Caging ruthenium complexes with non-toxic ligands for photoactivated chemotherapy

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Introduction

1.1 Cisplatin: the spearhead of metal-based chemotherapy

Cancer, also called malignant tumor or neoplasm, is a generic term for a wide group of diseases that involve an irregular growth of cells beyond their usual boundaries, which can then spread to adjoining or distant parts of the body. It is caused by alterations in oncogenes, tumor-suppressor genes, and microRNA genes. According to the World Health Organization (WHO), cancer was the second leading cause of death in 2015 with 8.8 million death.[1] Since the beginning of the 20th century, with the development of modern medicine, an enormous amount of resources has been dedicated to the understanding and cure of cancer. Although at first most efforts focused on the surgical removal of the tumor, chemotherapy received special attention after World War II, when the antitumor and antileukemic properties of mustine hydrochloride (the infamous mustard gas) and other poisonous gases were discovered.[2-3] This is how, within a program of the National Cancer Institute (US) to develop new chemotherapeutic agents, the antitumor activity of the complex *cis*-dichlorodiammineplatinum(II) (known as cisplatin, see Figure 1.1) was discovered in 1969 by Professor Barnett Rosenberg and Loretta van Camp at Michigan State University. This discovery gave birth to the first generation of metal-based chemotherapy drugs.[4] In 1978, cisplatin was approved by the US Food and Drug Administration for the treatment of testicular tumors and ovarian adenocarcinoma;[5] and with the development of carboplatin and oxaliplatin (two derivatives of cisplatin, see Figure 1.1) the use of platinum-based drugs was expanded to the treatment of other types of cancer.[6-8]
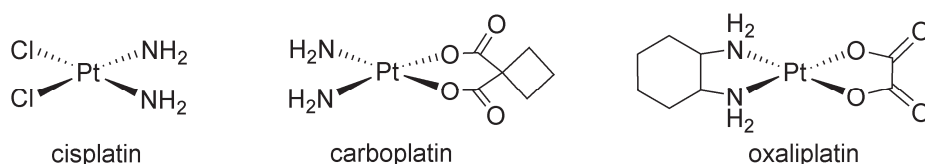

Figure 1.1. Platinum(II) complexes used in cancer chemotherapy.

Although the exact mechanism of action of platinum(II) complexes is not completely clear, the ultimate event that induces apoptosis in cancer cells is generally accepted to be the coordination of DNA to the metal center after aquation of one or two labile ligand(s). DNA binding to platinum inhibits DNA replication and transcription, ultimately leading to cell death.[9-11] In order to develop new platinum-based drugs that are able to bind to DNA, four classical rules are usually stated. First, the platinum complex should contain two monodentate or one bidentate labile ligand(s) that can be

replaced by water molecules; second, it should contain two (or one bidentate) kinetically inert amine ligands; third, the charge of the complex should be neutral; and fourth, it should have *cis* configuration, allowing DNA binding via two neighboring guanines on the same strand.[12-13] However, two important drawbacks of platinum drugs based on these principles can be mentioned: first, inherent or acquired resistances of the tumor cells to the drug are not uncommon,[14-16] and second, highly toxic side effects are typically experienced by the patients, for example hepato- and nephrotoxicity, which limits the long-term clinical use of these compounds in any given patient.[17-19]

1.2 Alternatives in the transition metal block: the case of ruthenium

In order to overcome the drawbacks generally associated with platinum-based drugs, a wide range of transition metal-based drugs has been investigated in the last decades, ranging from ruthenium to osmium, gallium, gold, or rhenium complexes.[20-22] Focusing on ruthenium, the flagship complexes in the field have been KP1019 and NAMI-A, which reached Phase I and II in clinical trials, respectively (Figure 1.2). Both compounds were developed in the late 80's and since they share certain structural similarities they have been often compared and extensively reviewed together.[23-24] In short, KP1019, a ruthenium(III) compound of formula [IndH][*trans*-RuCl$_4$(Ind)$_2$] (Ind = indazole), was developed within a series of azole-based ruthenium(III) complexes by Keppler *et al*.[25-26] It showed great activity against colon cancer in rat models, which allowed to undergo clinical trials. Although the conclusions of the results obtained in Phase I were positive,[27-28] clinical Phase II was never started due to the low solubility of the compound. The more water-soluble NKP-1339 (the sodium salt version of KP1019) has taken the leadership recently, concluding successfully Phase I.[23] The suggested mechanism of action involves the accumulation of the compound in transferrin receptors (which are overexpressed in certain tumor cells) and its subsequent reduction to ruthenium(II) species in the reductive environment characteristic of tumors. However, these hypotheses are controversial and the final biological target of the compound remains discussed.[24] Finally, it is believed that apoptosis of the cancer cells is achieved via mitochondrial damage by disruption of the redox balance, among other possible pathways.[29]

On the other hand, NAMI-A (Figure 1.2), a ruthenium(III) compound of formula [ImH][*trans*-RuCl$_4$(DMSO-κS)(Im)] (Im = imidazole and DMSO = dimethyl sulfoxide) was developed by Alessio *et al*. in the early 90's, and it was preceded by its sodium salt version (NAMI).[30] Despite its structural similarity with KP1019, NAMI-A

did not show any cytotoxicity *in vitro* but it showed antimetastatic activity *in vivo*, especially against non-small cell lung cancer, suggesting a different mechanism of action from that of cisplatin or KP1019. Although DNA binding is possible *in vitro*, such interactions are considered of no relevance in the cell due to the non-cytotoxic but antimetastatic effect of NAMI-A. Thus, the inhibition of cellular migration and invasion by modifying the actin cytoskeleton or selectively binding to collagen are the most likely antimetastatic modes of action.[31-32] This good *in vivo* data resulted in clinical trials, which failed in Phase I/II since NAMI-A appeared to be less effective than gemcitabine alone, a common chemotherapy medication.[33]
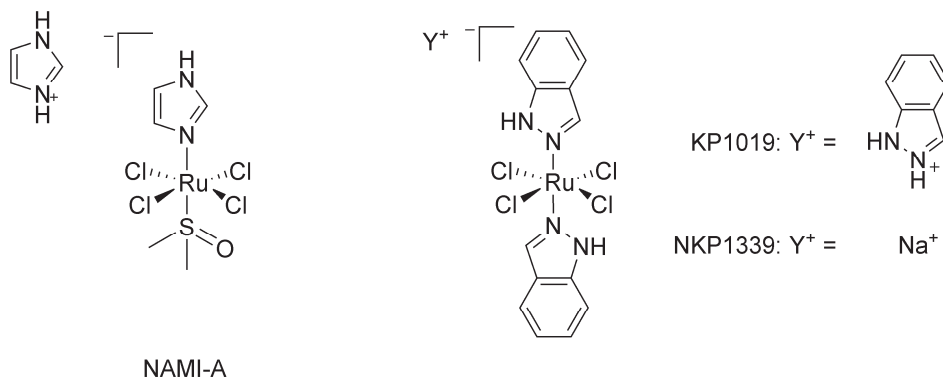

Figure 1.2. Formulae of the ruthenium(III) complexes NAMI-A and KP1019 that have undergone clinical trials for anticancer treatment.

In light of the relative success of NAMI-A and KP1019, many other ruthenium complexes have been developed as alternative antitumor drugs in the last two decades. A new group of complexes based on arene ligands was pioneered by Dyson and Sadler.[34] Their half-sandwich conformation leaves three free coordination sites to coordinate different kind of ligands (three monodentate ligands or one facial tridentate ligand), thus tuning its thermodynamic and kinetic properties to target different biomolecules. Furthermore, the hydrophobic arene ligand in conjunction with the hydrophilic metal center provides valuable amphiphilic properties.[35] One of the clinically most advanced arene complexes is RAPTA-C (Figure 1.3). This complex, which has a p-cymene and an 1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decanephosphine (PTA) as ligands, was first developed by Dyson and co-workers in 2001.[36] After aquation of chloride ligands and further substitution of the labile aqua ligand by biomolecules, it shows low *in vitro* cytotoxicity, but a good one *in vivo*.[37] Furthermore, studies have demonstrated its similarity to NAMI-A: DNA is an unlikely target, RNA and proteins are probable targets, and antimetastatic activity predominates. Scores of

structural variations, such as modification of the arene, halogen, or phosphine ligand were performed to study their influence and modulate the anticancer/antimetastatic activity of the complex. For example, RAPTA-B and RAPTA-T (Figure 1.3) inhibit metastasis growth and increase cytotoxicity, respectively.[38] Instead of p-cymene, they have a benzene and a toluene, respectively, coordinated to the ruthenium center.
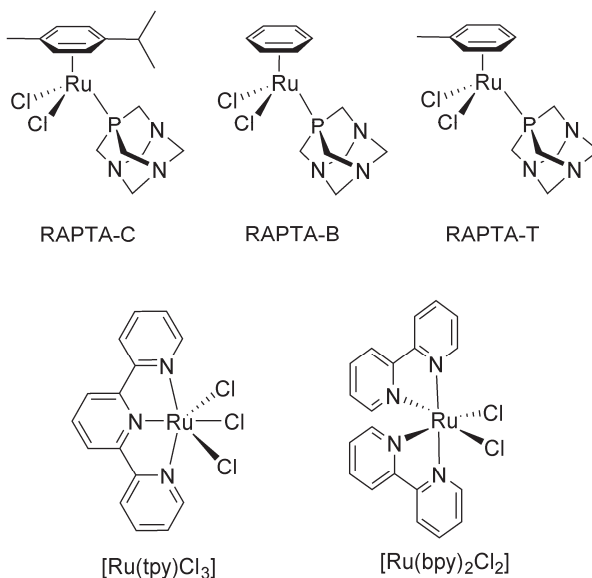

Figure 1.3. Top: Ruthenium(II)-arene complex RAPTA-C and its derivatives RAPTA-B and RAPTA-T with antimetastatic and enhanced cytotoxic activity, respectively. Below: ruthenium(II) and ruthenium(III) polypyridyl complexes studied by Reedijk and co-workers.[39]

Ruthenium polypyridyl complexes also caught the attention of researchers as possible cisplatin-like drugs. Like arene-based complexes, polypyridyl complexes can have coordinating sites available to interact with biomolecules after aquation of the chloride ligands. Thus, in principle, they are able to bind to DNA like cisplatin. Reedijk and co-workers studied the cytotoxicity of [Ru(tpy)Cl$_3$] (tpy = 2,2':6',2''-terpyridine), [Ru(tpy)(bpy)Cl]Cl (bpy = 2,2'-bipyridine), and *cis*-[Ru(bpy)$_2$Cl$_2$] (Figure 1.3) against HeLa and murine cancer cells. The cytotoxicity of a compound is expressed with the EC$_{50}$ value, which is the effective concentration of compound at which 50% of the treated cells are dead, compared to untreated control cells. For [Ru(tpy)(bpy)Cl]Cl and [Ru(bpy)$_2$Cl$_2$], EC$_{50}$ values 70 times higher than that for [Ru(tpy)Cl$_3$] were obtained.[39] In an attempt to increase the cytotoxicity but keep the two labile chloride ligands in *cis*, Reedijk and co-workers replaced the bpy ligands by 2-phenylazopyridine (azpy), a dissymmetric ligand which contains an azo group and is more lipophilic.[40] Due to the

dissymmetry of the azpy ligand, [Ru(azpy)$_2$Cl$_2$] has five different regioisomers (Figure 1.4), of which α, β, and γ were studied. Cytotoxicity studies against renal cancer (A498 cells), breast cancer (MCF-7 and EVSA-T cells), non-small cell lung cancer (H226 cells), ovarian cancer (IGROV cells), melanoma (M19 cells), and colon cancer cells (WIDR cells) showed lower EC$_{50}$ values for the α and γ isomers than for the β isomer. This result suggests a stereoselective coordination to biomolecules, and thus a different mechanism of action depending on the isomer.[41] Cytotoxicity studies of [Ru(tpy)(N-N)(L)]$^{n+}$ (where N-N = 2,2′-azobispyridine, azpy, or 2-phenylpyridinylmethylene amine, and L = Cl$^-$, H$_2$O, or CH$_3$CN) showed that the presence of an azo group is required for anticancer activity and that the nature of the labile ligand L does not have a significant effect on cytotoxcity.[42] Furthermore, the mixed-ligand complex α-[Ru(azpy)(bpy)Cl$_2$] shows an intermediate cytotoxicity: higher than that of [Ru(bpy)$_2$Cl$_2$] but lower than that of [Ru(azpy)$_2$Cl$_2$], reinforcing the idea that an azo group is necessary to reach a high cytotoxic effect.[43]
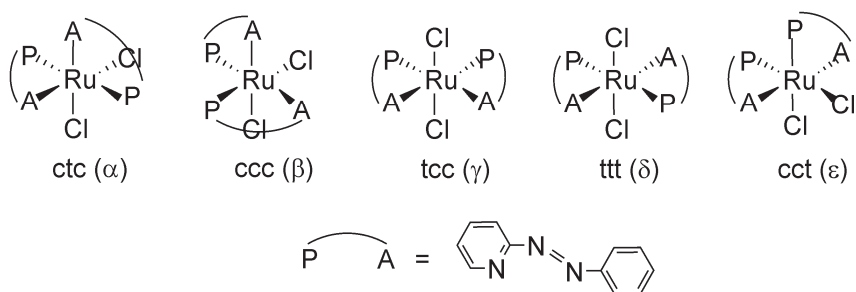

Figure 1.4. Structural representation of the five regioisomers (α, β, γ, δ, and ε) of the complex [Ru(azpy)$_2$Cl$_2$]. The three-letters code indicates the mutual cis (c) or trans (t) orientation of Cl, N-pyridine and N-azo donor atoms, respectively.[41]

1.3 And there was light

One of the first problems encountered with chemotherapy was the lack of selectivity towards cancer cells, inducing all kind of collateral toxicities. Different strategies to localize the administration of the drug and thus increase the selectivity have been developed over the years, from peptide targeting to specific drug delivery carriers.[44-46] One of these strategies consists in using visible light to activate a photosensitive drug with a precise spatial and temporal control.[47] In 1903, the treatment of skin cancer by application of eosin (a photosensitizer) followed by irradiation of the area was reported, establishing the relation between light, dioxygen, and the photosensitizer, and marking the scientific start of Photodynamic Therapy (PDT).[48] Although some work was performed in the PDT field in the following decades,[49-50] it was not until the early

1970's when Diamond, Dougherty, and Tomson reported, almost simultaneously, the use of PDT against malignant tumors.[51-53] Nowadays, several dyes are available on the market as PDT photosensitizers, most of them based on porphyrins (*e.g.* Photofirn, Verteporfin) or chlorins (*e.g.* Foscan, Figure 1.5).[48, 54]
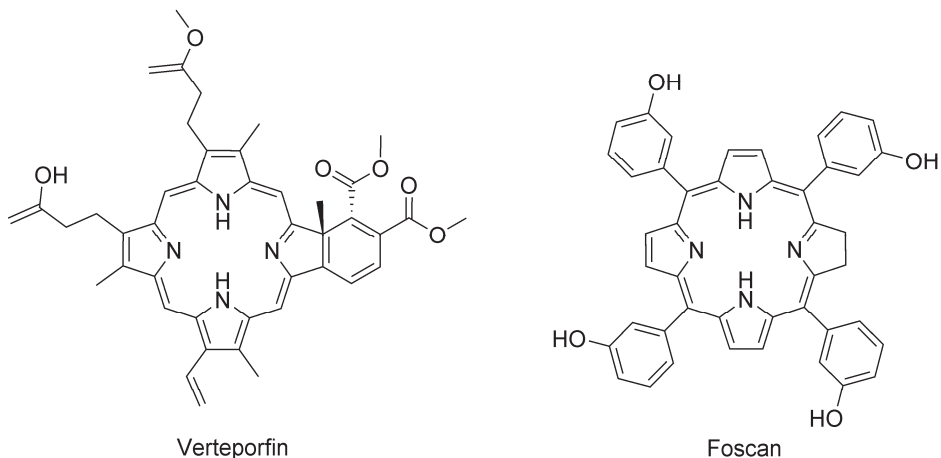

Verteporfin

Foscan

Figure 1.5. Chemical structure of Verteporfin and Foscan, two clinically used PDT photosensitizers.

In the most common form of PDT, called PDT type II, the photosensitizer is excited upon light irradiation to its singlet state and undergoes intersystem crossing (ISC) to a triplet state. As shown in Figure 1.7, in presence of ground state molecular oxygen ($^3O_2$) both molecules can collide to produce a triplet-triplet annihilation (TTA) event that transfers the energy from the photosensitizer to the molecular oxygen, which is excited to its singlet state ($^1O_2$). $^1O_2$ is a very reactive species that can oxidize many biomolecules like amino acids, DNA, or lipids, thereby causing oxidative damage and inducing cell death.[55-56] On the other hand, PDT type I involves the generation of free radicals through an electron (or proton) transfer reaction from the excited photosensitizer to a biological substrate. The radical further reacts with tissue dioxygen, generating reactive oxygen species (ROS) and oxidative stress.[48] Although PDT has been successfully used in the clinic to treat different cancer types, it also has two major limitations. First, it depends on the presence of dioxygen, while many regions in tumors are hypoxic;[57] second, the spectral range in which the photosensitizers absorb light should be in the so-called phototherapeutic window. This region of the spectrum consists of wavelengths that penetrate biological tissues deep enough without causing radiation damage. The range in which the phototherapeutic window is generally considered optimal is between 620 and 850 nm.[58]

Ruthenium(II) polypyridyl complexes are d^6 complexes with an octahedral geometry that can be potential photosensitizers for PDT due to their long-lived excited triplet state. In regular octahedral complexes of the type $[Ru(bpy)_3]^{2+}$, a singlet metal-to-ligand charge transfer (^{1}MLCT) state is populated upon irradiation, quickly evolving to a triplet metal-to-ligand charge transfer (^{3}MLCT) state via ISC. From this microsecond-lived triplet state, energy transfer to molecular oxygen can occur to produce the reactive species $^1O_2$ (Figure 1.7). Many examples of PDT-like ruthenium(II) complexes have been reported, of which TLD1433 has even reached clinical trials (Figure 1.6). TLD1433 is a complex having the formula [Ru(4,4'-dmbpy)$_2$(IP-TP)]Cl$_2$ (4,4'-dmbpy = 4,4'-dimethyl-2,2'-bipyridine, IP-TP = 2-(2',2'':5'',2'''-terthiophene)-imidazo[4,5-f][1,10]phenanthroline) developed by McFarland *et al.*[59] Preliminary *in vitro* studies against promyelocytic leukemia cells (HL-60) showed no cytotoxicity in the dark but a high cytotoxic effect upon red light irradiation. Last year, TLD1433 went to Phase I in clinical trials for the treatment of bladder cancer. [60]
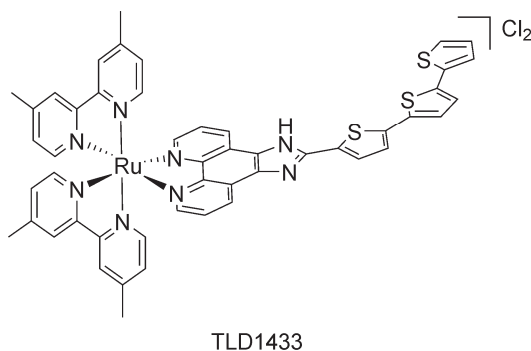

Figure 1.6. Chemical structure of TLD1433.

1.4 Photoreactivity of ruthenium polypyridyl complexes

Although many transition metal compounds have been explored as possible PDT photosensitizers, few have the versatile and tunable photochemistry of ruthenium(II) polypyridyl complexes. Indeed, from the ^{3}MLCT excited state generated upon light irradiation, the system can evolve following different pathways, as shown in the Jablonski diagram depicted in Figure 1.7. As mentioned before, one of the possible pathways is the relaxation of the system to the ground state via TTA with $^3O_2$. In this case, the ruthenium complex can be considered as a PDT photosensitizer. A second possible pathway is the relaxation via luminescence from the ^{3}MLCT state, with emission maxima in water generally in the 600 to 730 nm range. Luminescent

ruthenium(II) complexes form a large family of dyes for biological imaging.[61-63] Keyes *et al.* have reported several examples of complexes of the type $[Ru(bpy)_2(L)]^{2+}$ that target the nucleus, the endoplasmic reticulum, or the mitochondria, depending on whether L is a nuclear localization signal peptide, an endoplasmic directing sequence, or a mitochondrial penetrating peptide, respectively.[64-66]

If the ligand field splitting of the complex is small enough, the electron in the ligand-based π^* state can thermally populate a metal-based e_g orbital, generating a triplet metal-centered state (^3MC), which has dissociative character and may result in the photosubstitution of a ligand.[67] Smaller ligand field splitting can be achieved via distortion of the coordination sphere, for example using hindering ligands such as 6,6'-dimethyl-2,2'-bipyridine (dmbpy),[68] or via controlling the electronic properties of the ligands.[69] The photoreactivity of ruthenium(II) polypyridyl complexes was already reported by Bosnich *et al.* in 1966.[70] However, it was in the 1980's when Durham and Meyer pioneered the research in the field with, for example, the photoconversion of $[Ru(bpy)_2(py)_2]^{2+}$ (py = pyridine) to $[Ru(bpy)_2(Y)_2]^{n+}$ in dichloromethane or acetone in the presence of coordinating anions Y^- (Y^- = F^-, Cl^-, Br^-, or SCN^-).[71,72] Later, Sauvage *et al.* expanded the field with the introduction of hindering ligands to achieve controlled photosubstitution, which was applied in the design of light-driven molecular machines. His work in the field merited him the Nobel Prize in Chemistry in 2016.[73-76]
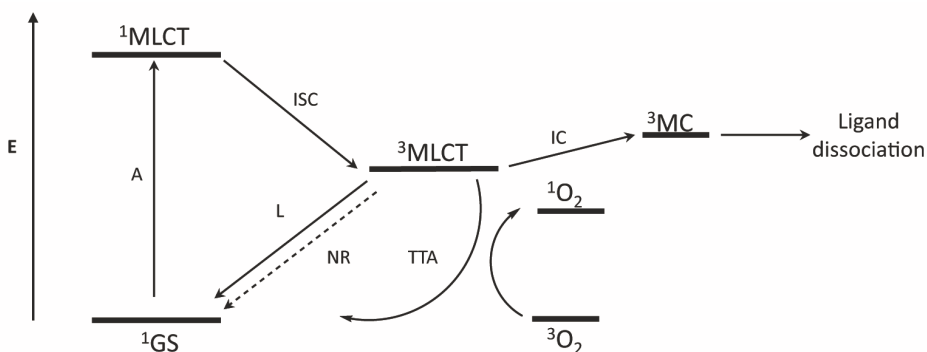

Figure 1.7. Jablonski diagram for ruthenium polypyridyl complexes. If the ^3MC is too high in energy and cannot be thermally populated, then ligand dissociation does not occur. A = absorption, ISC = intersystem crossing, L = luminescence, NR = non-radiative relaxation, TTA = triplet-triplet annihilaiton, IC = interconversion.

More recently, Turro and co-workers compared the ability of ruthenium polypyridyl complexes to undergo photosubstitution of thioether S-ligands, namely 3,6-dithiaoctane (bete) and 1,2-bis(phenylthio)ethane (bpte), *vs.* amine N-ligands, namely ethylenediamine (en) and 1,2-dianilinoethane (dae), by water and Cl⁻ (Scheme 1.1).

According to their work, the higher ligand-exchange quantum yields of S-complexes compared to N-complexes (Φ_{Cl} of 0.019, 0.016, 0.002, and 0.003 for bete, bpte, en, and dae complexes, respectively) are due to the greater elongation of the Ru-S bond in the triplet excited state. This elongation is a result of the transfer of electron density from the metal-based t_{2g} orbital to the bpy-based π^* orbital, which weakens the Ru-S bond in the excited state.[77] Thus, changing the nature of the ligand has an important effect on the photoreactivity. Overall, all the processes mentioned above (TTA, luminescence, and photosubstitution), as well as non-radiative relaxation, can in principle coexist in ruthenium polypyridyl complexes, and of course compete with each other.
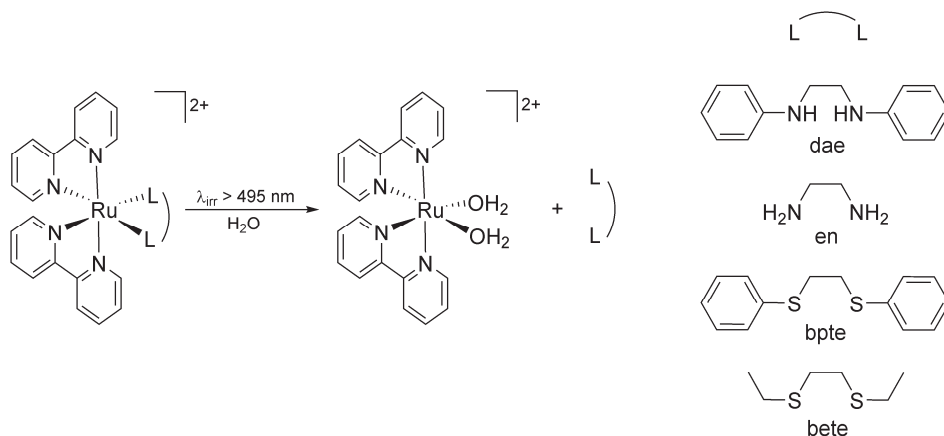

Scheme 1.1. Complexes studied by Turro and co-workers to compare photosubstitution efficiency of S-based vs. N-based bidentate ligands.[77]

1.5 Photoactivated Chemotherapy (PACT)

The photosubstitution properties of ruthenium(II) complexes can be combined with the idea of timely and spatially controlled delivery of a cytotoxic species, developed in PDT, into a new type of phototherapy called photoactivated chemotherapy (PACT).[78] In PACT, a cytotoxic compound is "caged" by linkage to a photocleavable protecting group, creating a prodrug in which the cytotoxic compound is not able to interact with its biological target. Upon light irradiation, the photocleavable group is released to recover the biologically active compound. Although photocaging is also applied for organic molecules,[79] in this thesis we will focus on the ruthenium-based PACT. Ruthenium-based PACT can be applied in two ways: either a non-toxic ruthenium complex is used as a cage for a bioactive organic molecule (one of the ligands), or one of the ligands is non-toxic and used to cage a ruthenium-based cytotoxic species. In any case, coordination of the ligand to the metal complex has to be strong and stable

enough in water for the prodrug not to be activated thermally. Meanwhile, the ligand-metal bond(s) should become weak enough upon low-energy light irradiation for the ligand to be photosubstituted by water molecules, thereby releasing the two photoproducts. Examples for the photocaging of bioactive organic molecules can be found in the work of Etchenique, Turro, or more recently Renfrew, who reported many examples of such compounds.[80-85] Etchenique and co-workers reported the caging of nicotine (Nic), a known addictive drug, in the complex $[Ru(bpy)_2(Nic)_2]^{2+}$. Upon violet, blue, or green light irradiation this complex photosubstitutes only one of the Nic ligands, yielding free Nic and $[Ru(bpy)_2(Nic)(OH_2)]^{2+}$ as side-product (Scheme 1.2).[86] This monosubtitution was also achieved for photocaged neurotransmitters such as tryptamine, serotonin, tyramine, and γ-aminobutyric acid (GABA) (shown in Scheme 1.2).[83] Another family of caged compounds, also developed by Etchenique $et\ al.$, have the formula $[Ru(bpy)_2(PMe_3)(L)]$, in which L is a biologically active amine, and PMe_3 is a non-labile ligand. Compounds like glutamate and GABA have been caged using this type of complexes.[86-87] In our group, Lameijer anc co-workers have reported the photocaging of a nicotinamide phosphoribosyl transferase (NAMPT) inhibitor STF-31 in the complex $[Ru(tpy)(biq)(STF-31)]^{2+}$ (biq = 2,2'-biquinoline). When tested against skin (A431 cells) and lung (A549 cells) cancer cells, a 3- to 4-fold increase in cytotoxicity was found upon red light irradiation.[88]
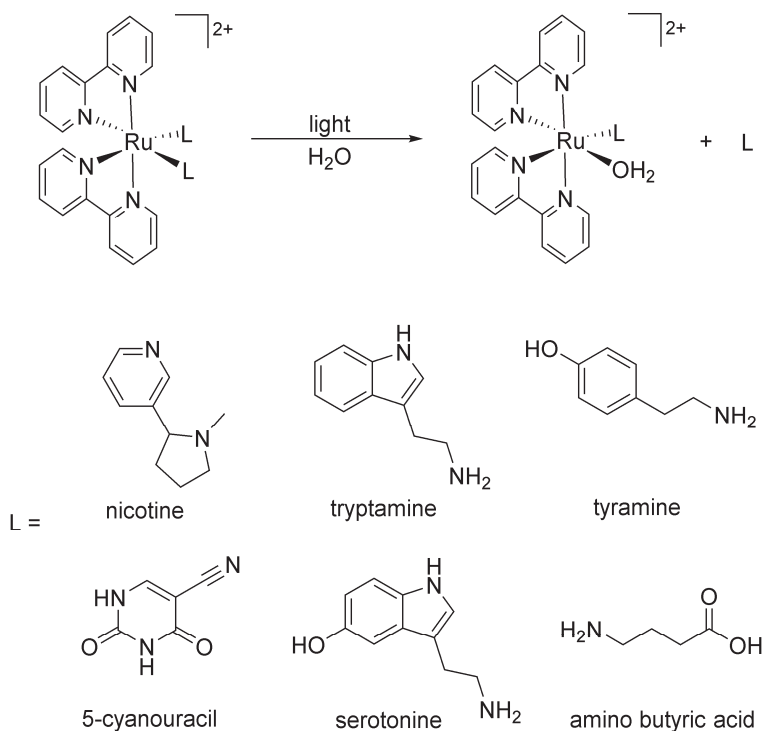
Scheme 1.2. Photosubstitution of a caged compound L by a water molecule reported by Etchenique or Turro et al. Coordination of the ligand L is established through the amine donor atom, except for 5-cyanouracil, which binds via the nitrile group.

On the other hand, there are many examples in literature in which the cytotoxicity is attributed to the photogenerated ruthenium complex. For example, Glazer and co-workers reported that after irradiation of [Ru(bpy)$_2$(dmbpy)]Cl$_2$ dmbpy is released, generating *cis*-[Ru(bpy)$_2$(OH$_2$)$_2$]$^{2+}$, which can bind to plasmid DNA (Scheme 1.3).[68] When A549 cells were treated with [Ru(bpy)$_2$(dmbpy)]Cl$_2$, the cytotoxicity was enhanced after light irradiation with a photo index (PI), *i.e.* the ratio of the EC$_{50}$ value obtained in a dark control and that after light irradiation, of 136, and an EC$_{50}$ value of 1.1 µM was found after light irradiation. Many have interpreted this result as a consequence of the cytotoxicity of *cis*-[Ru(bpy)$_2$(OH$_2$)$_2$]$^{2+}$, by analogy to the cytotoxic aquated form of cisplatin, *cis*-[Pt(NH$_3$)$_2$(OH$_2$)$_2$]$^{2+}$. Following the same scheme, Papish *et al*. reported the enhanced cytotoxicity upon blue light irradiation of [Ru(bpy)$_2$(dhbpy)]Cl$_2$ (dhbpy = 6,6'-dihydroxy-2,2'-bipyridine), which photosubstitutes dhbpy. Dhbpy cannot be photosubstituted at high pH due to the deprotonation of the hydroxyl groups, but becomes labile when protonated at lower pH. This property would allow for selective activation in the more acidic environment

of cancer cells, while healthy cells would not be harmed by the molecule even under light irradiation.[89] On the other hand, McFarland *et al.* reported the cytotoxic activity of a series of complexes of the type $[Ru(dmbpy)_2(IP\text{-}nT)]^{2+}$ (IP = imidazo[4,5-f][1,10]phenanthroline, n = 1-3, T = thiophenes), the strained form of TLD1433.[90] Upon visible light irradiation, one dmbpy is released, generating the bis-aqua $[Ru(dmbpy)(IP\text{-}nT)(OH_2)_2]^{2+}$, which is believed to be the cytotoxic species. The series of complexes showed low EC_{50} values of 1-2 µM against HL-60 cells after visible light irradiation, with PI's ranging from 22 to 166. However, since the $^1O_2$ generation quantum yields (Φ_Δ) were relatively high when n was 2 or 3 (Φ_Δ = 0.34 and 0.42, respectively), a PDT effect could not be excluded and a dual mode PACT/PDT was suggested. Finally, Turro *et al.* reported the caging of two 5-cyanouracil (5-CNU) molecules, an uracil derivative that inhibits the pyrimidine catabolism, in the complex $[Ru(bpy)_2(5\text{-}CNU)_2]^{2+}$. The bis-aqua complex is generated after photorelease of two 5-CNU via two consecutive photosubstitution reactions. The authors suggested that two biologically active species were generated, the bis-aqua ruthenium complex and the two cytotoxic 5-CNU ligands, and thus considered for this compound a dual mode of action. [84]
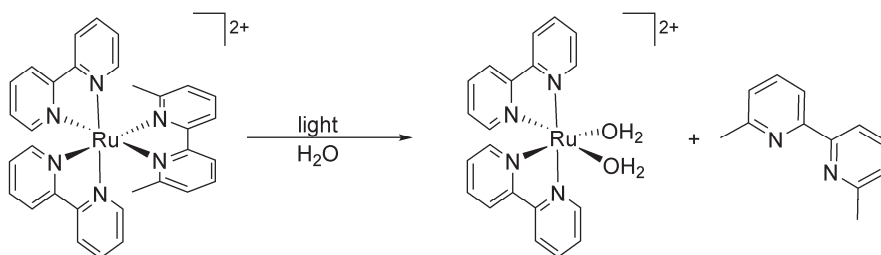
Scheme 1.3. Photoaquation of $[Ru(bpy)_2(dmbpy)]^{2+}$ upon irradiatin at λ>450 nm. Which of the photoproducts is the cytotoxic species?[68]

However, there is one major drawback for the application of ruthenium-based PACT in the clinic. As shown before, the photosubstitution mechanism starts via population of the ^{1}MLCT, which is generally achieved by light in the blue region of the spectrum (440-500 nm). However, blue light does not penetrate efficiently biological tissue and it can be toxic in high doses.[91] In other words, it is far from the phototherapeutic window (620-850 nm). In order to overcome this issue and to obtain photosusbtitution using red light, several strategies have been considered. One of the strategies is to shift the MLCT absorption band of ruthenium(II) complexes to the red part of the spectrum. Glazer and co-workers have done that by incorporating biq ligands in the complex $[Ru(phen)_2(biq)]^{2+}$ (phen = 1,10-phenanthroline), thus distorting the octahedral

geometry. This complex shows some absorption at a wavelength as high as 700 nm.[92]
On the other hand, Turro and co-workers have used negatively charged coordinating
atoms such as 2-phenylpyridine (phpy⁻) in the cyclometalated complex
[Ru(phen)(phpy)(CH$_3$CN)$_2$]PF$_6$ for the same purpose.[93] However, cyclometalated
complexes generally show limited photoreactivity. Another strategy is to "upgrade" red
light locally into blue light using an upconversion drug delivery system. For example,
Askes and Bonnet have developed TTA upconverting liposomes. Upon red light
irradiation (630 nm) an amphiphilic [Ru(bpy)(tpy)(SRR')]$^{2+}$ complex, also included in
the lipid bilayer of the liposome, photosubstitutes the lipophilic thioether ligand SRR'
by one water molecule, thereby detaching from the membrane.[94] A similar approach
was followed by Salassa and co-workers by using NaYF$_4$:Yb^{3+}/Er^{3+} upconverting
nanoparticles (UCNPs) to photoactivate *cis*-[Ru(bpy)$_2$(py)$_2$]Cl$_2$ in aqueous solution.
Upon near infrared light irradiation (980 nm) one pyridine is substituted by one water
molecule in a complex with an MLCT band in the blue region (λ_{max} = 455 nm).[95]

1.6 Aim and outline of the thesis

The goal of the research described in this thesis is the development of new PACT
ruthenium(II) complexes that, upon light irradiation, substitute a non-cytotoxic
bidentate chelating ligand by two solvent molecules to form a cytotoxic *cis*-
ruthenium(II) photoproduct. It should be noted here that the cytotoxicity of *cis*-
ruthenium(II) polypyridiyl complexes remains controversial. On the one hand, Reedijk
and co-workers reported the low cytotoxicity of [Ru(bpy)$_2$Cl$_2$], which hydrolyzes into a
bis-aqua complex, while Etchenique, Renfrew, and Kodanko claim the non-toxicity of
that same bis-aqua complex to cage bioactive ligands in living cells. In such
applications, it is of utmost importance that the ruthenium(II) caging agent is not toxic.
On the other hand, Glazer and Papish showed increased cytotoxicity with compounds
producing [Ru(bpy)$_2$(OH$_2$)$_2$]$^{2+}$ and dmbpy or dhbpy, respectively, and claimed that the
phototoxicity is caused by the bis-aqua complex. Thus, some questions were unsolved
when this PhD research started. In which case is a *cis*-ruthenium polypyridyl complex
cytotoxic? Is it possible to distinguish the photocytotoxicity of the aquated metal
complex from that of the released ligand? What is the role of the charge and
lipophilicity of the prodrug on the dark cytotoxicity and light activation of the
complex? And finally, is ^{1}O$_2$ generation a factor to take into account to understand the
phototoxicity of these light-activated compounds?

In Chapter 2 we have first studied whether the natural amino acid L-proline (L-prol) could be used as a photolabile ligand in a series of three complexes of the type $[Ru(N,N)_2(L\text{-}prol\text{-}\kappa N,\kappa O)]PF_6$ (N,N = bpy or dmbpy). In this series of complexes, the strain is systematically increased by adding zero, two, or four methyl substituents at the 6 and 6' position of the bpy ligand(s). In water, none of the complexes is photoreactive, whereas in CH_3CN, a less polar solvent and better coordinating molecule, the more strained complexes proved to be photoreactive. However, the photoreactivity is not selective and either L-prol or dmbpy are substituted in parallel by two CH_3CN molecules. The difficulty of selectively photosubstituting an anionic N,O chelating ligand made us investigate further sulfur-based neutral chelating ligands, some of which are known to be excellent photolabile ligands for ruthenium polypyridyl complexes.[96] Indeed, in Chapter 3 we show that the N,S chelating ligand 2-(methylthio)methylpyridine (mtmp) is a good photolabile ligand in $[Ru(bpy)_2(mtmp\text{-}\kappa N,\kappa S)]Cl_2$, which generates $cis\text{-}[Ru(bpy)_2(OH_2)_2]^{2+}$ upon light irradiation. Cytotoxicity assays against A549 cells show that the mtmp ligand itself is non-cytotoxic and that $[Ru(bpy)_2(mtmp)]Cl_2$ is non-cytotoxic in the dark and after light irradiation. By contrast, we verified Glazer's result that $[Ru(bpy)_2(dmbpy)]Cl_2$ shows an enhanced cytotoxic effect after light irradiation. However, we demonstrate dmbpy to be cytotoxic. As a consequence, due to the low lipophilicity and low cellular uptake of both ruthenium prodrugs, we attribute the photocytotoxic effect of $[Ru(bpy)_2(dmbpy)]Cl_2$ to the released dmbpy ligand, rather than to the bis-aqua ruthenium complex. These results contradict the available literature, in which the photocytotoxicity is attributed, based on the cisplatin analogy, to the metal-base photoproduct $[Ru(bpy)_2(OH_2)_2]^{2+}$.

In Chapter 4 the synthesis, photochemistry, and cytotoxicity of a series of ruthenium complexes bearing the non-cytotoxic N,S chelating ligand 3-(methylthio)propylamine (mtpa) is described. The series consists of complexes of the type $[Ru(N,N)_2(mtpa)]^{2+}$ (N,N = bpy or dmbpy) in which the distortion of the octahedral sphere and the lipophilicity of the complex are increased by addition of two or four methyl substituents at the 6 and 6' positions of the N,N ligand, $i.e.$ by using one or two dmbpy ligands instead of bpy. We show that an intermediate level of octahedral distortion, such as that in the complex $[Ru(bpy)(dmbpy)(mtpa)]^{2+}$, is necessary to obtain full photosubstitution of the N,S chelating ligand while keeping thermal stability.

In Chapter 5 we study cyclometalation as a strategy to increase the absorption wavelength of a PACT ruthenium compound. The synthesis and photochemistry of a

series of complexes of the type $[Ru(bpy)(phpy)(N,S)]^+$ (phpy = 2-phenylpyridine) is described, where the N,S chelating ligand is either mtpa, mtea (2-(methylthio)ethylamine), mtmp, or mtep (2-(methylthio)ethyl-2-pyridine). Mtpa and mtmp were already used in previous chapters, and by adding mtea and mtep in the series we investigate the influence of the size of the N,S chelating ring (five- or six-membered ring) and the nature of its coordinated nitrogen atom (pyridine *vs.* primary amine) on the stereoselectivity of the synthesis of these highly dissymmetric complexes, on their stability towards aerial oxidation, and on their photoreactivity. We show that complexes bearing ligands that form a six-membered ring (*i.e.* mtpa and mtep) are synthesized stereoselectively to obtain only one of the eight possible isomers, and that these complexes are photoreactive in CH_3CN. Furthermore, complexes bearing a pyridine-based N,S ligand (*i.e.* mtmp and mtep) are less prone to oxidize under air than amine-based complexes due to the π-acceptor properties of the pyridine.

Finally, the toxicity of a series of ruthenium complexes bearing a photolabile non-toxic N,S ligand is tested in human cancer cells under hypoxia (1% O_2) to investigate the oxygen dependency of their biological effect (Chapter 6). We show that the cytotoxicity of all compounds is lower under hypoxia compared to that under normoxia (21% O_2) probably due to the chemoresistance acquired by cancer cells under hypoxia. However, the cytotoxicity of some of the complexes is clearly enhanced upon green light irradiation, which is the first experimental demonstration of light-induced cytotoxicity under hypoxia for a metal-based PACT compound releasing a non-toxic organic ligand.

1.7 References

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