To cage or to be caged? The cytotoxic species in ruthenium-based photoactivated chemotherapy is not always the metal†

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In metal-based photoactivated chemotherapy (PACT), two photo-products are generated by light-triggered photosubstitution of a metal-bound ligand: the free ligand itself and an aquated metal complex. By analogy with cisplatin, the aquated metal complex is usually presented as the biologically active species, as it can typically bind to DNA. In this work, we show that this qualitative assumption is not necessarily valid by comparing the biological activity, log P, and cellular uptake of three ruthenium-based PACT complexes: [Ru(bpy)2(dmbpy)]2+, [Ru(bpy)2(mtmp)]2+, and [Ru(Ph2phen)2(mtmp)]2+. For the first complex, the photoreleased dmbpy ligand is responsible for the observed phototoxicity, whereas the second complex is not phototoxic, and for the third complex it is the ruthenium bis-aqua photoproduct that is the sole cytotoxic species.

Ruthenium polypyridyl complexes are well known for their versatile and tunable photophysical and photochemical properties.1 In recent years, they have attracted much interest for molecular imaging and photopharmacology,2 and in particular for photodynamic Therapy (PDT) and photoactivated chemotherapy (PACT).3

In PACT like in PDT, a non-toxic or poorly cytotoxic prodrug becomes much more cytotoxic upon light irradiation, allowing for a time- and spatially-resolved delivery of the toxicity of the anticancer drug. However, whereas in PDT the photocytotoxicity relies on the photochemical generation of reactive oxygen species (ROS) such as singlet oxygen (1O2), in PACT a photochemical bond-breaking reaction occurs, which for coordination compounds is often realized via the photosubstitution of one of the ligands by water molecules.4 To make PACT ruthenium-based compounds, [Ru(bpy)3]2+-like complexes must be modified so that the triplet metal-centered excited states (3MC) come in close proximity to the triplet metal-to-ligand charge transfer states (3MLCT).5 Such modification typically entails the use of sterically hindered bidentate ligands such as 6,6′-dimethyl-2,2′-bipyridine (dmbpy) and its derivatives.6 For example, the irradiation of [Ru(bpy)2(dmbpy)]2+ in water (bpy = 2,2′-bipyridine) leads to the photosubstitution of dmbpy by two water molecules, generating the aquated species cis-[Ru(bpy)2(OH2)2]2+ (Scheme 1) that was shown to bind to plasmid DNA.7 When performed in the presence of growing cancer cells, this photoreaction clearly leads to photocytotoxicity, which many have interpreted as a consequence of the cytotoxicity of cis-[Ru(bpy)2(OH2)2]2+. By analogy to the cytotoxic aquated form of cisplatin, cis-[Pt(NH3)2(OH2)2]2+. On the other hand, many ruthenium polypyridyl complexes have been used as photocaging groups for neurotransmitters and organic enzyme inhibitors,8 for which the absence of acute toxicity is a pre-requisite. The parent compound [Ru(bpy)2Cl2], which thermally hydrolyzes into cis-[Ru(bpy)2(OH2)2]2+, was shown by the group of Reedijk not to be cytotoxic.9 As several groups have developed analogues of [Ru(bpy)2(dmbpy)]2+ for developing new PACT compounds, we asked ourselves which photoproduct, from the two that are formed upon light irradiation, actually is cytotoxic enough to kill cancer cells: the cis bis-aqua ruthenium complex or the free ligand?

To address this question, we compared the known compound [Ru(bpy)2(dmbpy)]Cl2 ([1]Cl2) with a new photoactive

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**Scheme 1** Chemical structures of PACT ruthenium compounds [1]Cl2–[3]Cl2 and their reaction upon blue light irradiation in water.
compound \([\text{Ru(bpy)}_2(\text{mtmp})\text{Cl}_2]\) containing the bidentate chelate 2-methylthiophenylpyridine (mtmp).\(^7\) Thioethers are soft enough to coordinate well to ruthenium(II) in the ground state, but they can be photosubstituted more efficiently than pyridines due to the relative weakness of the Ru–S bond in the excited state, compared to Ru–N bonds.\(^10\) Indeed when a solution of dmbpy with \([\text{mtmp} + \text{H}^+]\) (calc. \(m/z = 140.2\), [\(\text{Ru(bpy)}_2(\text{OH}_2)\text{Cl}_2\)]\(^2+\) (calc. \(m/z = 225.0\), and [\(\text{Ru(bpy)}_2(\text{OH}_2)(\text{OH})\text{Cl}_2\)] (calc. \(m/z = 448.5\)), respectively. Thus, \([\text{2}]^{2+}\) like \([\text{1}]^{2+}\) leads upon light irradiation to the formation of the bis-aqua complex \(\text{cis-}[\text{Ru(bpy)}_2(\text{OH}_2)]^{2+}\), but the free ligand obtained as the second photoproduct is mtmp, instead of dmbpy with \([\text{1}]\text{Cl}_2\) (Scheme 1). The second photosubstitution is much slower, as usually reported,\(^11\) and leads to the final photoproducts mtmp and \(\text{cis-}[\text{Ru(bpy)}_2(\text{OH}_2)]^{3+}\). The quantum yield of this second process (\(\Phi_{\text{PR}}\)) was 0.0030 according to Glotaran global fitting (see the ESI†).

The cytotoxicities of the free ligands dmbpy and mtmp were first compared in an A549 lung cancer cell line (adenocarcinomic human alveolar basal epithelial cells). Both organic ligands are rather lipophilic, as demonstrated by the octanol/water partition coefficient values (log \(P\)) of +3.29 and +1.63 for dmbpy and mtmp, respectively (Table 2). Both ligands are therefore expected to be taken up at least passively by the cells. The cell growth inhibition effective concentrations (EC\(_{50}\)), i.e. the compound concentration at which the cell viability was reduced by 50% compared to the non-treated control, were measured following a protocol adapted from Hopkins et al. (see the ESI†).\(^12\) Clearly, dmbpy was found to be cytotoxic, with EC\(_{50}\) values of 8.7 and 6.5 \(\mu\)M in the dark and upon light irradiation, respectively (Fig. 2 and Table 1), whereas no cytotoxicity was observed for mtmp up to 200 \(\mu\)M. Although cellular localization of chemicals may differ whether they are simply incubated with the cells or generated inside the cells upon light irradiation of a prodrug such as \([\text{1}]\text{Cl}_2\), this result suggests that the photocytotoxicity reported for \([\text{1}]\text{Cl}_2\) may be at least partly due to the release of the dmbpy ligand.

In a second step, the EC\(_{50}\) values of complexes \([\text{1}]\text{Cl}_2\) and \([\text{2}]\text{Cl}_2\) were measured in A549 cells, both in the dark and upon blue light irradiation, and following the same protocol applied for the free ligand (Table 1). The selected light dose (6.5 \(\text{J cm}^{-2}\)) guarantees that no toxic effect for the cells occurs due to the irradiation itself.\(^12\) At that light dose, both \([\text{1}]\text{Cl}_2\) and \([\text{2}]\text{Cl}_2\) are fully activated below 40 \(\mu\)M (see Fig. S4 and ESI†). As shown in Fig. 3, no significant decrease in the cell population was observed after treatment with less than 100 \(\mu\)M of complex \([\text{1}]\text{Cl}_2\) or \([\text{2}]\text{Cl}_2\) in the dark (Table 1). Thus, these species can be considered to be essentially non-cytotoxic in the dark. After blue light irradiation, an EC\(_{50}\) value of 10.9 \(\mu\)M was found for \([\text{1}]\text{Cl}_2\), corresponding to a photoindex of 19, which qualitatively fits the data reported by Glazer et al. on this compound.\(^7\) However, no photocytotoxicity was observed for \([\text{2}]\text{Cl}_2\), in spite of the fact that this compound also delivers the \(\text{cis-}[\text{Ru(bpy)}_2(\text{OH}_2)_2]^{2+}\) species upon irradiation.

In order to explain these differences, the octanol/water partition coefficient (log \(P\) value; see the ESI† \(^{11}\)), the cellular uptake, and the quantum yield for singlet oxygen generation were measured for both complexes (Table 2). log \(P\) values of \(-1.42\) and \(-1.33\) were found for \([\text{1}]\text{Cl}_2\) and \([\text{2}]\text{Cl}_2\), respectively, which means that both complexes have similar hydrophilicities and are not prone to enter the cell by passive diffusion through the membrane. As expected from these high hydrophilicity values, the cellular uptake before light activation, measured by ICP-MS by incubating A549 cells with \([\text{1}]\text{Cl}_2\) or \([\text{2}]\text{Cl}_2\) at 20 and 80 \(\mu\)M, respectively, for 6 h in the dark, was found to be very low: 1.32 and 1.27 ng of ruthenium were found per million cells for \([\text{1}]\text{Cl}_2\) and \([\text{2}]\text{Cl}_2\), respectively, compared to values usually found above 10–20 ng Ru per \(10^8\) cells for compounds that are well taken up.\(^14\) Thus, the higher cytotoxicity found for \([\text{1}]\text{Cl}_2\) after light activation cannot be attributed to a higher uptake of the complex prior to irradiation.

Many published phototherapeutic ruthenium complexes are excellent PDT agents, i.e., they generate \({}^{1}\text{O}_2\) via energy transfer from the \(3\text{MLCT}\) to molecular oxygen present in the cells.\(^15\)
Although it is commonly admitted that photosubstitutionally labile ruthenium complexes are poor singlet oxygen generators, the experimental values of $^1O_2$ generation quantum yields ($\Phi_A$) are very rare in the literature for PACT compounds. In order to rule out that $[\text{Ru(bpy)}_3]\text{Cl}_2$ does not influence an already negligible cytotoxicity. 

To demonstrate this idea, we synthesized a much more lipophilic version of compound $[\text{Ru(Ph}_2\text{phen)}_2\text{Cl}_2]$ (see the ESI†). 

The photoreactivity of $[\text{Ru(Ph}_2\text{phen)}_2\text{Cl}_2]$ in water under blue light irradiation ($455$ nm) is similar to that of $[\text{Ru(bpy)}_2\text{Cl}_2]$ or $[\text{Ru(Ph}_2\text{phen)}_2\text{Cl}_2]$ after irradiation ($10.9 \mu M$), respectively, using $[\text{Ru(bpy)}_2\text{Cl}_2]$ as a reference ($\Phi_A = 0.73$).† Thus, both complexes are mediocre photosensitizers for $^1O_2$, the phototoxicity of $[\text{Ru(bpy)}_2\text{Cl}_2]$ cannot be a photodynamic effect.

To summarize, $[\text{I]}\text{Cl}_2$ and $[\text{II]}\text{Cl}_2$ have similar negative log $P$ values, similarly low cellular uptake after 6 h incubation in the dark, similarly low $^1O_2$ generation quantum yields, and they both form $[\text{Ru(bpy)}_2\text{Cl}_2]$ upon light irradiation. Their main difference is that they photochemically release either dmbpy or mtmp, respectively. Meanwhile, we also demonstrated three points. First, the light activation of $[\text{I]}\text{Cl}_2$ resulted in a 19-fold lower EC$_{50}$ value compared to that obtained in the dark, whereas the light irradiation of $[\text{II]}\text{Cl}_2$ does not influence an already negligible cytotoxicity. Second, dmbpy is cytotoxic to A549 cells, whereas mtmp is not. Third, the EC$_{50}$ value of $[\text{I]}\text{Cl}_2$ after irradiation ($10.9 \mu M$) is close, in the same protocol, to the EC$_{50}$ value found for dmbpy ($6.6 \mu M$). Altogether, these results strongly suggest that the phototoxicity observed with complex $[\text{I]}\text{Cl}_2$ is caused by the dmbpy ligand that is photorelease and taken up after extra-cellular activation, rather than by the cis-$[\text{Ru(bpy)}_2\text{Cl}_2]$ species. In other words, $[\text{Ru(bpy)}_2\text{Cl}_2]$ is a photocaging group for the cytotoxic dmbpy ligand, rather than the reverse!

These surprising results do not, in our eyes, discredit the concept of ruthenium-based PACT. The problem of compounds such as $[\text{I]}\text{Cl}_2$ or $[\text{II]}\text{Cl}_2$ is only that their ruthenium-based photoproductions, cis-$[\text{Ru(bpy)}_2\text{Cl}_2]$, is not lipophilic enough to cross membranes and cause significant damage inside the cells. To demonstrate this idea, we synthesized a much more lipophilic version of compound $[\text{II]}\text{Cl}_2$, i.e., $[\text{Ru(Ph}_2\text{phen)}_2\text{Cl}_2]$ or $[\text{Ru(Ph}_2\text{phen)}_2\text{Cl}_2]$, in ethylene glycol at $115$ °C (see the ESI†). 

$[\text{II]}\text{Cl}_2$ has a much higher log $P$ value of 0.28, as expected from the more lipophilic Ph$_2$phen spectrograph ligands. The photoreactivity of $[\text{III]}\text{Cl}_2$ in water under blue light irradiation ($445$ nm) is similar to that of $[\text{II]}\text{Cl}_2$: a shift of the $^1\text{MLCT}$ absorption maximum from 404 to 492 nm and two sequential isosbestic points at 447 and 472 nm were observed (Fig. 1b). Mass spectrometry after 70 min of irradiation (Fig. S1b, ESI†) also showed photosubstitution of the non-toxic mtmp ligand, with peaks at 140.2, 412.3, and 424.5, corresponding to mtmp + H$^+$, $[\text{Ru(Ph}_2\text{phen)}_2\text{MeCN)}(\text{OH}_2)]^{2+}$ (calc. m/z = 421.6), and $[\text{Ru(Ph}_2\text{phen)}_2\text{MeCN)}]^{3+}$ (calc. m/z = 424.1), respectively. The last two species are formed in the mass spectrometer and demonstrate the photochemical formation of the bis-aqua...
photoadduct [Ru(Ph2phen)2(OH2)]2+. The photosubstitution has a quantum yield of 0.0010, slightly lower than that found for [2]Cl2, and the 3I generation quantum yield was similar to that found for [1]Cl2 (i.e., ΦA = 0.020; see Table 2). Thus, [3]Cl2 is a bad PDT sensitizer but a good PACT compound, as like [2]Cl2 it photosubstitutes the non-toxic mtmip ligand to deliver [Ru(Ph2phen)2(OH2)]2+, a lipophilic analogue of [Ru(bpy)2(OH2)]2+.

In A549 cells, [3]Cl2 had a higher cytotoxicity in the dark (EC50 = 2.66 M), as expected from its higher lipophilicity. Critically, the EC50 value decreased 6-fold down to 0.48 M, because of other released dmppy ligand, that of compound [3]Cl2 demonstrates that PACT compounds where the Ru-based species is caged via coordination of the mtmip ligand. Overall, our results demonstrate that determining which photoproduct is the cytotoxic species is not straightforward, as factors such as ligand toxicity, lipophilicity of the produg, cellular uptake and localization, and/or singlet oxygen generation may all influence the photosensitivity of a given compound. Although we have demonstrated here that the photosensitivity of [1]Cl2 is not due to the ruthenium-based photoadduct but because of the released dmppy ligand, that of compound [3]Cl2 demonstrates that PACT compounds where the Ru photoadduct bears the toxic load can be made, provided its lipophilicity is high enough for the compound to enter the cell.

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