Photomedicine with inorganic complexes: a bright future

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Photomedicine with Inorganic Complexes: A Bright Future

34

Michael S. Meijer, Rose Maria Carlos, Mauricio S. Baptista, and Sylvestre Bonnet

Contents

Abstract

Photo-induced reactions have the potential to revolutionize the fields of photomedicine and intelligent drug delivery by providing means of specifically inducing a chemical transformation in biological environments. The molecule that absorbs light and engages in photo-induced reactions is called the photosensitizer, and is the key component in this process. It transforms photon energy into a variety of reactions, such as photosensitized oxidations in photodynamic therapy (PDT) and ligand exchange in photoactivated chemotherapy (PACT). Ruthenium complexes, in particular, offer the possibility to maximize and fine-tune each of these reactions by changing the electronic properties, hydrophobicity, and steric hindrance of the ligands, thus affecting the energy and reactivity of the excited states. The field has advanced immensely in the last decade and we aim here to report on major achievements of ruthenium compounds for phototherapy. We will also discuss the mechanism of light-induced toxicity, the potential of upconverting systems for the activation of this type of drugs, as well as initial steps towards commercial applications of ruthenium complexes as PDT agents.

Keywords

Ruthenium · Phototherapy · Photochemistry · Chemotherapy · Light activation

34.1 Photomedicine: Curing with Light

Over the years, light has been used to treat a wide variety of medical conditions. On its own, it has been shown to be effective in the treatment of seasonal affective disorder (SAD) [1], neonatal jaundice [2], and various skin diseases [3], among others. However, light can also be used to activate an otherwise biologically non-active chemical substance, i.e., a photo-activatable prodrug. As a trigger, it provides both spatial and temporal control over the prodrug activation, greatly increasing the potential selectivity of these prodrugs with respect to regular chemotherapeutics. Moreover, light can be administered to a patient in a noninvasive manner, by simply irradiating areas of the skin, or minimally invasive

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manner, using thin optical fibers or endoscopes. Over the last few decades, the potential benefits of phototherapy have sparked significant research efforts in the field of cancer treatment [4–8], one of the most prevailing causes of death in the developed world [9].

Several therapeutic anticancer treatment modalities based on light have been developed in recent years, all with their own merits. They include, among others:

- Photothermal therapy (PTT), in which the energy of the incoming light is converted to heat, conflicting thermal damage to the diseased tissue [10]
- Isomerization-based photopharmacology, in which the light activates photoswitches, based on the thermally reversible photo-isomerization reactions in azobenzenes or dithienylethenes, leading to the temporary activation of the prodrug [5]
- Photodynamic therapy (PDT)
- Photoactivated chemotherapy (PACT)

The latter two techniques are the main focus of this chapter and will be discussed in the upcoming sections, with particular focus on organic-inorganic hybrid systems consisting of porphyrins and ruthenium polypyridyl complexes as potential photosensitizers for light-activated anticancer treatments.

34.2 Photodynamic Therapy

34.2.1 Introduction

Photodynamic therapy is based on the interaction of light, dioxygen, and a photosensitizer, which can be either a molecular dye or an inorganic semiconductor particle. In the classical understanding of this treatment modality, the photosensitizer plays a catalytic role. Thus, the limiting factors to the efficiency of PDT are the photostability of the sensitizer, the light dose, and the concentration of dioxygen. After absorption of a photon by the photosensitizer, which brings it to its singlet excited state, intersystem crossing (ISC) takes place to the excited triplet state of the photosensitizer. This triplet state generally has a relative long lifetime, allowing for the excited photosensitizer to react with nearby molecules. In the most common type of PDT, called PDT type II, the excited photosensitizer is quenched by a ground state dioxygen molecule ($^3O_2$) via an energy transfer process called triplet-triplet annihilation (TTA) [11]. This process produces a highly reactive excited state of dioxygen, called singlet oxygen ($^1O_2$), which is able to oxidize nearby biomolecules or lead to the generation of secondary reactive oxygen species (ROS), e.g., hydroxyl radicals, superoxide anions, and peroxides [11, 12]. Contrarily, in PDT type I, the excited triplet state of the photosensitizer transfers an electron/hydrogen to/from a nearby molecule, which may be either a biological substrate or dioxygen [11, 12]. Both processes ultimately lead to the build-up of ROS, which generates oxidative stress, possibly causing cell death, most often via necrosis, but also through regulated cell death pathways. Recent evidence points to the stronger role of PDT type I processes in causing definitive damage in biological structures [13]. Figure 34.1 shows the photochemical processes that underlie PDT in transition metal-based photosensitizers containing 6 d electrons in their valence shell, such as ruthenium(II) or iridium(III). Note that the layout of Fig. 34.1 is fairly generic and does not differentiate between the roles of type I and type II, giving similar credit to both, without defining main biological targets, which are necessary to obtain a certain outcome. Thus, the mechanistic knowledge and the development of photosensitizers (hereafter, PS) that are intrinsically more efficient, seems to be the way to improve the PDT and make it more accepted in the medical environment.

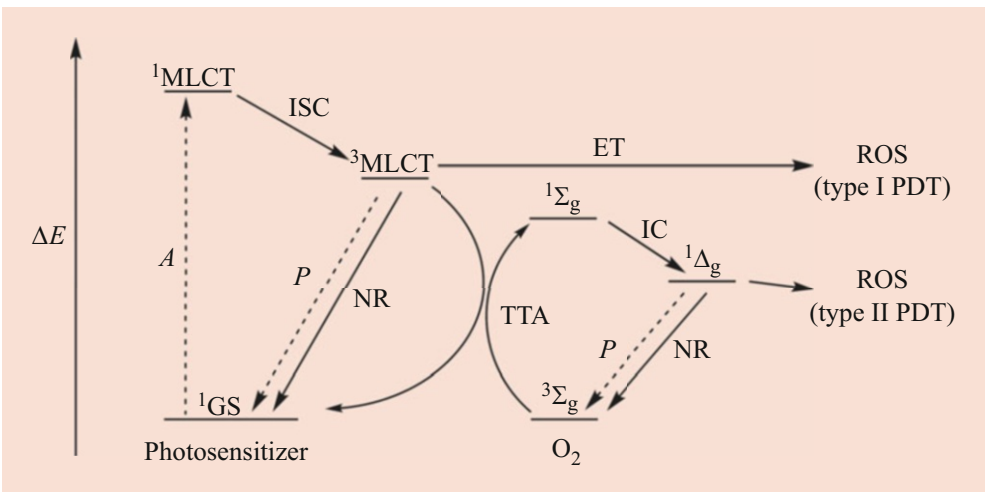

Fig. 34.1 Jablonski diagram of the principal photophysical and photochemical pathways in type I and type II photodynamic therapy (PDT) with d^6 transition metal-based compounds. Dashed arrows depict radiative transitions. Abbreviations: *A* absorption, *P* phosphorescence, NR non-radiative relaxation, GS ground state, MLCT metal-to-ligand charge transfer excited state, ISC intersystem crossing, IC internal conversion, TTA triplet-triplet annihilation, ET electron transfer, ROS reactive oxygen species

34.2.2 PDT: A Short History

Raab was the first to describe the photodynamic effect by observing that the combination of a dye (eosin) and light promoted the death of a microorganism (paramecium) [14]. One of the first published examples of photodynamic therapy is from 1903, when the same combination of the photosensitizer eosin and light was used to treat skin cancer [15]. After extensive research, it was proposed that this phenomenon was due to an energy transfer process from the fluorescent dye to molecular oxygen, forming singlet oxygen. Thus, the photodynamic process was established as a process highly dependent on singlet oxygen formation [16]. Therefore, it was reasoned that other dyes that generate singlet oxygen would also be potential anticancer agents, and the most obvious candidate was porphyrin.

Oxygen in its singlet-excited state ($^1O_2$) is a highly oxidative species, significantly more electrophilic than $^3O_2$, reacting rapidly with different organic substrates and exhibiting strong cytotoxic effects [11]. In mammalian physiology, $^1O_2$ present both positive and negative effects because it can act as a signaling and therapeutic molecule with antitumor and antimicrobial effects, but it can also directly cause cellular damage by rapidly oxidizing cellular components, including proteins and lipid membranes. For this reason, it has been implicated in many disorders caused by oxidative stress, such as aging and neurological diseases [17]. This contrasting behavior is controlled by the local concentration of singlet oxygen in the tissue and the biologic access of species that leads to ROS, namely, superoxide, $O_2^{\bullet -}$, hydroxyl radical, $OH^{\bullet}$, and hydrogen peroxide, H_2O_2 [18]. Singlet oxygen is also responsible for the effects of UVA and visible light in skin photosensitivity [19]. It is a diffusing species, but it has a relatively short lifetime (approximately 40 ns) and displays a radius of action in the order of 0.01–0.16 μm, which does not present a risk of toxicity to the microenvironment, except in the immediate vicinity of where it has been generated [20].

The great breakthrough in the field of PDT came more than half a century after its discovery, with the development of some of the major current clinically-used PDT agents. The photodynamic action of a mixture of porphyrins was tested against many neoplastic lesions. The studies demonstrated a strong phototoxicity and a high affinity for tumor tissue [21]. In 1970, Dougherty and collaborators demonstrated the efficacy and safety of this methodology in patients with skin tumors. Based on these results, porphyrin entered clinical trials and was approved in 1995 labeled as Photofrin® (porfimersodium) for the treatment of different tumors. Despite having been introduced to the market almost 20 years ago, Photofrin® remains among the most commercialized light-based cancer treatment drug worldwide. Its success is due to optimal responses in a high percentage of patients, with low recurrence, and cosmetic outcomes superior to those of traditional treatments.

Recognition of porphyrin-based photosensitizers has motivated research in this field. New generations of photosensitizers have been investigated. Many new drugs are commercially available, e.g., Levulan® (5-aminolevulinic acid) and Metvix® (methyl aminolevulinate), which are porphyrin precursors, and Foscan® (temoporfin), a chlorin compound. Many more are in clinical trials: synthetic hypericin (anthraquinone), phthalocyanine-4 (phthalocyanine), and chlorin e6-PVP (chlorin) [22–24]. These photosensitizers are mainly based on organic cyclic tetrapyrroles, which efficiently absorb green or red light in their Q absorption bands [12]. Unfortunately, some of these compounds are poorly soluble in aqueous media, and remain present in the body until long after the treatment.

Metallated tetrapyrrolic photosensitizers, based on zinc (II), tin(IV), palladium(II), and lutetium(III), have also been developed [11, 12, 25]. They offer a number of advantages over classical, organic PDT photosensitizers, such as increased water solubility, reduced photobleaching, low dark toxicity, highly efficient ISC due to the heavy metal ion, and long-lived triplet excited states. Besides tetrapyrroles, polypyridine complexes are also thermally and photochemically very stable in solution. Consequently, low spin d^6 – Ru(II), Os(II), Rh(III), Ir(III) – and low spin d^8 – Pt(II), Pd (II) – complexes have received most attention in the recent years. The full occupation of their d orbitals make them kinetically stable and thermodynamically inert; consequently, ligand dissociation and toxicity due to release of heavy metal into biologic medium is limited. The inertness of these complexes to ligand dissociation in both ground and excited states enables investigation of intra- and inter-molecular electron transfer reactions without interference of ligand exchange. Furthermore, the optical properties of these complexes relative to biological applications benefit from a large Stokes shift, which precludes self-quenching, and from long emission lifetimes, which enables direct imaging of biologic media without interference by the fluorescence of biomolecules [26, 27].

As ruthenium(II) polypyridyl complexes are also known for their excellent (photo)chemical stability and ability to sensitize the generation of singlet oxygen, they have also been very popular in the development of novel PDT agents. The group of Gasser developed a range of ruthenium PDT photosensitizers, based on the $[Ru(bpy)_2(dppz)]^{2+}$ (bpy = 2,2′-bipyridine, dppz = dipyrido[3,2-a:2′,3′-c]phenazine) scaffold, in which they modified the dppz ligand [28]. Especially the methoxy- and amine-modified complexes (see Fig. 34.2c, d) showed promising results, with a toxicity increase (or phototoxicity index, PI) in HeLa cervical cancer cells of 42 and >150 times upon irradiation with blue light, respectively [28, 29]. Arguably the most successful Ru^{2+}

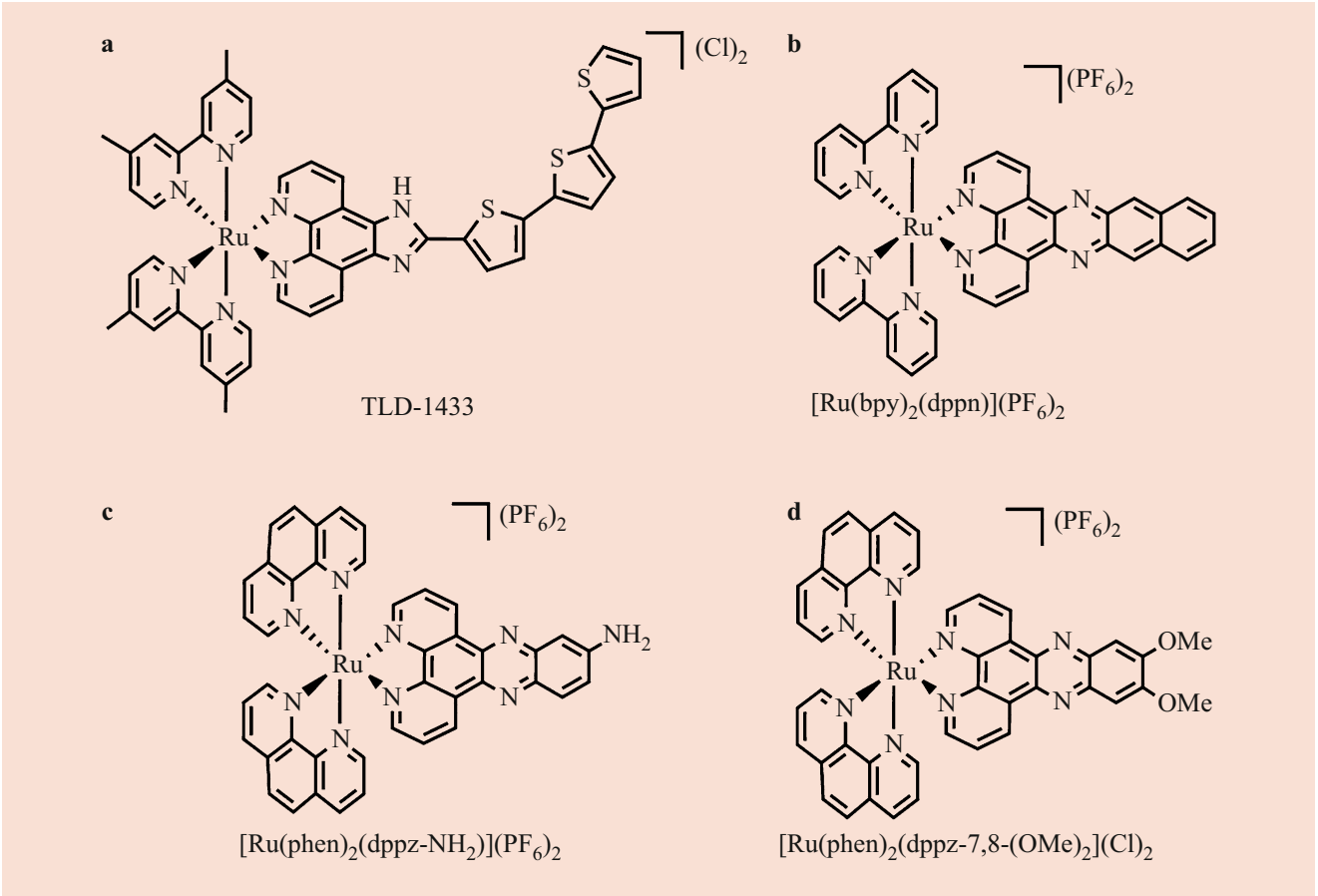

Fig. 34.2 Examples of ruthenium-based PDT photosensitizers, developed by the groups of Gasser and McFarland

PDT photosensitizer to date is TLD-1433 (Fig. 34.2a), developed by the group of McFarland. The drug has displayed PI values over 10,000, and in 2018, it has completed Phase Ib clinical trials for the treatment of non-muscle invasive bladder cancer (NMIBC) [30, 31].

Indeed, much attention has been directed to transition metal complexes as photosensitizers, in particular to polypyridine complexes containing low energy π* orbitals, mostly because of their attractive pharmacologic, spectroscopic, and electrochemical properties [32]. These complexes display strong visible absorption arising from metal-to-ligand charge transfer transition (^{1}MLCT, M,dπ → L,π*) and very weak metal-centered electronic transitions (MC, M,dπ → M,dπ*). Depending on the complex, intra-ligand (^{1}IL, L,π → L,π*) and/or ligand-to-ligand (^{1}LLCT, L,π → L',π*) charge transfer transition may also occur. Upon excitation in the ^{1}MLCT band, one electron of the metal dπ-orbital is excited to ligand-centered orbital (L,π*) and inter-system crossing (ISC) leads to 100% population of the long-lived emissive redox-active ^{3*}MLCT. The complex in its photo-excited states (^{3}*M^{n+}-$L^{\bullet-}$) is more oxidizing and more reducing than in its ground state (^{1}*M^{n+}-L), enabling participation in photoinduced electron transfer reactions in competition to energy transfer or nonradiative pathways [33, 34].

The emissive ^{3}MLCT excited state is very sensitive to the presence of $^3O_2$, which quenches its emission. For many complexes, emission suppression leads to nonradiative decay without parallel reactions with oxygen. However, interaction between the complex in its excited state and $^3O_2$ may activate the oxygen, and this process has two important consequences to PDT. First, formation of $^1O_2$ by energy transfer processes from ^{3}MLCT to $^3O_2$ (PDT type II), and secondly, the enhanced redox properties of these complexes in the excited states enable activation of ROS by an electron transfer process from its luminescent redox ^{3}MLCT (PDT type I). Cancer cells exhibit increased expression of the heme-transport protein (HCP1), which is associated with increased hypoxia, compared with normal cell. The presence of ROS derived from mitochondria would diminish the HCP1 expression by increasing the ROS scavenger enzyme manganese superoxide dismutase [35]. This process facilitates photodynamic activity in hypoxic conditions (see further discussion below).

Among the polypyridine metal complexes, the parent complex tris(2,2′-bipyridine)ruthenium(II) ($[Ru(bpy)_3]^{2+}$,

hereafter Rubpy) and its derivatives have been recognized and extensively investigated as PDT photosensitizers [8, 36, 37]. Similarly to porphyrins, the Rubpy complexes show advantageous pharmacological properties, such as phototoxicity, especially against cancer [37–39]. In contrast, these complexes present poor cell selectivity compared with that of porphyrins, which preferably accumulate in tumor cells [40, 41].

34.2.3 Chemical and Biological Mechanisms in PDT

Recent reports in terms of mechanistic understanding of PDT have shown the important role of direct-contact reactions in terms of causing irreversible damage in biological structures [13]. Contact-dependent reactions cause more severe damage to biomolecules and with a better chance of achieving specific targets when compared to the generated diffusive species, for example, singlet oxygen and anion radical superoxide. Formation of the excited triplet species is the key step in terms of PS performance. Contact-dependent reactions are also fundamental for generating vascular damage in a new modality of treatment of prostate cancer, which has already received approval in several countries [42]. In this case, the photosensitizer (or PS) is incorporated into proteins (human serum albumin) and there is a quick electron transfer reaction forming radical species in the hydrophobic protein pocket (Fig. 34.3) [43].

Membrane damage is of particular importance to the PDT efficiency, since photosensitizers accumulating in cell membranes and/or organelles are generally more efficient [44, 45]. The mechanism by which photosensitized oxidization causes membrane leakage has been recently described [13]. Photoinduced lipid peroxidation is usually initiated by the reaction between singlet oxygen and lipid double bond, forming a lipid hydroperoxide, which makes the membrane thinner but is not enough to make it leak. Irreversible damage occurs with the abstraction of a hydrogen atom from an unsaturated fatty acid (LH), leading to the formation of a lipid radical (L•) with consequent formation of peroxyl and alkoxyl radicals. These further react with the PS triplet species or naturally rupture the carbon chain by the known β-scission mechanism, forming truncated lipid aldehydes, which are the molecules responsible for the beginning of the leakage process. The fact that the most relevant damage occurs due to contact-dependent reactions, indicates that the damage can be confined to the nanometer scale.

Metal based complexes have the potential to act as PSs that do both, i.e., generate singlet oxygen, as well engaging in type I reactions. Many efforts have centered on the design of a functional porphyrin-Ru(bpy) hybrid system aiming to combine their pharmacologic properties into a single molecular system. Organic-inorganic hybrid materials consist of a combination of two or more active compounds covalently linked by a bridge, producing dyads, triads, etc. [46] Thus, the combination of the two moieties may trigger one target in preference to the other or bring new possibilities, such as dual targeting functions. This approach has been successfully applied in PDT and a synergistic effect is expected and indeed observed in molecular hybrids comprising a tetra(4-pyridyl) porphyrin scaffold linked to pendant Ru(II)-bipyridine, Ru (II)-arene. Os(III)-arene, Os(III)-bipyridine, Rh(III)-bipyridine, Rh(III)-arene moieties [47–49].

The uptake and sub-cellular localization of the photosensitizer in the biological system is another important challenge to be overcome. In general, organic photosensitizers accumulate preferentially in mitochondria, lysosomes, the endoplasmic reticulum (ER), the Golgi apparatus, and the plasma membrane [11]. The sites of intracellular damage of the PS define the type and intensity of the cellular responses, varying from survival to senescence, regulated cell death, or even unregulated necrosis [50, 51]. Positively charged photosensitizers accumulate in mitochondria [52], and the cationic Rubpy complexes are no different [28, 53, 54]. For example, in *meso*-tetra(4-pyridyl) porphyrin modified with four pendant Ru(II)-arene moieties,

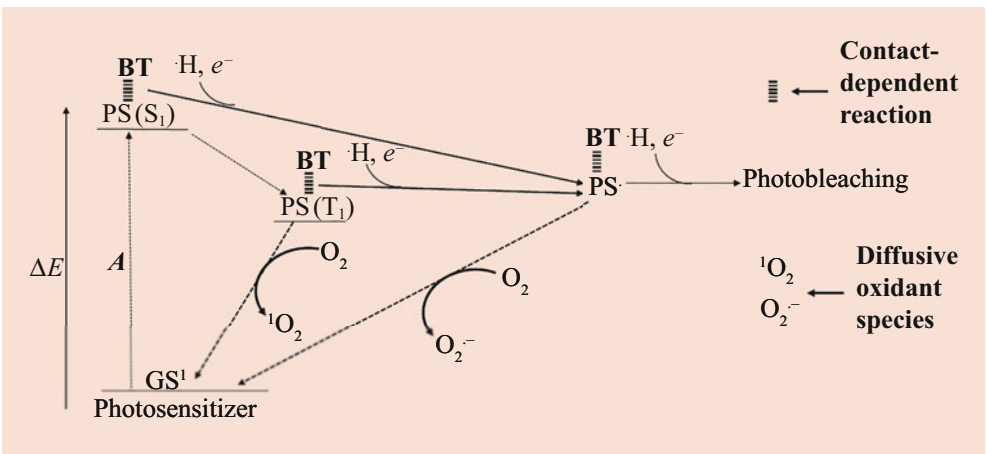

Fig. 34.3 Symbols are the same as in Fig. 34.1, PS refers to the photosensitizer, which can be either in the singlet (S_1) or in the triplet (T_1) excited state. BT is a biological target and $O_2^{\bullet -}$ is the superoxide anion radical

the presence of the Ru(II) moiety increases the hydrophilicity and cellular uptake of the PS in human Me300 melanoma cells. The hybrid material was found in non-lysosome granular structures at the cytoplasmatic domains. Further, the addition of the Ru(II) moieties resulted in a highly photocytotoxic hybrid system [55].

A critical element in planning organic-inorganic hybrid systems is the design of bridges to connect the different units and permit synergistic action. Wong and coworkers demonstrated a straight dependence of PDT treatment to physicochemical and electronic properties of the bridge ligand in a series of Rubpy complexes appended to a porphyrin [48]. Phototoxicity of 1 μM was obtained for a few compounds when yellow light was applied (500–600 nm) at different doses (from 2 to 11.5 J cm^{-2}). Confocal fluorescent microscopy revealed that many of these hybrid systems were incorporated into cells and enabled correlation of their sub-localization (cytoplasm, mitochondria, and lysosome) with cell mortality due to $^1O_2$-induced oxidative damage. In this study, the hydrophobic phenylethynyl ligand was the most promising linker. Following these studies, a number of porphyrins, chlorins, phthalocyanines, and corrole macrocycles functionalized with transition metal complexes have been developed, as well as other related systems designed to improve photodynamic therapy for cancer [48, 56–60].

The dyad $[(phen)_2Ru(II)(pPDIp)]^{2+}$, where pPDIp is a phenanthroline ligand functionalized with a pendant perylene group, also triggers the population of the triplet excited state of the perylene group that decays with a lifetime of 1.8 μs [61]. Upon light irradiation, the dyad produces large amounts of $^1O_2$, while presence of the metal ion leads to improved solubility in physiologic medium. Notably, in a neutral buffer solution (pH = 7.4) without oxygen bubbling and under green light irradiation (0.41 J/cm^2) $[Ru(phen)_2(pPDIp)]^{2+}$ ([Ru] = 10–150 μM) was found to generate a singlet oxygen concentration of 0.03–0.47 μM. No effects on B16F10-Nex2 murine melanoma cell viability were observed up to 10 μmolL^{-1} of dyad in the dark. However, under green LED illumination (dose = 0.41 J/cm^2), a strong photocytotoxic effect is detected, displaying IC_{50} = 1.2 μM [62].

Subcellular localization is perhaps the most important factor controlling the efficiency of PDT, since it defines the site of photodamage because $^1O_2$ can only diffuse up to 100 nm and contact dependent reactions are based on molecular level interactions [44, 63, 64]. More important is the fact that the site and the amount of photo-oxidative damage will define the cell death mechanism taking place. Positively charged photosensitizers are located mainly in mitochondria, since they are electrostatically attracted by the negative electrochemical transmembrane potential, leading to mitochondrial concentrations that are up to 100 times higher than in the cytoplasm [52]. On the other hand, anionic PSs tend to locate in lysosomes after their cellular absorption by endocytosis. PSs that are absorbed by endocytosis can be found in lysosomes because the endoscopes follow intracellular trafficking and end up merging with lysosomes. In addition, dyes with weak base amines may accumulate in these organelles. This happens because they enter the lysosomes in their uncharged form, but are trapped once they are protonated due to the low pH within this organelle [50].

Figure 34.4 describes the main scenarios in terms of photoinduced cell death, i.e., unregulated necrosis, apoptosis and autophagy. Cells can also simply survive by activation of survival mechanisms, or they can enter in senescence, a process in which the cell is not dead but it is not reproducing either. This picture is simplified, since there are several other scenarios of cell death, such as regulated necrosis and necro-apoptosis; it merely serves to explain our main intracellular targets, which are mitochondria and lysosomes. Note that it is not necessary to damage all targets to trigger cell death. High doses of PDT in organelles and photodamage of the plasma membrane cause ATP depletion, resulting in unscheduled cell death or unregulated necrosis. The production of reactive species in mitochondria and/or BCL-2 protein damage causes the release of cytochrome c and other apoptogenic factors, which classically trigger the caspases cascade, resulting in apoptosis. Low doses of PDT in organelles (mitochondria, ER, and lysosomes) can activate the autophagic process in an unbalanced manner (inhibition of the flow), resulting in autophagic cell death. Inhibition of the mTOR complex by photooxidation can also trigger autophagy as a cell death pathway. The parallel damage in lysosomes and mitochondria was shown to be a very efficient way to induce photoactivated cell death, because there is activation of mitophagy at the same time as interruption of the autophagic flux by the lysosome damage [51].

There are plenty of scientific studies correlating intracellular loci of organic PSs with the mechanisms of cell death they activate, allowing the establishment of structure versus activity correlations (see above). However, in the field of metallic complexes, the scenario is different and there is not yet enough evidence to establish clear correlations between the molecular structure and the photoinduced mechanisms of cell death. Since most complexes have some sort of dark cell toxicity, there is a growing body of evidence correlating the intracellular localization of these complexes to the induction of different mechanisms of cell death. Many complexes are known to target mitochondria or the ER have shown to induce apoptosis [65, 66]. It must be said that the concentrations used (micromolar to millimolar) are relatively large to allow for accurate information on the main sites of intracellular accumulation of these molecules. This is also true for inorganic complexes developed for PDT applications. In terms of mechanisms of regulated cell death, although some articles mention signs of apoptosis or necrosis, the evaluations were performed in a level that does not really permit details of the specific mechanisms of regulated cell death.

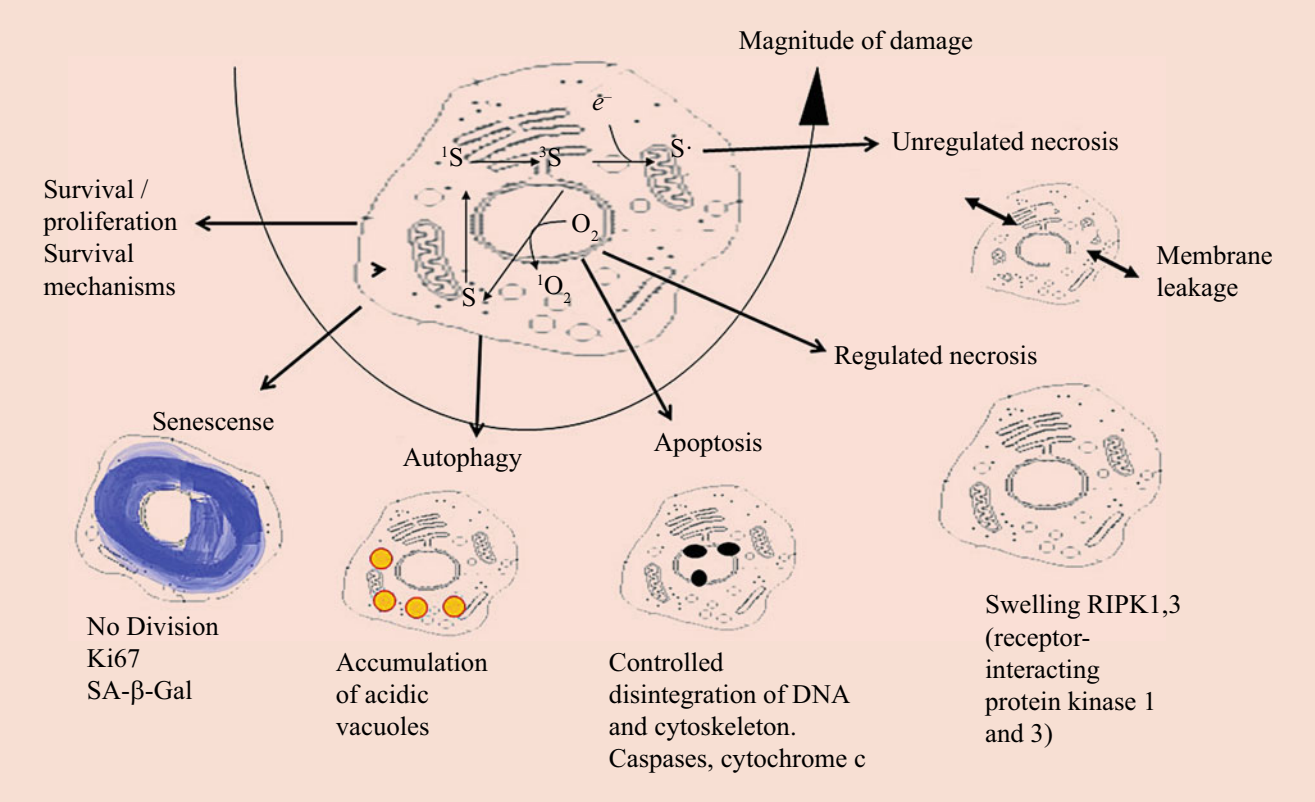

Fig. 34.4 Regulated and unregulated mechanisms of cell death. The increased severity of damage disable the survival mechanisms, allowing activation of regulated cell death mechanisms or of senescence. High levels of damage do not allow activation of any of these mechanisms, and thus cells die by uncontrolled necrosis, in which low ATP levels and damage to the cytoplasmic membrane simply make the cell lose its content to the microenvironment

Nevertheless, metal-centered inorganic complexes offer great potential in the development of improved PDT photosensitizers, and there are well-written reviews describing the recent development in the field [8].

The literature contains several reports discussing the role of intracellular localization of inorganic PDT complexes on their efficacy. Since organelle-targeted photosensitized oxidation represents a promising approach in PDT, it is important to understand whether the concepts of the intracellular localization well-described by organic dyes, holds for inorganic complexes as well. Initial description of intracellular localization of ruthenium complexes was shown by a group specialized in dye-sensitized solar cells that tested the photosensitizers. They showed that the hydrophobic complex showed clear accumulation in the cytoplasmic membrane [67]. A clear distinction of intracellular localization was shown by the Glazer group. Complexes having two positive charges localize in mitochondria and those with five negative charges accumulates in lysosome [68]. Similar differential accumulation in mitochondria and lysosomes was obtained by modifying a tetraphenylphosphine group to the iridium(III) complex, which drives accumulation in mitochondria and a simple alkyl derivative, which makes the complex accumulate in lysosomes [69]. TLD-1433, the first Ru(II)-based photosensitizer for PDT to enter a human clinical trial, seems to mainly accumulate in lysosomes [70]. Based on these results already published, it is possible to say that the localization concepts that have been raised by organic molecules also work for inorganic complexes. The usual entrance route in cells is by endocytosis. If there is no route of escape or signal to localize in other organelles, molecules will accumulate in lysosomes. Highly polar and charged molecules will remain in lysosomes as reported for some polypyridyl complexes [71]. Less charged ruthenium(II) polypyridyl complexes were shown to accumulate in mitochondria, following the negatively charged potential of breathing mitochondria [72]. Ruthenium complexes modified with specific mitochondria-targeting groups increases accumulation in this organelle and increases the efficiency of cell death by ten-fold [73].

34.3 Photoactivated Chemotherapy

As photodynamic therapy relies on the presence of molecular dioxygen, its efficacy is severely limited in the hypoxic environment that is often found in large tumors with poor

vascularization [74, 75]. This phenomenon sparked the development of oxygen-independent photo-activatable anticancer drugs, often called photoactivated chemotherapy (PACT) prodrugs. Although there are many examples of organic PACT prodrugs [5], we will limit ourselves here to those that involve metal ions.

The activation of PACT prodrugs always involves an irreversible structural change to the prodrug, typically through one of the following three photochemical reactions, photoreduction, C–C bond cleavage, or ligand substitution. Photoreduction reactions are mainly observed in Pt^{4+} and Co^{3+} compounds, such as *cis,trans*-[Pt(en)$(I)_2(OAc)_2$] (en = 1,2-ethylenediamine), which form toxic Pt^{2+} or Co^{2+} species upon reduction [76]. An example of the use of C–C bond breakage was reported by the group of Gasser, who attached the common organic *o*-nitrobenzyl caging moiety to one of the ligands of a ruthenium polypyridyl complex to decrease its dark toxicity. Irradiation of the complex with UV light led to cleavage of the *o*-nitrobenzyl group and a more-than-sixfold increase in cytotoxicity [77].

An overwhelming majority of PACT prodrugs has a ligand substitution activation mechanism, and the field is dominated by photo-activatable ruthenium(II) polypyridyl complexes. Most of these compounds have a broad visible-light absorption band. Excitation of the complex in this band leads to population of the singlet metal-to-ligand-charge-transfer (^{1}MLCT) excited state (Fig. 34.5), and via intersystem crossing, to population of the associated triplet state (^{3}MLCT). From this triplet state, the complex normally either relaxes back to the ground state, nonradiatively or via the emission of a photon (phosphorescence), or reacts with oxygen to form singlet oxygen and perform PDT. However, in some complexes, notably those involving sterically hindering ligands or distorted coordination spheres, a high-energy triplet metal-centered state (^{3}MC) becomes thermally accessible. This state is dissociative in nature, as one of the electrons resides in an antibonding dσ* orbital, and thus photochemical population of these states leads to substitution of one of the ligands for a solvent molecule. The energy level of this ^{3}MC state, and thus the photolability of the ruthenium complex, can be tuned by changing the electronic and steric properties of the ligands [78], which are usually bound to the ruthenium ion via thioether, sulfoxide, nitrile, amine, or pyridine functional groups [79–85].

Upon ligand photosubstitution two or more fragments are obtained, i.e., a metal-based fragment and one or more photoreleased ligands, all of which can be biologically active components. In many of the known PACT complexes, the photoreleased ligand is the biologically active component, e.g., in the work of the Etchenique group, who demonstrated the release of several neurotransmitters, such as γ-aminobutyrate ($GABA^-$) from [Ru(bpy)$_2$(PPh_3)(GABA)](PF_6) (Fig. 34.6a) or *cis*-[Ru(bpy)$_2$$(GABA)_2$] [80, 86]. Other examples of the uncaging of organic, biologically active molecules from ruthenium complexes were reported by the groups of Turro [83, 87], Kodanko [88], and Renfrew [89, 90]. It is worth mentioning that having both ligand exchange (i.e., PACT) and singlet oxygen generation (i.e., PDT) mechanisms upon light irradiation of a single ruthenium complex, may act synergistically [91].

The use of sterically demanding ligands was introduced by Sauvage in the design of metal-based catenanes, rotaxanes, and molecular machines, to induce photolability in ruthenium polypyridyl complexes by disturbing the octahedral geometry around the ruthenium ion [92–96]. Based on this idea, in 2012, Glazer et al. reported the photoactivation of [Ru(bpy)$_2$(dmbpy)]Cl_2, bearing the sterically straining 6,6′-dimethyl-2,2′-bipyridine (dmbpy) ligand, which is released upon irradiation (Fig. 34.6, bottom)

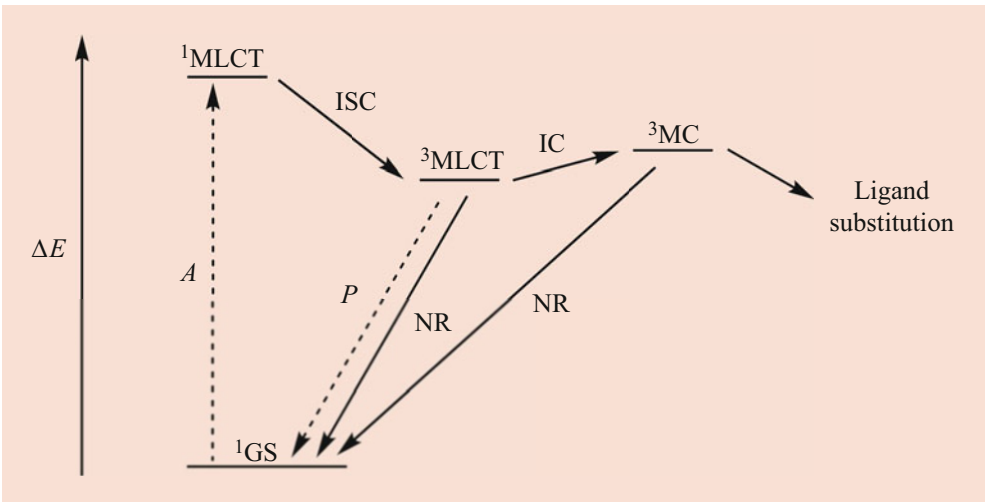

Fig. 34.5 Jablonski diagram of the principal photophysical and photochemical pathways involved in photosubstitution reactions in ruthenium(II) polypyridyl complexes. Dashed arrows depict radiative transitions. Abbreviations: *A* absorption, *P* phosphorescence, NR non-radiative relaxation, GS ground state, MLCT metal-to-ligand charge transfer state, MC metal-centered state, ISC intersystem crossing, IC internal conversion

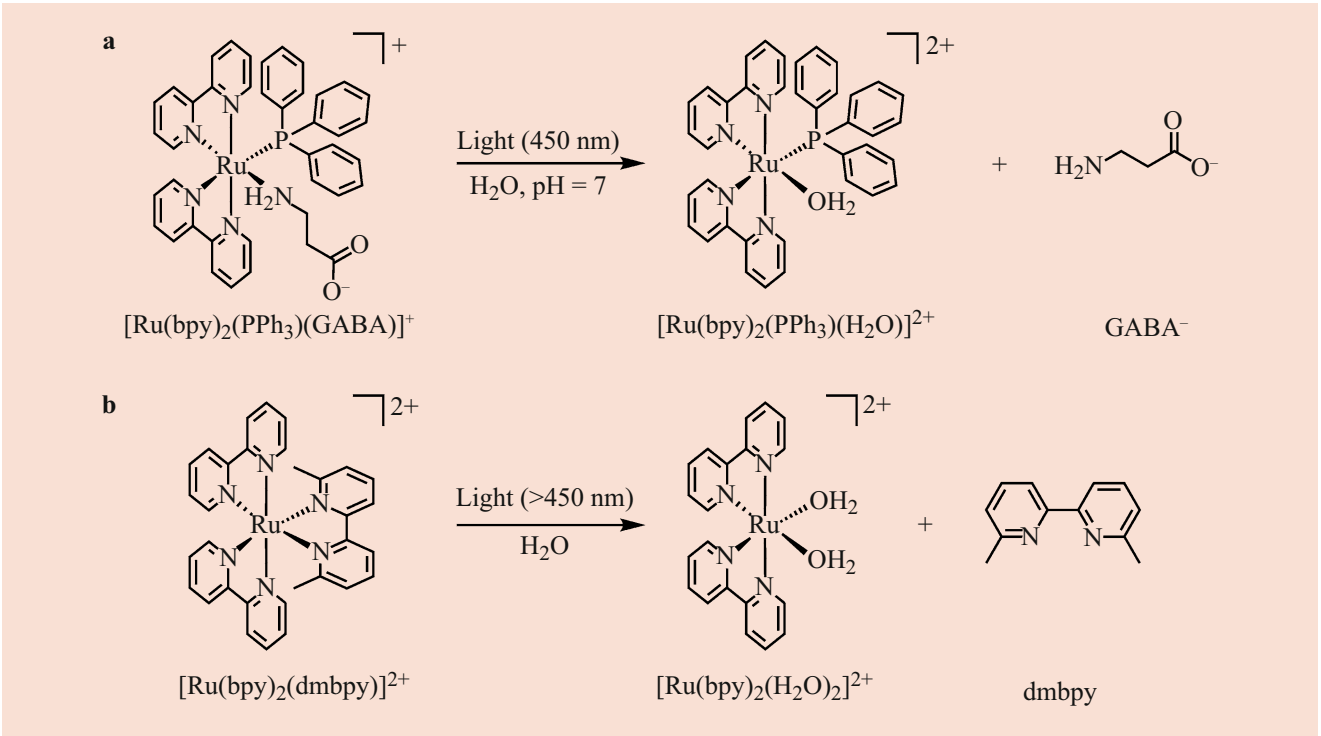

Fig. 34.6 Photosubstitution reactions in ruthenium-based PACT prodrugs developed by the groups of Etchenique (**a**) and Glazer (**b**), showing the release of the caged neurotransmitter γ-aminobutyrate ($GABA^-$) and the sterically demanding ligand 6,6′-dimethyl-2,2′-bipyridine (dmbpy), respectively

[81]. Glazer hypothesized that the observed phototoxicity stems from the ruthenium photoproduct *cis*-$[Ru(bpy)_2(H_2O)_2]^{2+}$, rather than the dmbpy ligand. However, in 2017, the groups of Bonnet and that of Khnayzer independently reported that the light-induced toxicity of this complex can be fully attributed to the photoreleased dmbpy ligand (IC_{50} = 6 μM), and that the ruthenium species are poorly taken up into cells, and are thus poorly toxic [97, 98]. Interestingly, a similar photosubstituted ligand, tmbpy (5,5′,6,6′-tetramethyl-2,2′-bipyridine), was later found to be nontoxic (IC_{50} > 30 μM), while its photolabile complex $[Ru(bpy)_2(tmbpy)]^{2+}$ showed a significant increase in toxicity towards leukemic HL60 cells upon the photorelease of tmbpy ($IC_{50,dark}$ >100 μM, $IC_{50,blue\ light}$ = 1.8 μM) [99]. Possibly, the difference in their biological activities can be explained by a difference in lipophilicity between the two beforementioned complexes, leading to a difference in cellular uptake. Over the past few years, the Bonnet group also published several examples of PACT prodrugs in which the ruthenium fragment is clearly the active species, namely, $[Ru(Ph_2phen)_2(mtmp)]Cl_2$, and *trans*-$[Ru(H_2biqbpy)(dmso)Cl]Cl$, shown in Fig. 34.7 [98, 100]. After irradiation, these compounds form the more toxic species *cis*-$[Ru(Ph_2phen)_2(H_2O)_2]^{2+}$, and *trans*-$[Ru(H_2biqbpy)(H_2O)_2]^{2+}$, respectively.

34.4 How to Quantify the Efficacy of Light Activation in PDT and PACT?

In anticancer phototherapy, the primary aim is to kill the light-irradiated cancer cells while leaving non-irradiated, healthy tissue intact. The parameter to optimize is hence the difference in toxicity between dark and light-irradiated conditions. The most used quantification for phototoxicity, called the photoindex (PI), is usually measured in vitro. The PI of a given compound is defined, in a set of conditions, as the ratio between the effective cell-killing concentration of this compound in the dark ($IC_{50,dark}$), and that measured after light irradiation ($IC_{50,light}$, see Fig. 34.8). When no $IC_{50,dark}$ can be derived from 2D cell monolayer toxicity data because the dark toxicity of a compound is too low, then the highest concentration used in the dark toxicity assay is taken as superior limit for $IC_{50,dark}$, and the corresponding minimum PI of the compound is reported. Basically, the PI measures how much more toxic a compound becomes after light irradiation, compared to dark conditions. Obviously, good PDT and PACT compounds have low dark toxicity and high light toxicity, i.e., high photoindices. However, other parameters, such as drug cellular uptake or the dark toxicity itself, are very important as well for in vivo efficacy and for further

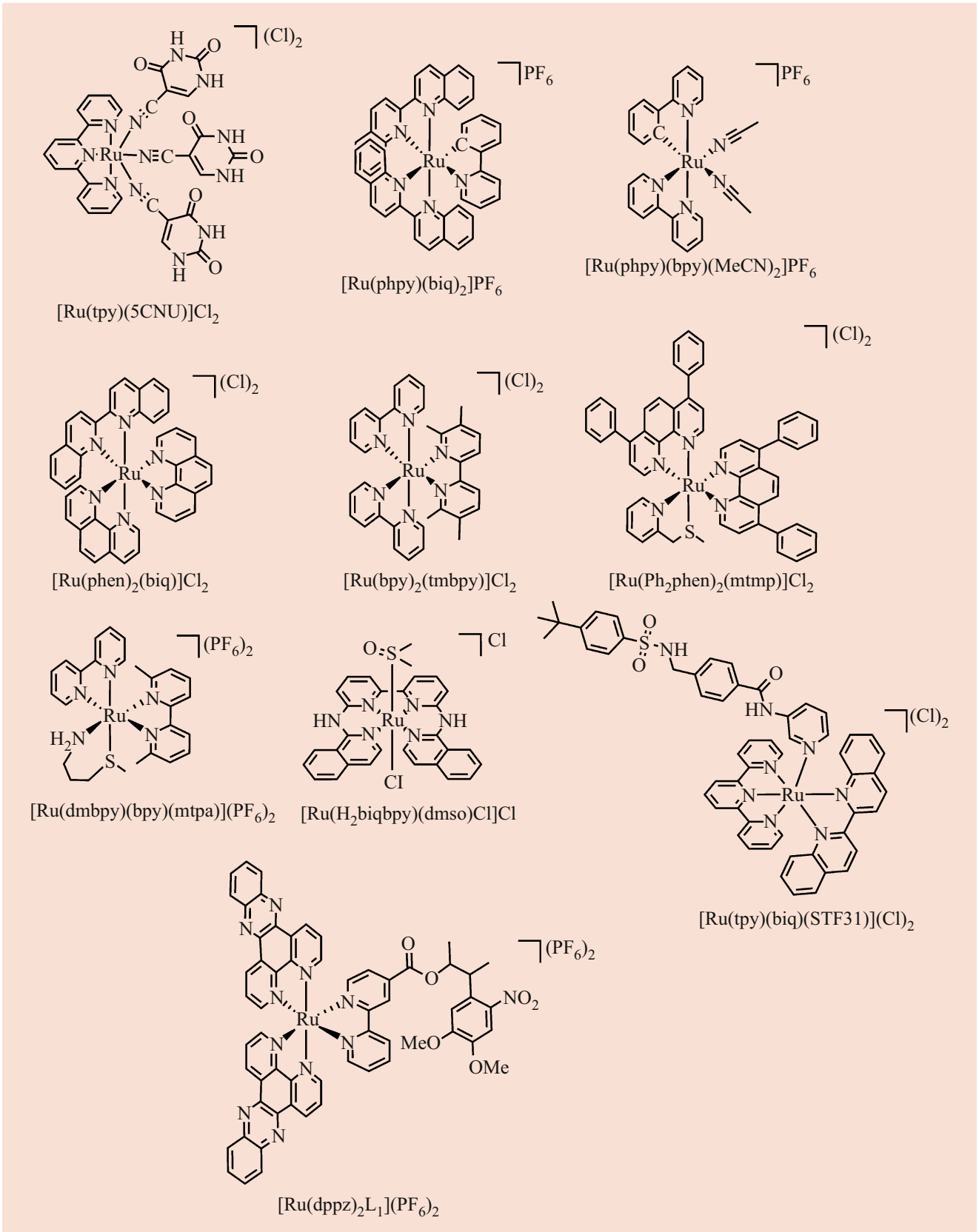

Fig. 34.7 Examples of ruthenium-based PACT prodrugs

clinical applications. It should also be noted that the PI of a compound is very specific for the conditions of the cytotoxicity assay, and will change upon alteration of one of many variables, such as the light dose, the light intensity, the irradiation time, the irradiation wavelength, the drug incubation time before irradiation, and the cell type. As an illustration, Table 34.1 lists a set of ruthenium-based PDT and PACT compounds in different conditions, together with photoindices reported in the literature. The chemical formulae of these compounds are shown in Fig. 34.2 for PDT and Fig. 34.7 for PACT.

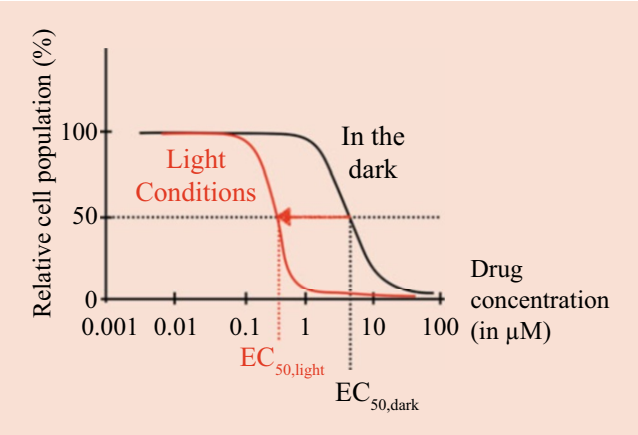

Fig. 34.8 Dose-response curves of a photoactivated (PDT or PACT) compound in 2D cell monolayer model of cancer, defining the effective cell-killing concentration in the dark and under light irradiation. The arrow represents the shift of the dose-response curve upon light irradiation

34.5 PDT or PACT?

When a light-sensitive compound is phototoxic in cancer cells, i.e., when light irradiation leads to enhanced cytotoxicity (PI >1), it is not always straightforward to know whether the compound is a PDT or PACT compound. Several experiments can be performed to discriminate between these modes of action.

Firstly, the singlet oxygen generation quantum yield φ_Δ can be measured in air, either via direct spectroscopic detection of its 1275 nm phosphorescence ($^1O_2 \rightarrow {}^3O_2 + h\nu$) or using a chemical probe specific to $^1O_2$, such as tetrasodium 9,10-anthracenediyl-bis(methylene)-dimalonate. Obviously, bad $^1O_2$ generators have less chance to become phototoxic in cells via a PDT type II mechanism. It is tempting, yet incorrect, to assume that all such compounds are PACT prodrugs, as PDT type I compounds, which generate ROS

Table 34.1 Photoindices (PI) of a selection of ruthenium-based PDT and PACT compounds measured in 2D cell monolayers. The effective cell-killing concentrations (IC_{50}) in the dark and under light irradiation, as well as the light dose and irradiation wavelength (λ_{irr}) used for the IC_{50} measurements, are also reported

Compound	PDT or PACT	Cell line	$IC_{50,dark}$ (μM)	$IC_{50,light}$ (μM)	PI	Light dose (J/cm^2)	λ_{irr} (nm)	References
$[Ru(tpy)(5\text{-}CNU)](Cl)_2$	PACT	HeLa	>300	156	>1.9	n.r.	>400	[87]
$[Ru(phpy)(biq)_2]PF_6$	PACT	HeLa	7	1	7	n.r.	633	[101]
$[Ru(bpy)(phpy)(MeCN)_2]PF_6$	PACT	OVCAR-5	1.0	0.070	14	n.r.	690	[102]
$[Ru(bpy)_2(dmbpy)](Cl)_2$	PACT	A549	150	1.1	136	n.r.	>450	[81]
$[Ru(bpy)_2(tmbpy)](Cl)_2$	PACT	HL-60	>100	3.9	26	28	>600	[99]
$[Ru(phen)_2(biq)](Cl)_2$	PACT	HL-60	47	2.4	20	n.r.	>450	[103]
$[Ru(Ph_2Phen)_2(mtmp)](Cl)_2$	PACT	A549	2.7	0.48	6.0	6.5	454	[98]
$[Ru(dmbpy)(bpy)(mtpa)](PF_6)_2$	PACT	A549	110	14	8.0	6.5	454	[104]
$[Ru(H_2biqbpy)(dmso)Cl]Cl$	PACT	A549	9.30	0.58	16	75	520	[100]
$[Ru(tpy)(biq)(STF31)](Cl)_2$	PACT	A431	23.6	7.1	3.3	21	628	[105]
$[Ru(tpy)(biq)(STF31)](Cl)_2$	PACT[a]	A431	34.6	9.6	3.6	21	628	[105]
$[Ru(dppz)_2(L_1)](PF_6)_2$	PACT	HeLa	>100	17	>5.9	2.6	350	[77]
TLD-1433	PDT	HL-60	>300	16	19	7.0	400–700	[31]
TLD-1433	PDT	HL-60	>300	0.2	>1500	100	400–700	[31]
$[Ru(bpy)_2(dppn)](PF_6)_2$	PDT	HL-60	282	0.30	931	100	400–700	[106]
$[Ru(bpy)_2(dppn)](PF_6)_2$	PDT	HL-60	137	1.5	91	100	625	[107]
$[Ru(bpy)2(dppz\text{-}NH_2)](PF_6)_2$	PDT	HeLa	>300	2.0	>150	9.7	420	[28]
$[Ru(phen)_2(dppz\text{-}7,8\text{-}(OMe)_2)](Cl)_2$	PDT	HeLa	37	3.1	12	9.3	420	[108]
$[Ru(phen)_2(dppz\text{-}7,8\text{-}(OMe)_2)](Cl)_2$	PDT	HeLab	104	9.5	11	9.9	800 [b]	[108]

n.r. non reported
[a]Measured in hypoxic conditions (1% O_2). All other data were measured using 21% O_2
[b]Two-Photon PDT in 3D tumor spheroids

via electron transfer rather than energy transfer, may be very bad $^1O_2$ generators but still show excellent PDT properties in cells. Some authors have also claimed that compounds capable of doing both photosubstitution and $^1O_2$ generation may work better for phototherapy, as synergies between PDT-based oxidative stress and ligand- or metal-based toxicity may act synergistically [109–111].

Secondly, if the PI of a compound measured in normoxic cancer cells drops dramatically when measured under hypoxic conditions, there is a high chance that the compound operates almost exclusively via a PDT type II mechanism. Notably, some compounds may have a different mode of action in different tumor environments. For example, the clinically tested compound TLD-1433 is an excellent $^1O_2$ generator under normoxia [31], but it has been shown to be an excellent ROS generator in hypoxic conditions as well [112].

In general, since PDT is a catalytic process, prolonged light irradiation should lead to increased ROS production inside the cells, and thus to an increase in PI, as light-induced ROS production is more or less the signature of the photodynamic effect. Contrarily, the PI of a pure PACT compound will not increase any further with increasing light dose once enough light has been administered to convert all of the compound to its activated form.

To further demonstrate that a photoactivated compound performs PACT, it is essential to conduct a photosubstitution assay. Basically, a combination of UV-vis spectroscopy under light irradiation, and mass spectrometry, thin layer chromatography, HPLC, and/or ^1H NMR before and after the irradiation experiment, can unravel whether ligand exchange reactions took place under the action of light. As $^1O_2$ generation and photosubstitution processes are competing pathways for the decay of the same excited states, the quantification of the inherent photosubstitution quantum yield must ideally be realized under inert atmosphere (Ar or N_2), where no $^1O_2$ generation takes place. Under normoxic conditions, both reactions ($^1O_2$ and photosubstitution) may occur at the same time, which in cells may lead to a "double mode of action," i.e., PDT and PACT simultaneously [109, 111]. PACT and PDT may even occur sequentially, if the generated photoproducts are good ROS generators where the original prodrug is not.

An additional argument that has been used in the Bonnet group to demonstrate that a photoactivated compound works via PACT [105], is that for a PACT compound the photoindex in normoxic and hypoxic cancer cells should be comparable, because photosubstitution reactions are essentially insensitive to the presence of O_2. For example, [Ru(tpy)(biq)(STF31)]Cl_2, which is a caged version of the cytotoxic NAMPT inhibitor STF31, has a PI of 3.6 in hypoxic A431 skin cancer cells, very similar to its PI of 3.3 in normoxic A431 cells (Table 34.1). It should be noted, though, that even if the ratio between $IC_{50,dark}$ and $IC_{50,light}$ remains the same when the dioxygen concentration was lowered, both $IC_{50,dark}$ and $IC_{50,light}$ were higher under hypoxia compared to normoxia, because hypoxic cells express a whole range of cell survival mechanisms triggered by the HIF1α gene [113–115].

In cells, the list of biological assay, that may be needed to unambiguously differentiate a PDT from a PACT mechanism, is difficult to establish as it cannot be exhaustive enough. As discussed, in PDT intracellular ROS usually increase upon increased light irradiation, which is a signature of the PDT effect. In PACT, the metal-based photoproduct may bind to nuclear DNA, in which case a DNA metalation assay can be realized. However, many cytotoxic metal compounds kill cells by increasing the ROS production in a light-independent manner, so that it may be tricky to distinguish the ROS directly generated by the photodynamic effect of a photostable PDT dye, from the ROS generated by the metal-based photoproduct deriving from an irradiated PACT prodrug via photosubstitution. On the other hand, PACT compounds are sometimes phototoxic because of the biological action of the photoreleased organic photoproduct (i.e., one of the ligands of the prodrug). In such a case, previous knowledge on the mode-of-action of that ligand is required to understand – and demonstrate – the reason why cells die under the combined action of light and a photoactivated prodrug. With the [Ru(tpy)(biq)(STF31)]Cl_2 compound, for example, phototoxicity comes primarily from the NAMPT-inhibiting action of the photoreleased STF31 inhibitor. Overall, distinguishing PDT from PACT requires careful examination of the biological and chemical effects of the prodrug under light irradiation altogether.

34.6 From Blue to Near-Infrared: The Phototherapeutic Window

Most metal-based phototherapeutic agents are activated with ultraviolet or high-energy visible light, ranging from UV or blue light for the photoreduction of Pt^{4+} prodrugs to the blue or green light (400–550 nm) used to drive ruthenium-based PACT or PDT. Unfortunately, this high-energy light can be harmful to cells at high light doses [116], and penetrates human tissue poorly (Fig. 34.9) [117]. In imaging applications, the use of high-energy visible light can also lead to poor contrast, caused by significant amounts of autofluorescence, i.e., emission from naturally occurring chromophores in the tissue that absorb light of the same wavelength as the dye used. For biological applications, three optimal wavelength regions, i.e., "phototherapeutic windows" have

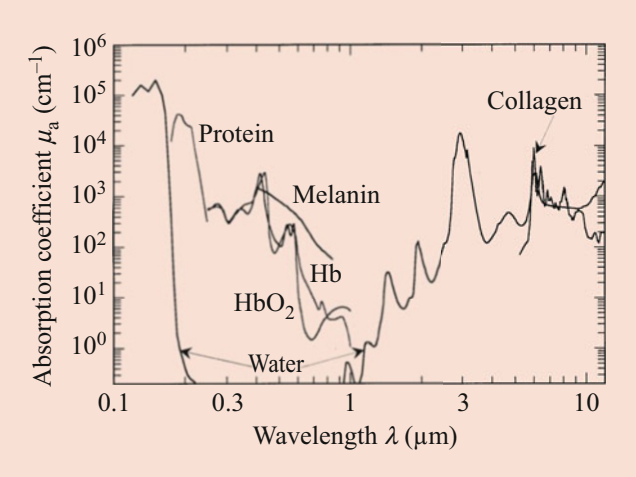

Fig. 34.9 Optical absorption coefficients of the major human body chromophores. (Reprinted with permission from Vogel and Venugopalan [119]. © American Chemical Society, 2003)

been identified in the near-infrared (NIR), namely, a first window at 650–950 nm, a second window at 1000–1350 nm, and a third phototherapeutic window at 1550–1870 nm [118]. In these wavelength ranges, absorbance by water and biomolecules is minimal, ensuring maximal penetration of the incoming light.

Activation of ruthenium-based photoactivatable complexes by light in the first phototherapeutic window has been achieved via several strategies. Firstly, several groups have shown ways to induce a bathochromic shift in the absorption bands of ruthenium complexes through the modification of some of the ligands, allowing excitation of the complex with light ≥650 nm. Usually, these modifications involve the extension of the aromatic core of the ligand, as demonstrated by the group of Glazer with $[Ru(phen)_2(biq)]^{2+}$, where phen = 1,10-phenanthroline, and biq = 2,2-biquinoline. Upon irradiation with NIR light (≥650 nm), the complex substituted the biq ligand for two solvent molecules, and was shown to be phototoxic towards leukemic HL-60 cells [103]. The same biq ligand was recently used by several groups in red light-driven photo-uncaging strategies involving the $[Ru(tpy)(biq)(L)]^{2+}$ scaffold, where tpy = 2,2′;6′,2″-terpyridine, and L is either a cytochrome P450 inhibitor [88], a NAMPT inhibitor [105], or a hydrogen sulfide releasing prodrug (Fig. 34.10) [120]. The scaffold was also used by Wu et al. for the preparation of ruthenium-loaded polymer micelles, which release their ruthenium payload upon red-light irradiation [121, 122]. The most recent development has been the introduction of the strongly donating mono-anionic acetylacetonate (acac) ligand, which destabilizes the HOMO of the ruthenium complex, thus causing a red-shift in the absorption spectrum [123, 124]. Combined with a tridentate ligand with an extended aromatic system, dqpy (2,6-di(quinolin-2-yl)pyridine), Turro et al. managed to create a photocleavable complex, $[Ru(dqpy)(acac)(CH_3CN)]PF_6$, with a ^{1}MLCT absorption band centered at 770 nm, with the tail of the absorption band extending to 950 nm [123].

Another strategy employed to activate ruthenium polypyridyl complexes with NIR light, especially used in PDT systems, is simultaneous two-photon absorption (2PA) [125]. Here, the excitation of the ruthenium complex is performed by the simultaneous absorption of two low-energy NIR photons, rather than by a single high-energy blue photon. The modification of ruthenium polypyridyl complexes with oligofluorenes or tertiary alkyl ammonium groups does not change their one-photon absorption bands, but significantly increases the chance of absorbing two photons simultaneously, expressed as the two-photon cross section σ^2, as shown by the groups of Lemercier and Gasser [25, 71, 126]. As 2PA requires both photons to be absorbed simultaneously, it demands very high instantaneous excitation power densities, which makes applicability in a therapeutic context quite challenging. However, recent progresses in the field suggest that 2PA phototherapy might have a bright future as well [108, 127].

A final strategy is to use upconversion to generate high-energy visible light in situ from low-energy red or NIR photons, so that the locally generated high-energy excitation drives prodrug activation (Fig. 34.11). Two types of upconverting systems have been proposed, namely, triplet-triplet annihilation upconversion (TTA-UC) and lanthanoid-doped upconverting nanoparticles (UCNPs). Bonnet et al. have shown that the activation of ruthenium polypyridyl prodrugs is possible with a combination of a liposomal TTA-UC system and red light (630 nm), even if irradiated through a 7-mm layer of pork fillet [128, 129]. The upconversion quantum yields measured for TTA-UC (up to 14%) [130] are significantly higher than those for UCNP-based upconversion [131], and thus the required excitation power density is relatively low (typically 100 mW/cm^2). However, TTA-UC is notoriously sensitive to the presence of molecular oxygen, requiring the addition of anti-oxidants to suppress quenching of the upconversion by dioxygen [132, 133], and its organic components are susceptible to photobleaching. The groups of Salassa and Bednarski demonstrated that such inconveniences can be circumvented by using lanthanoid doped upconverting nanoparticles [134–137]. The upconverting nanocrystals are photostable, oxygen-insensitive, and their surface can be modified with light-sensitive platinum [136, 137] or ruthenium [134] PACT complexes. Although the clinical application of such PACT nanoconjugates is still in its infancy, primarily because of the low upconversion quantum yields of UCNPs and their challenging synthesis, these systems are developing quickly and may allow activation of PACT compounds for curing cancer using NIR light in the near future.

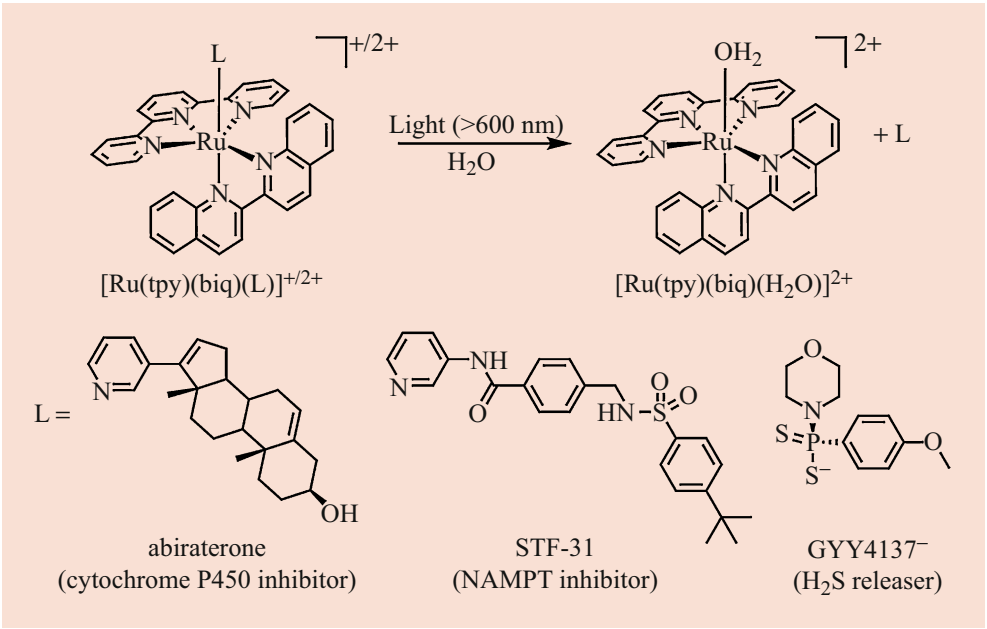

Fig. 34.10 Examples of ruthenium polypyridyl complexes that release a biologically active molecule upon irradiation with red light (>600 nm). Abiraterone and STF-31 coordinate via their pyridine nitrogen atoms, whereas GYY4137 coordinates through the anionic sulfur of its phosphinodithioate group

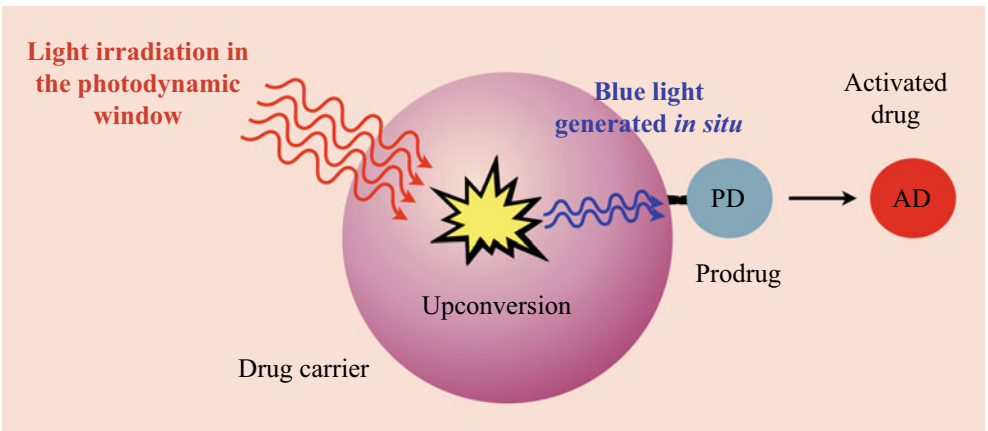

Fig. 34.11 Upconversion strategy for activating UV- or blue-light sensitive PDT or PACT compounds using light in the photodynamic therapy window. The prodrug (PD) attached to the surface of the drug delivery nanosystem is either a PDT dye that generates $^1O_2$ as activated drug (AD) or a PACT compound that simultaneously activates and detaches from the drug delivery nanosystem via a bond cleavage photoreaction releasing the activated drug (AD) inside the irradiated tissue

34.7 Conclusions and Perspectives

Compared to mainstream anticancer treatment modalities such as surgery, chemotherapy, immunotherapy, or radiotherapy, PDT is still poorly disseminated in the clinics, while PACT is new and not yet in clinical trials. Such limitations can be attributed in part to the high number of parameters involved for optimizing treatment efficacy. Not only the concentration and pharmacodynamics of the drug but also the light delivery inside the body must be considered. Market-related problems should also be solved: big pharma companies often consider the need for nurse training to phototherapeutic treatment, for modifications of operating rooms, or for selling laser devices together with a medicine, as too complicated, or out of their comfort zone, compared to traditional medicines sold "off the shelf". Some forms of conservatism have also limited the clinical development of PDT, as many physicians remain unaware for the curing potential of PDT, claim that humans are not transparent, or that phototherapy is only interesting for very small tumors. Overall, the number of randomized clinical trials for PDT should also be increased.

These challenges have led the phototherapy community into developing highly innovative inorganic compounds that can cure patients. While new metal-containing PDT sensitizers have been either approved (padeliporfin, for prostate cancer) or are currently in clinical trial (TLD-1433, for bladder cancer), many new PACT compounds are also being developed that offer more selective light-activation mechanisms, i.e., targeted to proteins involved in cancer development; activation in oxygen-poor areas; and/or improved light absorption in the PDT window. These chemical developments will make use of the new medical devices that nowadays allow for shining light in almost any part of the body. For example, interstitial phototherapy delivers light in internal organs such as the pancreas, where no other therapy works [138], or to address tumors that are as big as a fist [139]. Two-photon PDT and upconversion technologies allow for the activation of inorganic compounds with light that penetrates up to more than 1 cm into tumor tissue. Light-delivering balloons or fabrics allow for delivering light very homogeneously and over large areas, for PDT treatment of brain tumor cavities [140] or extended skin diseases [141]. These technological developments justify the testing of inorganic light-activated compounds in almost any forms of cancer, which predicts a bright future for PDT and PACT with inorganic compounds.

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