{abs}}\)) of 0.5–2 percent, leading to photosubstitution reactivities (\(\xi = \Phi_{\text{abs}} < \Phi_{\text{exc}} > \)) in which \(\epsilon_{\text{abs}}\) is the molar absorption at 450 nm on the order of ten to hundreds (\(\xi = 11–256\)). Changing the bidentate ligand has thus a significant influence on the photosubstitution rates. Interestingly, the dppz complex 4b\(^{2+}\) has the highest photosubstitution quantum yield of the series, which is also about 20-fold higher (\(\Phi_{\text{abs}} = 0.020\)) than that of the structurally similar dpp analogue 5b\(^{2+}\); which showed the lowest \(\Phi_{\text{abs}}\) (0.00095).\textsuperscript{[14b]} Furthermore, 4b\(^{2+}\) produces minimal amounts of \(\pi\pi^*\) excited states located on the spectator bidentate ligand with the dppz complex such \(\pi\pi^*\) states are either too high in energy to be populated, or outcompeted by a rather quick conversion to the photodissociative metal-centered triplet state (3MC).

Another interesting observation concerned the difference in reactivity between 7b\(^{2+}\) and 8b\(^{2+}\). Whereas 7b\(^{2+}\) displayed ligand dissociation efficiency comparable to that of the bpy complex 1b\(^{2+}\), the azpy compound 8b\(^{2+}\) did not show any ligand photodissociation, indicating a strong electronic effect of the azo ligand on the photoreactivity of its ruthenium complex. The \(\pi\pi^*\) MLCT absorption maximum for 8b\(^{2+}\) is significantly lower in energy (505 nm) than that of 7b\(^{2+}\) (472 nm), which points to the low energy of the azo-based \(\pi^*\) orbital of the azpy ligand, leading to a low-lying \(\pi\pi^*\) excited triplet state (3MC).

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**Figure 3.** Electronic absorption spectra of 1b\([PF_6]_2\), 4b\([PF_6]_2\), 6b\([PF_6]_2\), and 7b\([PF_6]_2\) in deoxygenated \(H_2O\) upon irradiation at 450 or 470 nm for 5 min at \(T = 298\) K. Spectra measured every 30 s. a) 1b\([PF_6]_2\), \(\lambda_{\text{exc}} = 450\) nm, photon flux \(= 1.71 \times 10^{-7}\) mol s\(^{-1}\), \(\lambda_{\text{abs}} = 450\) nm, photon flux \(= 6.83 \times 10^{-7}\) mol s\(^{-1}\); b) 2b\([PF_6]_2\), \(\lambda_{\text{exc}} = 450\) nm, photon flux \(= 5.29 \times 10^{-7}\) mol s\(^{-1}\), \(\lambda_{\text{abs}} = 450\) nm, photon flux \(= 5.92 \times 10^{-7}\) mol s\(^{-1}\); c) 3b\([PF_6]_2\), \(\lambda_{\text{exc}} = 450\) nm, photon flux \(= 5.18 \times 10^{-7}\) mol s\(^{-1}\), \(\lambda_{\text{abs}} = 450\) nm, photon flux \(= 4.97 \times 10^{-7}\) mol s\(^{-1}\); d) 4b\([PF_6]_2\), \(\lambda_{\text{exc}} = 450\) nm, photon flux \(= 5.29 \times 10^{-7}\) mol s\(^{-1}\), \(\lambda_{\text{abs}} = 450\) nm, photon flux \(= 5.92 \times 10^{-7}\) mol s\(^{-1}\); e) 5b\([PF_6]_2\), \(\lambda_{\text{exc}} = 450\) nm, photon flux \(= 5.29 \times 10^{-7}\) mol s\(^{-1}\), \(\lambda_{\text{abs}} = 450\) nm, photon flux \(= 5.92 \times 10^{-7}\) mol s\(^{-1}\); f) 6b\([PF_6]_2\), \(\lambda_{\text{exc}} = 450\) nm, photon flux \(= 5.29 \times 10^{-7}\) mol s\(^{-1}\), \(\lambda_{\text{abs}} = 450\) nm, photon flux \(= 5.92 \times 10^{-7}\) mol s\(^{-1}\). Inset depicts the evolution of ln \([Ru]_{\text{tot}}\) vs. irradiation time in s, in which [Ru]\(_{\text{tot}}\) represents the concentration of ruthenium-thioether complex at time t, and [Ru]\(_{\text{tot}}\) the total ruthenium concentration.
Cytotoxicity

The cytotoxic properties of the chloride complexes [1a][Cl],[8a][Cl] and their caged analogues [1b][PF6],[8b][PF6] were evaluated against two different human cell lines: A549 (human lung carcinoma) and MCF-7 (human breast adenocarcinoma). Considering the photo-substitution properties of some of these complexes, their photocytotoxicity was also tested under blue light irradiation (3.2 ± 0.2 J cm⁻² at 454 ± 11 nm), as described previously for [5b][PF6].[14b] Cells were seeded at t = 0, treated after 24 h with a concentration gradient of each ruthenium complex, irradiated or maintained in the dark after replacing the media, and further incubated in the dark for 48 h. At t = 96 h cell viability was determined using the sulforhodamine B (SRB) assay.[24] The effective concentrations (EC₅₀), defined as the concentration at which a 50% survival rate on cell viability is observed, are reported in Table 3. Most chloride complexes were found to be non-cytotoxic, with the exception of [8a][Cl] that was found moderately cytotoxic (EC₅₀ = 28 μM) against the MCF-7 cell line, in agreement with the value reported by Reedijk and co-workers.[25] The values for [4a][Cl] (59 μM and 34 μM against A549 and MCF-7, respectively) were found similar to that observed for [Ru(bpy)(dppz)]⁺⁺ analogues reported by the group of Schatzschneider.[26] Based on their results, it was expected that the structurally similar but more lipophilic dppn complex [5a][Cl] would be cytotoxic, but no significant toxicity was observed for this complex. On the other hand, its EC₅₀ could not be clearly determined due to the poor solubility of this complex in cell culture medium.[14b] Interestingly however, [5a][Cl] was to be found cytotoxic upon blue light irradiation, with EC₅₀ values of 9.7 and 3.2 μM for A549 and MCF-7 cells, respectively, corresponding to photoin dexes (PI) of more than 2.6 and 7.9, respectively. This result is unexpected, because the O₂ quantum yield of [5a][Cl] (0.023) is much lower than that of its glycoconjugated analogue [5b][PF6] (0.71). A possible explanation would be the partial conversion, after uptake, of the chloride complex to its aquated counterpart [Ru(tpy)(dppn)(H₂O)]⁺⁺ (Figure 4a), which has been demonstrated to be a good O₂ sensitizer (its close analogue [Ru(tpy)(dppn)(CD₃OD)]⁺⁺ has a O₂ production quantum yield under air of φO₂ = 0.43).[14b]

Table 3. Cytotoxicity of compounds [1a][Cl],[8a][Cl] and [1b][PF6],[8b][PF6] towards A549 and MCF-7 cells in the dark and upon blue light irradiation (454 nm, 3.2 J cm⁻²). Cell-growing inhibition effective concentrations (EC₅₀) are reported in μM with 95% confidence interval (CI) in μM. Data is the mean over three independent experiments. Photocytotoxicity index (PI) = EC₅₀m/EC₅₀d (dimensionless).

| Complex | Light dose [J cm⁻²] | A549 EC₅₀ [μM] CI | MCF-7 EC₅₀ [μM] CI | PI |
|---------|---------------------|---------------------|---------------------|-----|
| [1a][Cl] | 0                   | >100                | >100                | –   |
|          | 3.2                 | >100                | 64 +12 1.2          | –   |
|          | 3.2                 | >100                | 52 +15 – 10         | –   |
| [2a][Cl] | 0                   | >100                | >100                | –   |
| [3a][Cl] | 0                   | >100                | >100                | –   |
| [4a][Cl] | 0                   | 59 +31 1.3          | 34 +6.0 1.1         | – 21 |
|          | 3.2                 | 9.7 +4.4            | 3.2 +1.3 – 0.67     | –   |
| [5a][Cl] | 0                   | >25                 | >25                 | >7.9|
|          | 3.2                 | >25                 | >25                 | –   |
| [6a][Cl] | 0                   | >25                 | >25                 | –   |
| [7a][Cl] | 0                   | >100                | >100                | –   |
| [8a][Cl] | 0                   | >100                | >100                | –   |
| [1b][PF6] | 0                   | >100                | >100                | –   |
| [2b][PF6] | 0                   | >100                | >100                | –   |
| [3b][PF6] | 0                   | >100                | >100                | –   |
| [4b][PF6] | 0                   | >100                | >100                | –   |
| [5b][PF6] | 0                   | 64 +17 2.4          | 52 +12 2.6          | – 13 |
|          | 3.2                 | 27 +6.4             | 20 +2.5             | –   |
| [5b][PF6] | 0                   | 19 +4.0 26 9.6     | +2.9 11             | – 3.3 |
|          | 3.2                 | 0.72 +0.16          | 0.86 +0.21          | – 0.13 |
| [6b][PF6] | 0                   | >100                | >100                | –   |
| [7b][PF6] | 0                   | >100                | >100                | –   |
| [8b][PF6] | 0                   | >100                | >100                | –   |

[a] Standard protocol: Cells were incubated with compound for 24 h, followed by replacement of the media, kept in the dark, or irradiated with blue light (5 min at 454 nm, 10.5 mW cm⁻², 3.2 J cm⁻²) and further incubated in the dark for 48 h. [b] As in standard protocol, but without replacing media during treatment (cells are irradiated in the presence of compound). [c] Ref. [14b].
None of the glycoconjugated complexes were found to be photocytotoxic except \([5b](PF_6)_2\), which was recently reported to enter passively into the cells and to destroy mitochondrial DNA by singlet oxygen generation.\(^{[14b]}\) In our standard treatment protocol, media is replaced before light irradiation. In such conditions, photocytotoxicity can solely rely on the molecules that have been taken up by the cells during incubation, which may be a problem for highly hydrophilic glucose-conjugates such as \([1b](PF_6)_2\)?\(^{[8b]}\)(PF_6)_2\)(PF_6)_2\), see below).

For compound \([4b](PF_6)_2\), an adjustment of the protocol, consisting in irradiating the cells without media refreshing, led to a modest but clearly improved PI (2.4 and 2.6 for MCF-7 and A549, respectively). With such a protocol the full dose of compound added to each well remains present during and after irradiation, and most importantly activation may occur outside the cell, and be followed by cellular uptake of the activated photoproduct. For \([4b](PF_6)_2\), the observed phototoxicity might thus be explained by the formation of the aquated species \([Ru(tpy)(dppz)(H_2O)]^2+\) outside the cell, followed by in situ conversion to the chloride species \([4a]Cl\) due to the high chloride content in media (>100 mM), followed by cellular uptake (Figure 4b). This interpretation is supported by the EC50 values found for \([4a]Cl\), which were not impressive but could clearly be measured (59 and 34 \(\mu\)M for A549 and MCF-7 respectively).

Not refreshing the media before light activation did not lead to enhanced toxicity for \([1b](PF_6)_2\)?\(^{[3b]}\)(PF_6)_2\)(PF_6)_2\) and for \([6b](PF_6)_2\)?\(^{[7b]}\)(PF_6)_2\)(PF_6)_2\), showing that keeping high concentrations of the prodrug during and after light irradiation does not necessarily lead to enhanced phototoxicity. Overall, these results demonstrate that \([4b](PF_6)_2\) is a moderately effective PACT agent,\(^{[3b]}\) whereas the dppn analogues \([5a]Cl\) and \([5b](PF_6)_2\) are catalytic PDT sensitizers, which can be activated using a low dose of blue light. They also demonstrate that apparently minor differences in the treatment protocol of light-activated drugs may lead to very different interpretation of the cytotoxicity of light-activated compounds.

**Log \(P_{ow}\) and uptake**

To acquire more insight on the effect of glycoconjugation on the solubility, cellular uptake, and toxicity of these complexes, the water-octanol partition coefficients (log \(P_{ow}\)) were determined for all complexes according to reported standards (Figure 5b).\(^{[28]}\) As shown in Figure 5b (left), the chloride compounds with the smallest bidentate ligands, that is, \([1a]Cl\)?\(^{[3a]}\)(PF_6)_2\)(PF_6)_2\) and \([7a]Cl\) and \([8a]Cl\), have log \(P_{ow}\) values ranging from −0.81 to −1.1, while \([7a]Cl\) and \([8a]Cl\) have log \(P_{ow}\) values of −1.60 to −1.80. For these five complexes, the chloride counter anion provides appreciable water solubility. By contrast, the chloride compounds with the largest bidentate ligands, that is, \([4a]Cl\)?\(^{[6a]}\)(PF_6)_2\)(PF_6)_2\), are much more hydrophobic with log \(P_{ow}\) values ranging from −0.10 to +1.0. Although one may expect that the dicationsic nature of \([1b](PF_6)_2\)?\(^{[8b]}\)(PF_6)_2\)(PF_6)_2\) and glycoconjugation should necessarily improve water solubility compared to their chloride analogues, we found that \([1b](PF_6)_2\)?\(^{[3b]}\)(PF_6)_2\)(PF_6)_2\) had similar log \(P_{ow}\) values (−0.11 to −0.51, respectively) compared...
to their analogues [1a]Cl–{3a}Cl, whereas [7b](PF₆)₃ and [8b](PF₆)₃ were slightly more hydrophilic (log $P_{ow} = -0.20$ and $-0.18$, respectively) than [7a]Cl and [8a]Cl. This result points to the critical influence of the counterions, as the two hexafluoridophosphate anions of the glycoconjugate compounds increase lipophilicity, compared to chlorides. Furthermore, the chloride complexes are not stable in water, resulting in (partial) conversion to the [Ru(tpy)(N–N)(H₂O)]Cl₂ species which are more soluble in water than the hexafluoridophosphate salts of the R-substituted ruthenium complexes. The most hydrophobic chloride complexes [4a]Cl–{6a}Cl, that were much more difficult to dissolve in water, profited most from the glycoconjugation because [4b](PF₆)₃–{6b}(PF₆)₃ indeed became water soluble (log $P_{ow} = -0.84$ to $-0.50$, respectively). Overall glycoconjugation allowed for investigating the photochemistry of all thioether complexes [1b](PF₆)₃–{8b}(PF₆)₃ in water.

To check whether the low toxicity of the thioether-glucose conjugates was not simply due to a low uptake, cellular uptake was studied for all sixteen complexes in A549 cells at a concentration of 25 μM, using an incubation time of 24 h and measuring intracellular ruthenium concentrations by ICP-MS. Although no general correlation could be found between the log $P_{ow}$ values for these complexes and their cellular uptake, very strong differences in metal uptake were observed depending on the ligands and counterions (Figure 5 a). The most hydrophobic chloride compounds [4a]Cl, [5a]Cl and [6a]Cl displayed very high metal uptake (> 1000 ng Ru per million cells), whereas their glycoconjugates [4b](PF₆)₃, [5b](PF₆)₃ and [6b](PF₆)₃ displayed cellular uptake that was much lower (10–20 ng Ru per million cells, for example, 250 times lower for [5b](PF₆)₃ compared to [5a]Cl). Of course, this lower uptake can partially be explained by the lower log $P_{ow}$ values of the glycoconjugates, and at least for [5b](PF₆)₃, by the absence of GLUT-based active uptake. However, [4b](PF₆)₃–{6b}(PF₆)₃ are also taken up in 10-fold higher amounts than [1b](PF₆)₃–{3b}(PF₆)₃, which have comparable log $P_{ow}$ values. These results may not necessarily represent the conditions experienced by these compounds at the cell membrane, for which it is more likely that the lipophilic PF₆⁻ counterions are already exchanged for the more abundant and more water soluble chloride or phosphate anions in the buffer, canceling the effect of the PF₆⁻ anion on lipophilicity.

**Discussion**

Some of the chloride complexes [1a]Cl–{8a}Cl were thermally unstable and therefore no photodissociation quantum yields were determined, whereas their singlet oxygen properties were in general very low. The phototoxicity in the series of the most lipophilic compounds [4a]Cl–{6a}Cl cannot be explained by the trends observed in cell uptake and singlet oxygen generation. [6a]Cl has indeed a higher singlet oxygen quantum yield (0.082) than [4a]Cl and [5a]Cl (0.005 and 0.023, respectively), but it is not phototoxic, whereas [4a]Cl and [5a]Cl are, and all three complexes are taken up in high amounts. In this series of complexes, different intracellular localization or biological targets, coupled to unknown photo reactions of [5a]Cl,
must explain the differences in photocytotoxicity between [6a]Cl on the one hand and [4a]Cl and [5a]Cl on the other.

An opposite conclusion can be drawn for the glycoconjugates series [4b][PF$_6$]$_2$, [5b][PF$_6$]$_2$ and [6b][PF$_6$]$_2$. The only phototoxic agent of this series, [5b][PF$_6$]$_2$, has by far the highest singlet oxygen quantum yield (0.71 vs. 0.0010 and 0.0020), whereas all three compounds are taken up in similar amounts (10–20 ng Ru per million cell). Hence, [5b][PF$_6$]$_2$ is at least an excellent PDT agent, whereas a PACT mode of action cannot be ruled out considering the photocytotoxic properties of [5a]Cl and its low singlet oxygen quantum yield. The phototoxicity observed for [4b][PF$_6$]$_2$ when the protocol is slightly modified, suggests that this compound may act as a cytotoxic PACT agent. Furthermore [4b][PF$_6$]$_2$ showed the highest photosubstitution quantum yield (0.02) and no significant singlet oxygen production. When cell-culture media was replaced before light irradiation, the glycoconjugate compound was not taken up in high amounts, and given the poor photodynamic properties of the photoproduct ([4a] + [Ru(tpy)(dpdz)(OH)$_2$])$^{3-}$) not enough reactive oxygen species could be generated to kill the cells. This example demonstrates that the potential of [4b][PF$_6$]$_2$ as a PACT agent is determined by the treatment protocol, which should be taken into account in further PACT studies. Furthermore, this complex has been shown to act as a DNA light-switch in the presence of DNA, which might be useful for theranostic applications.[29]

**Conclusion**

Overall eight chloride terpyridine complexes [1a]Cl–[8a]Cl with eight different bidendate spectator chelating ligands, and their eight thioether-glucose conjugates, were synthesized to compare the corresponding photophysical properties, photoreactivity, water solubility, cellular uptake, and photocytotoxicity.

Depending on the bidentate ligand, these complexes can be considered either for photocaging, or for PACT and/or PDT. Compound [8a]Cl is not suitable for photocaging or phototherapy because the azo group of the azpy spectator ligand stabilizes the MLCT states too much and prevents thermal population of the MC state, thereby quenching photosubstitution. Singlet oxygen generation was also fully quenched in [8a]Cl and [8b][PF$_6$]$_2$, emphasizing the poor photosensitizing properties of this compound. The five complexes [1a]Cl–[3a]Cl, [6a]Cl, and [7a]Cl are non-toxic, and once substituted by thioethers, they form complexes with similar photosubstitution quantum yields ($\Phi_{650} \approx 0.01$) and low $^{1}O_2$ production quantum yields ($\Phi_{\lambda} < 0.10$). As a consequence, they are excellent candidates for the photocaging of thioether-based biologically active compounds, such as the antibiotics amoxicillin and clindamycin. The exceptionally high cellular uptake measured for [6a]Cl is worth noticing (5220 ± 737 ng Ru per million cells), considering that this compound did not show any measurable cytotoxicity at concentrations lower than 25 µM. It can even turn highly hydrophilic compounds such as R into species such as [6b][PF$_6$]$_2$ that are still lipophilic enough to enter into cancer cells. Finally, [4a]Cl and [5a]Cl show similar lipophilicity compared to [6a]Cl and comparatively high cellular uptake, but they also showed some toxicity both in the dark and after light activation. They are therefore less interesting as PACT carriers and instead have better potential as a either a cytotoxic PACT agent or for PDT, as we have recently demonstrated for [5b][PF$_6$]$_2$.[14b] Overall, this work demonstrates that complexes based upon the [Ru(tpy)(NN)(R)]$^{3-}$ scaffold are good photocaging agents but poorly (photo)cytotoxic unless DNA intercalators such as dppz and dppn are chosen as a bidentate ligand, in which case they could serve as photocytotoxic agents.

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**Conflict of interest**

The authors declare no conflict of interest.

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