The synthesis and biological applications of photo-activated ruthenium anticancer drugs

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Chapter 7:

Summary, conclusions and outlook

7.1 Summary

7.1.1 General introduction

Conventional chemotherapy suffers from poor selectivity leading to adverse side-effects in patients using these drugs. A possible solution to overcome these selectivity issues is by local activation of a drug with light, providing spatio-temporal control over drug activity. In the field of bioinorganic chemistry, ruthenium(II) polypyridyl prodrugs Ru-L have been investigated as potential light-induced drug delivery devices where photo-activation leads to bond-cleavage and the release of an aquated metal species Ru-OH_2 and a ligand L (Figure 7.1 left) or as PDT sensitizer (Figure 7.1 right). In theory, the aquated metal complex, for example $[Ru(tpy)(bpy)(H_2O)]^{2+}$, is thermally reactive towards species bearing donor atoms such as amines, thioethers or aromatic imines, present in amino acids, RNA, and DNA. Under physiological conditions, the reactivity of these substrates towards $[Ru(tpy)(bpy)H_2O]^{2+}$ may lead to similar adducts as observed for cisplatin, potentially leading to cell toxicity. However, not all ruthenium compounds are toxic and the nature of the spectator ligands remaining bound to the metal after photosubstitution, play a critical role on toxicity of the metal-based photoproduct. Simultaneously, an organic ligand L is released as well, which can be a drug with a defined target and known biological mode of action.

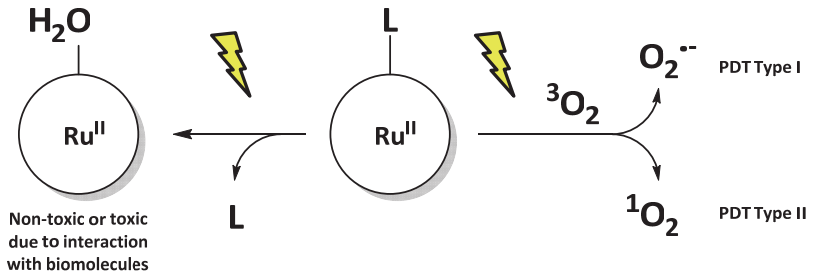


Figure 7.1. Simplified diagram with principle of photoactivated chemotherapy (PACT) in inorganic systems based on ruthenium(II). Two dominant mechanisms are illustrated: Left: Upon light irradiation, a ligand or drug (L) is released resulting in an aquated ruthenium species which is either non-toxic (PACT carrier) or toxic (PACT drug). Right: The ruthenium(II) polypyridyl species act as a PDT type I or II photosensitizer.

The research described in this thesis aimed at the development of new photoactivated chemotherapy (PACT) drugs against cancer based on the $[Ru(tpy)(NN)(L)]^{2+}$ scaffold, which has well-defined photosubstitution properties.[1]

7.1.2 Ruthenium polypyridyl D-glucose glycoconjugates

In Chapter 2 we described a synthetic approach towards every positional isomer of D-glucose bearing a methylthioether functional group. These compounds were used as ligands and conjugated to a non-toxic photosubstitutionally active ruthenium complexes

with the formula $[Ru(tpy)(bpy)(L)]^{2+}$, $[Ru(S\text{-}tpy)(bpy)(L)]^{+}$ (S-tpy = [2,2':6',2''-terpyridine]-4'-sulfonic acid) and $[Ru(bpy)_2(L)]^{2+}$. The idea behind this work was to determine which modifications of D-glucose are tolerated by glucose transporters without impairing active uptake. Most challenging was the synthesis of 2-*O* and 4-*O* alkylated derivatives, since the use of the benzyl(idene) protecting group(s) was unfavored due to the presence of sulfur donor atoms in ligand L.

7.1.3 D- versus L glucose conjugation

Conventional methods to determine glucose uptake via glucose transporters (GLUTs) use competitive inhibition with GLUT inhibitors such as phloretin, which often require conditions that are very different from those used in cytotoxicity assays. In Chapter **3** we describe a new method to investigate whether or not a glycosylated compound is taken up by GLUT transporters, which consists in comparing two conjugates bearing either a D- or an L-glucose moiety. The synthesis of two D/L enantiomers of a thioether-functionalized glucose ligand and their coordination to the highly lipophilic ruthenium complex $[Ru(tpy)(dppn)(OH_2)]^{2+}$ ($[1]^{2+}$, tpy = 2,2':6',2''-terpyridine, dppn = benzo[*i*]dipyrido-[3,2-*a*:2',3'-c]phenazine) is presented, together with toxicity, uptake, and intracellular localization studies. The use of enantiomers allowed for an unbiased comparison between cytotoxicity of the conjugates, while comparing a glycon versus an aglycon would not account for their different physical properties and in particular their different Log $P_{o/w}$ values. Submicromolar cytotoxicity values were found for $[Ru(tpy)(dppn)(L)]^{2+}$ after blue light irradiation, which was attributed to the photorelease of $[Ru(tpy)(dppn)(OH_2)]^{2+}$. This activated species showed a remarkably high affinity for DNA while generating high amounts of ROS under light irradiation. Interestingly, the D- or an L-glucose-ruthenium conjugates showed different cytotoxicity in the dark, but this difference could not be attributed to GLUT-mediated uptake. The DNA light switch properties of these compounds revealed identical localization in the mitochondria for both enantiomers. Independent of the cell confluence, of the addition of an ATP-blocker (sodium azide), or of incubation time, both compounds were taken up in a similar manner. Therefore, uptake occurs *via* passive diffusion for these compounds, while the difference in cytotoxicity is most likely related to an enantioselective, post-uptake enzymatic process, such as active efflux or enzymatic breakdown of the β-glycosidic bond of the ligand by a β-glucosidase. Although the ruthenium-glucose conjugates in this chapter were not taken up by GLUT transporters, the selective localization of the prodrug, the very high affinity for mitochondrial DNA (400:1 bp:Ru), and their high singlet oxygen quantum yield (0.71) make them excellent candidates for PDT.

7.1.4 Photodynamic therapy or photoactivated chemotherapy?

The findings described in the previous chapter provided an incentive for a thorough investigation of sixteen different complexes based upon the $[Ru(tpy)(NN)(L)]^{2+}$ scaffold.

The difference between photodynamic therapy and photo-activated therapy is often unclearly defined in the literature or poorly demonstrated. By measuring both the $^1O_2$ generation quantum yield and the photosubstitution quantum yield of glycoconjugates of this series of complexes, some insight is provided in this chapter between these two different modes of action. Structural modifications of the bidentate spectator ligands NN in $[Ru(tpy)(NN)(L)]^{2+}$ lead to completely different photochemical and biological activity. One of the most important findings described Chapter 4 is that highly similar analogues $[Ru(tpy)(dppz)(L)]^{2+}$ and $[Ru(tpy)(dppn)(L)]^{2+}$ induce photocytotoxicity *via* PACT and PDT, respectively. Also, the compound [Ru(tpy)(dppn)Cl]Cl, was found to be very cytotoxic against A549 and MCF-7 cancer cells after blue light activation, which, given the low singlet oxygen quantum yield of this compound is most likely due to its hydrolysis *in vitro*, since we have demonstrated in Chapter 3 that the aqua compound $[Ru(tpy)(dppn)(OH_2)]^{2+}$ is an excellent PDT sensitizer. Other findings of Chapter 4 are that only one of the analogues $[Ru(tpy)(azpy)(L)]^{2+}$ and $[Ru(tpy)(pymi)(L)]^{2+}$ is photoactive, and that increased cellular uptake due to increased lipophilicity does not necessarily warrant (photo)cytotoxicity in A549 and MCF-7 cancer cells. Overall, this study emphasizes that $[Ru(tpy)(dppn)(SRR')](PF_6)_2$ (described in Chapter **3**) is a unique prodrug characterized by two modes of action , i.e. PACT and PDT.

7.1.5 Cyclometalated complexes based upon $[Ru(tpy)(bpy)(L)]^{2+}$

Due to the poor cytotoxicity of most complexes described in Chapter 4, cyclometalated complexes derived from the $[Ru(tpy)(NN)(L)]^{2+}$ scaffold were designed as alternative, monocationic PACT agents. Cyclometalated complexes often absorb at higher wavelengths than their non-cyclometalated analogues, and they are often more cytotoxic. The plane of symmetry in $[Ru(tpy)(NN)(L)]^{2+}$ complexes, which includes the bidentate ligand, is lost when replacing one nitrogen atom of the terpyridine with a carbon atom. Such replacement induces hence chirality, which was confirmed by the synthesis and separation of the two diastereoisomers of $[Ru(phbpy)(phen)(SORR')]^+$ (Hphbpy = 6'-phenyl-2,2'-bipyridyl, SORR' = (*R*)-methyl *p*-tolylsulfoxide), where the chiral cyclometalated complex is bound to an enantiomerically pure chiral sulfoxide ligand. Meanwhile, the thermal and photophysical properties were investigated of racemic complexes $[Ru(phpy)(NN)(dmso\text{-}kS)]^+$ with increasing annulated bidentate ligands (NN = bpy, dpq, phen, dppz and dppn). Compared to the non-cyclometalated analogons described in Chapter **4**, these cyclometalated complexes showed a much lower ligand photosubstitution efficiency, and the ones bearing the dppz or dppn ligand even completely lacked the ability to photorelease the monodentate ligand. Density functional theory calculations and cyclic voltammetry further revealed that the diminished photoreactivity of these complexes is most likely the result of a larger gap between the ^{3}MLCT and ^{3}MC states, making thermal population of the ^{3}MC state from the photogenerated 3MLCT state more unlikely. The broader absorption bands of these

complexes allowed three of them to be activated using green light (520 nm) in A549 and MCF-7 cells, reaching photocytotoxicity at sub-micromolar concentrations. Since both the $^1O_2$ production and ligand photosubstitution quantum yields were found to be very low, the photocytotoxicity of these compounds is attributed to a PDT type I mechanism, although the localization, target, and mode of action of these compounds remains largely unknown.

7.1.6 Red light and hypoxia

A current drawback of light-activated ruthenium(II) polypyridyl prodrugs are that these drugs are usually activated with wavelengths that fall outside the range of phototherapeutic window (600 – 850 nm). In Chapter 3 – 5 it is demonstrated that non-sterically hindered complexes based upon the $[Ru(tpy)(NN)(L)]^{2+}$ or $[Ru(phbpy)(NN)(L)]^{+}$ architecture can be activated with blue to green light. Still, these wavelengths do not penetrate through biological tissues very well. Although upconverting drug delivery systems are currently being developed to overcome this problem,[2] they often have very low overall efficiency, making them complicated to use for therapy. In the past, Bonnet's group has studied series of sterically congested ruthenium complexes $[Ru(tpy)(NN)(L)]^{2+}$ using dmpby or biq as bidentate ligand NN.[3] When L is a thioether ligand, very high ligand photosubstitution quantum yields were observed. However, these complexes were also thermally unstable, which prevents their use as photoactivated prodrugs. Sterically non-hindered pyridine ligands L offer lower photosubstitution quantum yields than thioether ligands, but much higher stability in the dark. In Chapter **6**, two complexes $[Ru(tpy)(dmbpy)(L)]^{2+}$ and $[Ru(tpy)(biq)(L)]^{2+}$ were used as PACT carriers to photocage the pyridine-containing ligand STF-31, a known NAMPT inhibitor. Pyridine binding to the ruthenium center generated new MLCT bands in the red region, which allowed for activating these complexes using red light. We demonstrated that the molar absorption coefficients and photosubstitution quantum yield at body temperature (37 °C) is high enough for these compounds to be fully activated within 10 min irradiation. Most importantly, for the first time a demonstration of PACT under hypoxia is given. $[Ru(tpy)(biq)(STF\text{-}31)]^{2+}$ was tested under low dioxygen concentration (1%) using a specific irradiation setup for hypoxic cells recently developed in the group. The caged STF-31 compound had a photocytotoxic effect both under hypoxia and normoxia, whereas in the latter condition traditional PDT would not work.

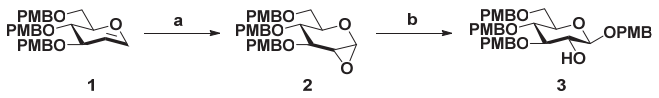
7.2 General conclusions

In this thesis new light-activated compounds based upon the $[Ru(tpy)(NN)(L)]^{2+}$ or $[Ru(phbpy)(NN)(L)]^{+}$ scaffold are described, which were tested against human cancer cell lines. The use of a number of bidentate ligands led to essentially non-toxic compounds, but improving lipophilicity of the complexes either by cyclometalation, or by extension of

the aromatic backbone of the NN ligand, led to cytotoxic and/or phototoxic species. New synthetic routes were developed towards positional isomers of a thioether-functionalized D-glucose ligand, after coordination to ruthenium, leading to the corresponding ruthenium(II) polypyridyl glycoconjugates. GLUT-mediated uptake was not detected for any of these complexes, which may be due either to a too short or too long linker between the thioether donor atom and the glucose moieties, or to the charge and overall hindrance of the ruthenium fragment that may prevent, in the conjugate, interaction of glucose with the GLUT transporter. Notwithstanding, the glucose moiety significantly improved the water solubility of these compounds, allowing for the study of the photoreactivity of these complexes independent of the lipophilicity of the bidentate ligand. By studying a wide variety of ligands, we found that complexes based upon the $[Ru(NNX)(NN)(L)]^{2+/1+}$ (X=N or C^-) scaffold can act either as a PACT drug, or as a PACT carrier, or as a PDT drug, while apparent minor modifications of these complexes had major impact on their photoreactivity and (photo)cytotoxicity. Last but not least, we provided the first demonstration that PACT is applicable under hypoxic conditions in which traditional PDT does not work.

7.3 Outlook

A major drawback of the synthetic route presented for the liberation of the 2-*O* position in D-glucose in Chapter **2** is the use of freshly prepared dimethyldioxirane (DMDO). The synthesis of this compound is arduous and only relatively small quantities can be made safely at a laboratory scale, therefore preventing the synthesis of this compound on a large scale. A proposed alternative route would proceed via the stereospecific *in situ* epoxidation of protected D-glucal **1** (Scheme 7.1), allowing access to larger amounts of **3** (Scheme 7.1).



1 2 3

Scheme 7.1. Alternative 2-*O* modification: a). Oxone, aq. $NaHCO_3$, DCM, 0° to rt; b). PMB-OH, $ZnCl_2$ in THF, -78 °C to rt.

We have demonstrated in Chapter **3** that glycoconjugates based upon the $[Ru(tpy)(dppn)(SRR')]^{2+}$ scaffold where SRR' bears a triethyleneglycol bridge between the thioether ligand and the glucose moiety are not transported *via* GLUT. However, the D- and L-glucose derivatives showed different cytotoxicity in the dark. These findings implicate that a post-uptake process such as hydrolysis by a β-glucosidase is responsible for this difference in cytotoxicity. This observation opens up new routes towards the use of glucosidase inhibitors in PACT. For example, the cyanogenic glycoside D-amygdalin could be coordinated via its nitrile group to [Ru(tpy)(bpy)Cl]Cl or to one of the PACT

carriers described in Chapter **4**. One of the fundamental reasons this cytotoxic compound (**6**, Figure 2) is not used in chemotherapy is the ubiquitous expression of glucosidases in both normal and cancer cells. An idea is proposed here, where modification of this structure by coordination to ruthenium would prevent enzymatic processing by a β-glucosidase in the dark, whereas release of this 'prodrug' with light would release the β-1,6-linked D-glucose disaccharide (Figure 2), where enzymatic breakdown of this molecule would lead to the release of hydrogen cyanide, the latter inducing cell-death.

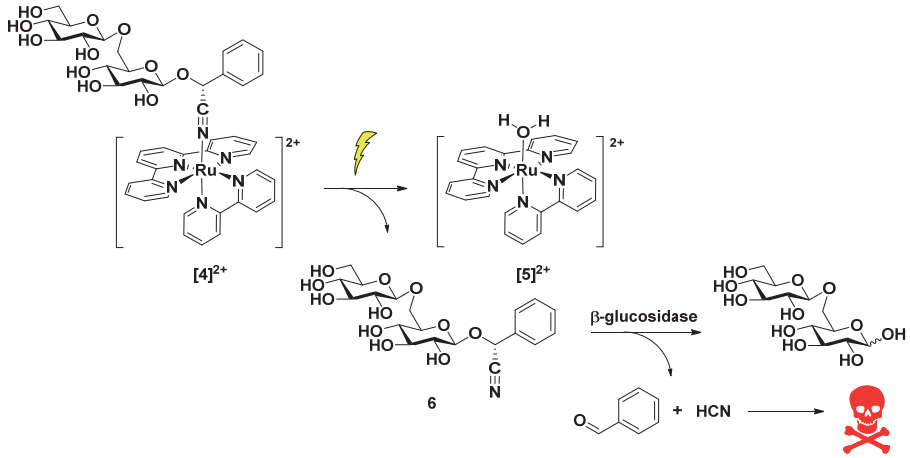


Figure 2. Proposed two-step mechanism for controlled release of D-amygdalin cell-death using a β-glucosidase.

The observed photocytotoxicity for cyclometalated compounds [**1**]PF_6 – [**3**]PF_6 in Chapter **5** are promising for future use in PACT. However, a current drawbacks of these compounds is their low ligand photosubstitution quantum yield. A proposed improvement would be to introduce a substituted bidentate ligand that leads to a more sterically congested complex, as demonstrated for polypyridyl complexes bearing biq and dmbpy in Chapter **6**. As depicted in Figure 3, introducing steric hindrance in cyclometalated complexes [**7**]$^+$ and [**8**]$^+$ may reduce the ligand field splitting and hence lower the ^{3}MC state, making the latter more easily (thermally) accessible from the photochemically generated ^{3}MLCT state and leading to higher ligand exchange efficiency. The observed (photo)cytotoxicity for these complexes could not be attributed to DNA interaction and/or $^1O_2$ generation. Future fractionation experiments to determine the location of these complexes *in cellulo* combined with experiments to prove the generation of reactive oxygen species of these complexes, could provide insight in the mode of action of these drugs.

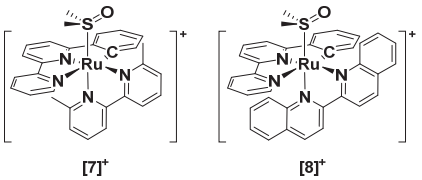


Figure 3. Proposed sterically hindered ruthenium(II) cyclometalated PACT compounds.

As described in Chapter **6** one of the observed disadvantages of the compound $[Ru(tpy)(biq)(STF\text{-}31)]^{2+}$ was its relatively low photocytoxicity index. Although the cytotoxicity under irradiation cannot be improved, the high cytotoxicity in the dark could possibly be reduced by further lowering the log $P_{o/w}$ of the prodrug. A general trend observed throughout this thesis is that more lipophilic ruthenium drugs usually induce higher cytotoxicity in the dark. Therefore, a modification on the lead PACT drug described in Chapter **6** is proposed, where a sulfonate group is introduced on the 4' position (Figure 4) of the terpyridine, which should increase the overall water solubility, possibly reducing the dark cytotoxicity, and thereby increasing the photoindex.

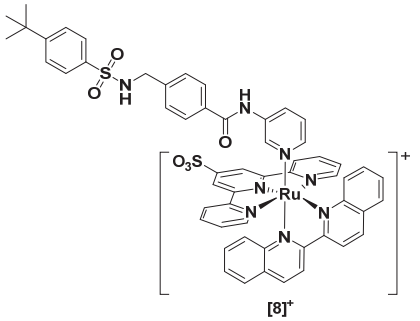


Figure 4. Proposed modification of ruthenium-photocaged caged NAMPT inhibitor.

Overall, the suggestions in this chapter may contribute to the advancement of photo-activated chemotherapy. This might make future clinical application of this new potential therapy possible.

References

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[2] S. H. Askes, W. Pomp, S. L. Hopkins, A. Kros, S. Wu, T. Schmidt, S. Bonnet, *Small* **2016**, *12*, 5579-5590.

[3] A. Bahreman, B. Limburg, M. A. Siegler, E. Bouwman, S. Bonnet, *Inorg Chem* **2013**, *52*, 9456-9469.